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Study on Reproductive Potencial of *Merluccius merluccius* in the Galician Shelf.

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Autoriza a la presentación de la memoria adjunta, titulada "Study on Reproductive Potential of *Merluccius merluccius* in the Galician Shelf", realizada por Dña. Rosario Domínguez Petit bajo su dirección, para optar al grado de Doctora en Biología.

Y para que así conste, se expide el presente certificado en Vigo, en Noviembre de 2006.

Juan Francisco Saborido Rey

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Y para el final dejo lo mejor de mi vida. La ilusión de despertarme cada mañana, el que me hace vivir la vida hasta la última gota, el que alegra mis días. Puede que él llegara después y a lo mejor no participó en toda esta historia desde el principio, pero es el pilar sobre el que quiero construir mi futuro. Por su eterna paciencia, por su amor, su cariño, su admiración, por su respeto. Porque me lo da todo. Porque es mi batería inagotable, mis pilas alcalinas, no conoce el desánimo, ni el imposible. Su fe en mí desborda todo lo imaginable y es mi impulso para seguir, porque con él todo merece la pena. Siempre me apoya, no se interpone nunca y renuncia a lo que haga falta por mí. En él encuentro el reposo y la calma y sólo él es capaz de hacerme reír con el corazón. Mi elfo, mi duende, mi vida. Te quiero.

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CHAPTER 1

INTRODUCTION

El hombre del conocimiento disfruta sobre el mar,
y el hombre de la virtud goza sobre las montañas;
porque el sabio es inquieto y el virtuoso pacífico.
(Confucio).

More than a dozen species of demersal marine fish are known by the common name of hake, all of them belonging to the genus *Merluccius*; they are distributed almost all round the world in both hemispheres. Hake species constitute very important resources, both in terms of landings and economic value; they are, in all seas, a highly appreciated food for human consumption. More than one million tons of hake have been caught annually worldwide in the last three decades (Alheit and Pitcher, 1995), although specific composition of catches has been changing, North Atlantic species decreasing with respect to South American hakes (Figure 1.1). Since 1983 total world landings have fluctuated, but some species such as European hake (*Merluccius merluccius*) have suffered a steady decrease.

Traditionally, for European hake, two management units have been differentiated, the Northern stock that covers II, IIIa, IV, VI, VII, VIIIa, b and d ICES divisions, and the Southern stock that covers VIIIc and IXa ICES divisions. The main argument for this choice was that the Cap Breton canyon (close to the border between the Southern part of Division VIIIb and the more Eastern part of Division VIIIc, i.e. approximately between the French and Spanish borders) could be considered as a geographical bound limiting exchanges between the two populations. More recent discussion has asked whether the Mediterranean Sea has an independent stock, even while more than one stock can be identified in this Sea.

Total landings of European hake in the Southern Stock have decreased markedly in recent decades, from 22,376 t in 1983 to 5,617 t in 2003 that is the lowest recorded value in the historical data. In 2005, however, landings increased slightly, reaching 7,437 t. Regarding discards, at present with available data from Spanish research surveys, it has been estimated that hake discards by the Spanish fleet are between 200 and 900 t, and for the Portuguese fleet equal to 650 t in 2004 and 1,064 t in 2005; but discard estimates are characterised by low precision and data have to be interpreted with caution. In the Northern stock, landings decreased steadily from 66,500 t in 1989 to 35,000 t in 1998. From 1998 to 2003, landings have fluctuated around 40,000 t, increasing in 2004 to 47,123 t and reaching 46,300 t in 2005 (ICES, 2006a; 2006b). Both stocks are outside safe biological limits, or overexploited, and recovery plans have been created for both.

European hake fisheries in Galician Shelf waters, i.e. part of the southern stock, are basically mixed fisheries. The trawl fleet is rather homogeneous and mainly uses two gears: pair trawl and bottom trawl. The proportion of hake present in the landings of these fleets is relatively low (6%), and accompanies another commercially important and targeted species. The trawl fleet focuses on small hake, ranging between 25 and 50 cm length, with a mode of 29-31 cm. In contrast, the artisanal fleet is very heterogeneous, comprising different boat and gear types. The main gears targeting hake are small and large gillnets and long lines. While small gillnets also target small hake, both large gillnets and longlines target mainly adults, but with different length compositions, longliners catching larger fish. Considering the length composition, in general, there has been a decrease in mean size of hake in the landings;

specifically, landings of smaller specimens have diminished since 1989, because of the ban on fishing hake smaller than 27 cm established that year. This decrease has occurred independently of the gear employed.
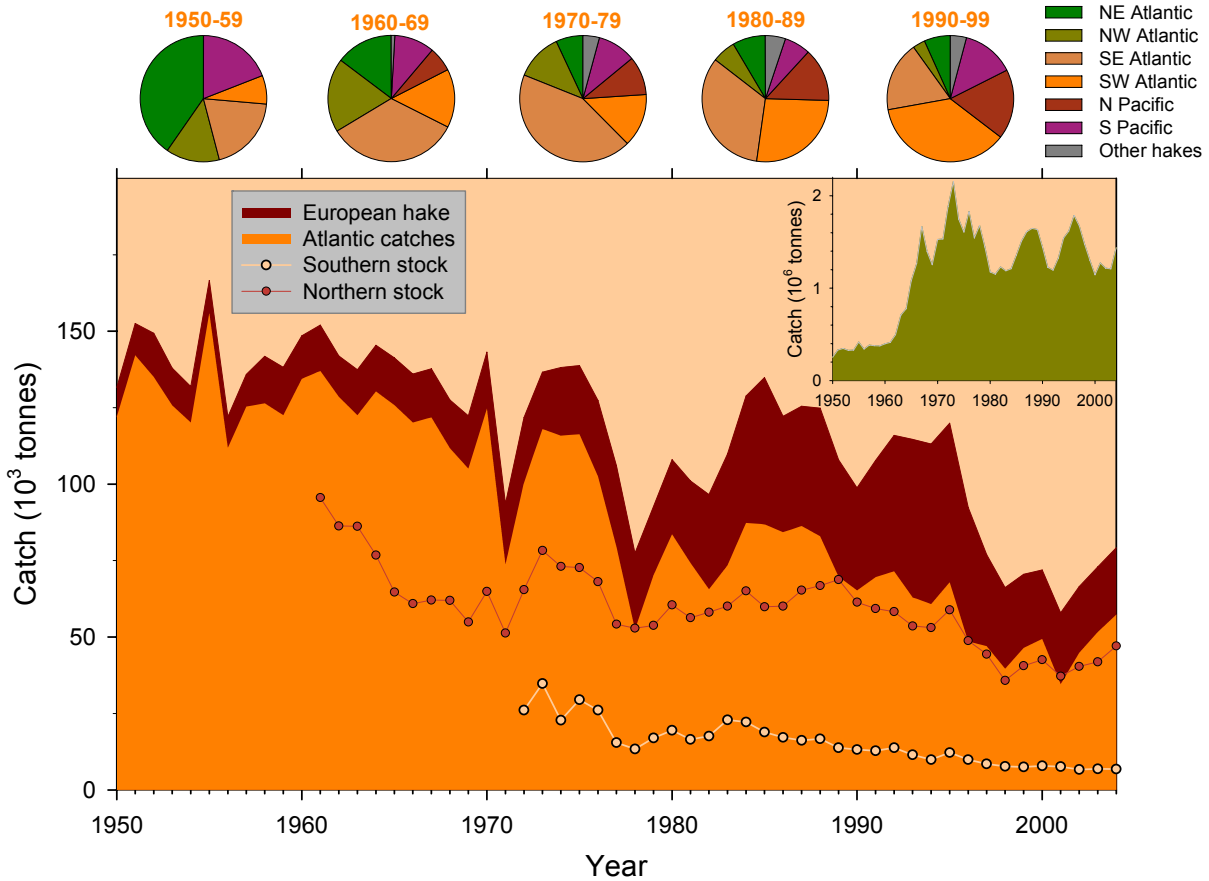
Figure 1.1: Pie graph represents specific composition of world catches of genus Merluccius classified by areas. Upper panel represents world catches of hake in millions of tonnes since 1950 until 2004 (based on FAO data). Main graph shows total catches of European hake (Mediterranean Sea: red, and Atlantic Ocean: orange), catches from Atlantic populations (Northern and Southern Stock together), and catches of each Atlantic stock separately; data from ICES WG.

In the Southern Stock, total hake biomass, spawning stock biomass and recruitment index, based on VPA values (ICES, 2006a; 2006b), showed a steady decrease in all indices in the historical series from 1982 to 2001, with a slight increases during recent years in all indices, although a slight recover has taken place in the last years in total and spawning biomass. Some peaks of recruitment have been observed, probably associated with strong year-classes that may be related to good environmental conditions (Figure 1.2).
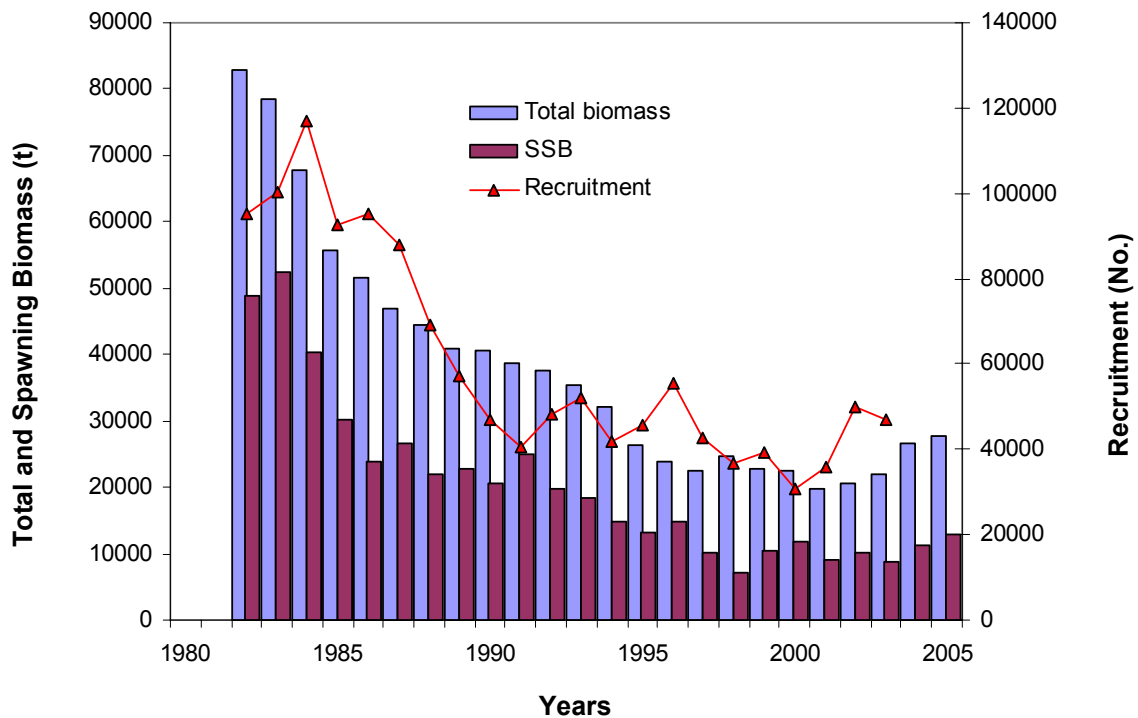
Figure 1.2: Historical series of Total and Spawning Biomass and Recruitment in Southern Stock based on VPA data.

In spite of the economic and ecological importance of hake and the depletion of the European stocks, studies of this species, especially of its reproductive strategy, are scarce. Its particular reproductive characteristics and the impossibility of keeping hake in captivity make its study difficult. The objective of the present study is to improve knowledge of the reproductive ecology of European hake in Galicia Shelf waters, and of the factors that determine its reproductive potential, as a basis for better assessment and management of this resource.

1.1   TAXONOMY AND DESCRIPTION OF GENUS MERLUCCIUS

The generic name of hake includes 14 species of marine fish whose taxonomic classification is:

Subphylum: *Vertebrata*
Superclass: *Gnathostomata*
Class: *Actinopterigii*
Division: *Teleostei*
Subdivision: *Euteleostei*
Superorder: *Paracanthopterigii*
Order: *Gadiformes*
Family: *Merluccidae*
Genus: *Merluccius*

Family *Merluccidae* is characterized by having two dorsal fins. The first one has 8 to 13 rays, the first ray being a pseudo-spine. The second fin has between 34 and 46 soft rays. Both fins are separated by an incomplete nick. The anal fin, also divided in two portions, has between 35 and 46 rays. All rays of the dorsal and anal fins are segmented. The pelvic fins are in a thoracic or jugular position. The caudal fin is separated from both dorsal and anal fins, and is relatively short (Nelson, 1994; Bauchot and Pras, 1993; Alheit and Pitcher, 1995).

These fish have a symmetric body covered by small scales. The lateral line contains from 101 to 171 scales that form a black line along body. Head and mouth are large; the mouth is located in a terminal position, with two rows of sharp conical teeth. Head of vomer is also provided with teeth, although these are retractile, and not present in the palate. The spinal column is made up of 48-58 vertebrae. Fixed ribs extend from the cervical vertebrae, while the rest are floating. Flat bones of tail are fused, this feature, combined with the high amount of white muscle that hake has and its hydrodynamic shape, suggest a high capacity of this genus to accelerate quickly (Alheit and Pitcher, 1995).

The back is normally black, grey or brown and the abdomen pale, white or silver. Hake can reach lengths of 1.5 m and weights of 15 kg. Hakes are demersal species that live near the sea bottom, over continental shelf and slope, between 30 and 500 m deep or even more, and are widely distributed worldwide (Figure 1.3).
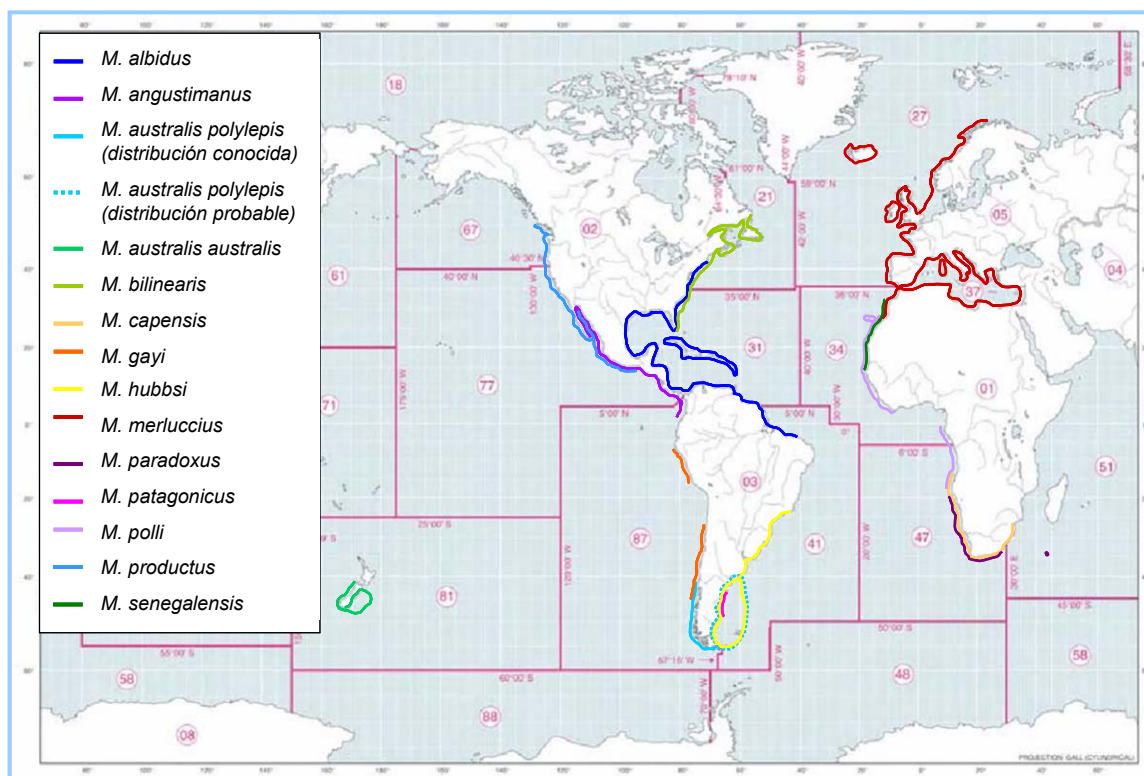
Figure 1.3: Distribution of different species of genus Merluccius

This work is focused in *Merluccius merluccius* (Linnaeus, 1758) or European hake that is distributed from Northeast Atlantic to the Mediterranean Sea.
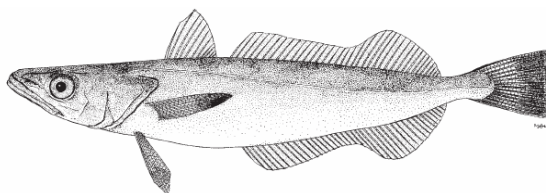
1.2   DISTRIBUTION AND ECOLOGY OF MERLUCCIUS MERLUCCIUS

In general, European hake, from now on simply hake, is a demersal and bathypelagic species. It lives over muddy or muddy-sandy bottoms on the continental shelf and slope.

M. merluccius is found between 27ºW and 40ºE of longitude and between 16ºN and 70ºN of latitude, in temperate areas of the Eastern Atlantic (Figure 1.4), i.e. from the coasts of Norway and Iceland to Mauritanian waters, although in the extreme latitudes hake is not common. Some specimens that are being studied have been discovered in the Azores (Lloris and Matallanas, 2003b). Its distribution area overlaps with those of *Merluccius capensis* and *Merluccius senegalensis*. It is also very common in the Mediterranean Sea.

Figure 1.4: Distribution of Merluccius merluccius. Dotted line: Northern stock; Solid line: Southern stock.

Bathymetrically, hake lives from 30 to 1000 m deep, developing its daily activity mainly between 30 and 370 m. In the Mediterranean Sea, the highest concentrations of individuals are found from 100 to 200 m deep, increasing in abundance from West to East (Orsi-Relini *et al*., 2002; Maynou *et al*., 2003; Oliver, 1991). In the Gulf of Lyon area, the highest concentration is between 200 and 400 m (Recasens *et al*., 1998).

Hake distribution by depth depends on its development stage and its distribution area. Eggs and larvae usually are concentrated over the continental shelf. In the North-western Mediterranean, on the Catalonian coast, maximum density is recorded at 100 m deep, on the shelf edge (Olivar *et al*., 2003). However, in the Bay of Biscay, larvae change their distribution depending on their size, moving towards the coast as they grow (Álvarez *et al*., 2001). Juveniles concentrate in shallower waters than adults. On the coasts of Catalonia,

juveniles are distributed between 60 and 160 m deep in autumn-winter, descending as deep as 300 m in spring-summer (Maynou *et al*., 2003; Oliver, 1991) and between 100 and 200 m in the Gulf of Lyon (Recasens *et al*., 1998; Oliver, 1991). In Galician shelf waters, age 0 individuals are distributed following cold water upwelling fronts. So, they can be observed deeper than 300 m from autumn to spring, ascending over 100 m in the summer months. Adults are found at all depths, generally on the edge of continental shelf, although their highest abundance is recorded between 150 and 300 m deep, rising to hatchery areas during the spawning season, located between 50 and 250 m (Maynou *et al*., 2003; Recasens *et al*., 1998; Alheit and Pitcher, 1995; Oliver, 1991). Due to hydrographic mesoscale structures, the distribution of eggs, larvae and recruits can vary. In the Bay of Biscay, adult hakes spawn from December to April at 200 m depth. Eclosion takes place a few days after spawning. The planktonic stage of larval development lasts two months and takes place between 50 and 150 m. Larvae and pre-recruits are transported offshore from spawning areas to the open sea by the eastward shelf-edge current, especially when this inflow is massive, coinciding with low recruitment years. On the contrary, larvae can be transported by anticyclonic mesoscale structures moving towards the recruitment areas. Orography may act as a physical barrier to prevent the drift of pre-recruits, and provoke a patchy distribution of recruits (Sánchez and Gil, 2000). Recruitment begins when juveniles swim towards the sea bottom, in September-November (Figure 1.5).
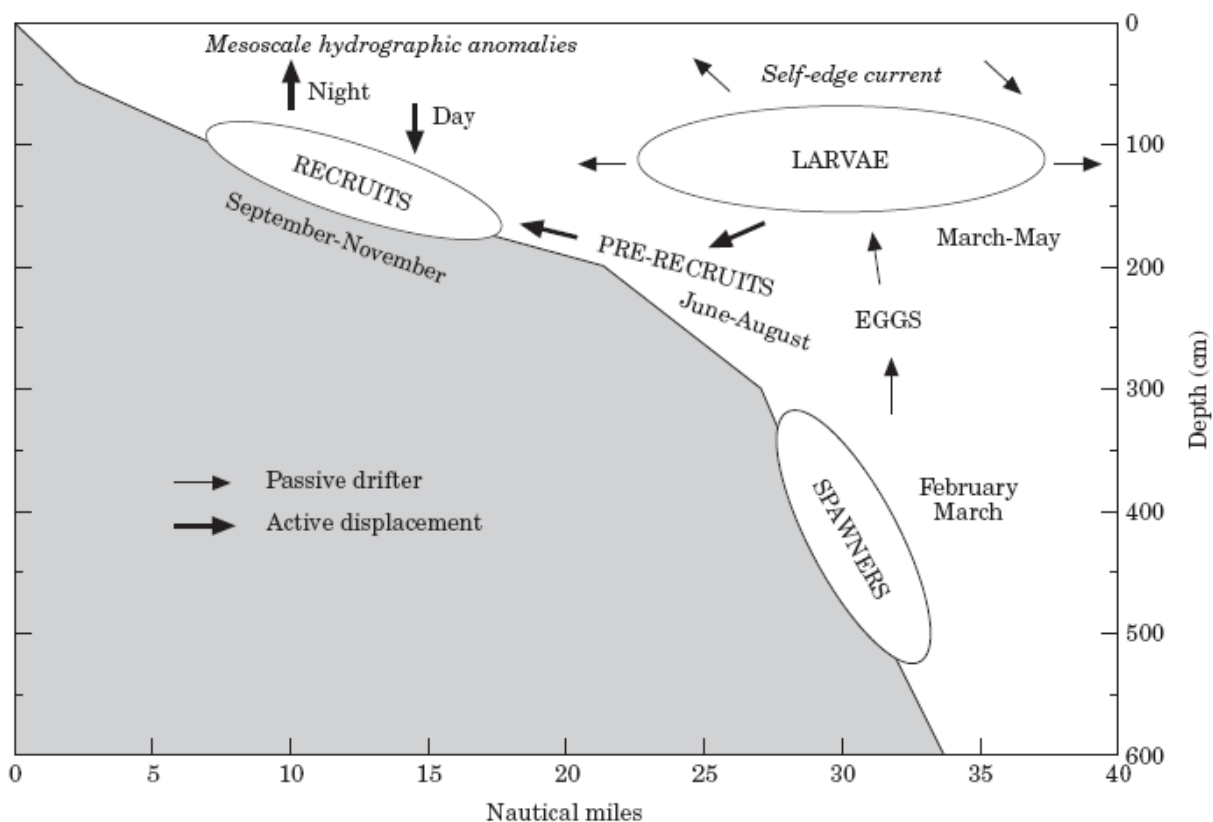
Figure 1.5: Recruitment process of European hake in the Bay of Biscay (from Sánchez and Gil, 2000)

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1.3 BIOLOGY OF MERLUCCIUS MERLUCCIUS

1.3.1 FOOD AND FEEDING

Hake is one of the largest predators in the ecosystems it inhabits. It is mostly piscivorous, i.e. feeds mostly on fish, but also eats crustaceans, molluscs, etc. Hake is ecologically a key-species in these ecosystems and adults, as top predators, are only preyed on by marine mammals, sharks and human beings.

Hake is an active, carnivorous and opportunistic predator, that changes its diet depending on seasonal and environmental variations in accordance with prey resource availability (Roel and MacPherson, 1988; Ruiz and Fondacaro, 1997), for example, *M. hubbsi* consumes high amount of *Engraulis anchoita* during spring-summer, preying mainly on Crustaceans and Cephalopods during winter (Galli, 2002). However, in specimens of the same species that live in Escondida Island waters, the diet consists basically on only one species, *Peisos petrunkevitchi* or white shrimp, because of the high abundance of this species in this area, which may be due to the high productivity of the tidal front off Valdés Peninsula, that has its southern boundaries in this island (Ruiz and Fondacaro, 1997).

In general, as in many fish species, the diet of hake varies according to size. Early life stages prey on zooplankton and crustaceans and change toward a more ichthyophagous diet as they grow. So, Carpentieri *et al*. (2005) differentiated between 4 food groups of *M. merluccius* in the Central Mediterranean, depending on length: i) group A (5-10.9 cm) in which euphausiids and mysids predominated in the diet; ii) group B (11-15.9 cm) with high abundance of euphausiids and decapods; iii) group C (16-35.9 cm) with increased importance of teleosts, mainly Clupeiforms and iv) group D (>36 cm) with a piscivorous diet based on Centracanthidae. Similarly, in the Bay of Biscay a trend from a diet based on crustaceans at early life stages to predominantly piscivorous diet in adults is also observed (Bozzano *et al*., 1997; Guichet, 1995; Auster *et al*., 2003). This ontogenetic change of diet is due, not only to size increase, but also to changes in energy requirements, because sexual maturity carries higher energy needs (Carpentieri *et al*., 2005). Besides, hake dietary variations may depend on the distribution patterns of prey organisms, and on the breeding season during which it is usually thought that females reduce ingestion rates (Bowman, 1984; Velasco and Olaso, 1998), as observed in other species (Iles, 1984; Wootton, 1990; Slotte, 1999). In the Bay of Biscay, practically no seasonal variation in the dietary composition of hake has been observed, increasing slightly on the second quarter (Velasco and Olaso, 2000). The main prey organisms of hake are blue whiting, horse mackerel and silver pout for adults, and Crustaceans for younger individuals (age-0 and age-1; Velasco and Olaso, 1998; 2000). In offshore waters of Portugal, hake has a high impact on populations of sardine, blue whiting and, in lower proportion, small hake; nevertheless, in this area seasonal changes in diet composition have been reported, and hake prey mainly on

snipefish in autumn, although the highest abundance of this species in Portuguese waters is recorded during summer (Cabral and Murta, 2002). Differences between sexes have been detected in other species of *Merluccius*, probably related to sexual differences in size or energy requirements associated with reproduction; e.g., Roel and MacPherson (1988) described for a higher feeding intensity on crustaceans for male than female *M. capensis*, the latter preyed mainly on fish. In contrast, no difference was detected for *M. paradoxus* in the same area. On the other hand, Bowman (1984) observed that at the same size, females of *M. bilinearis* presented higher feeding intensity than males.

Hake carries out daily vertical migrations. During the day, it lives near the sea bottom, moving into shallower water at night to feeding (Bozzano *et al*., 2005; Mackas *et al*., 1997; Reiss *et al*., 2004). Moreover, some cannibalism has been observed by adults on juveniles, whose importance varies depending on species. In Argentine hake (*M. hubbsi*), Galli (2002) and Ruiz and Fondacaro (1997) considered cannibalism as an alternative strategy to the lack of other prey resources; the same occurs in European hake (*M. merluccius*) in which cannibalism reaches 20.8% of diet, and for *M. bilinearis* cannibalism reaches a maximum of 9.2% of diet weight (Bowman, 1984; Bozzano *et al*., 2005; Carpentieri *et al*., 2005; Velasco and Olaso, 1998). For all these species, cannibalism is a punctual phenomenon that may be due to different distributions of juveniles and adults. On the other hand, for *M. capensis* it could be a mean 50% of its diet, being in this case an important source of natural mortality (Roel and MacPherson, 1988).

1.3.2 AGE AND GROWTH

Hake lives an average of 10 years, although the oldest specimen recorded was 20 years (Cohen *et al*., 1990). However, the process of ageing of this species is not free of controversy due to the complexity of interpretating growth patterns in otoliths, which reflect environmental, ontogenic and phylogenetic changes, as well genetic variability (Alheit and Pitcher, 1995; Piñeiro and Saínza, 2003; Lombarte, 2003). In young specimens, it is possible to distinguish daily growth rings.

The main difficulties of *Merluccius* otolith reading are (1) definition of the nucleus; (2) presence of false rings (checks) that can be confused with annual rings; (3) interpretation of otolith border (Alheit and Pitcher, 1995; Piñeiro and Saínza, 2003; Morales-Nin and Moranta, 2004; Kacher and Amara, 2005). Besides, environmental factors also determine growth rates of otoliths, Lombarte (2003) notes the influence of temperature on juveniles growth, Norbis *et al*. (1999) discusses the importance of physical processes on the growth rate of *M. hubbsi*, such as variations in the thermocline. Also, migrations of adults could determine latitudinal changes in nursery and hatchery areas, and consequently variations in growth patterns, as has been observed in *M. productus* (Benson *et al*., 2002). For *M. merluccius* from the Mediterranean, Morales-Nin and Moranta (2004) found a decrease in growth rate during

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autumn that becomes stable in winter-spring and increases in summer. In summary, standard ageing criteria does not exist for hake, although some interpretatitive criteria have been accepted internationally for hake otoliths up to age 5. Recent studies, based on tagging experiments, have shown that hake probably grow faster than was considered until now (De Pontual *et al*., 2006; Uçkun *et al*., 2000), at a rate twice as fast as previously reported. These results are still under debate.

This complex grow pattern of hake otoliths indicates that hake grow in an intermittent way, suffering a series of growth interruptions during early life (Piñeiro and Saínza, 2003). Sexual differences in growth are also detected. In general, males grow faster than females until sexual maturity, and then female growth is higher; in consequence, it is more probable to find females among larger specimens (Lucio *et al*., 1998). Besides sexual differences, density-dependent factors also determine grow patterns of *Merluccius* (Helser and Almeida, 1997; Helser and Brodziak, 1998). Even differences between stocks can influence growth as Helser (1996) questions for *M. bilinearis*. Table 1.1 summarizes some of the observed differences in growth rates among areas.

Table 1.1: Growth rates estimated by different authors for some species of Merluccius

Author	Species	Area	Growth rate
Morales-Nin & Moranta, 2004	*M. merluccius*	Western Mediterranean	1.2-2.5 cm/month
Morales-Nin & Aldebert, 1997	*M. merluccius*	NW Mediterranean	1.15 cm/month
Orsi-Relini *et al*., 1989	*M. merluccius*	Ligurian Sea	0.7-1.2 cm/month
Kacher & Amara, 2005	*M. merluccius*	Bay of Biscay	0.71-0.74 mm/day
Woodbury *et al*., 1995	*M. productus*	--------	0.73-1.1 mm/day
Arneri & Morales-Nin, 2000	*M. merluccius*	Central Adriatic Sea	0.35-0.6 mm/day

1.3.3 REPRODUCTION

Although nowadays, the reproductive strategy of hake is a target of studies and discrepancies, it is accepted by most authors that fecundity is indeterminate, i.e., oocytes continuously recruit to the secondary growth stage during the breeding season. This type of fecundity is associated with asynchronous development of the oocytes, which is the same as saying that oocytes of all stages of development are present without dominant populations as shown in Figure 1.6, except when hydration occurs producing a dramatic increase of the size of oocytes that are going to be laid, and creating an hiatus between advanced yolked oocytes and hydrated oocytes.

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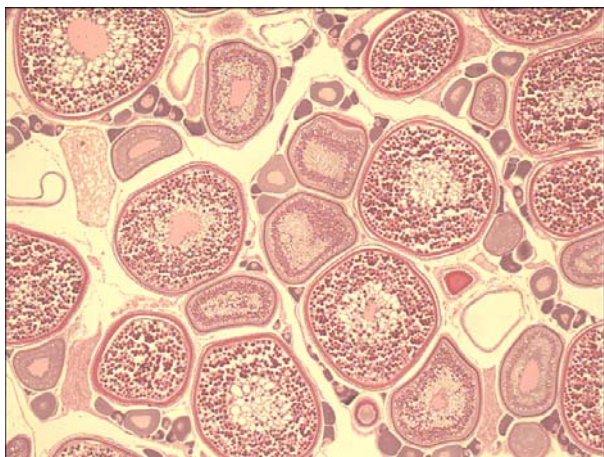
Figure 1.6: Histological slice of M. merluccius ovary.

Hake is a serial or batch spawner (Murua *et al*., 1996), which may increase the chances of survival of the offspring in fluctuating environments and decrease natural mortality of the cohort. According to Piñeiro and Saínza (2003), hake in Iberian waters spawn from December to May with a peak in February, but two peaks of spawning have been reported in the Adriatic Sea (Ungaro *et al*., 2001), one on winter and another in summer. Alheit and Pitcher (1995) differentiated clearly the spawning seasons of *M. merluccius* in the NW Atlantic and Mediterranean Sea. In the former, he records high intensity from December to April, with a peak in February-March in Iberian waters, and in March-July in the Celtic Sea. For Western Mediterranean individuals, he observes higher intensity of spawning between May and February, with a peak of oocyte production between October and January. What can be observed is a latitudinal gradient such that the latest peaks of spawning occur in higher latitudes. In general, adults breed when water temperatures reach 10º or 12ºC, changing their bathymetric distribution depending on the region they are in and the local current pattern, releasing eggs at depths from 50 to 150m (Murua *et al*., 1996 ; 1998; Alheit and Pitcher, 1995).

Males and females mature at different sizes, and then probably at different ages, too; males maturing earlier than females. Table 1.2 presents size at maturity (length at which 50% of individuals are mature) estimated for different areas.

Table 1.2: Length at maturity (L_{50}) of M. merluccius estimated by different authors in different areas.

Author	Species	Area	L_{50} male (cm)	L_{50} female (cm)	L_{50} both (cm)
Piñeiro and Saínza, 2003	*M. merluccius*	Iberian Peninsula	32.8 ± 4.2	45.4 ± 6.2	37.9 ± 11.5
Ungaro *et al*., 2001	*M. merluccius*	Adriatic Sea	20-30	26-33	25-30
Alheit and Pitcher, 1995	*M. merluccius*	E Mediterranean	18-35	18-41	-------
Alheit and Pitcher, 1995	*M. merluccius*	W Mediterranean	22-32	30-39	-------

These data translated to age indicate that males mature between 2 and 4 years, while females do so between 4 and 9 years (Piñeiro and Saínza, 2003; Alheit and Pitcher, 1995). Differences are due to males growing slightly faster than females in early life stages, reaching sexual maturity earlier. Also some geographic differences in size at maturity have been detected, probably because of phenotypic responses to variations of environmental

factors, mainly temperature and food availability. It has been observed that cold waters rich in nutrients allow larger and later mature individuals to exist than those that live in warmer waters. Hake fecundity is high, based on estimated realized fecundity, since potential fecundity cannot be estimated. Realized and batch fecundity varies considerably in relation to size and weight (Murua *et al*., 2006). High egg production is typical of species releasing pelagic eggs in open seas.

1.3.4 REPRODUCTIVE POTENTIAL

One of the main purposes of stock assessment is to evaluate the spawning stock size in order to conserve sufficient reproductive potential to allow for sustainable exploitation. Spawning stock biomass (SSB) has been generally used as an indicator of spawning stock size. However, SSB is a short term indicator of spawning stock size, which estimates the magnitude of spawning in the coming spawning season, but is not exact because it has been observed that some mature fish do not spawn annually, or their fecundity is extremely low because of environmental and physiological conditions are not suitable (Fedorov, 1971; Burton, 1999; Kraus, 2002; Kurita and Kjesbu, 2003; Saborido-Rey *et al*., 2004; and Jørgensen *et al*., 2006). On the other hand, factors such as spawning experience, sex-ratio, growth, or offspring survival also determine reproductive success and future recruitment (Wigley, 1999; Marteinsdottir and Begg, 2002; O´Brien, 2003 and Morgan and Brattey, 2005), i.e. larger female offspring quality is higher than that of small females, which is an important limitation in stocks that have been rejuvenated as a consequence of overfishing, although some species show some resilience in their life history characteristics and are able to develop compensatory responses to environmental changes including high fishing pressure (Junquera *et al*., 1999).

Because SSB cannot account for the long-term reproductive ability of the stock, fisheries management based on SSB may fail to avoid or predict overfishing. Trippel (1999) emphasized the importance of integrating basic reproductive biology such as spawners' ages, size, maturation, condition and reproductive history into stock assessment, and introduced the new term of Stock Reproductive Potential (SRP) that "represents the annual variation in a stock's ability to produce viable eggs and larvae that may eventually recruit to adult population or fishery". SRP is beyond the SSB, as there is evidence indicating that SSB may not be directly proportional to reproductive potential (Marteinsdottir and Begg, 2002; Marshall *et al*., 2003). Besides, not only it is necessary to take into account spawners' reproductive characteristics, but also their spatio-temporal variation (Godø and Haug, 1999; O´Brien, 1999; Morgan and Brattey, 2005).

In consequence, stock-recruitment relationships normally improved when the SRP index is used instead of SSB (Wigley, 1999; Marshall *et al*., 2000; O´Brien, 2003 and Tomkiewicz *et al*., 2003). Moreover, the reproductive potential can be used to evaluate the

long-term reproductive ability of the standing stock, considering the biological parameters and the age composition (Katsukawa and Matsumiya, 1998).

Improvement of SRP estimates involves acquisition of reliable time series of data on population maturity and weight-at-age. In marine fish species, growth, maturation and mortality are often different among sexes, and males mature earlier than females. Consequently, the sex ratio in the SSB varies depending on the age/size composition of the spawning stock, which compromises the reliability of SSB as an index of SRP. In such cases, female SSB based on time series of sex ratios and female maturity ogives, is a better but rarely used index of SRP. Further, some male reproductive aspects may be considered in assessment models (Trippel, 2003).

Because of this, estimates of the stock potential egg production are necessary, and these require information about fecundity relationships as well as time series of female spawning stock size or biomass. Most studies estimate fecundity from female body weight or length, but do not account for variations in size, quality and viability of eggs and larvae that may be influenced by female characteristics such as size/age and condition (Hislop, 1988; Trippel, 1998). The importance of some SRP parameters varies depending on the stocks, and sometimes it is possible to use alternative variables that represent SRP indices (Lambert *et al*., 2003; Marshall *et al*., 2003 and Morgan and Brattey, 2005). Two general categories of indices that could be combined to improve evaluation of the SRP can be distinguished:

- **Stock-based indices**: estimated from attributes of the spawners which approximate potential total egg production (TEP); i.e., fecundity-based estimates of potential TEP by the stock; bioenergetic indices (Lambert and Dutil, 1997b); refinements of SSB through the incorporation of year-, area- and/or gender-specific information on maturity and weight-at-age; proxies derived from long term series describing the condition of individual spawners and proxies such as age diversity indices, derived from basic demographic data provided by stock assessments.

- **Survey-based indices**: estimated from egg abundances measured during ichthyoplankton surveys which approximate realized total egg production: e.g.: estimates of egg production; relative egg abundance and proportions of tows containing eggs.

Eight population parameters are considered critical for estimating potential egg production: stock size and composition, age, weight, sex ratio, sexual maturity, fecundity and condition (Tomkiewicz *et al*., 2003). In this sense, variation in relative or size specific fecundity and viability of eggs and larvae can be substantial due to changes in female growth and nutritional condition (Kjesbu, 1998). Fecundity is of interest to fishery scientists as both a critical parameter of stock assessment based on egg production methods (Lasker, 1985), and as a basic aspect of population dynamics (Hunter *et al*., 1992).

The assessment of population reproductive potential and its impact on recruitment variability or the assessment of fish stocks by Egg Production Method must be included in management of fisheries stock (Lockwood *et al*., 1981; Parker, 1980 and Lo *et al*., 1992). With this aim, it is necessary to study the development and resorption of ovarian follicles that make oocyte development possible. After hydrated oocytes are released at ovulation, follicles remain in the gonad as a group of cells with characteristic morphology; these post-ovulatory follicles (POF) are indicative of the time elapsed since the last ovulation event. Some oocytes do not complete the maturation process and are not ovulated; in this case follicles are reabsorbed by atresia, that is a fecundity regulation mechanism, and the extent to which this occurs is a characteristic of the spawning strategy of each species (Witthames and Walker, 1995). Fish species have different patterns of follicle development (Wallace and Selman, 1981), and it is essential to understand the implications this has for the perceived temporal pattern of egg production and the seasonality of spawning. Two spawning strategies may be differentiated, i) indeterminate spawning in which follicles are continuously recruited into vitellogenesis, e.g. anchovy (Hunter and Macewicz, 1980), and ii) those in which oocyte recruitment is discontinuous. The last group may produce synchronous follicles, when all oocytes are recruited and developed at the same time and released in a single ovulation (semelparous species), or group synchronous follicles, in which all oocytes are recruited at the same time but are developed and ovulated in groups or batches (cod; Kjesbu, 1989).

In addition, individual fecundity changes within a single spawning season are influenced basically by the availability and quality of energetic reserves or food (Tyler and Colow, 1985) directly affecting spawners' condition, and at the same time determining maturation of individuals (Morgan, 2004; Morgan and Lilly, 2004). In this sense, allocation of energy to reproduction comprises a substantial investment in teleost fishes, and varies among species and populations; large females in better condition tend to produce larger eggs. Variation in egg size can have a direct influence on larval energy stores, resistance to starvation, and initial feeding success (O´Brien, 2003 and Abdoli *et al*., 2005). Egg production and egg size, viability and seasonal patterns of release are therefore important to understanding several key processes that shape early growth, survival and recruitment (Trippel and Neil, 2004).

Considering all the factors involved in the concept of reproductive potential, it can be used not only as an indicator for stock assessment, but also as a reference point for fisheries management (Katsukawa and Matsumiya, 1998). Some reproductive potential indexes may be simple indicators like condition factors (Fulton´s K, hepatosomatic index, etc.), and are correlated with fecundity or egg size. In other cases, however, SRP indices need to be elaborated from parameters which are more difficult to obtain, such as realized fecundity. According to Tomkiewicz *et al*. (2003), it is necessary to encourage efforts to improve SRP indices for potential application in assessment and management in order to establish

reference points, which are basic to a precautionary approach to fisheries management and sustainable fisheries. Ignorance of variability in SRP adds uncertainty to stock-recruitment relationships, which affects the reliability of associated reference points. It would be wise to separate out which components need to be sampled annually, e.g. age, maturation and condition, and which components can be estimated from routine monitoring, such as fecundity and size and viability of eggs and larvae (Trippel, 1999). In any case, it is indispensable to understand the main factors that affect SRP in each species, and even each stock, not only to the extent that they are genetically intrinsic, but also as affected by the population dynamics and the environment. To achieve this, precise studies on reproductive ecology should be initiated.

This work springs from the need to improve our knowledge of the reproductive dynamics of European hake to provide more information for assessment and management. On one hand, we describe the reproductive cycle of European hake. In addition, we detail new factors that improve the determination of stock reproductive potential, such as condition, proximate composition, fecundity estimated by new methods, and the atresia process. The main handicap to this study was asynchrony of the reproductive process of hake, not only at the individual, but also at the population level; because of this, we study new approaches to the classification of ovary developmental stages using information obtained from proximate composition and the distribution of oocytes size during the spawning season, and applying factorial analysis and analysis of variance.

CHAPTER 2

MATERIAL AND METHODS

Parece, mar, que luchas
-¡oh desorden sin fin, hierro incesante!-
por encontrarte o porque yo te encuentre.
(Juan Ramón Jiménez)

2.1 MATERIAL

The collection of samples followed a general agreement among the researchers involved in the 5th Framework RASER project (QLRT-2001-01825) regarding the optimum number of samples to be collected by year, season and size, providing, thus, good temporal and population coverage.

With this purpose, 69 sampling surveys were carried out from November 2002 to November 2004. Sampling area covered the Galician shelf, although highest sampling intensity was on the Costa da Morte and Ribeira (Figure 2.1). Adult specimens from the same area but caught between 1999 and 2000 were also studied (Galicia Government Project XUGA-40201B98).

For adult catches, we counted on local traditional fleet help. Gillnets were used, because this type of net is more selective for larger hake. These sampling surveys were focused on the Costa da Morte area (NW Galician shelf). To obtain juveniles, commercial trawlers of Ribeira (W of Galician shelf) were employed whose target species are younger specimens. In neither of the two ways of sampling did more than 24 hours elapse from the time fish were caught to sample preservation in the laboratory; they were kept refrigerated during this period in order to avoid tissue degradation. Some samples of adults were made onboard, the rest samples were bought in the fish market directly.

Figure 2.1: Sampled areas of Galician Shelf from 1999 to 2000 and from 2002 to 2004.

Sampling frequency between 2002 and 2004 was established depending on the breeding season; the aim of every survey was to intensify sampling during the spawning season and to stratify it by length class (Table 2.1). In previous years (1999-2000), a fixed pattern of sampling was not established.

In total, 3,416 hakes were caught, 865 in the 1999 and 2000 surveys and 2,551 in 2002-2004. Of these, 2,779 were females, 689 from the first sampling period and 2,090 from the second one (Table 2.2 and Table 2.3). From every specimen of the 1999-2000 periods, the following data were taken: length (cm), total weight, gutted weight, sex, macroscopic maturity stage (Figure 2.2) and gonad weight. In addition, in the second sampling period (2002-2004), liver weight and gut weight were measured. It was also noted if regurgitation had occurred.

Table 2.1: Number of sampled females for each length class and frequency of sampling surveys marked as objective from 2002 to 2004.

Length (cm)	Oct-Nov (Monthly)	Dec (Bimonthly)	Jan-May (Weekly)	Jun-Jul (Bimonthly)	Aug-Sep (Monthly)
30-35	……..………………20 monthly………………………………				
35-40	6	6	6	6	6
40-45	7	7	7	7	7
45-50	7	7	7	7	7
50-55	7	7	7	7	7
55-60	7	7	7	7	7
>60	6	6	6	6	6
Total	40	40	40	40	40

Table 2.2: Monthly sampled females for each length class in 1999 and 2000.

Length (cm)	1999									2000					Total
	Feb	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Jan	Feb	Mar	Apr	May	
35-39									1						1
40-44	7	1	1	4					10	1	1	1	1	2	29
45-49	28	9	6	19	11	1	3	2	4	14	10	13	10	20	150
50-54	60	18	24	15	16	11	10	6	6	10	29	29	14	35	283
55-60	29	12	16	14	7	7	4	4	6	15	9	15	12	16	166
>60	9	4	6	5	3				1	11		13	3	5	60
Total	133	44	53	57	37	19	17	13	27	51	49	71	40	78	689

Some females from the 2002-2004 periods were selected depending on length, maturity stage and date, to make bioenergetic analyses (Table 2.4.a and b). Gonads were removed in every female. One lobe was fixed in 4% buffered formaldehyde to determine maturity stage microscopically and to calculate fecundity. The other lobe was frozen for bioenergetic analysis.

Whole liver and one muscle slice from the middle part of the body were also frozen. From the rest of females (without bioenergetic analysis), only whole gonad was fixed in 4% buffered formaldehyde. Otoliths were removed from all individuals.

Table 2.3: Monthly sampled females for each length class from 2002 to 2004.

Length (cm)	2002		2003											Total
	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Oct	Nov	Dec	
<35			2	8		1	15	20	4	1	14	21	3	89
35-40		2	11	5	3	15	7	11	20	6	5	3	10	98
40-45		1	6	3	10	14	5	2	1	7	3	2	11	65
45-50		1	1	16	20	11	9		2	1	5	1	12	79
50-55	9	1	13	63	50	45	51	13	11	5	11	5	8	285
55-60	5		14	26	31	40	71	35	33	21	19	6	9	310
60-65			11	4	25	17	22	26	23	14	8	3	9	162
>65				6	5	5	9	3	1	4	1	1		35
Total	14	5	58	125	145	148	185	116	97	56	69	42	63	1123

Table 2.3 (Cont.): Monthly sampled females for each length class from 2002 to 2004.

Length (cm)	2004 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Total
<35	15	3	11	20	17	21	19			6	4	116
35-40		1	5		16	11	10			5	15	69
40-45			9	2	11	5	2		1	6	5	41
45-50	8	7	10	14	35	8	9	6	10	6	5	118
50-55	29	39	37	36	40	16	16	7	12	13	12	257
55-60	34	33	42	27	24	10	10	7	12	6	19	227
60-65	18	23	24	10	12	1	3	3	1	2	3	90
>65	8	11	7	7	6	2	1	2	1	2	1	48
Total	112	117	145	116	161	74	70	25	37	46	64	967

Table 2.4: Number of females with bionergetic analyses from each length class a) by month, b) by microscopically determined ovary developmental stages (I – immature, M – mature, H – hydrated, IM – inactive mature, RC – recovering).

a)

Length (cm)	2002 Nov	Dec	2003 Jan	Feb	Mar	Apr	May	Jun	Jul	Total
40-45						1				1
45-50				5	3	1	3		1	13
50-55	9	1	2	2	3	3	2	2	1	25
55-60	5		2	2	2	2	3	3	3	22
60-65			2	1	3	3	2	3	3	17
>65				2	1	2	1			6
Total	14	1	6	10	13	11	12	9	8	84

b)

Length (cm)	I	M	H	IM	RC	Total
40-45		1				1
45-50		9	4			13
50-55	1	16	6	1	1	25
55-60	1	14	3	2	2	22
60-65		9	3	4	1	17
>65		4	2			6
Total	2	53	18	7	4	84
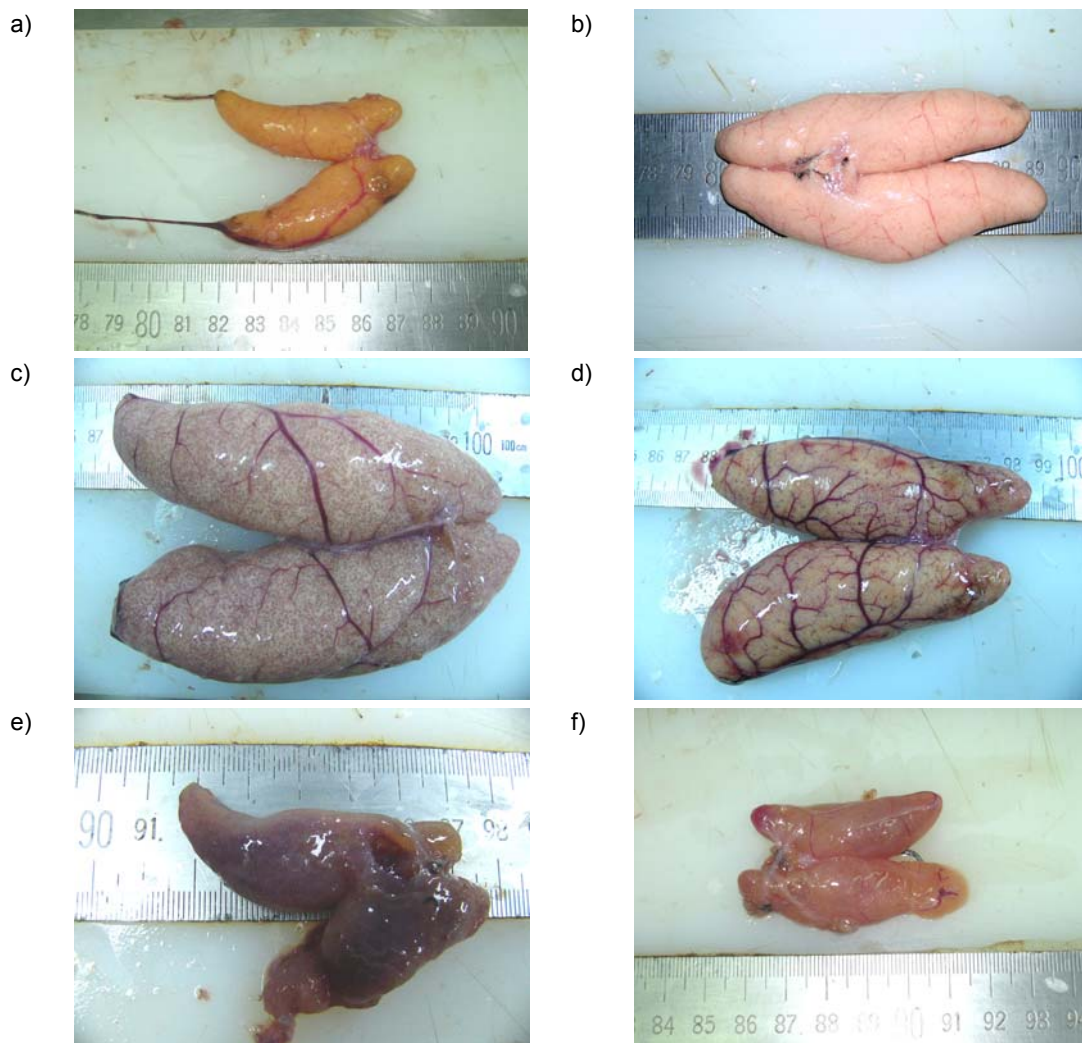
Figure 2.2: Macroscopic classification of hake ovaries: a) incipient maturation; b) matures; c) hydrated; d) advanced mature e) spent and f) recovering.

2.2 METHODS

2.2.1 GENERAL INDEX: GSI, HSI AND K

Three general indices were calculated in order to have an approximation of maturity and condition stage of females: gonadosomatic index (GSI), hepatosomatic index (HSI) and condition factor (K). These indexes are defined by the following equations:

$$GSI = \frac{gonadW}{guttedW} \cdot 100 \qquad HSI = \frac{liverW}{guttedW} \cdot 100 \qquad Fulton´sK = \frac{guttedW}{length^3}$$

2.2.2 ESTIMATES OF MATURITY OGIVES

One slice per ovary of all females was embedded in paraffin and 3 μm sections were cut and stained with haematoxylin and eosin. One section per ovary was examined with light microscope to identify different stages of development.

Different stages were classified based on criteria of Saborido-Rey and Junquera (1998), with some modifications carried out for this work:

- **Primary growth**: This covers two phases: the chromatin nucleolar phase and the perinucleolar phase. These are the first signs of the primary development of the teleost oocyte. As the oocyte grows, both the cytoplasm and the nucleus increase in size and multiple nucleoli appear in the periphery of the nucleoplasm, which is the perinuclear stage. (Figure 2.3.a)

- **Cortical alveoli (CA)**: When the ovary begins to mature, oocytes that were in primary growth phase enter the trophoblastic growth phase. Initially the mitochondria move to the periphery of the cell and a protective outer membrane forms. In this stage, very electron dense material was closely associated with the nuclear pores and accumulated near the mitochondria; this material is believed to be RNA. The cell wall acquires its characteristic multilayer structure during this phase. These layers are, from outside to inside: theca, granulosa and zona radiata. Then vesicles or droplets are observed in the cytoplasm forming a ring surrounding the nucleus whose outline is very irregular, and some nucleoli are detached from the nuclear membrane. This vesiculated ring moves to the periphery of the oocyte (Figure 2.3.b).

After this stage, the vitellogenesis process starts. In this study, vitellogenic oocytes have been divided in two major groups (vitellogenensis 1 and vitellogenesis 2) each of which includes 3 divisions, as follows:

- **Vitellogenic 1.1 (Vit 1.1)**: An inner layer made up of a number of ridges is present in the periphery of the oocyte as vitellogenesis begins. The inner ridged layer is more eosinophilic than the outer one in hematoxylin-eosin stained preparations, and a peripheral magenta ring is detected (Figure 2.3.c).

- **Vitellogenic 1.2 (Vit 1.2)**: Oocyte grows and yolk droplets are larger and more visible, although they continue in a peripheral position (Figure 2.3.d).

- **Vitellogenic 1.3 (Vit 1.3)**: Oocyte size increases and vitellogenic vesicles are more abundant, beginning to be distributed everywhere throughout the cytoplasm (Figure 2.3.e).

- **Vitellogenic 2.1 (Vit 2.1)**: Yolk droplets are larger than previously and are well distributed throughout the cytoplasm. Cell size increases, the chorion swells and becomes more eosinophilic and is coloured an intense magenta by hematoxylin-eosin stain (Figure 2.3.f).

- **Vitellogenic 2.2 (Vit 2.2)**: Oocyte size and vitellogenic vesicles continue increasing. Oil droplets appear around the nucleus which will make possible migration of the nucleus in a later phase (Figure 2.3.g).

- **Vitellogenic 2.3 or coalescence (Vit 2.3)**: Oocyte grows and chorion is thicker than in previous stages. Yolk vesicles start to fuse together and oil droplets are larger (Figure 2.3.h).

- **Migratory nucleous (MN)**: Vitellogenic vesicles continue fusing together whereas oil droplets have formed a single drop that displaces the nucleus which becomes irregular in shape towards the the animal pole (Figure 2.3.i). At this stage, yolk in the oocytes makes them opaque and easily seen by naked eye.

- **Hydrated (H)**: Oocytes enlarge due to a massive intake of water and become transparent. Cytoplasm is homogeneous and no cell structures can be identified. Chorion is thinner than in previous stages (Figure 2.3.j).

After the hydrated eggs are released, the granulosa and theca of the follicle separate and the theca cells contract reducing follicle size. Cells of some follicle remnants hypertrophy. These structures are known like postovulatory follicles (POFs), and can be dated depending on their morphologic characteristics. In the present work, 4 POFs ages have been described (Figure 2.4):

- **POF-0**: POFs younger than 24 hours. POFs have an irregular, convoluted shape; the granulosa cells are aligned, and many folds and the lumen are clearly visible.

- **POF-1**: POFs from 24 to 48 hours. POFs show degenerative process, the linear appearance of the granulose cells can be still identified but the lumen becomes reduced.

- **POF-2**: POFs older than 48-72 hours. Follicular cells are dissociated from their neighbours and detached from the basement membrane; nucleus shows pycnosis and some vacuoles are observed.

- **POF-3**: POFs older than 72 hours. The lumen is minimal or inexistent, there are no eosinophilic granules and walls of granulose cells are not distinguished. There are no

differences between the theca and connective tissue. At this age, POFs can be confused with β-atresia.

Those oocytes that are not going to be released are resorbed by females to recover their energetic compounds. This resorption is common during fish reproductive cycles, and is more intense during skip spawning episodes and when the spawning season finishes like a part of recovery process. Atresia consists in an autolysis of oocytes and a hypertrophy of follicular cells. Organization of the follicle is lost in atretic oocytes, and there is an accumulation of macrophages and fibroblasts around the degenerating follicle. Finally, the oocyte is enclosed in a more or less thick fibrous capsule. In this thesis, atretic follicles have been divided depending on their age into two stages:

- **Alpha (α)**: Is the initial phase of oocyte atresia. This phase starts when the chorion breaks. At the beginning of the phase, cellular structures are not distorted and only some gaps in the oocyte wall are detected. Subsequently, cytoplasmic disorganization and nuclear distortion progress, some vacuoles appear in the cytoplasm, and cellular size diminishes. This phase continues until no remains of the chorion can be recognized (Figure 2.5.a-b).

- **Beta (β)**: During this phase, cytoplasmic structures are not recognizable, vacuoles occupy most of the cell, there is no trace of nucleus or chorion, and cell size is considerably reduced. When this phase is very advanced, it can be confused with very late POFs (Figure 2.5.c-d).

Females were considered immature when their oocytes were in primary growth stage. In mature females, cortical alveoli, vitellogenic oocytes, post-ovulatory follicles and/or different levels of atresia could be identified. For hake, it is assumed that fecundity is indeterminate, and reproduction is asynchronous, and thus all developmental stages can be observed at the same time in the ovary during the spawning season. Besides, some spawning activity is observed throughout the year, so all specimens sampled during the four seasons have been employed to estimate the length at which 50% of the population is mature (L_{50}).

The percentage of mature females for every length class was fitted to a logistic equation as described by Ashton (1972):

$$\hat{P} = \frac{e^{a+bL}}{1 + e^{a+bL}}$$

And the logarithmic transformation:

$$\ln \frac{\hat{P}}{1-\hat{P}} = a + bL$$

where $\hat{P}$ is the predicted mature proportion, a and b the estimated coefficients of the logistic equation, and L the length.

The maximum-likelihood method was considered to be the most satisfactory methodology for estimations. Statistica 6.0 for Windows software was used to calculate predicted values and coefficients.

Size at maturity was estimated as the negative ratio of the coefficients (-a/b), by substituting $\hat{P}$ = 0.5 in the second equation.

To evaluate differences in size at maturity between years, the varince of those parameters each year was calculated from the variance and covariance of the maturity curve coefficients (Ashton, 1972):

$$V(L_{50}) = \frac{1}{b^2}\left[V(a) + \frac{a^2}{b^2}V(b) - \frac{2a}{b}\text{cov}(a,b)\right]$$

where L_{50} is the length at maturity.

Assuming that L_{50} estimates are normally distributed, then Z statistic can be computed as:

$$Z = \frac{\dfrac{a_1}{b_1} - \dfrac{a_2}{b_2}}{\sqrt{V_1 + V_2}}$$

where *a* and *b* are the logistic regression coefficients and V_i the L_{50} variances of each year compared. *Z* values can be used to test the null hypothesis of parameter equality (Gunderson, 1977).

To compare maturity ogives estimated by the micro- and macroscopic methods, we had the collaboration of the Spanish Institute of Oceanography (IEO) that shared with us its data about maturity ogives estimated macroscopically during its annual research surveys in 2003 and 2004.
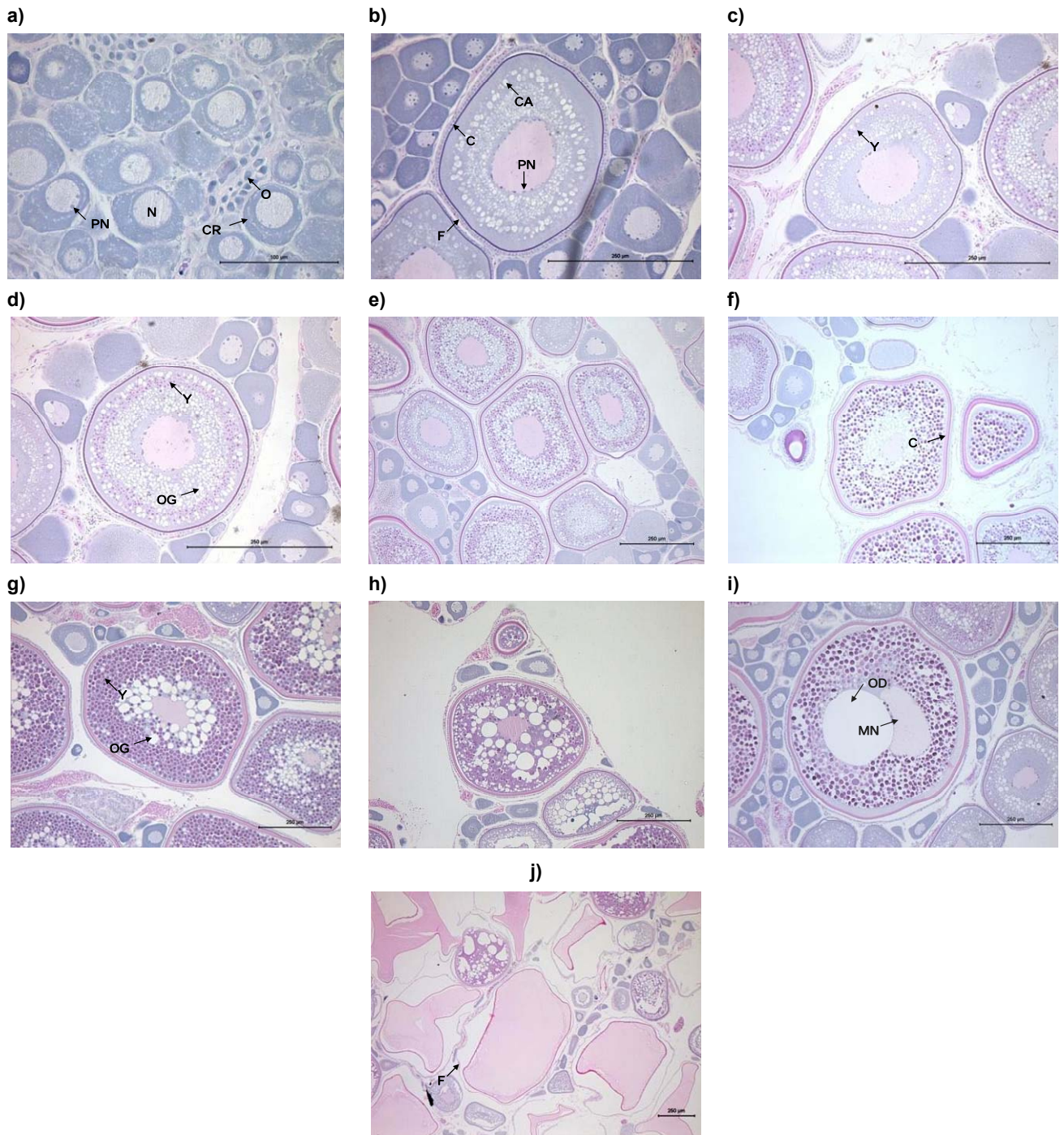
Figure 2.3: Oocyte developmental stages a) Primary growth, b) Cortical alveoli (CA), c) Vitellogenesis 1.1, d) Vitellogenesis 1.2, e) Vitellogenesis 1.3, f) Vitellogenesis 2.1, g) Vitellogenesis 2.2, h) Vitellogenesis 2.3, i) Migratory nucleus, j) Hydrated. PN=Peripheral nucleoli; N=Nucleus; O=Oogonia; CR=Circumnuclear ring; CA=Cortical alveolus; F=Follicular cells; C=Chorion; Y=Yolk globules; OG=Oil globules; OD=Oil droplet; MN=Migratory nucleus. Microns bar corresponds to 250 μm except in a) where microns bar corresponds to 100 μm.
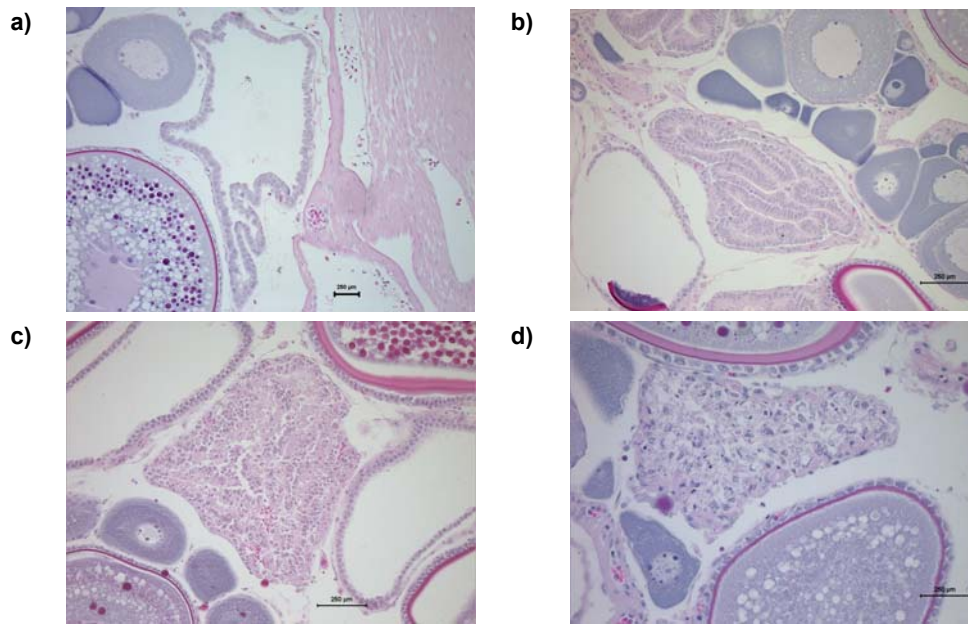
Figure 2.4: Microphotograps showing the typical aspect of hake POFs in each of the three stages identified in hake a) POFs 0-24h; b) POFs 24-48h and c) POFs 48-72h and d) POFs >72h. Microns bar corresponds to 250 µm.
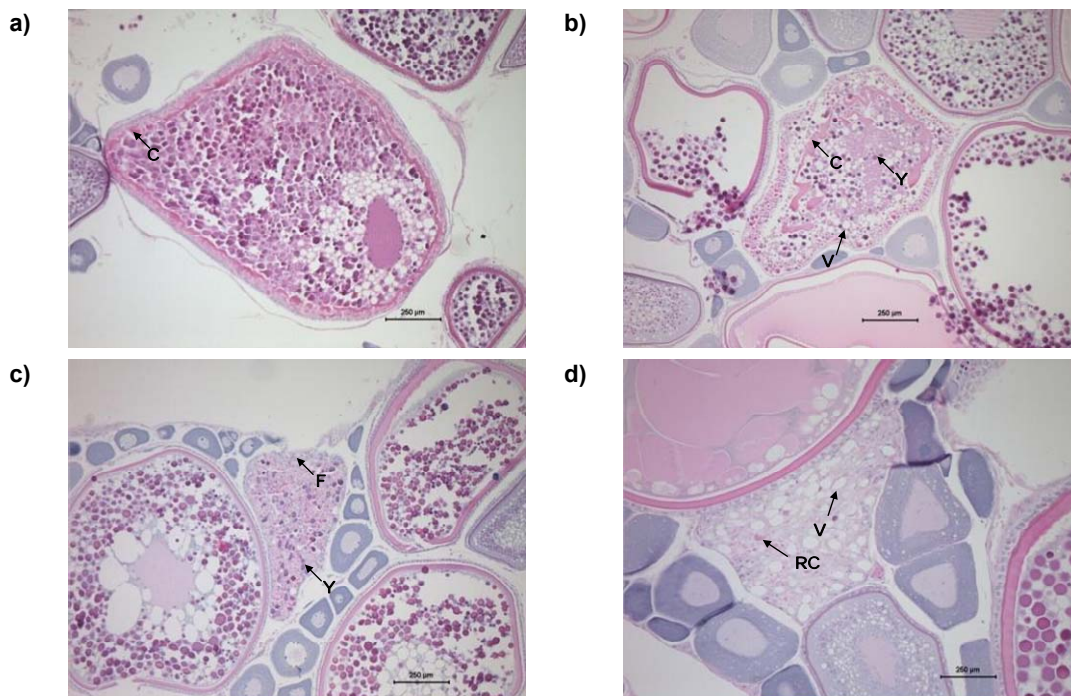
Figure 2.5: Diferent images of a-b) alpha and c-d) beta atresia. C=Chorion; Y=Yolk granules; V=Vacuoles; F=Follicular cells; RC= Rest of cytoplasm. Microns bar correspond to 250 µm.

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2.2.3 SPAWNING FRACTION AND SPAWNING FREQUENCY

To estimate the spawning fraction, post-ovulatory follicles (POFs) were aged from histological sections. As explained previously, four different types of POFs were identified.

The fraction of females spawning was assessed from the prevalence of the different spawning stages for each month of the reproductive season. The duration of the spawning stages of European hake is still unknown because daily spawning is asynchronous. Nevertheless, the relative rates of spawning could be assessed by comparing the prevalence of different spawning stages during the spawning season.

To determine the spawning fraction, we assumed duration of 24 hours for the hydration stage and for the day-0 postovulatory follicle, following the criteria for European hake (Murua *et al.*, 1998; Murua and Motos., 2006). The calibration of histological readings carried out by different readers showed that hydrated ovaries and day-0 POF were identified consistently between readers, and they were the easiest spawning stages to classify. Consequently, we considered that both stages could be used in order to estimate spawning fraction. However, we consistently found that the proportion of hydrated females were slightly larger than the proportion of females with day-0 POF or with day-1 POFs. The oversampling of hydrated females could be a consequence of the actively spawning females being more vulnerable to capture by fishing nets. Therefore, we determined the spawning fraction as the average of the incidence of hydrated and day-0 POF females from a random sample of sexually mature females since both are an estimate of the number of females spawning on any given day.

Picquelle and Stauffer (1985) presented an equation to calculate mean and variance of the spawning fraction, where each haul is weighted by its subsample size. This equation assumes that fishing activities sampled the population of mature females by judgement, in proportion to the local abundance of mature females.

$$\overline{S} = \frac{\sum_{i=1}^{n} m_i * y_i}{\sum_{i=1}^{n} m_i}$$

$$\hat{V}ar(S) = \frac{\sum_{i=1}^{n} m_i^2 \left(y_i - \overline{S}\right)}{\left(\sum_{i=1}^{n} m_i / n\right)^2 * n(n-1)}$$

where

$\bar{S}$ is the estimate of the mean spawning fraction

y_i is the average percentage of females with hydrated oocytes and with day-0 POF in the sample of sexually mature females from the ith station

m_i the number of sexually mature female sampled in the ith station

n is the total number stations

Once the spawning fraction is estimated, spawning frequency was calculated as the inverse of the spawning fraction at each sample (Fitzhugh *et al*., 1993; Hunter and Goldberg, 1980; Lasker, 1985; Macchi and Acha, 2000).

$$Frequency = \frac{1}{\% \, females.with.H + POF - 0} = \frac{n^{\circ} \, total.vit.females}{n^{\circ} \, females.with..H + POF - 0}$$

Then, monthly spawning frequency average was calculated for every year.

2.2.4 PROXIMATE COMPOSITION

In total, 84 female hakes were selected for bioenergetic analyses depending on their size and maturity stage. One ovary, liver and muscle slice from the posterior area of the dorsal fin were removed from each of them. These samples were preserved frozen at -22ºC until their analysis in the laboratory. In the case of immature and spent females, it was only possible to analyze a small number of individuals because gonad size did not allow us to extract adequate samples for this kind of analysis.

LIPID QUANTIFICATION

To determine lipid contents in each tissue, sub-samples between 1.5±0.4 g were taken, depending on the tissue; for tissues with lower lipid content (i.e. muscle), it is necessary to take larger sub-samples to avoid precision errors. Lipid extraction followed the method developed by Bligh and Dyer (1959). For lipid quantification, the gravimetric method of Herbes y Hallen (1983) was applied. Quantification was made in duplicate. The method involves transferring 200-500 μl of lipid extract to a small capsule of aluminum paper that is weighed previously. This capsule is placed on a heater board proceeding to evaporation at mild temperature. Samples are transferred to a dessicator and weighed at ambient temperature (0.001 g) until their weight is constant. The difference between capsule weight and weight after evaporation provides the lipid content in the extract.

PROTEINS DETERMINATION

100 mg of frozen tissue were taken to analyze protein content using Bovine seroalbumin (BSA) concentrated at 0.33 mg/ml as standard (Lowry *et al*., 1951).

GLYCOGEN CONTENT

Sub-samples of each tissue previously lyophilized for 2 days were taken (22.27±3.99 mg), and glycogen content determined according to the method of Strickland and Parsons (1968). For analysis, reagents used were concentrated sulphuric acid 95% (NORMAPUR ref. 20 700.298), hydrazine sulphate 130.12 g/mol (MERCK ref. 1.04603.0500 K22663003 606) and phenol 5%. The base solution to elaborate the glucose standard was obtained from D(+)-anhydrous glucose 180.16 g/mol (MERCK ref. 8337.0250 145 K16373937). Due to differences in glycogen concentration between tissues, it was necessary to create two different standards, one for muscle, with values of D(+)-anhydrous glucose from 0.003 mg/ml to 0.1 mg/ml and another , for gonad and liver, with values that cover from 0.01 mg/ml to 0.2 mg/ml, so that tissue glycogen concentration values were between detection limits of the standard. Reading was made in a spectrophotometer (Beckman Coulter DV 640) at 490 nm.

DRY WEIGHT AND ASHES

From each tissue, 3-5 g samples were taken, skin and scales were removed from muscle, and parasites were also removed from gonad and liver. Then, samples were manually homogeneized. These samples remained in the oven 24 hours at 100ºC, and were weighed at ambient temperature (0.001 g). After drying the samples, they were put in a furnace muffle. For approximately 12 hours. To avoid tissue projections, temperature was raised gradually, from 100ºC to 500ºC (100ºC every 60 minutes). Samples were weighed at ambient temperature (0.001 g).

ENERGY CONTENT

To summarize information of all biochemical components of every tissue, two new variables were created for each tissue: total energy and energy density content, defined as total kJ and kJ per gram of each tissue respectively. Equivalences used to transform biochemical composition into energy values were:

Lipids → 39.5kJ/g
Proteins → 23.6kJ/g
Glycogen → 17.1kJ/g

In addition, total energy values of females were also calculated by adding up total kJ of each analyzed tissue:

$$Fish\_energy(kJ) = total\_gonad\_kJ + total\_liver\_kJ + total\_muscle\_kJ$$

STATISTICAL ANALYSIS

Simple regression analysis was used, not only to establish existing relationship between somatic variables and general condition index, but also to check if there was any relationship between tissues' energetic compositions.

Analysis of variance was used to check if variation in energy storage with month and spawning season existed. Mobilization of energy from liver and muscle to gonad during the spawning season was also studied.

2.2.5   FECUNDITY CALCULATION

DEFINITIONS (From Murua *et al.*, 2003)

Annual realized fecundity: The total number of eggs released per female in a year.

Potential annual fecundity: The total number of advanced yolked oocytes matured per female and year, uncorrected for atretic losses.

Total fecundity: The total number of vitellogenic or advanced yolked oocytes at any time in the ovary.

Residual or remnant fecundity: The number of vitellogenic or advanced yolked oocytes in ovaries showing postovulatory follicles. This indicates that these females had already spawned some eggs.

Batch fecundity: The number of eggs spawned per batch. The sum of batch fecundities represents the realized annual fecundity.

FECUNDITY ESTIMATION METHODS

Different methods suggested by Murua *et al.* (2003) were tested to estimate fecundity:

Combined Gravimetric Method and Image Analysis: This method was used to determine potential and batch fecundity (Bagenal and Braum, 1978). The ovary was weighed in the laboratory with a precision of 0.01 g, subsamples of about 40 mg were taken from the central section, oocytes were separated from connective tissue using a washing process (modified from Lowerre-Barbieri and Barbieri, 1993) and sorted by size with sieves into those larger or smaller than 800 µm. After this, they were put in eppendorf tubes with 3.6%

buffered formaldehyde. Before they were counted, and to improve safety conditions due to the toxicity of formaldehyde, oocytes were washed with water.

Counts and measurements of oocyte size were obtained at the same time using a computer-aided image analysis system. Measurements were performed using the software QWin (© Leica Imaging Systems) on a PC (AMD Athlon XP 3000+) connected to a video camera (Leica IC A) on a stereo microscope (Leica MZ6). Camera and light settings were established at the beginning of the analysis and kept constant throughout the whole analysis. Microscope zoom was set at two values of 2.5x or 3.2x, depending on subsample size, with an objective of 0.32x for bigger oocytes and 1x for smaller ones to amplify the field of view adequately. Thus the total magnification in the first case were 0.8x and 1.024x corresponding to 27.623 and 21.929 µm/pixel respectively and 2.5x and 3.2x with the second objective, corresponding with 8.839 and 7.017 µm/pixel respectively. Due to the amount of oocytes measured per sample, and to allow a rapid and precise image analysis, each sample was divided in 4-8 subsamples, each of them analyzed in sequence as described below. The subsample was placed in a watch glass under the microscope objective; it was not necessary that oocytes were separated from each other, so it was enough to place the subsample under the camera and allow the oocytes to remain together (Figure 2.6). In this way, considerable time is saved in comparison with other methods where egg separation is required. Particles (oocytes) were measured using a semiautomatic routine developed by Saborido-Rey for a previous *Sebastes* fecundity study. The routine consists in the use of different algorithms provided by most image analyses software. First, a threshold for selecting the oocytes was defined manually by the user. The system selects all the pixels with a grey value above the threshold. When the threshold that selects all the oocytes in the image is defined, usually another particle's end extremities are also selected. Then, several algorithms of mathematical morphology operation (erosion and dilation) and segmentation algorithms were used automatically, although the possibility for manual intervention was implemented in the routine. As result, in a few minutes a binary image is obtained that represents exclusively the pixels defined by the oocytes. Then, many different measurements can be performed on each particle (oocyte). The longest and shortest diameters, the area and roundness were measured. Roundness (R) is a shape factor which gives a minimum value of unity for the circle. This is calculated from the ratio of perimeter squared to area.

$$R = \frac{Perimeter^2}{4 \cdot Perimeter \cdot Area \cdot 1.064}$$

The adjustment factor 1.064 correct the perimeter for the effect of the corners produces by the digitisation of the image.

For each subsample, oocytes with a measured diameter (longest or shortest) out of the range defined by the mean±1.5*SD were considered as outliers and completely excluded from the analyses. Occytes with a roundeness value higher than 1.15 were considered as departing from the sphericity and excluded from the diameter estimation, but included for the fecundity estimation. Something similar occurs with those oocytes that where cut in half, they were included for the fecundity estimations, not for diameter, but they were counted as 0.5 oocyte. Finally, oocyte diameter was defined as the ratio between the longest and shortest diameter of each oocyte.
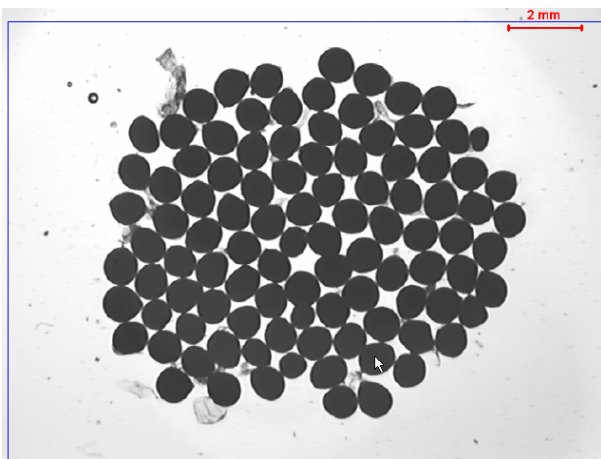
Figure 2.6: Original image of oocytes taken with a camera using a stereo microscope for fecundity analysis

Fecundity was then estimated using the equation:

$$F = \frac{OW \cdot N_s}{SW}$$

Where F is the potencial fecundity for each individual analyzed, OW is the ovaries weight after fixation, SW th subsample weight and N_s the total number of oocytes in the subsample. Relative fecundity was estimated as the number of developed oocytes in a female divided by the total gutted weight of that female.

Gravimetric Method: This method is the most commonly used to estimate fecundity. It is based on the relation between ovary weight and the oocyte density in the ovary. This method was employed in the present study to calculate batch fecundity. Previously, hydrated ovaries were weighed after fixation (0.01 g) and screened histologically to check for the occurrence of post-ovulatory follicles (POF). Ovaries that contained early stage POFs were eliminated. After this, three subsamples of 0.150 g (aprox.) were taken from the anterior, central and posterior part of each ovary. Subsamples were put on microscope slides and two glycerine (ANALEMA, P.m. 92.09, d: 1.26) drops added. So hydrated oocytes became transparent and were easily identified, separated manually and counted with a manual counter using a stereo microscope (Nikon 45245).

Then, fecundity was determined with this equation:

$$F = \frac{\sum_i \frac{o_i}{w_i}}{n} \cdot GW$$

where F is fecundity, o_i is the number of oocytes in a weighed subsample of ovarian tissue, w_i is the subsample weight (0.0001 g), n the number of subsamples (three in this case) and GW is gonad weight.

Stereometric method: Stereology involves several mathematical methods and relates tridimensional parameters that define a structure with bidimensional measures obtained from sections of this structure; thus stereology is the tridimensional interpretation of bidimensional sections of a structure (Weibel, 1979). It is very useful in several fields of science such as medicine, geology, ecology, pathology, metallurgy, etc. This method allows us to estimate, among other things, the number of particles within a structure, what is called *Numeric Density*. For this, it is necessary to apply the *Delesse Principle*, one of the most important principles of stereology. In 1847, the french geologist Delesse, studying numeric density of rock components, proved that one section taken randomly could be quantitatively representative of the material it comes from, understanding section as a plane that intersects a tridimensional space. This section, that is infinitely thin because it is a plane, is the object of study in stereology. In practice, it is impossible obtain a true plane (bidimensionality) from a tridimensional structure, so a slice is considered infinitely thin when its thickness is minimal with respect to the objects embedded in it that are to be studied. Then, if we have a structure that contains embedded objects with irregular form and the same nature, with any size and shape, separated or connected, but occupying the entire structure, the volume they occupy with respect to the total volume of the structure can be calculated from sections cut randomly. Reliability of results will be highest when more sections are taken, although a single section can be representative of the whole structure.

Stereological methods are statistical by nature: we cut a structure randomly and make our observations on these sections, although it is assumed that each shape appears with a well defined probability. Whereas we work with geometric elements, the probability of they are considered will be also geometric.

Another basic principle to estimate numerical density of particles within a structure is that the larger the number of particles embedded in a unit of volume is, the larger the number of shapes observed per unit volume unit in the section. On the other hand, large particles will have higher probabilities to be cut by a random section than small ones. *A priori*, the number of particle shapes per unit area in each section (N_A) will depend on the number of particles per unit volume (N_V), and on the particle size.

Weibel and Gómez (1962) developed a method for particles with constant shape. This method is commonly used in biology and relates N_V with N_A through a nondimensional shape coefficient (β), so that

$$N_V = \frac{1}{\beta} \cdot \frac{N_A^{3/2}}{V_V^{1/2}}$$

where V_V is the partial area of oocyte in the histological section and β is defined by the non-dimensional relation between particle volume and mean sectional area. The main virtue of this coefficient is that it scarcely changes for short axis ellipsoids, so its use is unrestricted for particles like oocytes which are almost spherical.

However, variation of particles size can introduce some systematic error. This error can be considered in the equation introducing coefficient K that is proportional to the ratio of the third and first moment of particle size distribution. The complete equation is

$$N_V = \frac{K}{\beta} \cdot \frac{N_A^{3/2}}{V_V^{1/2}}$$

For distributions of particle sizes with standard deviation less than 20% of the mean, K values vary between 1 and 1.05. Rejection of this coefficient does not introduce more than 5% error when N_V is estimated. For many biological estimates, K can be rejected.

The method most commonly used to estimate N_V in biology is the *Optical Fractionator* that is used to estimate numbers of objects within a structure, like organ tissue. Optical fractionator requires that tissue sections be very thin, and that the cuts are made randomly but orientated (follows orientation axis of the organ, i.e. antero-posterior); these are known as *Cavalieri sections* (Figure 2.7). The method consist in overlaying the image of the histological section studied with a point grid constructed from hexagonal cells with known size, the distance between each point being less than the size of the smallest particle to be counted. This grid must be placed over the tissue image randomly. Once in place, the points that are over a particle of interest must be counted.

Then, the number of particles (not points) that are inside the grid must be counted too (profile method; Figure 2.8). For those particles that coincide with grid borders, the counting frame method has to be applied (Figure 2.9; Weibel, 1979); accordingly, it was determined that those particles that touch the right and lower limits of the grid (red lines), or their extensions, are not counted; particles that touch the left and upper borders are counted.
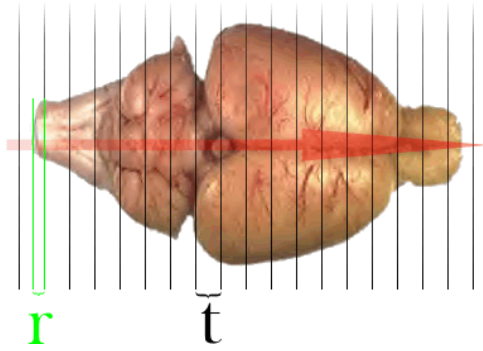
Figure 2.7: Cavalieri sections of a mouse brain.

In the present work, stereology has been applied to estimate the number of atretic, developing and hydrated oocytes, following Emerson's method (Emerson *et al*., 1991) based on the Delesse principle, and using the Weibel equation (Weibel *et al*., 1966) presented by Murua *et al.* (2003).

$$N = O_v \cdot \frac{K}{\beta} \cdot \frac{N_a^{3/2}}{V_i^{1/2}}$$

where β is the ratio between the longest and shortest axis of oocytes transected through the nucleus, O_v is the ovary volume, N_a is the number of oocyte transections per unit area, V_i is the partial area of oocyte in the histological section, and K a size distribution coefficient.

The partial area (V_i) was estimated from the proportional area determined by counting the number of points of the Weibel grid that overlay oocytes of interest and dividing it by the total number of points on the grid. The number of oocytes per unit area (N_a) was estimated counting the number of oocytes of each stage within the total grid area; exclusion rules explained previously were applied (Emerson *et al.*, 1991).

Figure 2.8: Weibel grid overlaying histological section of hake ovary.

The K coefficient is calculated from the equation

$$K = \left[\frac{M_3}{M_1} \right]^{3/2}$$

where M_1 is the mean diameter of the oocytes estimated by averaging the longest and shortest axes. M_3 is defined by:

$$M_3 = \left(\frac{\sum_{i=1}^{n} D_i^3}{n} \right)^{1/3}$$

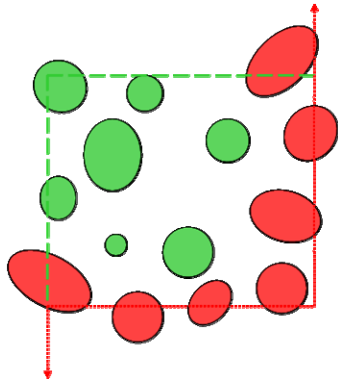*Figure 2.9: Counting frame applied in stereological analysis. Red lines represent borders of forbidden and green lines borders of counting.*

This estimate of the oocyte size distribution assumes that the nuclear diameter is constant over the whole size range of measured oocytes. However, this is not true for hake, and it was therefore necessary to apply a correction factor based on the relation between oocyte diameter and its nuclear diameter. This was done by measuring 750 occytes in 15 individual histological sections with image analysis software. The achieved hake correction factor is presented in Table 2.5.

The Weibel grid employed in this work had 168 points and a total size of 0.047 cm^2 and it overlayed an image of 1392 x 1040 pixels. Estimates were based on 4 microscope fields per ovary section, two fields near to the ovary wall and the other two near the lumen (Figure 2.10).
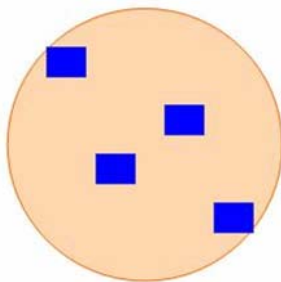
Figure 2.10: Microscope fields per ovary section selected to applied stereological method.

The Image analyzer comprised a Leica DMRE microscope connected to a Leica DFC320 FX (3.3Mp) digital video camera. The software used was Leica IM50 4.0 Release 97 (© Leica Microsystems Imaging Solutions Ltd.) and AnalySIS 3.2 (© Soft Imaging System GmbH). This method provides fecundity per unit of volume (ml); because of this, and to extrapolate to the whole ovary, it was necessary to estimate total volume of ovaries (O_V) volumetrically. Scherle's method for complex shaped objects was applied (Scherle, 1970). This method is based on the hydrostatic balance of Mohr-Westphal which, for its part, is based on principle of Archimedes; according to it the buoyant force on a submerged object is equal to the weight of the liquid displaced by the object. If the density (G_L) of the liquid is known, measurement of buoyancy (B) of the object permits the determination of its volume (V) according to the following equation

$$V = \frac{B}{G_L}$$

For this purpose, it would be necessary have a Mohr-Westphal balance, but this kind of balance is not very common. However, a common balance can be used if we establish a double weighing procedure for determination of buoyancy which is the weight of the object in air (W_A) minus its weight in water (W_L)

$$V = \frac{B}{G_L} = \frac{W_A - W_L}{G_L}$$

A simple modification can be applied to simplify the procedure: the volume of the object is measured as the weight increase of the immersion liquid (precision of 0.01 g) after immersion of the object, which is held in suspension by an external force. If the density of the liquid used differs from that of water (1 g/cm^3), then a correction factor is necessary:

$$V = W \cdot \frac{1}{G_L}$$

Table 2.5: Correction factor based on relation between oocyte diameter and its nucleus diameter.

Oocyte diam	Nucleus diam	Factor	Oocyte diam	Nucleus diam	Factor
125	57.79	3.14	575	133.78	1.36
150	63.88	2.84	600	136.95	1.32
175	69.54	2.61	625	140.06	1.30
200	74.84	2.42	650	143.12	1.27
225	79.84	2.27	675	146.12	1.24
250	84.61	2.14	700	149.07	1.22
275	89.16	2.03	725	151.98	1.19
300	93.54	1.94	750	154.84	1.17
325	97.75	1.86	775	157.66	1.15
350	101.81	1.78	800	160.44	1.13
375	105.75	1.72	825	163.17	1.11
400	109.57	1.66	850	165.88	1.09
425	113.29	1.60	875	168.54	1.08
450	116.91	1.55	900	171.18	1.06
475	120.44	1.51	925	173.77	1.04
500	123.88	1.46	950	176.34	1.03
525	127.25	1.43	975	178.88	1.01
550	130.55	1.39	1000	181.39	1.00

where G_L is the specific gravity (density) of the liquid. In our work, volumes of 64 ovaries fixed in 3.6% buffered formaldehyde were estimated. To avoid distortions on volume, the same buffered formaldehyde was used as immersion liquid (G_L=1.029). Then, a jar containing sufficient formaldehyde was placed on a common laboratory balance which was subsequently adjusted to zero. Every gonad was immersed completely in the liquid using a hook hanging from a laboratory stand by means of a thread, and taking care that there was no direct contact between the object and the walls of the jar (Figure 2.11)

Linear regression was forced to pass through the origin and the equation obtained between the two variables was

$$V = 0.9263 \cdot GW \qquad (r^2 = 0.9921)$$

where V is the volume of gonad and GW the fixed gonad weight.

STATISTICAL ANALYSIS

Simple Regression Analysis was used to establish relationships of the number of developing oocytes (NDO), relative number of developing oocytes (NDO/g of female), batch fecundity (BF) and oocytes density in gonad with somatic and condition variables: length (L), gutted weight (gut W), gonad weight (gonad W), liver weight (liver W), gonadosomatic index (GSI), hepatosomatic index (HSI) and condition factor (K).

Figure 2.11: Volumetry of hake gonads

To learn more about the relationship between between somatic and condition variables, and each fecundity variable, and which of these predictor variables had more effect on each fecundity variable, multiple regression analysis was carried out, employing the forward stepwise method. Stepwise Regression is a model-building technique that finds subsets of predictor variables that most adequately predict responses to a dependent variable by linear regression, given the specified criteria for adequacy of model fit. Specifically, the forward stepwise method employs a combination of the procedures used in the forward entry and backward removal methods. At Step 1, the procedures for forward entry are performed. At any subsequent step where 2 or more effects have been selected for entry into the model, forward entry is performed if possible, and backward removal is performed if possible, until any procedure can not be performed and stepping is terminated. Stepping is also terminated if the maximum number of steps is reached. The criteria to enter or remove an independent variable are based on critical F and p values. Both of them can be specified and used to control entry and removal of effects from the model. If p values are specified, the actual values used to control entry and removal effects from the model are 1 minus the specified p values. The critical value for model entry must exceed the critical value for removal from the model.

Temporal trends of fecundity variables were also studied by using Analysis of Variance (ANOVA) whose purpose was to test for significant differences between yearly and monthly means.

2.2.6 REALISED FECUNDITY AND POPULATION EGG PRODUCTION

The individual spawning season of European hake is not known; however, assuming a 2-month spawning period for individual hake based on data for other gadoids such as cod (Kjesbu, 1989), female realized fecundity could be estimated by combining the mean spawning fraction, mean batch fecundity, and the duration of individual spawning season.

Population Relative Egg Production (EP=number of eggs spawned per unit of mature active female weight) per month was estimated as the product of the mean number of batches per month (NB), relative number of eggs per batch (BF$_{rel}$), and percentage of active females (AF) from January to December in 2003 and from January to October in 2004:

$EP = NB \cdot BF_{rel} \cdot AF$

The mean number of batches per month was estimated as

$NB = S^{-1} \cdot n° \ days \ of \ each \ month$

where S is the spawning fraction estimated as is explained in point 3.2.3 of this work.

2.2.7 ESTIMATES OF INTENSITY AND PREVALENCE OF ATRESIA

In this case, the same stereometric method that was applied to calculate fecundity was used to estimate intensity of atresia that is defined as the proportion of atretic mature oocytes that are present in the ovary.

$$Intensity = \frac{N° \ atretic \_ follicles}{Total \_ n° \_ mature \_ follicles} \cdot 100$$

Prevalence of atresia was calculated as number of females with signs of atresia regarding the total number of mature females, i.e. it was estimated based on presence/absence of atretic follicles.

$$\Pr evalence = \frac{N° \ mature \_ females \_ wtih \_ atresia}{Total \_ n° \_ mature \_ females} \cdot 100$$

STATISTICAL ANALYSIS

Simple regression analysis was made to detect relationships between intensity of atresia and somatic and condition variables. Temporal trends (yearly and monthly) were analyzed through analysis of variance (ANOVA), both for atresia intensity and prevalence. In

addition, regression analysis was applied to establish both the relationship between these two variables and oocyte diameter effect on intensity.

According to previous studies in cod, the profile method underestimates levels of atresia (RASER, 2006), so regression calculated between profile and dissector methods for cod was used in hake to correct this mistake. This regression is:

Intensity_profile = -1.1668 + 0.7352 · Intensity_dissector

2.2.8 OVARY DEVELOPMENTAL STAGES ESTABLISHMENT

Because the hake is an indeterminate spawner, it is difficult to characterise exactly where the specimen resides in the spawning period. However, this information is essential to estimate fecundity, spawning rates, etc. One of the principal objectives of this study was to look for new methods to determine as exactly as possible the ovary developmental stage of each female in order to establish at which moment of the spawning season a female hake is (beginning, halfway or end).

First of all, analysis of variance was carried out to study variations of biochemical components in every tissue (lipids, proteins, glycogen, etc.) in relation to spawning season progress, using as grouping variable histologically determined ovarian developmental stages. The aim of this analysis was try to find a criterion to distinguish between maturity stages.

The main disadvantage was considered the likely high number of biochemical variables that had to be analyzed, making interpretation of results difficult. Thus, factorial analysis was applied to study if proximate composition is a good index of maturity stage. The main applications of this multivariate analytic technique are 1) to reduce the number of variables and 2) to detect structure in the relationships between variables, that is, to classify variables. Therefore, factor analysis is applied as a data reduction or structure detection method. The correlation between two or more variables can be summarized in a scatter plot. A regression line can then be fitted that represents the best summary of the linear relationship between the variables. If we could define one or more variables that would approximate the regression line in such a plot, then these variables would explain most of the variance of the items. Subjects' single scores on these new factors, represented by the regression line, could then be used in future data analyses to represent that variance. In a sense, several variables would be reduced to new factors that would actually be a linear combination of variables. In PCA, after the first factor has been extracted, another line (factor) that maximizes the remaining variability is defined, and so on. In this manner, consecutive factors are extracted. Because each consecutive factor is defined to maximize the variability that is not captured by the preceding factor, consecutive factors are independent of each other; i.e. they are uncorrelated or orthogonal to each other.

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With this aim, factorial analyses were conducted using GSI, HSI and K of each specimen, combined with contents of lipid (mg/g lyophilized), protein (mg/g lyophilized), glycogen (mg/g lyophilized), water and ash of gonad, liver and muscle. Analyses of variance between obtained factors and ovary developmental stages were carried out.

Similarly, factorial analyses were conducted with 23 different parameters of oocyte size distribution, namely, mean, harmonic mean, geometric mean, median, mode, frequency of mode, minimum, maximum, -95% confidence, +95% confidence, lower quartile, upper quartile, 10 percentile, 90 percentile, range, quartile range, variance, standard deviation, standard error, skewness, standard error of skewness, kurtosis and standard error of kurtosis. The most explanatory distribution variable was mode, and both regression analysis and analysis of variance were employed to study its relation with NDO, ovary developmental stages and temporal trends. Oocyte diameter was measured for every oocyte counted for fecundity estimation. Diameters were estimated using image analysis software as described in Section 3.2.4.

Another advantage of factorial analysis is that the extraction of principal components amounts to a variance maximizing rotation of the original variable space (*varimax*; Bisquerra, 1989). For example, in a scatter plot we can think of the regression line as the original x-axis, rotated so that it approximates the regression line. This type of rotation is called variance maximizing, because the criterion for the rotation is to maximize the variance (variability) of the "new" variable (factor), while minimizing the variance around the new variable. This kind of rotational strategy has been used in this work.

2.2.9 REPRODUCTIVE POTENTIAL

Stock reproductive potential depends on female status, proximate composition and energy storage that, in combination with environmental conditions, act on potential fecundity to determine realised egg production. To analyze dependence of the Number of Developing Oocytes (NDO), the relative NDO (NDO_{rel}) and mean oocyte diameter on biochemical composition and energy content of each tissue (gonad, liver and muscle), linear regressions were carried out. To detect which of these components have more influence on NDO and NDOrel, Multiple Regression Analysis was used. In European hake, oocyte diameter is not an estimate of egg diameter related to offspring quality and its probability of survival. In any case, it is not possible to know egg diameter, because egg surveys have not been done and there is no possibility to maintain specimens in captivity. Under these circumstances, the best and almost the only proxy to egg size, an important factor that affects reproductive potential, is hydrated oocyte diameter. So, Multiple Regression Analysis was carried out combining different groups of biochemical and energetic variables to create models that explain hydrated oocyte size variability.

CHAPTER 3

RESULTS

No sabe perdonar, su ley de siempre
es que el pez grande ha de comerse al chico
nosotros pasaremos pero el mar
quedará como sábana y testigo
(Mario Benedetti)

3.1 Reproductive cycle of *Merluccius merluccius* in Galician Shelf waters

A total of 2,779 female hake were collected during the sampling period of this study. To assess the reproductive cycle, ovaries of 2,673 females were histologically analysed (Table 3.1). Histology allows a better classification of the oocyte development stage than macroscopic inspection and hence it is possible to establish a reliable maturity classification of females.

Table 3.1: Number of ovaries histologically analyzed by year and developmental stage.

Maturity	1999	2000	2002	2003	2004	Total
i	22	5		247	207	481
m1	80	58	4	252	211	605
h	42	38	2	135	106	323
m2	161	190	1	270	195	817
m3	1	1	7	92	108	209
im	18	18		78	61	175
rc	7	4	2	18	32	63
Total	331	314	16	1092	920	2673

When progression of oocyte developmental stages (CA, vit 1.1, vit 1.2, etc.) with time was investigated, it was observed that not only were all stages present at the same time in spawning females, but also at practically any time of the year.

Regarding the cortical alveoli (CA) stage, around 80-90% of females showed oocytes at this stage in each month, except June, July, October and December when percentages of females with CA diminished to around 60%. The lowest percentage of females with CA was detected in November (23.4%; Figure 3.1.a). Percentages of females with vitellogenic oocytes in stage 1 (vit 1.1, vit 1.2 and vit 1.3) ranged between 15.6% in November and 88% in February, the month with the highest values. After February, the percentage of females with vitellogenic oocytes in stage 1 decreased progressively and reached the minimum value in November (around 15%; Figure 3.1.b). These stages overlapped with stage 2 of vitellogenic oocytes (Vit 2.1, vit 2.2 and vit 2.3) that presented a similar pattern to previous stages, and reached a maximum in February (88% of females) and minimum in November (15% of females), being basically in all months more than 30%. Vitellogenic oocytes in stage 2.3 are scarce because the coalescence process takes place in a few hours, and it is difficult to find females with this oocyte developmental stage (Figure 3.1.c). Something similar happened with the migratory nucleus stage, the highest proportion of females with oocytes at

this stage was reached in February (44.2%), then this proportion progressively decreased to minimum values in November (3.1%), rising slightly in December (7.9%; Figure 3.1.d). Percentage of females with hydrated oocytes was maximum in January (10.8%), decreasing until April (4.9%), then increased in May (7.6%), and showed a sharp decrease in June, remaining around 2% except September, when percentage of females with hydrated eggs increased to 5.4% (Figure 3.1.e).

Post-ovulatory follicles (POFs) were present all year round. POFs 0-24h reached their maximum in February (26%) and their minimum in September-October (2.7%), and decreased progressively from February to October. No POFs 0-24 h were observed in November or December. POFs 24-48 h females followed a similar trend as previous ones, save September, when they reached 16.2%. POFs older than 48h are the most abundant and in some months exceeded 40%, but it is necessary to note that these POFs are so distorted that they can be confused with atretic oocytes and consequently, the proportion of POFs older than 48h females could be overestimated (Figure 3.1.f).

On the basis of these results, it can be postulated that European hake is an asynchronous species because all oocyte developmental stages are present at the same time in the ovary. This asynchrony is not only seen at the individual level, but also at the population level since they are present all year round. Besides, the European hake spawning season is protracted through the year, although a peak of spawning can be identified in the first quarter of the year (January-March). Consequently, it is very difficult to determine which females have started to spawn and which not, and to differentiate which females were at the beginning, the midpoint, or the end of the spawning season. This makes it very difficult to study fecundity, an essential component of stock reproductive potential studies.

Thus, one of the main objectives of the current thesis is to establish new criteria to differentiate ovary developmental stages of hake females. With this aim, histological, biochemical, and other techniques were applied, as detailed in subsequent sections.

With respect to histological investigations, ovarian development was divided into seven stages:

- **Immature** (**i**): All the oocytes in the ovary were in primary growth stage (Figure 3.2.a).

- **Ripening** (**m1**): the occurrence of cortical alveoli and/or vitellogenic oocytes (From vit 1.1 to migratory nucleus) is observed, but post-ovulatory follicles are not present, and no signs of advanced spawning process like thick ovary wall, high vascularization of gonad and/or disorganization of lamellae, are observed either (Figure 3.2.b and c).

- **Spawning** (**m2**):   Post-ovulatory follicles younger than 72 hours are observed throughout the ovary together with vitellogenic oocytes in different stages. Signs of advanced spawning process are not necessarily observed (Figure 3.2.d).

- **Late spawning** (**m3**): Ovary with vitellogenic oocytes and without post-ovulatory follicles younger than 72 hours but with signs of advanced spawning process such as high number of blood vessels, swelling ovary wall, atresia, disorganization of ovary structures, etc. (Figure 3.2.e).

- **Ovulating-Hydrated** (**h**): There is a high percentage of hydrated oocytes or at the beginning of the hydration process (Figure 3.2.f).

- **Inactive mature** (**im**): Females at this stage will no longer produce more oocytes to be released during the current breeding season. They are characterized by high level of atresia, disorganization of ovary structures, numerous blood vessels, thick ovary wall and absence of yolked oocytes groups (excepting some atretic yolked oocytes). The cessation of egg production may be due to the end of the spawning season (spent females) or an earlier interruption of it (skip spawners; Figure 3.2.g).

- **Recovery** (**rc**): Ovary is without mature oocytes, with wide ovary wall, lamellae are not so compact as in immature ovaries, and blood vessels could be more abundant too. These structures indicate that this ovary has produced eggs in the previous spawning season and that it is recovering for the next one (Figure 3.2.h).

All these different ovary developmental stages were observed through the year, indicating clearly the population asynchrony of the reproductive cycle. Thus, ovulating-hydrated females were found every month, although a high percentage were recorded from January to March in all years studied (Figure 3.3).

This asynchronicity in both the oocyte development within the female, and the ovarian development across females within the population, resulted in two important handicaps to analysis of hake reproductive ecology; on one hand, it was not possible to determine which mature females were in pre-spawning condition and which of them had already initiated egg release (batch production) as discussed later. On the other hand, both features prevent a normal estimation of spawning duration, which is usually estimated as the time lapsed from the point when 50% of mature females are in pre-spawning condition to the point when 50% of them are in post-spawning stage. However, these levels of spent females were never reached in the study period. Consequently, duration of spawning season could not be estimated. But it could be discerned that although the European hake spawning season covered several months, the spawning intensity is different depending on the month/season

of the year, and seemed to be higher in the first quarter of every year, although a secondary peak of spawning was observed in some years, as explained below.
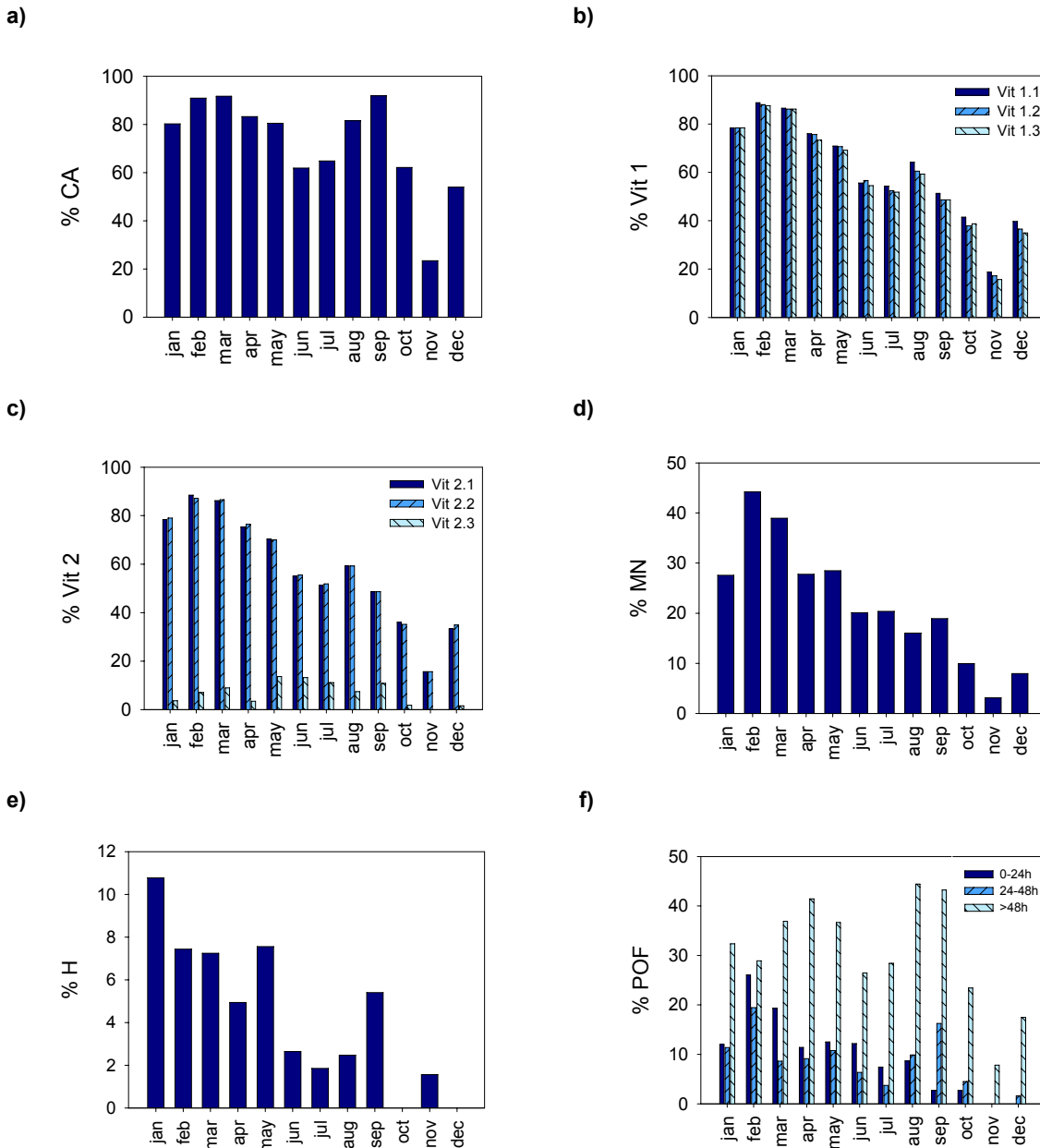

Figure 3.1: Incidence of different oocyte developmental stages during the year: a) Cortical alveoli (CA); b) Vitellogenesis 1 (Vit 1.1, vit 1.2, vit 1.3); c) Vitellogenesis 2 (vit 2.1, vit 2.2, vit 2.3); d) Migratory nucleus (MN); e) Hydrated (H) and f) Post-ovulatory follicles (POFs 0-24h, POFs 24-48h and POFs>48h).
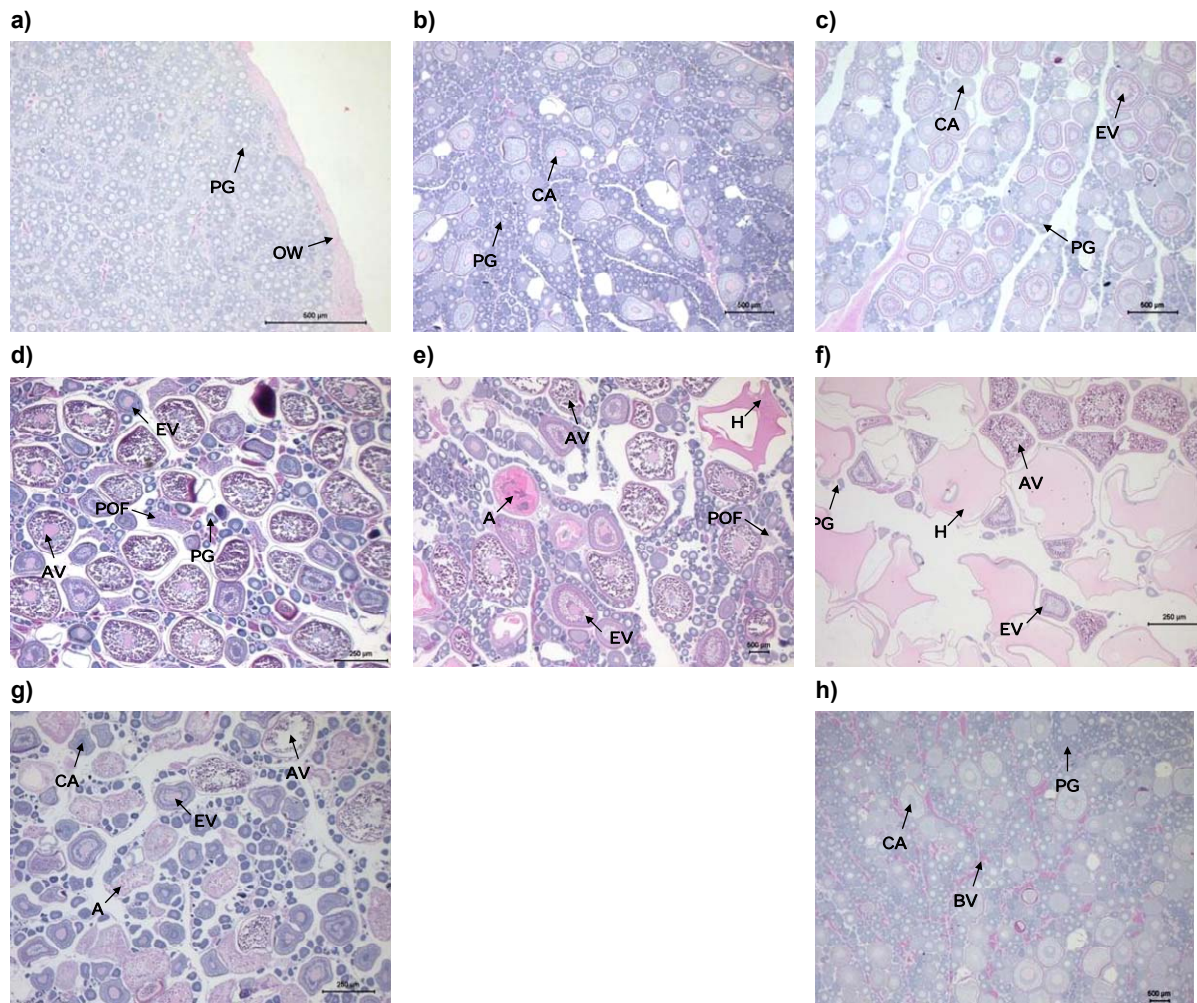
Figure 3.2: Ovary developmental stages that have been histologically determined; a) immature ovary (i, microns bar = 500 μm); b) early ripening ovary (m1, microns bar = 500 μm); c) ripe ovary (m1, microns bar = 500 μm), d) ovary after batch release (m2, microns bar = 250 μm), e) late maturing ovary (m3, microns bar = 500 μm), f) ovulating-hydrated ovary (h, microns bar = 250 μm), g) inactive mature females (im, microns bar = 250 μm) and h) recovering female (rc, microns bar = 500 μm). PG=Primary growth; OW=Ovary wall; CA= Cortical alveoli; EV=Early vitellogenesis; AV=Advanced vitellogenesis; POF= Postovulatory follicles; H=Hydrated; A=Atretic; BV=Blood vessel.

Figure 3.3: Monthly proportion for all years combined of the mature female ovary developmental stages. Stages ripening (m1), spawning (m2) and late spawning (m3) are grouped in "Mature" class.

The spawning fraction (S) was estimated as the mean prevalence of females with hydrated oocytes and females with presence of POFs younger than 24 hours (Figure 3.4). In 1999, the spawning fraction was highest in February (S=0.23±0.04) with a batch interva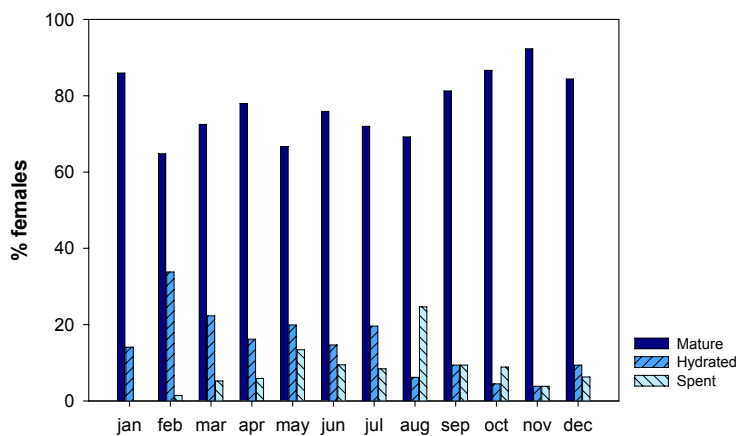l of 4.4 days, and then continuously decreased to the lowest values of April (S=0.06±0.017) with a batch interval of 17 days. Afterwards, the spawning fraction slightly increased in May and June (S=0.14±0.08 and S=0.27 respectively) with batch interval of 7.1 and 3.8 days respectively. In October, spawning fraction decreased to 0.11, and the batch interval was 9 days. A similar pattern of spawning fractions was observed in 2000, increasing from January (S=0.11±0.02 and 9.2 days between batches) to February (S=0.15±0.03 and 6.6 days of batch interval) and then decreasing continuously to April (S=0.07) when batch interval was estimated as 14.4 days. It increased again in May (S=0.20±0.01), when the highest values was observed with a batch interval of 5.1 days, to further decrease in July (S=0.12 and 8.7 days between batches). However, the spawning fraction of 1999 and 2000 should be taken with caution since the sampling was somewhat limited (n=331 and n=314 in 1999 and 2000 respectively; Figure 3.4).

In 2003, the spawning fraction increased from January (S=0.20), with a batch interval of 4.9 days, to the highest spawning intensity of February (S=0.34±0.04), with the batch interval around 3 days. Subsequently, the spawning fraction decreased in March to 0.23±0.08 and a batch interval of 4.3 days but still being at the same level as January. Then it dropped dramatically to the lowest observed figure of April (S=0.06±0.03) with a batch interval of around 15.8 days. A subsequent increase in spawning fraction was observed in May (S=0.23±0.06) and remaining at around that level in June and July, which corresponded to a batch interval for the period of May-July of around 4.4 days. The spawning fraction further diminished in August, October and November to around 8% of females spawning on a given day (with a batch interval of 12 days; Figure 3.4).

The spawning fraction pattern in 2004 was, somewhat, similar to that of 2003. The spawning fraction increased from January (S=0.13±0.02) to the highest figure of February (S=0.29±0.04), with a batch interval of 3.5 days, and then it slightly decreased in March (S=0.18±0.04), rising again in April (S=0.28±0.07). Subsequently, the spawning fraction dropped significantly in May to the value of 0.13±0.04 and a batch interval of 7.8 days and it remained around that level in June–July. However, contrary to what was observed in 2003, there was not a second peak of spawning activity in June-July 2004 and, moreover, there was not a dramatic drop in spawning fraction in April as seen in 2003. Further, the spawning fraction decreased until August when the lowest levels within the year were observed (S=0.06), which corresponded to a batch interval of 18 days. Surprisingly, spawning fraction increase in September and October (S=0.08 and S=0.12, respectively) with batch intervals of 12 and 8.5 days respectively.
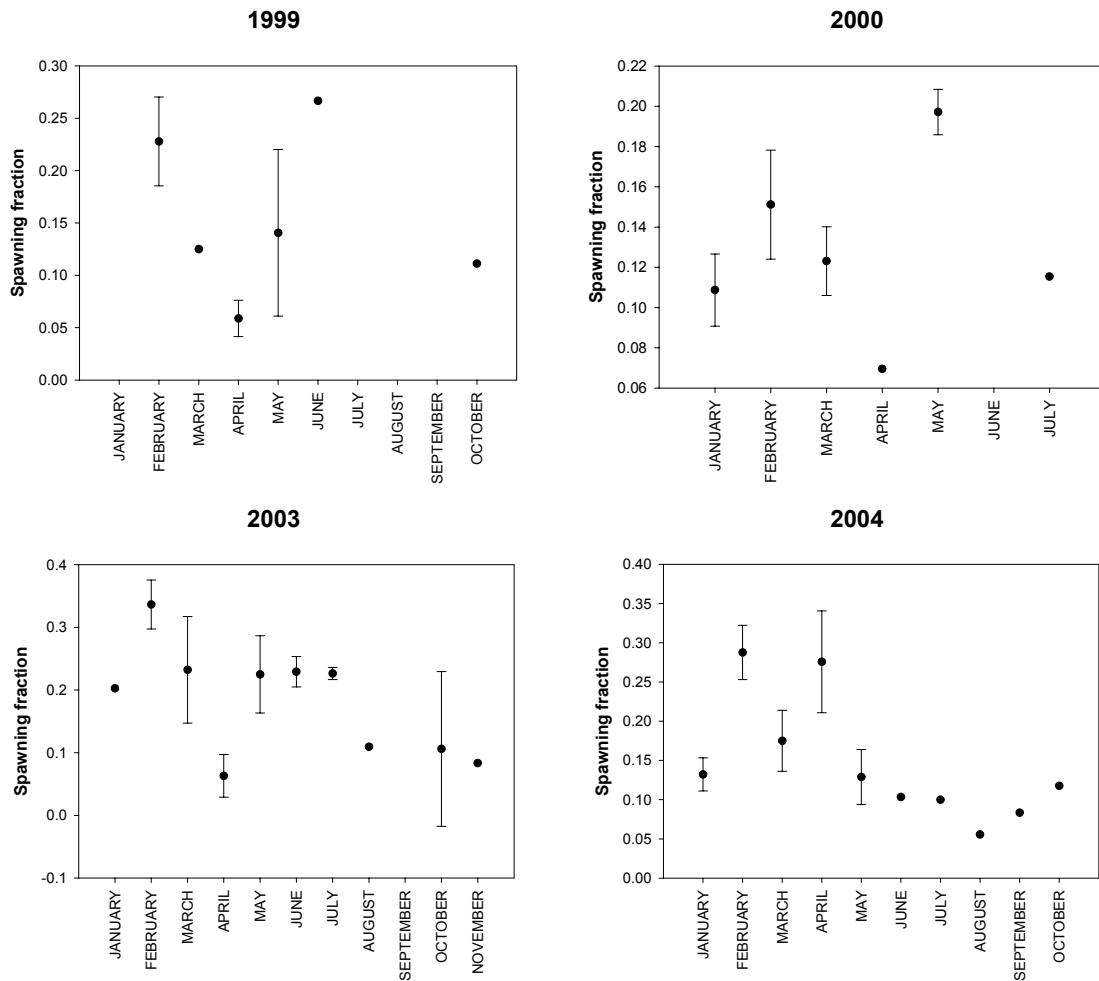
Figure 3.4: Monthly average spawning fraction for each year analyzed. Bars represent standard deviation.

These results basically agree with the observed temporal evolution of the gonadosomatic index (GSI) of mature females. GSI decreased slightly from January month in which it reached the maximum mean value of 6.8%, to February (6.5%), and it further decreased progressively to a minimum mean value of 0.6% recorded in November, to increase again, more than twice, in December (Figure 3.5). In 1999, 2000 and 2003 a slight upturn of GSI was observed in May-June (Figure 3.6), that was not observed in 2004, perhaps due to the lack of samples in June-July. Anyhow, the GSI varied significantly between months in all years except 2000 (Table 3.2). A progressive increase of mean annual female GSI took place from 1999 to 2004 (annual mean values 3.64% and 6.68% respectively), 2003 and 2004 being significantly different from the other years (Figure 3.7).
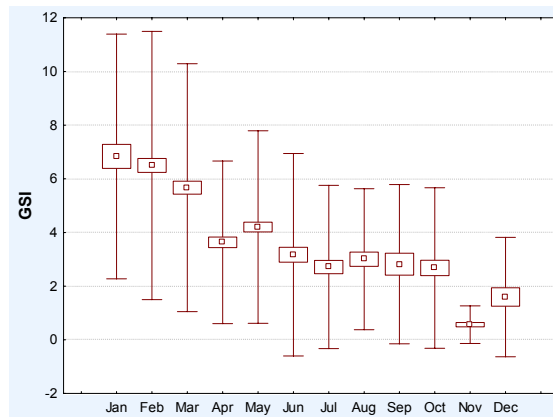
Figure 3.5: Monthly variation of mature female mean GSI (Mid point) for all years combined. Standard error (box) and standard deviation (wishker) are also represented.

Figure 3.6: Temporal trend of mature female mean GSI (mid point) in every analyzed year. Standard error (box) and standard deviation (wishker) are also represented.

Figure 3.7: Inter-annual variations of mature female mean GSI. Standard error (box) and standard deviation (wishker) are also represented.

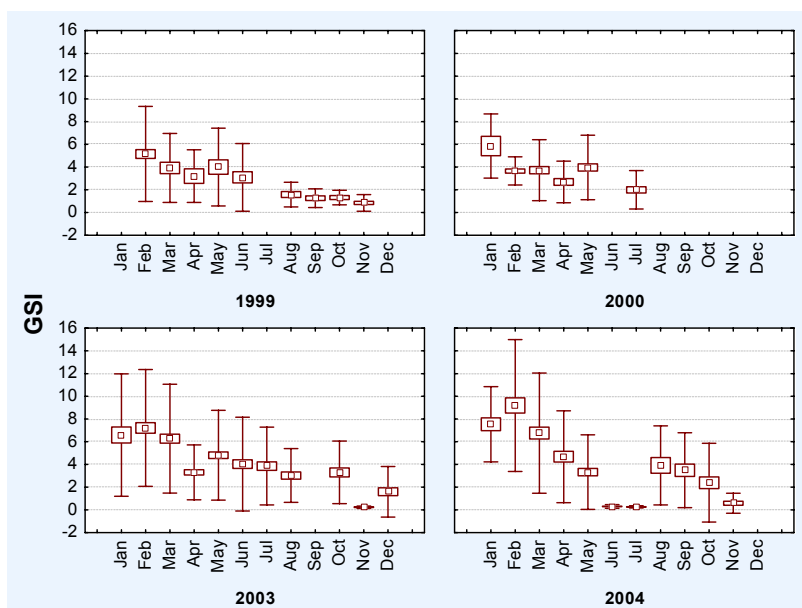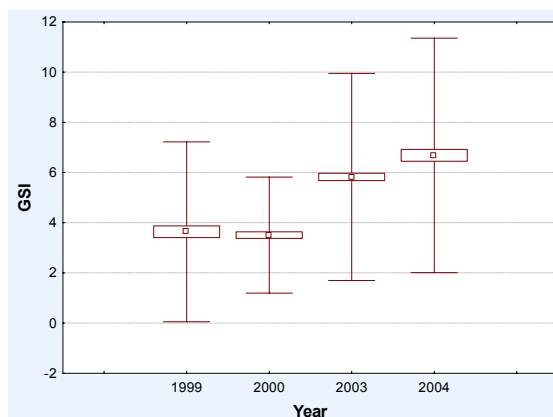Table 3.2: Results of ANOVA performed to compare GSI of mature females by month in each year and considering all years together.

	F	p-value
1999	7.57	≤0.001
2000	1.65	0.179
2003	14.12	≤0.001
2004	10.93	≤0.001
All years	16.16	≤0.001

In summary, the spawning fraction followed a similar trend from 1999 to 2004 on the Galician Coast, although the spawning activity was somewhat different between years. In 1999 and 2003, there seem to have been two peaks of spawning, one in February and the other in May and July for 1999 and 2003 respectively; this did not happen in 2004, whereas in 2000 this peak is not detected by the spawning fraction trends although a slight increase in GSI was observed. In 2003 the spawning fraction was higher than in the other years, except for the month of April when the value of 2004 was much higher than the value of 2003 and May when the value of 2000 is higher than the others.

3.2 MATURITY OGIVES OF MERLUCCIUS MERLUCCIUS IN GALICIAN SHELF

Maturity ogives were estimated both macro- and microscopically (based on histological examination of the ovarian tissue) in 2003 and 2004. Total numbers of macroscopically sampled females for each year were 1,089 and 919 respectively. Macroscopic stages were estimated by different observers but always involved in the research group and following a maturity staging protocol as described before.

The proportion of mature females by length interval is shown in Table 3.3 for both years and methodologies. The respective maturity logistic curves fit significantly to the observed data (X^2=959.13, p<0.001 and X^2=819.42, p<0.001 for 2003 and 2004 respectively). According to microscopic ogives, hake matures roughly between 40 cm (21.9% mature female) and 50 cm (84.6% mature females) although smaller mature female hake were found at 39 cm and 37.1 cm in 2003 and 2004 respectively. Conversely, the largest immature females were found at 60.5 cm in 2003 and at 54 cm in 2004. These largest immature females could be actually in recovering stages as sometimes these fish presented such compact gonad structure that they could be confused with immature ones, although it was not common. As regards the macroscopic classification of females, high number of medium-large (40-55c m) immature females was observed in both years, whereas small mature ones were detected only in 2004 (<35 cm). Taking into account that these observations were made on board commercial ships, high loadings and the lack of resources could limit

observer capacity to differentiate between immature and recovering females, because all of them showed small ovaries, with neither visible mature oocytes nor capillaries or veins. This could explain the high number of large immature females and presence of mature ones in the smallest length classes in both years when the macroscopic classification method was used.

Significant differences between micro- and macroscopically estimated ogives were observed in 2003 and 2004 (Figure 3.8). In the first year, size at maturity estimated macroscopically was significantly higher than if estimated microscopically (Z=4.95, p<0.001; Table 3.4). However, both curves (macro and microscopically estimated for 2003) were basically parallel, indicating that a systematic error in maturity assignation was produced for all sizes analyzed, probably because of differences in sampling methodology (date, length, etc.). Similarly, in 2004, the size at maturity estimated with different methods varied significantly (Z =4.51, p<0.001; Table 3.4). Nevertheless, the main difference in 2004 was the shape of the macroscopic curve. This curve was not parallel to the microscopic estimated curve or to the other two curves in 2003 (Figure 3.8). This probably represented a misclassification of maturity stage when the macroscopic method was used, most likely because of confusion between immature and recovering females.

Size at maturity varied significantly between 2003 and 2004 for both types of curves (Z=2.68; p<0.01 and Z=2.23; p<0.05) as shown in Figure 3.8. In 2003 microscopically estimated size at maturity was 44.45 cm whereas in 2004 it was 42.97, almost 2 cm lower than the previous year. The same was observed for macroscopically estimated size at maturity, that in 2003 was 47.05 cm, almost 2 cm higher than in 2004 (45.73 cm. Table 3.4).

3.3   CONDITION INDEX ANALYSIS

Several indices of female condition have been addressed in this study, from simple morphometric measures to physiological and biochemical measures. Given the extension and complexity of the biochemical measures, these are described in a different section (Section 3.4: Proximate composition of *Merluccius merluccius* on Galician shelf). In the following subsections are described the analyses performed with the so-called somatic condition factors, i.e. simple morphometric measure (length-weight relationship or K) and physiological measures, i.e. liver index or hepatosomatic index (HSI).

3.3.1   SOMATIC CONDITION FACTORS

The relationship between HSI and K with female size and weight, gonad and liver weight and GSI were first analyzed with all data combined, irrespective of the collection time (month or year), to search for particular trends related to female attributes. Both HSI and K showed significant relationships with most of the female features, although coefficients of

determination (r^2) were very low in most cases (Table 3.5). Thus, HSI increased significantly with female length (r^2=0.03, p<0.001) as shown in Figure 3.9.a. A similar positive significant relationship was found between gutted weight and HSI (r^2=0.20, p<0.001; Figure 3.9.b) but not with gonad weight (r^2=0.05, p=0.06; Figure 3.9.c). Similar relationship was observed between HSI and GSI, because the latter depends directly of gonad weight (r^2<0.01; p=0.74; Figure 3.9.d). As expected, HSI was positive and significantly related to liver weight (r^2=0.52, p<0.001; Figure 3.9.e).

Table 3.3: Number of immature/mature, total number and percentage of mature females sampled by length class and year for both micro- and macroscopical classification methods.

	Length (cm)	Microscopical				Macroscopical			
		I	M	Total	%	I	M	Total	%
2003	<30	11	-	11	0	226	-	226	0
	30-35	75	-	75	0	142	-	142	0
	35-40	93	2	95	2.1	160	-	160	0
	40-45	50	14	64	21.9	93	22	115	19.1
	45-50	12	66	78	84.6	54	59	113	52.2
	50-55	4	266	270	98.5	14	45	59	76.3
	55-60	1	299	300	99.7	2	33	35	94.3
	60-65	1	154	155	99.4	-	9	9	100
	65-70	-	32	32	100	-	3	3	100
	70-75	-	5	5	100	-	-	-	-
	>75	-	2	2	100	-	-	-	-
	Subtotal	247	842	1089	-	691	171	862	-
2004	<30	45	-	45	0	319	2	321	0.6
	30-35	70	-	70	0	125	5	130	3.8
	35-40	58	4	62	6.5	127	14	141	9.9
	40-45	25	16	41	39	111	51	162	31.5
	45-50	7	104	111	93.7	57	68	125	54.4
	50-55	2	241	243	99.2	16	60	76	78.9
	55-60	-	205	205	100	2	29	31	93.5
	60-65	-	91	91	100	-	11	11	100
	65-70	-	31	31	100	-	3	3	100
	70-75	-	14	14	100	-	2	2	100
	>75	-	6	6	100	-	-	-	-
	Subtotal	207	712	919	-	757	245	1002	-
	Total	454	1554	2008		1448	416	1868	
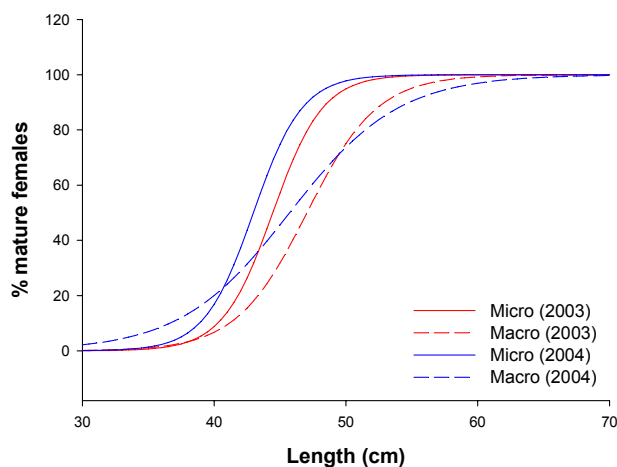
Figure 3.8: Comparison of micro- and macroscopically estimated hake maturity ogives for 2003 and 2004.

Table 3.4: Parameters of curve (a and b), size at maturity (L_{50}), variance (var) and covariance (cov) of curve parameters for both years (2003 and 2004) and methods (micro- and macroscopical). Statistics of analysis to analyse differences between years and methods (V and Z) and significance levels (p-value) are also included.

Year	Method	a	b	L_{50}	var(a)	var(b)	cov(a,b)
2003	Micro	-23.38	5.26E-01	44.45	3.59	0.0017	-0.08
2004	Micro	-23.08	5.37E-01	42.97	5.08	0.0026	-0.11
2003	Macro	-17.56	3.73E-01	47.05	2.08	0.0010	-0.05
2004	Macro	-11.05	0.24	45.73	0.55	0.0002	-0.01

Analysis	V	Z	p-value
2003 vs 2004 (micro)	0.13	2.68	<0.01
2003 vs 2004 (macro)	0.17	2.23	<0.05
micro vs macro (2003)	0.15	4.95	<0.001
micro vs macro (2004)	0.20	4.51	<0.001

Table 3.5: Coefficients of determination and p-values for the overall F-test obtained in simple linear regressions of K and HSI with female body attributes.

	HSI		K	
	r^2	p-value	r^2	p-value
Length	0.03	≤0.001	0.03	≤0.001
Gutted W	0.20	≤0.001	≤0.01	0.90
Gonad W	0.05	0.06	0.03	≤0.001
GSI	≤0.01	0.74	0.05	≤0.001
Liver W	0.52	≤0.001	0.01	≤0.001

Figure 3.9: Linear regressions between hepatosomatic index (HSI) and a)length; b) gutted weight; c) gonad weight; d) gonadosomatic index (GSI); e) liver weight.

With regard to K, it decreased with female size (r^2=0.03, p<0.001; Figure 3.10.a). However, there was no significant relationship between gutted weight and K (p=0.9; Figure 3.10.b). On the contrary, gonad weight was significantly related with K (r^2=0.03, p<0.001; Figure 3.10.c) and similar relationship was observed between K and GSI, because of the direct dependence of GSI on gonad weight as has been said before (r^2=0.05, p<0.001; Figure 3.10.d). K is also significantly and positively related to liver weight like HSI, although in this case the coefficient of determination was rather lower (r^2=0.01, p<0.001; Figure 3.10.e).

In summary, although most of the comparisons showed significant relationships following the overall F-test, the variability explained by the model was always extremely low indicating also a poor goodness of fit. The only exception was HSI and liver weight that, as

expected, showed a positive significant relationship explaining 52% of the total variability. In general, somatic condition factors are not related with female attributes and hence any observed trend should be caused by other factors, such as seasonality.

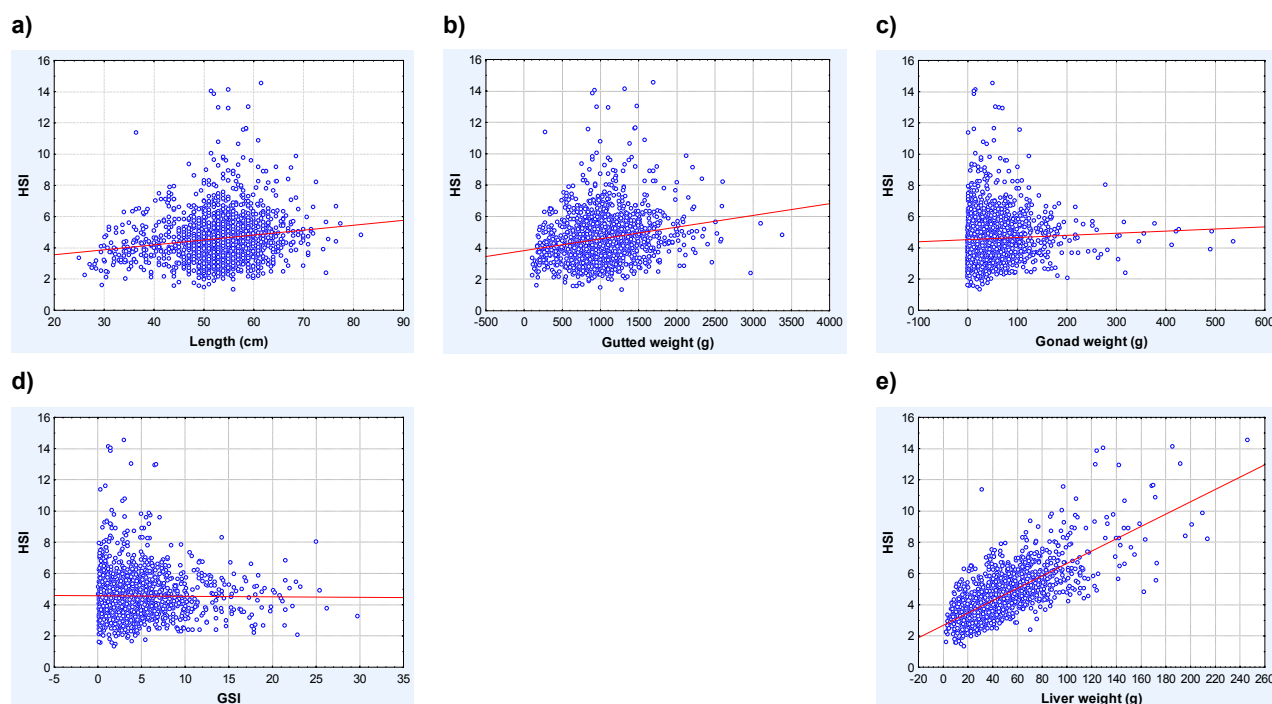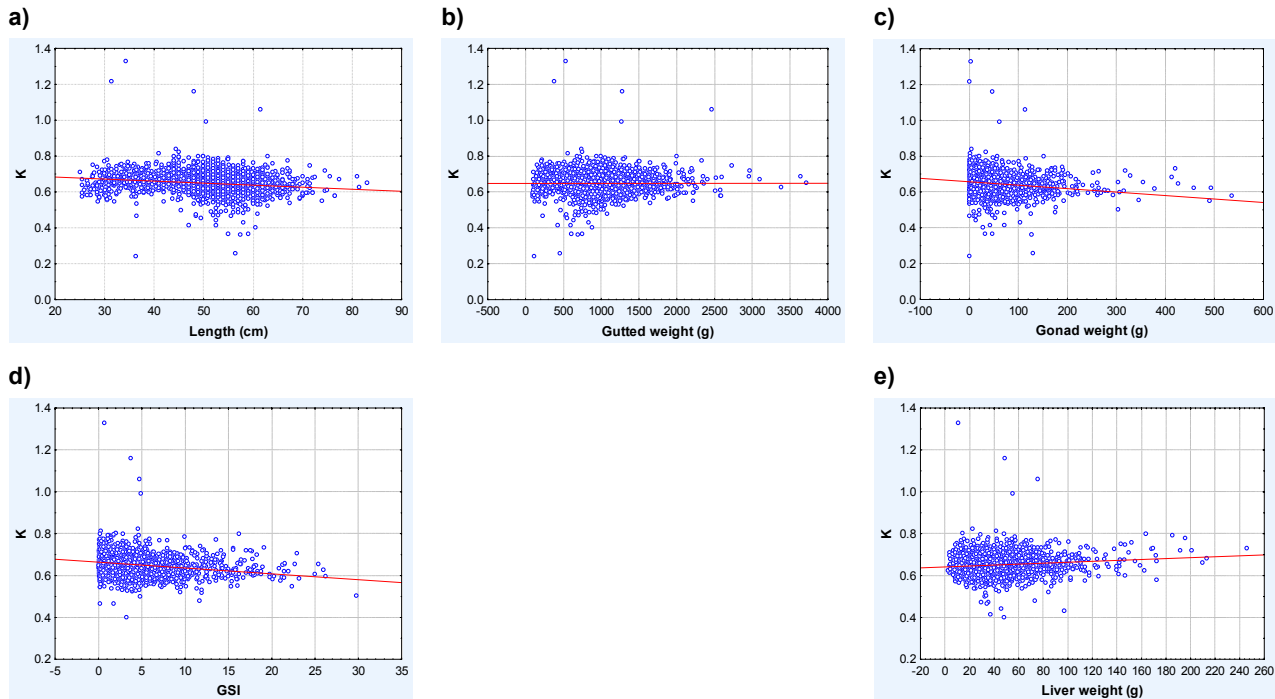*Figure 3.10: Linear regressions between condition factor K and a) length; b) gutted weight; c) gonad weight; d) gonadosomatic index (GSI); e) liver weight.*

3.3.2 TEMPORAL TRENDS

Temporal trends of HSI and K were studied. First of all, inter-annual variations of mean values of both indices were analysed, then mean values by month for all years combined; within year values were also studied. In both cases, HSI and K showed significant inter- and intra-annual differences (Table 3.6). Regarding the inter-annual study, significant differences were detected in both HSI (F=7.73, p<0.001) and K (F=46, p<0.001) annual mean values, which could be due to the fact that of the total of sampled females (2,585), most were captured in 2003 and 2004 (1,075 y 880 respectively). Besides, not all months of 2000 were sampled. In any case, the HSI annual average of 2003 was significantly above the values of the other years (Figure 3.11), while K annual mean values showed significant differences between all analysed years, with a clear trend to diminish from 1999 when the mean value was 0.67, to 2004 when it was 0.63 (Figure 3.11).

When the combined monthly variation of all years was analysed, it was observed that HSI month mean values slightly decreased from January to March from 4.5% to 3.9% respectively, then increased to reach 5.3% in August, decreased slightly in September,

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followed by a sharp rise reaching a maximum estimated value of 6.2% in October, and diminishing again to 5.5% in December (Figure 3.12).

Table 3.6: Results of the ANOVA performed to compare HSI and K by month within each analyzed year.

Year	Analysis	HSI		K	
		F	p-value	F	p-value
All	Inter-annual	7.73	≤0.001	46	≤0.001
All combined	Monthly	13.93	≤0.001	10.26	≤0.001
1999	Monthly	16,88	≤0.001	2.42	0.067
2000	Monthly	2,01	0.078	4.27	≤0.001
2003	Monthly	22,49	≤0.001	7.24	≤0.001
2004	Monthly	8,71	≤0.001	7.56	≤0.001
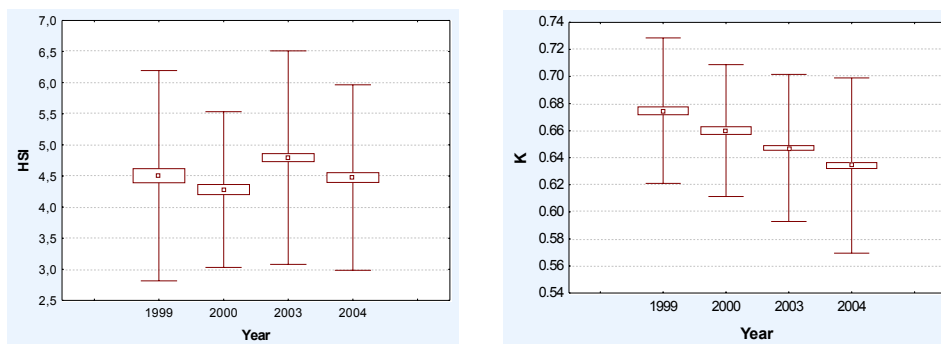
Figure 3.11: Inter-annual variation of mean HSI and K (mid point). Standard error (box) and standard deviation (wishker) are also represented.
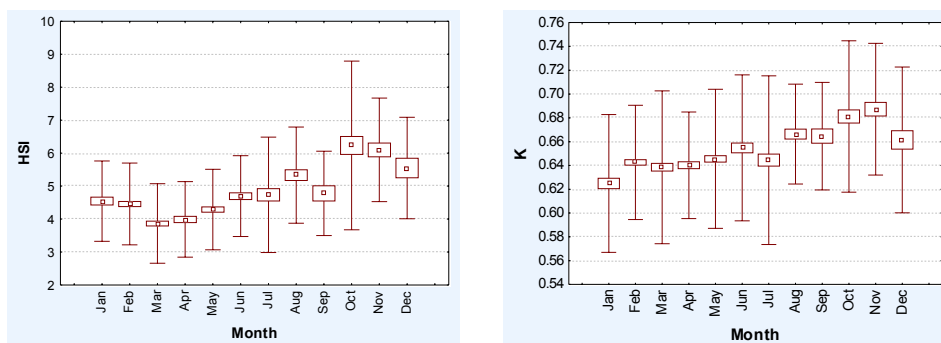
Figure 3.12: Monthly variation of mean HSI and K (mid point) for all years combined. Standard error (box) and standard deviation (wishker) are also represented.

The K monthly average value passed from 0.62 in January to 0.64 in February, and remained around this value in March. Later a progressive increase took place until it reached the highest value in November (0.69), except in July when the mean value of K decreased. In December a new slight decrease took place (Figure 3.12).

Figure 3.13 shows monthly variations recorded within each year and it is observed that intra-annual HSI and K trends are similar to those detected when all years are

combined. The exception is HSI in 2000 whose monthly mean values decrease from January to July, probably because of the scarce number of samples. It should be taken into account that only the first half of 2000 was sampled and trends of HSI monthly mean values from June to December are unknown. A similar situation is observed for K, although this time monthly mean values followed the expected pattern, increasing form January to July.

3.4 PROXIMATE COMPOSITION OF MERLUCCIUS MERLUCCIUS ON GALICIAN SHELF

A third set of condition indices was analysed, the biochemical indices. Analytical results were divided into biochemical components: lipids, proteins, glycogen, ashes and water content. The relative amount of each component within each tissue (gonad, liver and muscle) was estimated to address: a) their relationships with female attributes (length, gut, gonad and liver weight, and GSI); b) with somatic condition factors (HSI and K); c) to assess the seasonal variation of proximate composition between tissues, the progression of the monthly mean proportion of each biochemical component was analyzed; d) analyses of the influence of female attributes and somatic condition factors (all combined) on each tissue biochemical components using multiple regression analysis and finally, e) relationship of biochemical composition between tissues was also studied for every biochemical component. In case c), the proportion of each biochemical component was used because its interpretation was considered more intuitive. Additionally, the changes of these components in each tissue in relation to histologically determined ovary developmental stages were studied.

Proximate composition was estimated in samples collected from November 2002 to July 2003.

3.4.1 LIPIDS

Female attributes: No significant relation was observed between lipid content in any of the tissues and female length, gutted or gonad weight, the p-values in all cases were greater than 0.05. In the particular case of the gonad, no significant relationship was detected either with any of the other female attributes, i.e. GSI and liver weight as shown in Table 3.8 and Figure 3.14. Liver lipid content (Table 3.8 and Figure 3.15) was, however, positive and significantly related to liver weight ($r^2=0.24$, $p<0.001$), while negative but still significantly related with GSI ($r^2=0.05$, $p<0.05$). Regarding muscle lipid content (Table 3.8 and Figure 3.16), a significant increase was observed with liver weight ($r^2=0.25$, $p<0.001$).
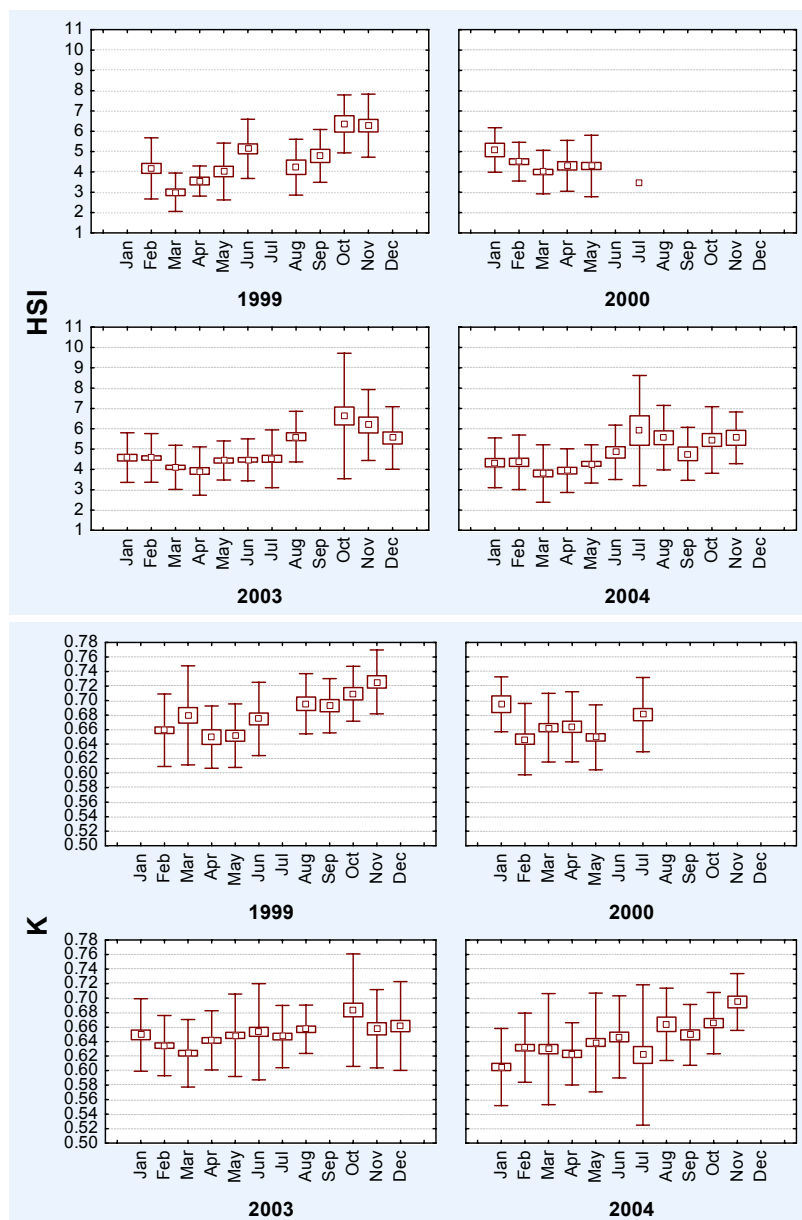
Figure 3.13: Temporal trend of mean HSI and K (mid point) in every studied year. Standard error (box) and standard deviation (wishker) are also represented.

Influence of somatic condition factors: Liver and muscle lipid contents were significantly related to HSI (r^2=0.33, p<0.001 and r^2=0.29 p<0.001 respectively; Table 3.8 and Figure 3.15 and Figure 3.16). Both components increased with HSI, although data did not fit adequately. Lipid contents of the three tissues did not show significant relationships with K (Table 3.8 and Figure 3.14 to Figure 3.16).

Seasonal variation: Liver and muscle lipid contents showed significant differences between months (F=8.67, p<0.001 and F=8.43, p<0.001; Table 3.7). In liver, a decrease in monthly average lipid content was recorded from January to April, going from 356.93 mg/g to 263.02 mg/g. This decline was followed by an increment until July, when the

maximum average was reached (513.60 mg lip/g). In muscle, a similar pattern was observed, the minimum mean value recorded in February (6.81 mg/g), a maximum in June (11.97 mg/g), and it decreased slightly but not significantly in July (10.93 mg/g). No particular trend in lipid content in gonads was observed. When monthly means of tissue lipid contents as percentages of total energetic component were represented (Figure 3.18), it was observed that percentages of lipids in gonad, liver and muscle followed the same trend from January to February and decreased from 7.41% to 6.47% in gonad, from 35.62% to 30.81% in liver, and from 1% to 0.79% in muscle. In March gonad lipids increased abruptly (8.77%), while liver and muscle lipids continued decreasing or remained constant (29.92% and 0.79% respectively). From April to June, lipids of liver and muscle increased to 40.68% in the first case and 1.23% in the second one, whereas in gonad lipid content decreased progressively until June (7.40%). In July, both liver and gonad lipids rose slightly (to 42.28% and 7.77% respectively), but muscle lipid decreased considerably (to 1%).

Multiple regression: About gonad lipid content, multiple regression by forward stepwise method analysis included only gutted weight in the model that explained only a 2% of data variability. For liver, three variables were included explaining a 36.6% of total variability. HSI was the first variable included in the model and explained by itself 33.03% of variance. The other two variables included in the model were the K and GSI. Regarding muscle lipid content, two variables were included in it: HSI and length that explained 30.4% of variability, although only HSI was significantly related to dependent variable (Table 3.9).

Relationships between tissue composition: Pearson correlations between gonad with muscle and liver lipid content was not significant (Table 3.10 and Figure 3.19). However, muscle and liver lipids showed a positive and significant correlation (r=0.70, p<0.001; Table 3.10 and Figure 3.19).
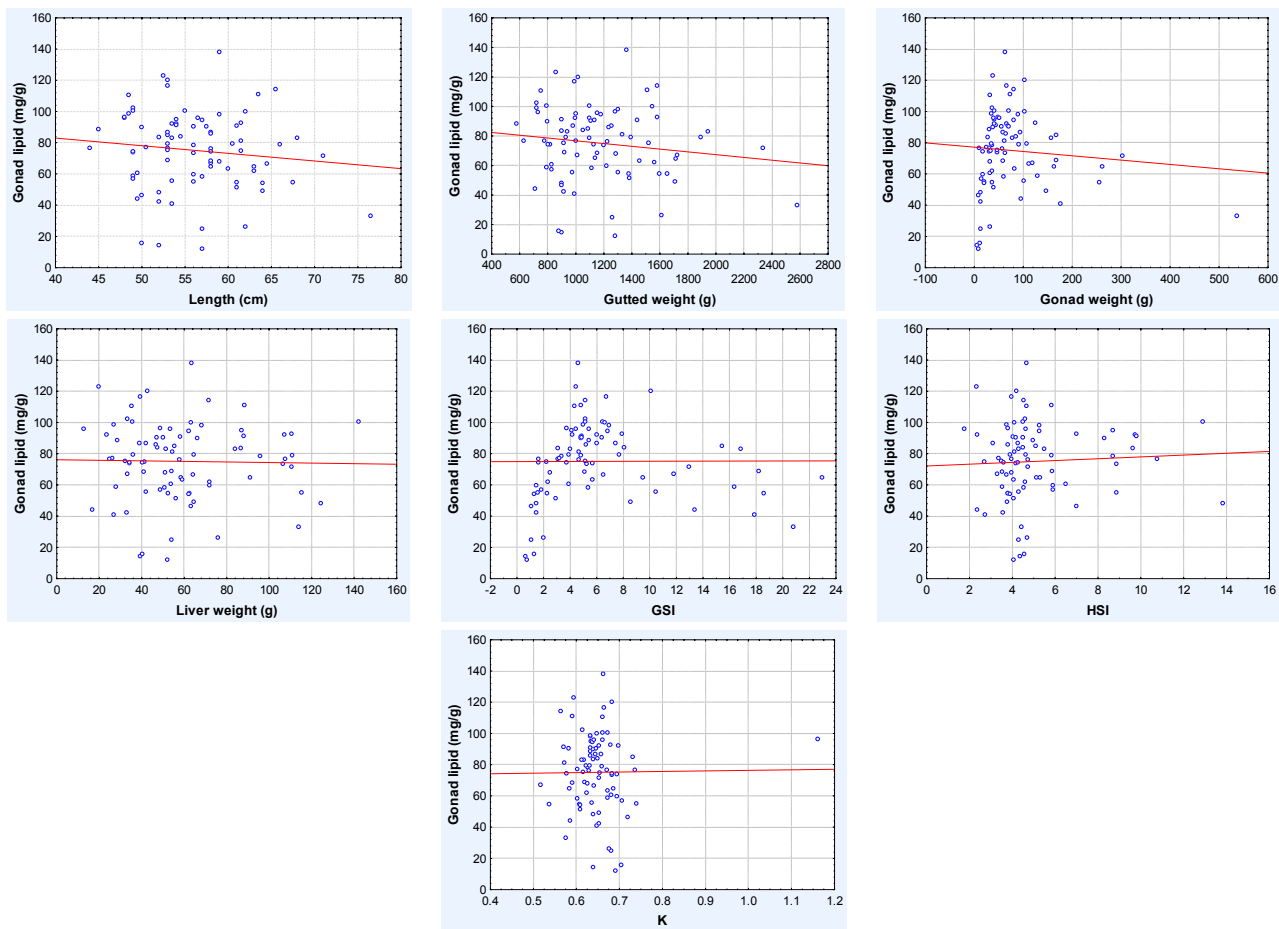
Figure 3.14: Gonad lipid content (mg/g) versus female attributes and somatic condition indices.
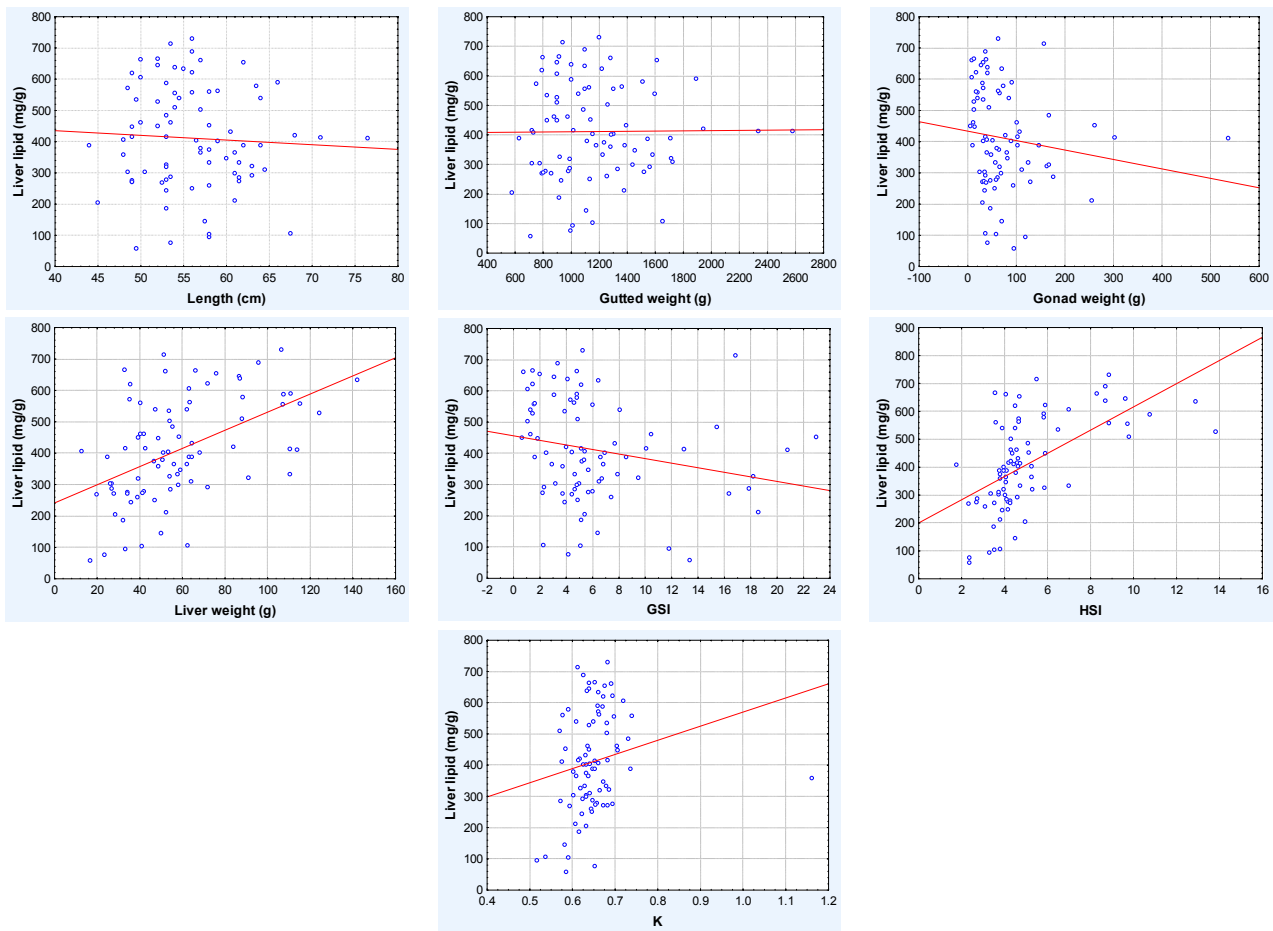
Figure 3.15: Liver lipid content (mg/g) versus female attributes and somatic condition indices.
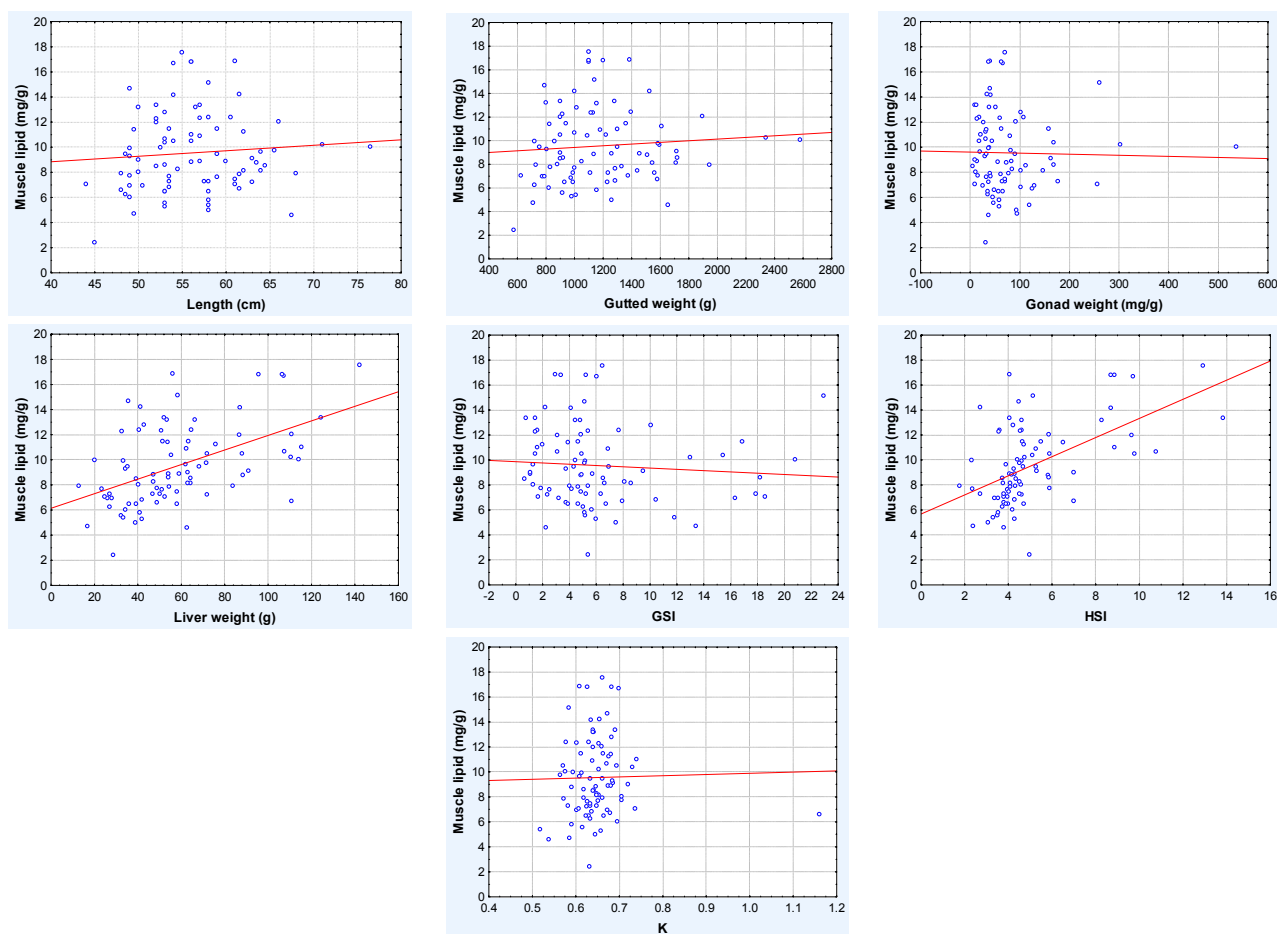
Figure 3.16: Muscle lipid content (mg/g) versus female attributes and somatic condition indices.

Table 3.7: Results of the ANOVA made to study intra-annual changes of every biochemical component in each tissue.

	Gonad			Liver			Muscle		
	N	F	p	N	F	p	N	F	p
Lipid	83	0.40	0.897	82	8.67	≤0.001	83	8.43	≤0.001
Protein	79	1.89	0.084	79	4.30	0.001	65	3.54	0.003
Glycogen	57	1.29	0.276	59	1.08	0.389	60	0.73	0.650
Water	52	1.08	0.391	55	1.93	0.085	55	1.09	0.381
Ash	82	3.35	0.004	83	3.74	0.002	81	1.18	0.323

Table 3.8: Determination coefficient and p-values of correlations between biochemical components and somatic factors in every tissue.

		Length		Gutted W		Gonad W		GSI		Liver W		HSI		K	
		r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
Lipid	**Gonad**	0.01	0.286	0.02	0.212	≤0.01	0.445	≤0.01	0.976	≤0.01	0.858	≤0.01	0.640	≤0.01	0.926
	Liver	≤0.01	0.622	≤0.01	0.939	0.02	0.199	0.05	0.049	0.24	≤0.001	0.33	≤0.001	0.04	0.075
	Muscle	≤0.01	0.461	≤0.01	0.466	≤0.01	0.856	≤0.01	0.485	0.25	≤0.001	0.29	≤0.001	≤0.01	0.849
Protein	**Gonad**	≤0.01	0.558	0.01	0.400	0.02	0.271	≤0.01	0.408	≤0.01	0.643	≤0.01	0.847	≤0.01	0.678
	Liver	0.02	0.256	0.03	0.144	≤0.01	0.925	0.01	0.345	0.02	0.196	≤0.01	0.715	≤0.01	0.878
	Muscle	0.03	0.166	0.04	0.116	≤0.01	0.982	≤0.01	0.459	≤0.01	0.583	0.02	0.210	≤0.01	0.975
Glycogen	**Gonad**	0.02	0.250	0.02	0.343	0.02	0.346	0.03	0.167	0.02	0.243	0.02	0.245	≤0.01	0.859
	Liver	0.10	0.017	0.12	0.008	0.01	0.397	≤0.01	0.877	0.11	0.009	0.01	0.417	≤0.01	0.719
	Muscle	0.08	0.032	0.08	0.025	≤0.01	0.931	≤0.01	0.627	0.04	0.128	≤0.01	0.773	≤0.01	0.760
Water	**Gonad**	0.03	0.201	0.03	0.244	0.19	0.001	0.23	≤0.001	0.03	0.200	0.02	0.348	0.03	0.193
	Liver	≤0.01	0.691	≤0.01	0.676	≤0.01	0.734	0.01	0.394	0.08	0.039	0.19	≤0.001	0.04	0.159
	Muscle	0.05	0.096	0.02	0.320	0.01	0.385	0.04	0.167	≤0.01	0.901	0.02	0.341	0.07	0.049
Ash	**Gonad**	0.03	0.122	0.03	0.110	0.06	0.025	0.11	0.002	≤0.01	0.786	≤0.01	0.438	≤0.01	0.769
	Liver	0.02	0.215	0.03	0.138	≤0.01	0.954	≤0.01	0.809	0.08	0.010	0.21	≤0.001	≤0.01	0.658
	Muscle	0.03	0.103	0.03	0.109	≤0.01	0.823	≤0.01	0,491	0.02	0.264	≤0.01	0.801	≤0.01	0.689
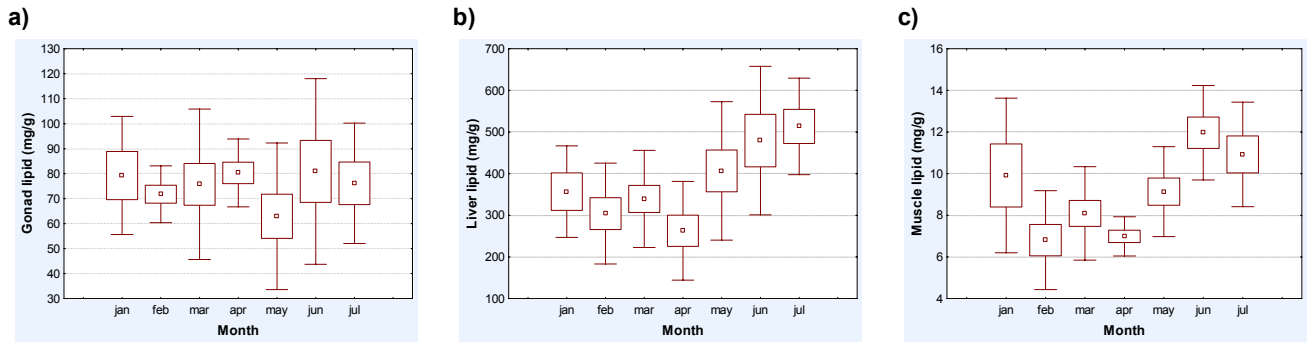
igure3.17: Monthly variation of mean lipid content(mid point) in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.
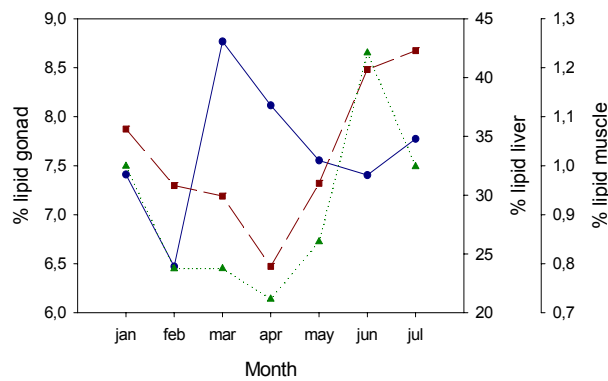
Figure 3.18: Intra-annual variation of mean lipid content as percentage of total biochemical components in every tissue. Gonad = solid blue line, liver = dash red line and muscle = dotted green line.
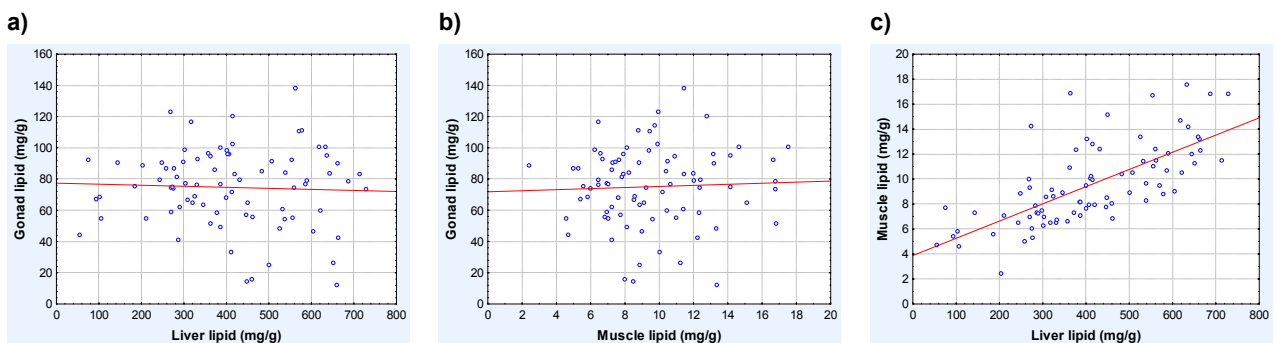
Figure 3.19: Relationship of lipid content among tissues: a) gonad versus liver; b) gonad versus muscle and c) liver versus muscle.

Table 3.9: Determination coefficient and p-values of multiple regression by forward stepwise method carried out to analysis the influence of each maternal attributes and somatic condition factors on biochemical composition of each tissue.

|		Gonad			Liver			Muscle		
		r^2	F to enter	p	r^2	F to enter	p	r^2	F to enter	p
Lipid	**Gutted W**	0.02	1.58	0.212	-	-	-	-	-	-
	HSI	-	-	-	0.33	39.45	<0.001	0.29	32.47	<0.001
	K	-	-	-	0.35	2.93	0.091	-	-	-
	GSI	-	-	-	0.37	1.48	0.228	-	-	-
	Length	-	-	-	-	-	-	0.30	2.09	0.152
Protein	**Gonad W**	0.02	1.23	0.271	-	-	-	-	-	-
	Gutted W	-	-	-	0.03	2.18	0.144	0.04	2.54	0.116
	GSI	-	-	-	0.04	1.37	0.245	-	-	-
	HSI	-	-	-	-	-	-	0.08	2.78	0.100
Glycogen	**GSI**	0.03	1.96	0.167	-	-	-	-	-	-
	HSI	0.06	1.22	0.274	-	-	-	-	-	-
	Length	-	-	-	0.15	10.55	<0.01	-	-	-
	Gutted W	-	-	-	-	-	-	0.08	5.30	<0.05
Water	**GSI**	0.23	14.57	<0.001	0.23	2.90	0.094	0.13	3.24	0.078
	Length	0.25	1.84	0.182	-	-	-	0.16	1.35	0.251
	HSI	-	-	-	0.19	12.41	<0.001	-	-	-
	K	-	-	-	0.25	1.39	0.243	0.08	4.60	<0.05
	Liver W	-	-	-	-	-	-	0.18	1.64	0.206
Ash	**GSI**	0.11	9.82	<0.01	-	-	-	-	-	-
	Length	0.16	5.02	<0.05	-	-	-	0.03	2.72	0.103
	Gonad W	0.18	1.99	0.163	0.24	2.45	0.122	0.05	1.27	0.263
	Gutted W	0.20	1.79	0.185	0.22	1.67	0.200	-	-	-
	Liver W	0.21	1.12	0.294	-	-	-	-	-	-
	HSI	-	-	-	0.21	20.90	<0.001	-	-	-

Table 3.10: Pearson's correlation coefficients and significance level between each tissue's biochemical components.

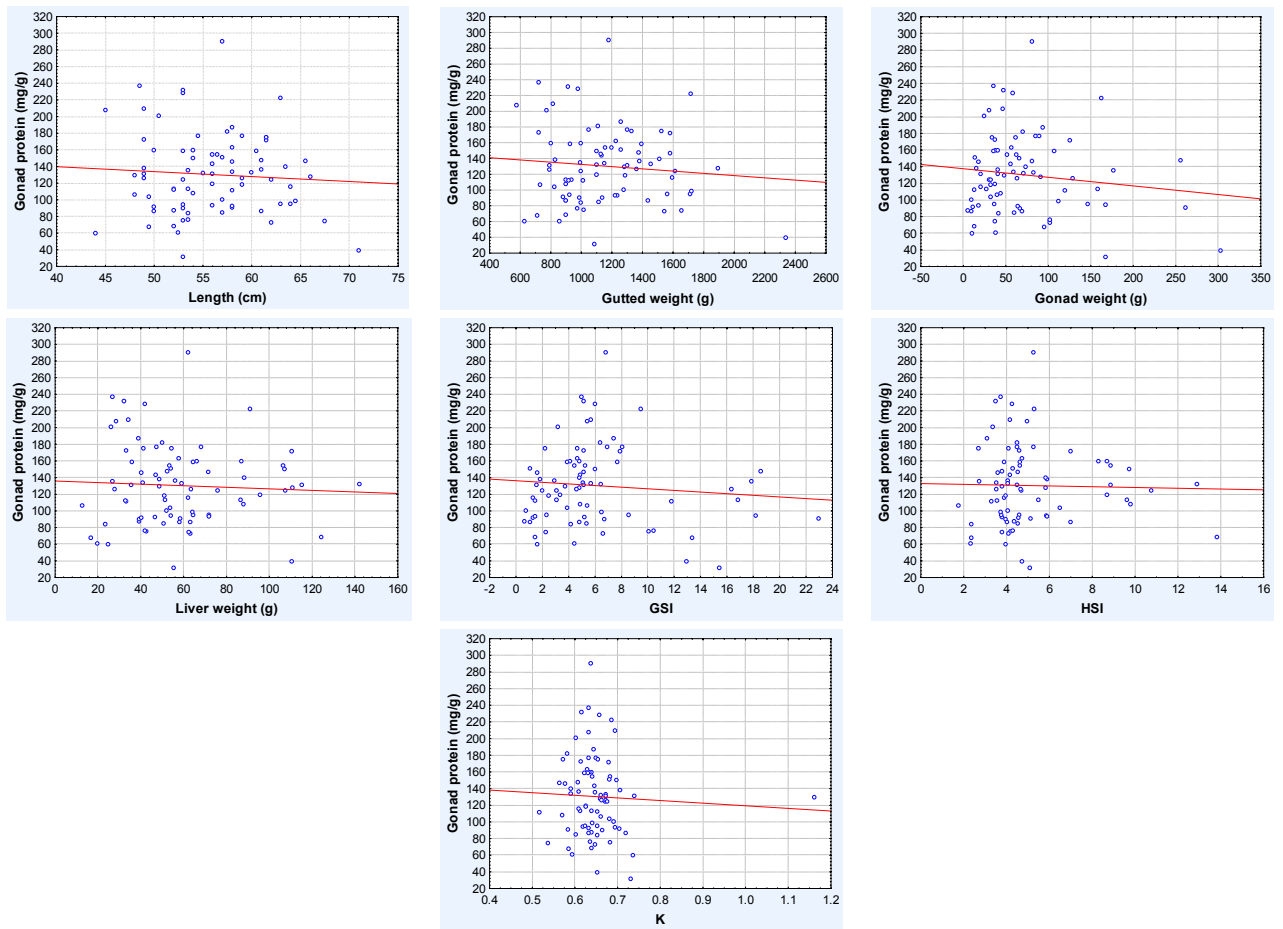|	gonad vs liver		gonad vs muscle		liver vs muscle	
	r	p	r	p	r	p
Lipid	0.04	0.692	0.04	0.693	0.70	≤0.001
Protein	0.51	≤0.001	0.55	≤0.001	0.77	≤0.001
Glycogen	0.08	0.555	0.17	0.233	0.04	0.762
Water	0.31	0.035	0.01	0.961	0.28	0.057
Ash	0.47	≤0.001	0.47	≤0.001	0.16	0.165

Figure 3.20: Gonad protein content (mg/g) versus female attributes and somatic condition indices.
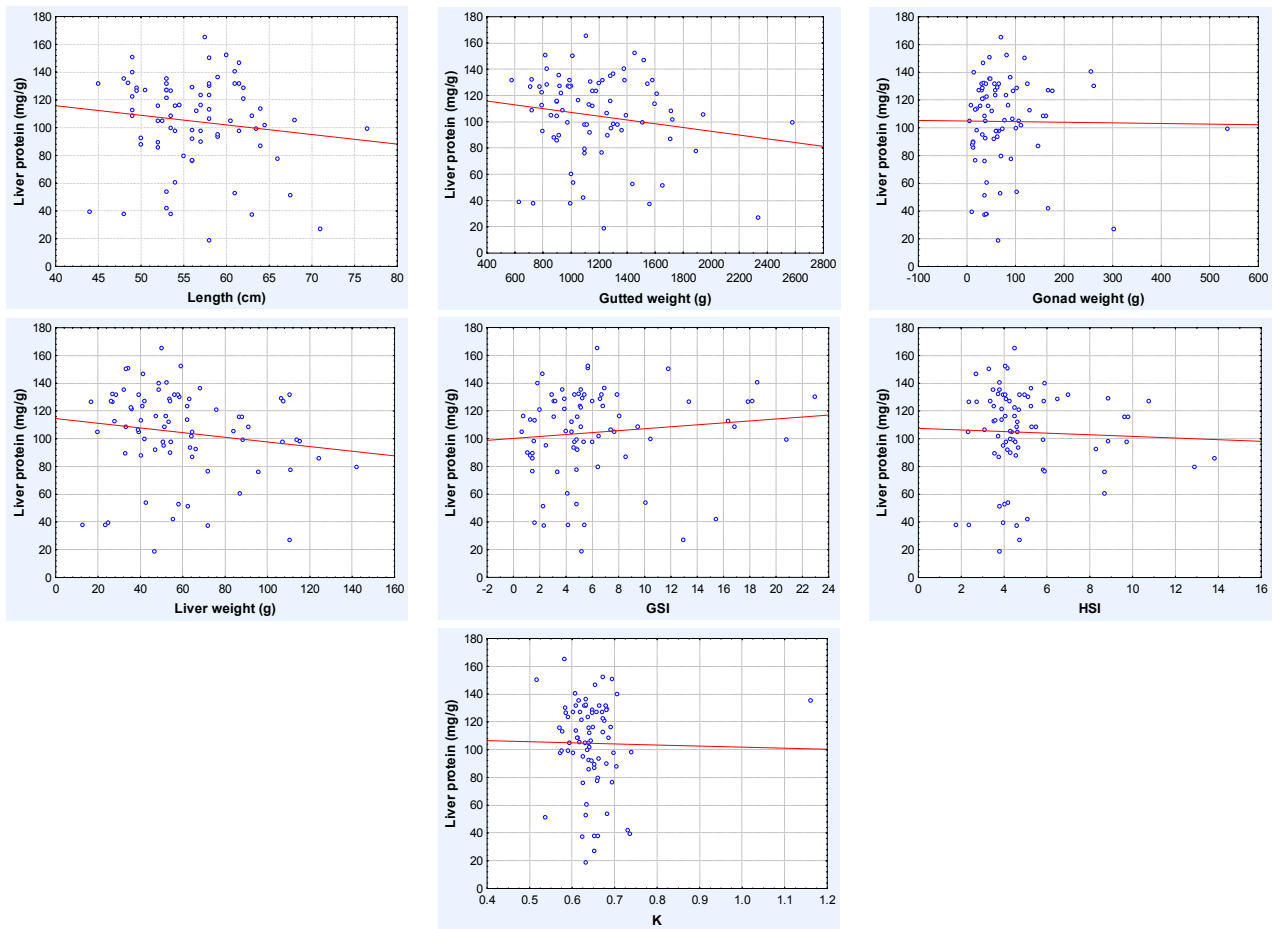
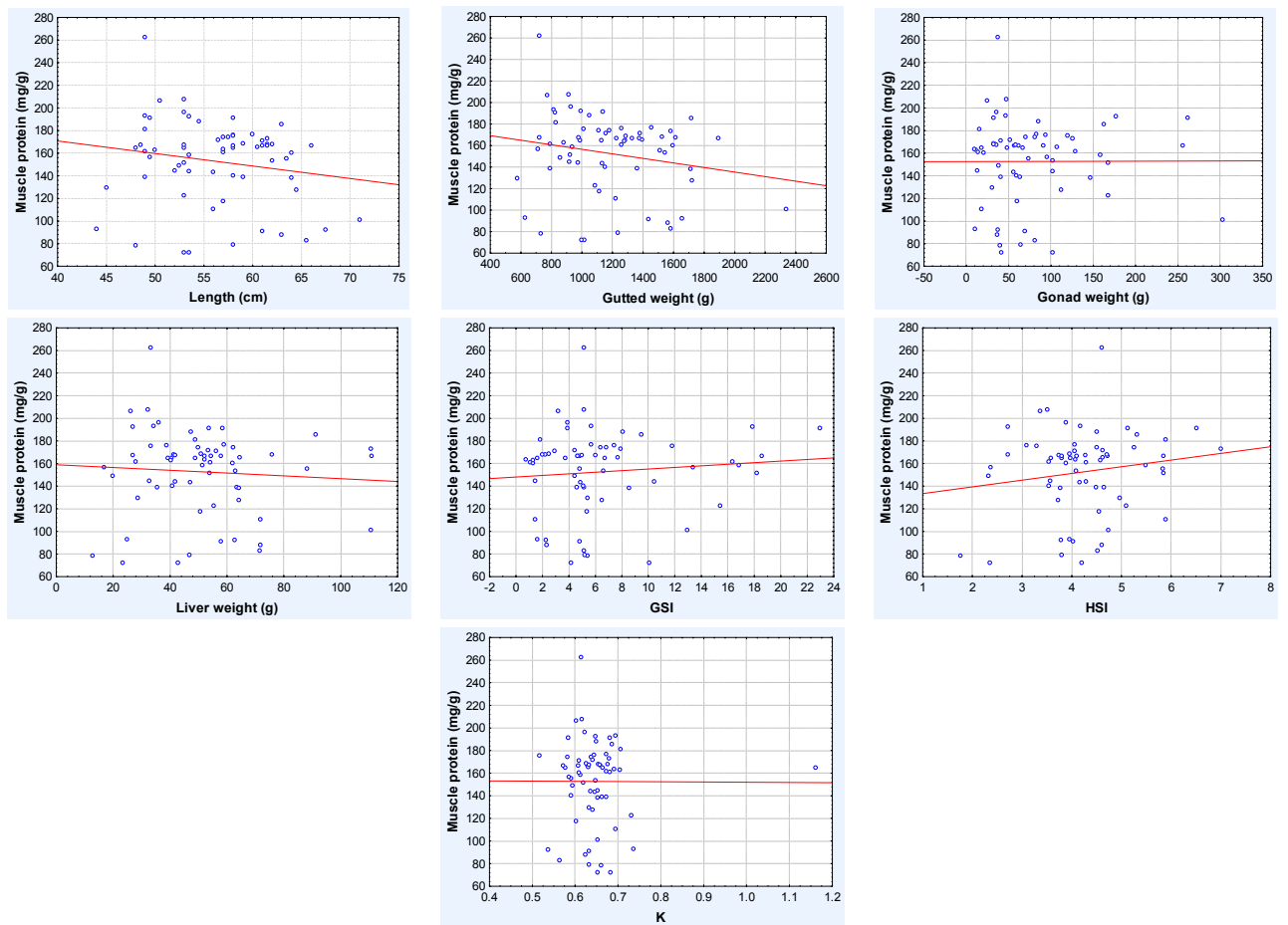*Figure 3.21: Liver protein content (mg/g) versus female attributes and somatic condition indices.*

Figure 3.22: Muscle protein content (mg/g) versus female attributes and somatic condition indices.

3.4.2 PROTEINS

Female attributes: Protein content of the three tissues analyzed were not related significantly to any of the female attributes considered in analysis (Table 3.8 and Figure 3.20, Figure 3.21 and Figure 3.22).

Influence of Somatic Condition factors: Neither HSI, not K, showed significant influence on the protein content of any of the three tissues analyzed (Table 3.8).

Seasonal variations: As with the lipid, protein contents in liver and muscle showed significant intra-annual differences (F=4.30, p<0.01 and F=3.54, p<0.01 respectively), while in gonads these remained statistically unchanged during the year (F=1.89, p=0.084; Table 3.7). Liver protein mean value by month increased from January (96.80 mg proteins/g of liver) to February (129.66 mg/g; Figure 3.23.b), followed by a sharp decrease until April, when a minimum value was recorded (mean=68.7 mg/g). Then a steady increase in liver protein content took place to reach an average of 111.96 mg/g in July, before returning to previous values. Significant differences were observed basically between April and other months, and between February-March and April-May. Muscle proteins (Figure 3.23.c) showed a similar trend to liver proteins, rising from January to March (from 152.24 mg/g to 179.68 mg/g), and an abrupt decrease in April, where again the minimum value was reached (mean=116.91 mg/g). Afterwards, protein amounts increased progressively until July with values of around 160 mg/g. Significant differences were detected by LSD test between April and other months (except May and June), and between March and the next three months (April, May and June). Although the overall F-statistic showed no significant differences throughout the year in gonad protein content, a clear trend can be observed (Figure 3.23.a) which is similar to that of liver and muscle, but with a month delay in the maximum and minimum values. A steady increase until March (161.43 mg/g) was followed by a sharp decrease until May (103.33 mg/g), and a progressive recovery in gonad protein content follows. In fact LSD *post hoc* test reveals significant differences between February with April-May and March with April-May-June. The proportion of protein content estimated by tissue (Figure 3.24) showed a similar pattern in the three tissues until April. Thus from January to March, the proportion of protein in the three tissues increased from 12.78% to 18.17% in gonad, from 9.17% to 11.85% in liver, and from 16.96% to 14.27% in muscle, droping sharply in April (12.53%, 7.20% and 12.61% respectively). Since then, while liver and muscle protein proportion increased continuously to reach 10.34% and 15.79% respectively, protein in gonads remained at low levels as earlier in the year, between 10.5% and 12.5%.

Multiple regression: Considering gonad protein content as the dependent variable, analysis introduced only gonad weight as predictor in the model that explained 12.5%

of total variability, although it was not significantly related with the dependent variable. Regarding liver protein content, two variables were included in the multiple regression model: gutted weight and GSI. Together they explained 21.2% of the total variability of the dependent variable, but were not correlated with the dependent variable. Finally, concerning muscle proteins, gutted weight and HSI were the variables selected by the stepwise regression, but only 8% of variance was explained; again none of the included variables showed a significant relationship with the dependent variable (Table 3.9).

Relationships between tissue composition: All correlations between the protein content in the three tissues were positive and significant. The highest Pearson correlation occurred between muscle and liver protein (r=0.77, p<0.001), followed by gonad and muscle (r=0.55, p<0.001) and finally, between gonad and liver (r=0.51, p<0.001; Table 3.10 and Figure 3.25).
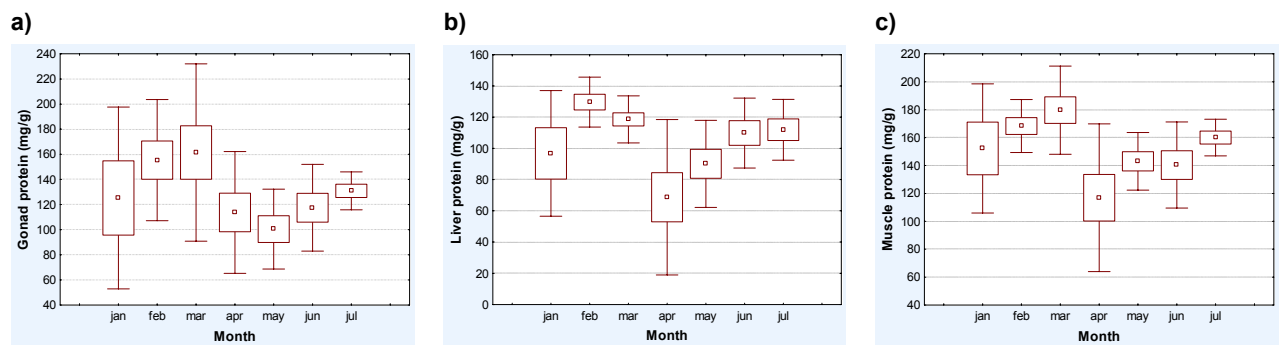

Figure 3.23: Monthly variation of mean protein content (mid point) in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.
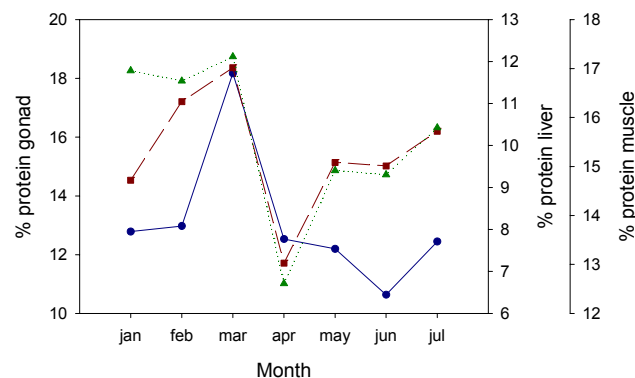

Figure 3.24: Intra-annual variation of protein content as percentage of total biochemical components in every tissue. Gonad = solid blue line, liver = dash red line and muscle = dotted green line.
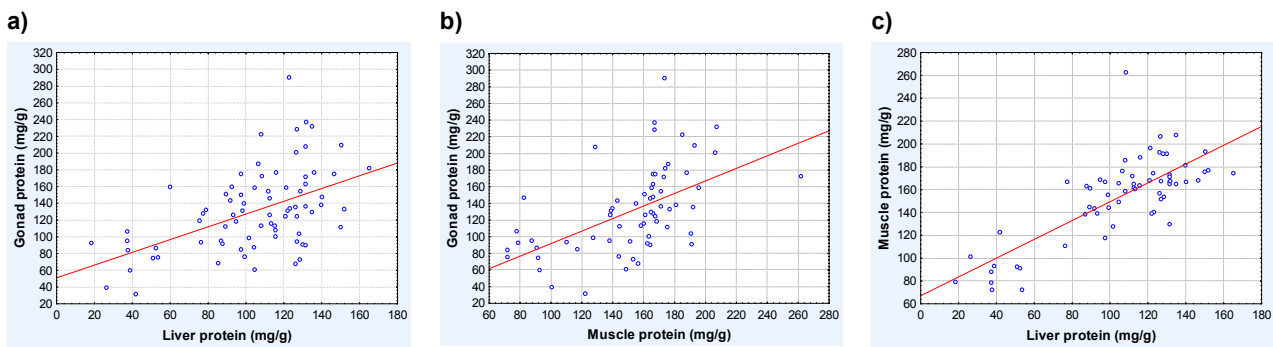
Figure 3.25: Relationships of protein content among tissues: a) gonad versus liver; b) gonad versus muscle and c) liver versus muscle.

3.4.3 GLYCOGEN

Female attributes: No significant relationship was detected between gonad glycogen content and female attributes (Figure 3.26). On the contrary, liver glycogen content was significantly and negatively related to female length ($r^2=0.10$, $p<0.05$), gutted weight ($r^2=0.12$, $p<0.01$) and liver weight ($r^2=0.11$, $p<0.01$; Table 3.8 and Figure 3.27). In spite of p-values lower than 0.05, regression coefficients were rather low ($r^2<0.2$). Regarding muscle glycogen content, it was negatively and significantly related to length and gutted weight, though determination coefficients in both cases was very low ($r^2=0.08$, $p<0.05$ and $r^2=0.08$, $p<0.05$ respectively; Table 3.8 and Figure 3.28).

Influence of somatic condition factors: Neither gonad nor liver nor muscle glycogen content were significantly related to HSI or K ($p>0.05$ in all cases and $r^2<0.1$; Figure 3.28 and Figure 3.27).

Seasonal variation: Gonad, liver and muscle glycogen content did not change significantly with month (Table 3.7 and Figure 3.29). However certain trends could be identified, i.e. From January to April gonad glycogen content showed the highest month mean values, then a significant decrease (LSD test) took place in May, remaining more or less constant from then until July (Figure 3.29.a). In muscle, a progressive decrease of glycogen content mean values was observed from January to July, although minimum values were around 0.5 mg/g in all months (Figure 3.29.c). Figure 3.30 shows glycogen contents of each tissue as percentages of fresh tissue, but no clear trend could be defined. In liver glycogen percentages showed two peaks, one in February (4.20%) and another in April (4.18%); the second coincided with a peak of glycogen percentage in gonad (2.36%). Muscle glycogen percentage increased from January (0.25%) to March (0.30%), decreasing progressively until June (0.23%), then, in July, increased considerably (0.30%). In all cases, differences observed between minimum and maximum values of percentage in all tissues were insignificant.

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Multiple regression: Multiple regression by forward stepwise method analysis included GSI and HSI in the model of gonad glycogen content. Those two variables explained 5.6% of its variability, although they were not related to the dependent variable. For liver glycogen content, the model included length and explained 15.4% of data variability. This somatic predictor was related with the dependent variable in a significant way. In the model for muscle glycogen content, only gutted weight was included by multiple regression by forward stepwise method and explained 8.4% of total data variability (Table 3.9).

Relationships between tissue composition: No significant correlations were observed between any tissue glycogen content ($p > 0.05$; Table 3.10 and Figure 3.31).
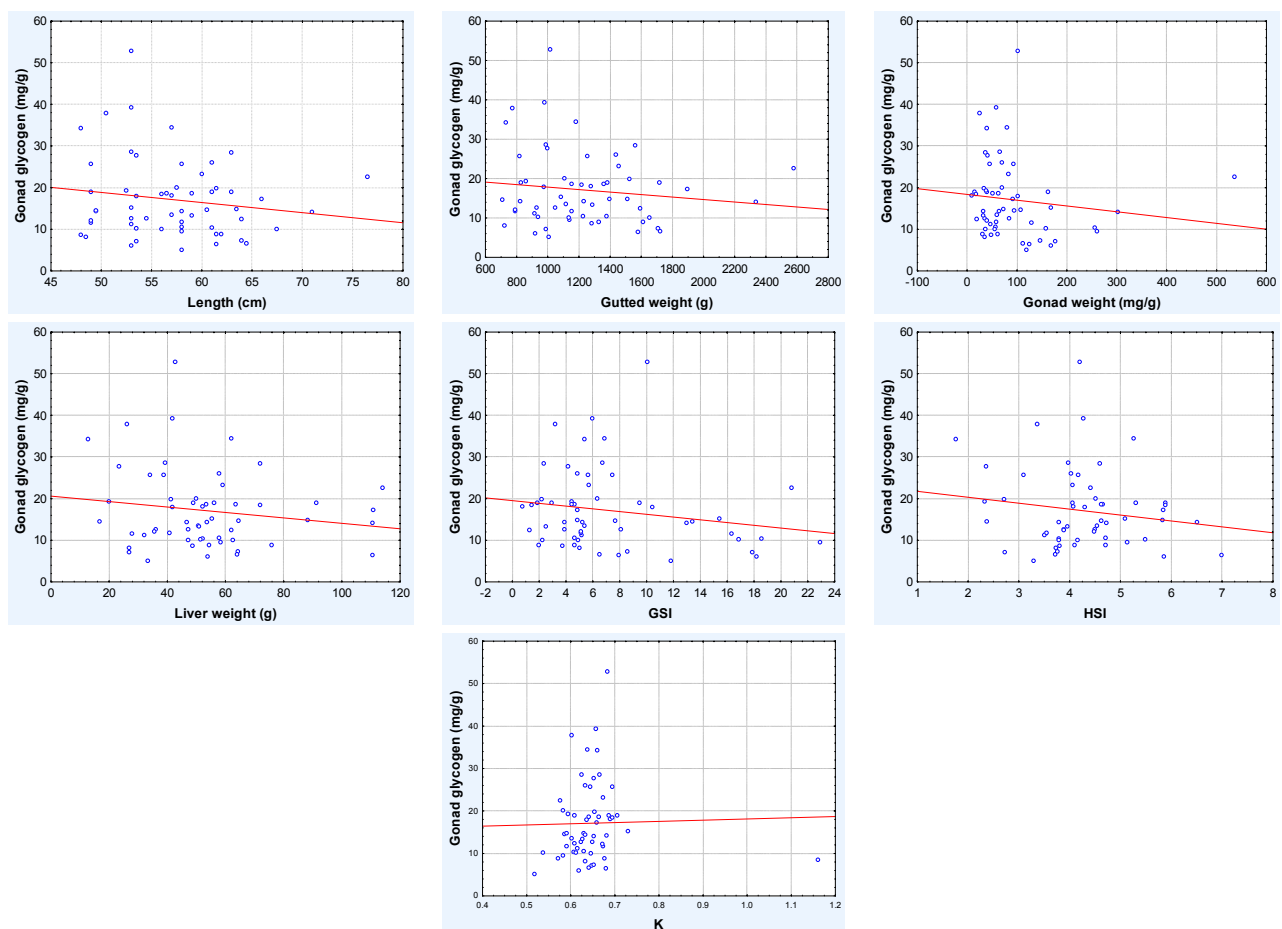

Figure 3.26: Gonad glycogen content (mg/g) versus female attributes and somatic condition indices.
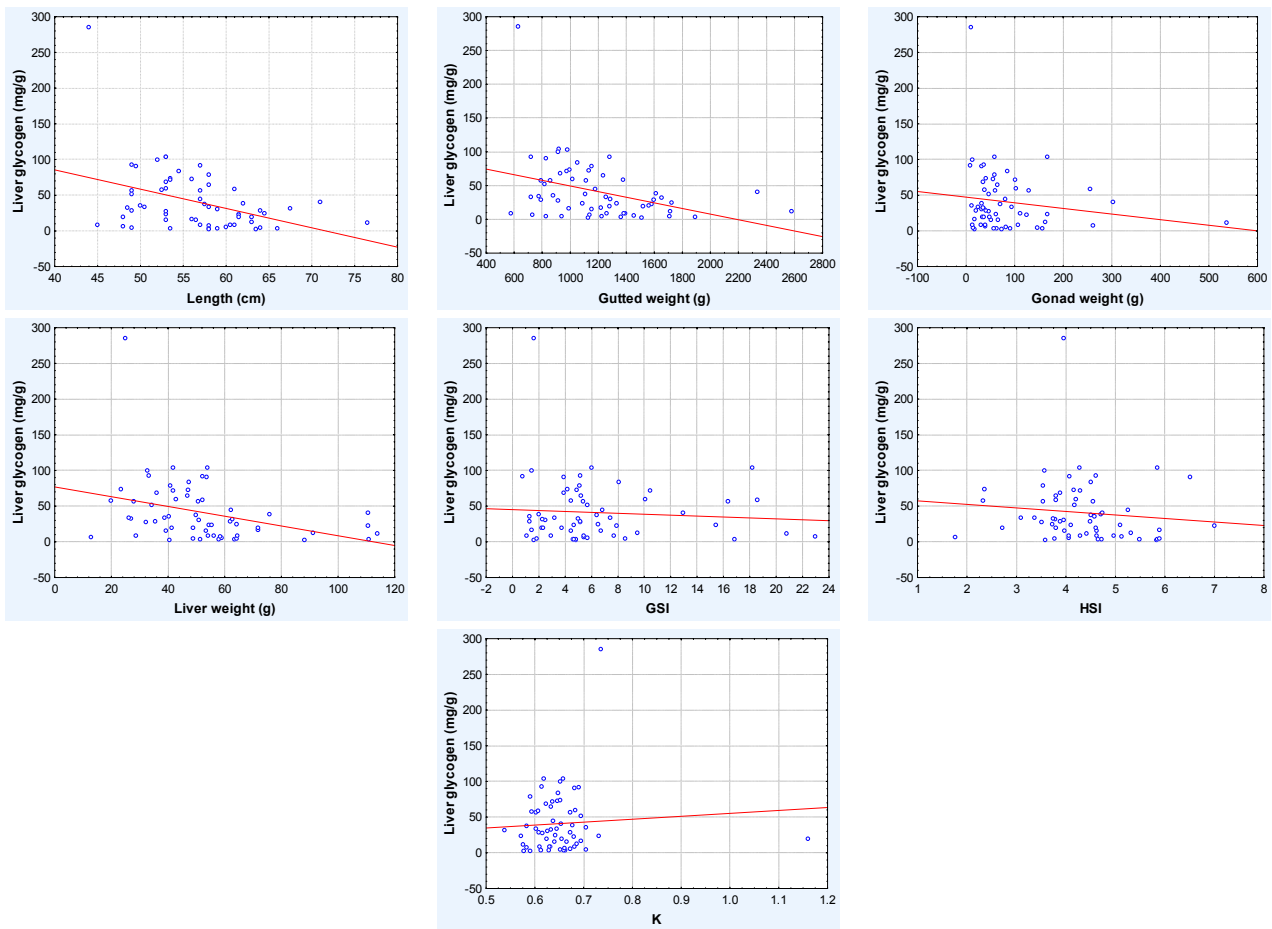
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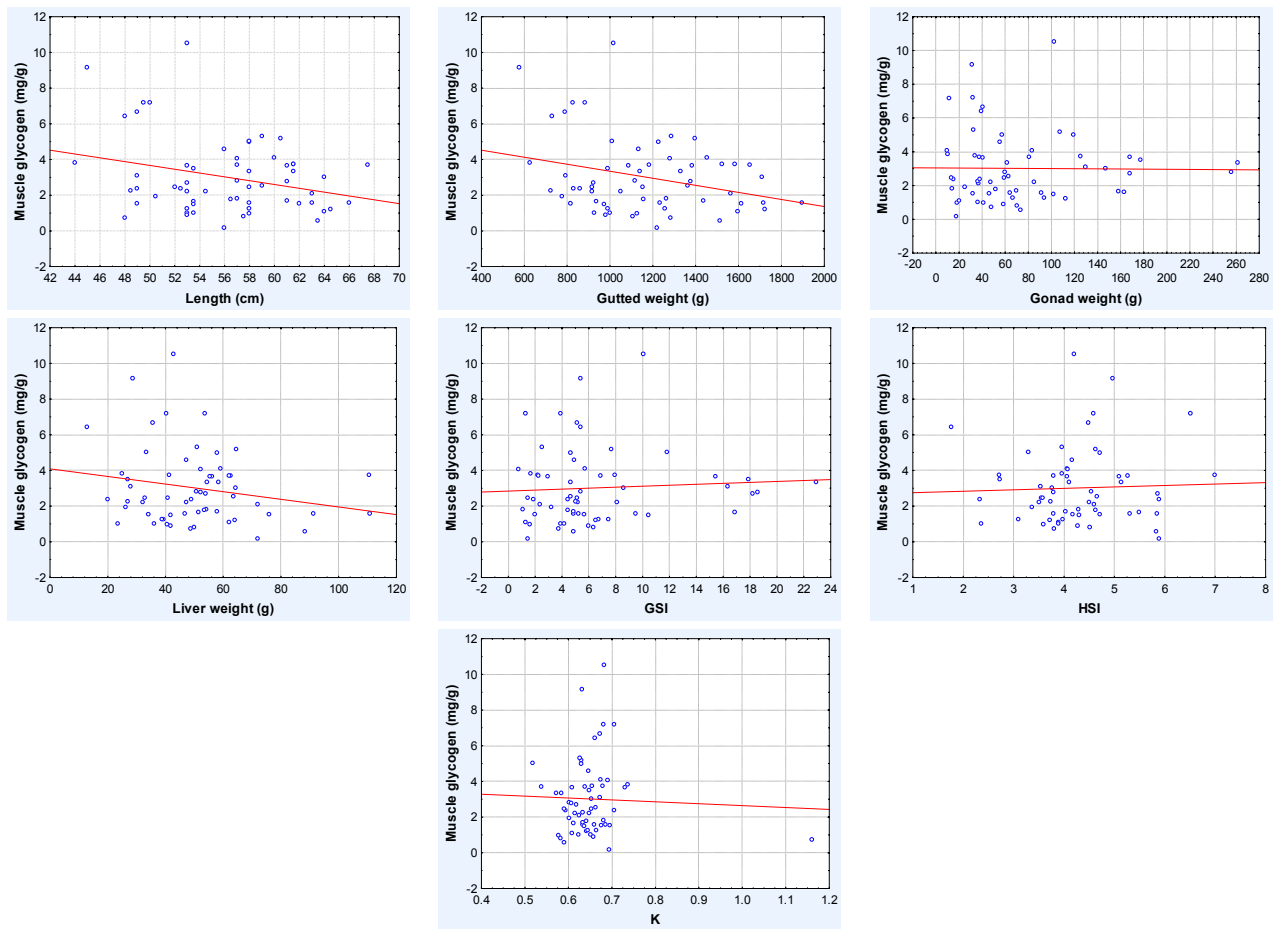*Figure 3.27: Liver glycogen content (mg/g) versus female attributes and somatic condition indices.*

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Figure 3.28: Muscle glycogen content (mg/g) versus female attributes and somatic condition indices.
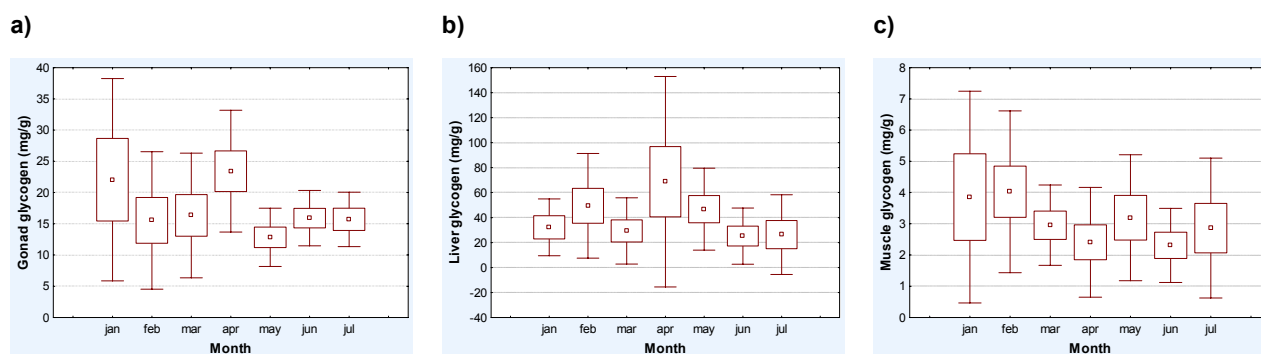
Figure 3.29: Monthly variation of mean glycogen content (mid point) in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.

3.4.4 WATER

Female attributes: Gonad water content was significantly and positively related to gonad weight (r^2=0.19, p<0.01); and GSI (r^2=0.23, p<0.001; Table 3.8 and Figure 3.32), although Pearson´s correlation coefficient was low. Liver water content was related to liver weight in a significant and negative way (r^2=0.08, p<0.05; Figure 3.33) but the correlation coefficient was low again.

Influence of somatic condition factors: Gonad water content did not show any significant relationship with any condition index, neither HSI nor K. In contrast, water content of liver was significantly related to HSI (r^2=0.19, p<0.001; Figure 3.33). And finally, muscle water content was also significantly and negatively related to K (Figure 3.34).

Seasonal variation: No significant monthly differences were established between the water contents of tissues (p>0.05; Figure 3.35). However, when monthly mean of tissues water content as a percentage of total components was represented, it was observed that liver and muscle water proportion increased from January (51.77% and 80.55% respectively) to April (62.83% and 85.01% respectively), decreasing subsequently to reach their minimum values in July (44.46% for liver and 77.59% for muscle). Gonad water proportion followed practically the opposite trend although its minimum was reached in March (69.20%), one month before liver and muscle values reached their maxima, and the maximum mean value of gonad water content was detected in February (77.64%; Figure 3.36).

Multiple regression: The gonad model made by multiple regression by forward stepwise method included two variables: GSI and length, although only GSI was significantly related to the dependent variable. Between all these variables, 25.4% of variability was explained, mostly by GSI (22.7%). For liver, three variables were included: HSI, GSI and K, but HSI was the only variable that was significantly related to water content.

They explained 25.3% of its variance, and HSI alone was able to explain 19% of it. Regarding muscle water content, four variables were included in the model: K, GSI, length and liver weight; of these variables, only K was significantly related to the dependent variable. All variables together explained 18.3% of muscle water content variability (Table 3.9).

Relationships between tissue composition: Significant and negative correlation was established between water content of gonad and liver, although determination coefficient was low (r^2=0.10, p<0.05; Figure 3.37).
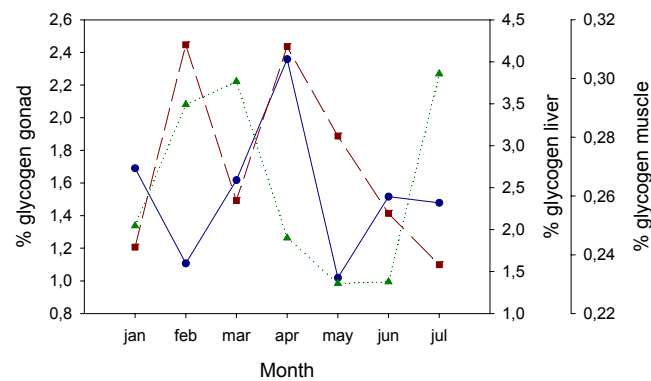
Figure 3.30: Intra-annual variation of mean glycogen content as percentage of total biochemical components in every tissue. Gonad = solid blue line, liver = dash red line and muscle = dotted green line.

Figure 3.31: Relationship of glycogen content among tissues: a) gonad versus liver; b) gonad versus muscle and c) liver versus muscle.

Figure 3.32: Gonad water content (mg/g) versus female attributes and somatic condition indices.

Figure 3.33: Liver water content (mg/g) versus female attributes and somatic condition indices.

Figure 3.34: Muscle water content (mg/g) versus female attributes and somatic condition indices.
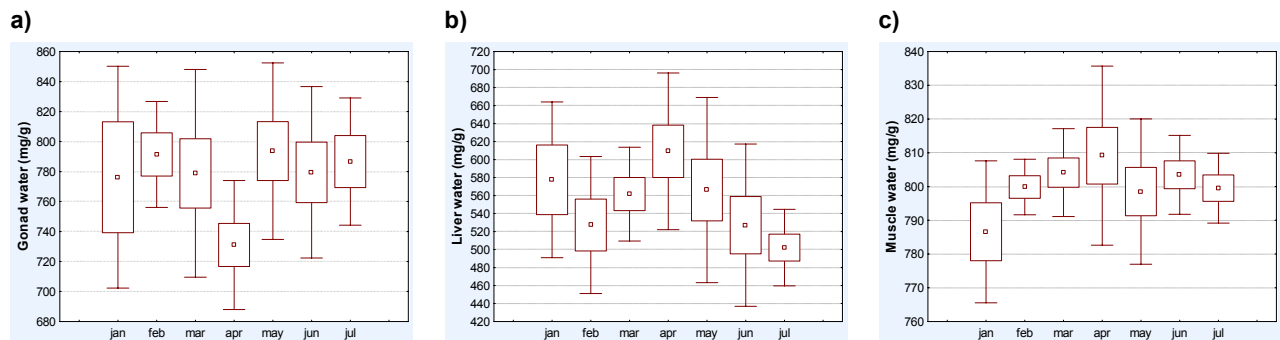
Figure 3.35: Monthly variation of mean water content (mid point) in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.
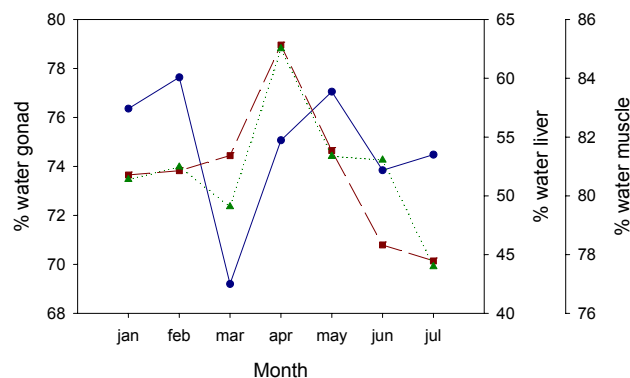
Figure 3.36: Intra-annual variation of mean water content as percentage of total biochemical components in every tissue. Gonad = solid blue line, liver = dash red line and muscle = dotted green line.

3.4.5 INORGANIC MATTER CONTENT

Females attributes: Gonad ashes content was significantly and negatively related to gonad weight (r^2=0.06, p<0.05) and with GSI (r^2=0.11, p<0.01; Figure 3.38); and liver inorganic matter content to liver weight was also (r^2=0.08, p<0.05; Figure 3.39). In all cases, determination coefficients were always lower than 0.25. On the contrary, no significant relationship between muscle inorganic matter content and females attributes was detected (Figure 3.40).

Influence of somatic condition factors: Significant relationship was only detected between inorganic matter content of liver and HSI (r^2=0.21, p<0.001; Figure 3.39) that were negatively related.

Seasonal variations: No intra-annual differences were observed in muscle inorganic matter content. On the contrary, monthly differences were significant for gonad (F=3.35, p<0.01) and liver (F=3.74, p<0.01; Table 3.7 and Figure 3.41). In gonad, inorganic

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matter content mean values remained constant during all months, around 16-19 mg/g excepting June, in which ash content of gonad increased abruptly (36.1 mg/g). In liver, ash content average values increased progressively from 16.53 mg/g in January to 20.7 mg/g in March, remained more or less constant until June, and then fell to 13.1 mg/g during July. When results were presented as percentages of fresh weight, it was observed that inorganic matter proportion was practically constant in gonad (around 2% mean value) save in June when gonad inorganic matter content reached the maximum mean value (3.33%). In liver the trend was similar to that in gonad, the maximum mean values were recorded in March (2.46%) and May (2.43%) and the minimum in July (1.32%), remaining the rest of year around 1.8%. Nevertheless, in muscle, inorganic matter content showed a progressive increased from 1.24% in January to more than 5% in July (Figure 3.42).

Multiple regression: The model obtained as output of the multiple regression analysis by forward stepwise method for gonad inorganic matter content included five variables: GSI, length, gonad weight, gutted weight and liver weight that explained 21.3% of ashes variability in this tissue. GSI and length were significantly related to the dependent variable. For liver ash content, HSI, gutted and gonad weight were the variables included in the model. Together they explained 24.5% of total inorganic content variability, and HSI was the only variable significantly related to the dependent variable, explained 20.5% of it. Regarding muscle inorganic matter content, length and gonad weight were included in the model, but they only explained 4.9% of muscle ash variability, and none of them were significantly related to the dependent variable (Table 3.9).

Relationships between tissue composition: Gonad inorganic matter content was positively and significantly related to liver and muscle ash content. The highest regression coefficient was observed for gonad-muscle relationship (r^2=0.22, p<0.001), followed by gonad and liver relationship (r^2=0.22, p<0.001; Table 3.10 and Figure 3.43).
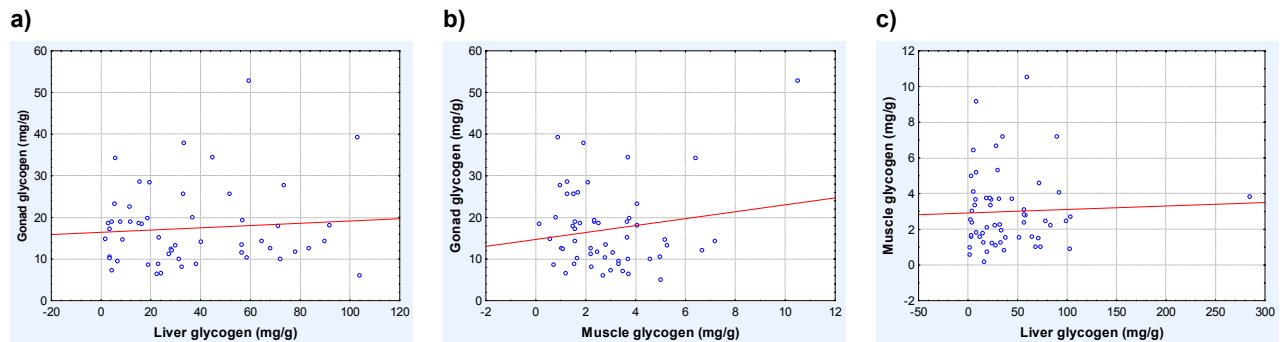

Figure 3.37: Relationship of water content among tissues: a) gonad versus liver; b) gonad versus muscle and c) liver versus muscle.
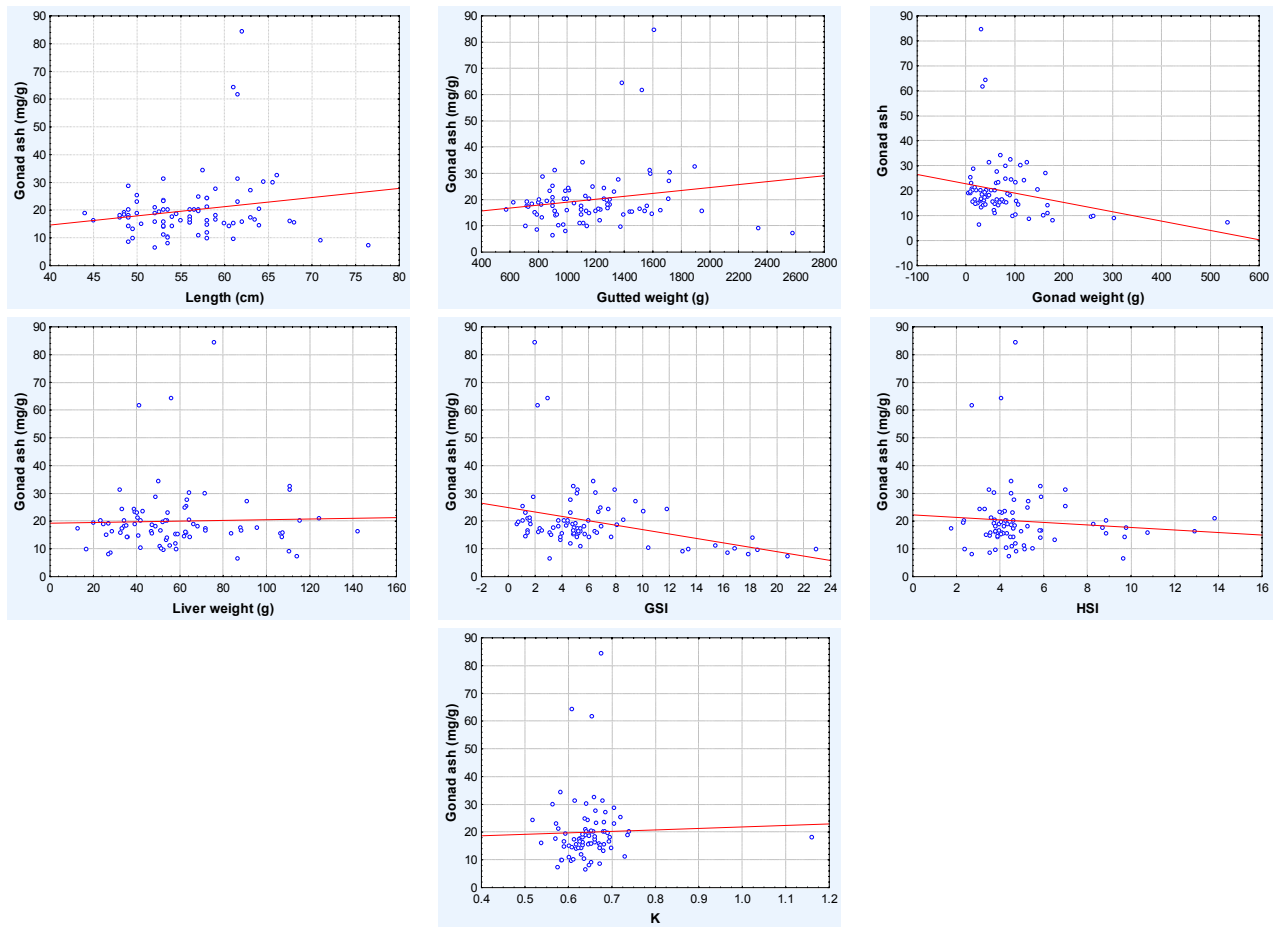
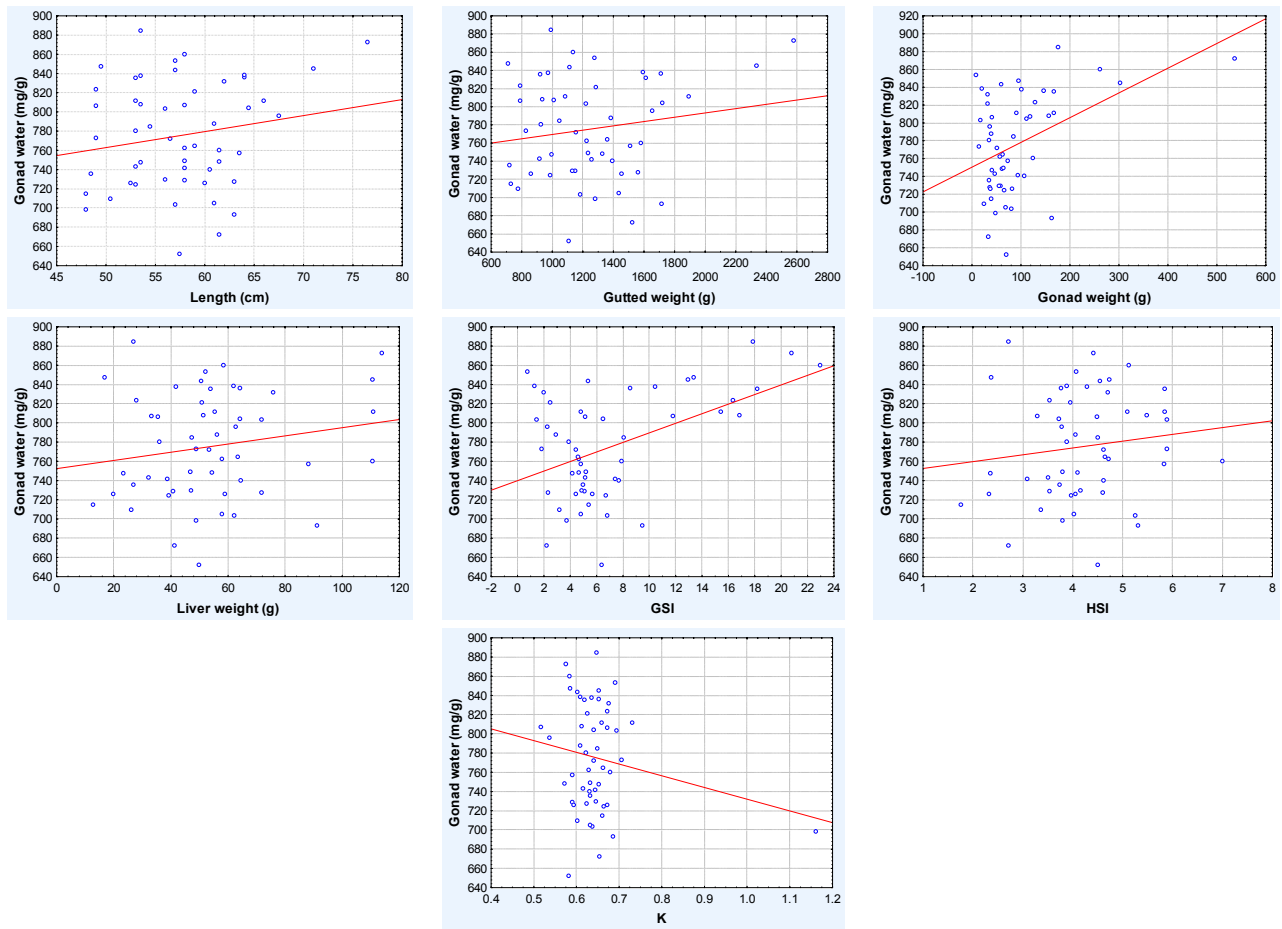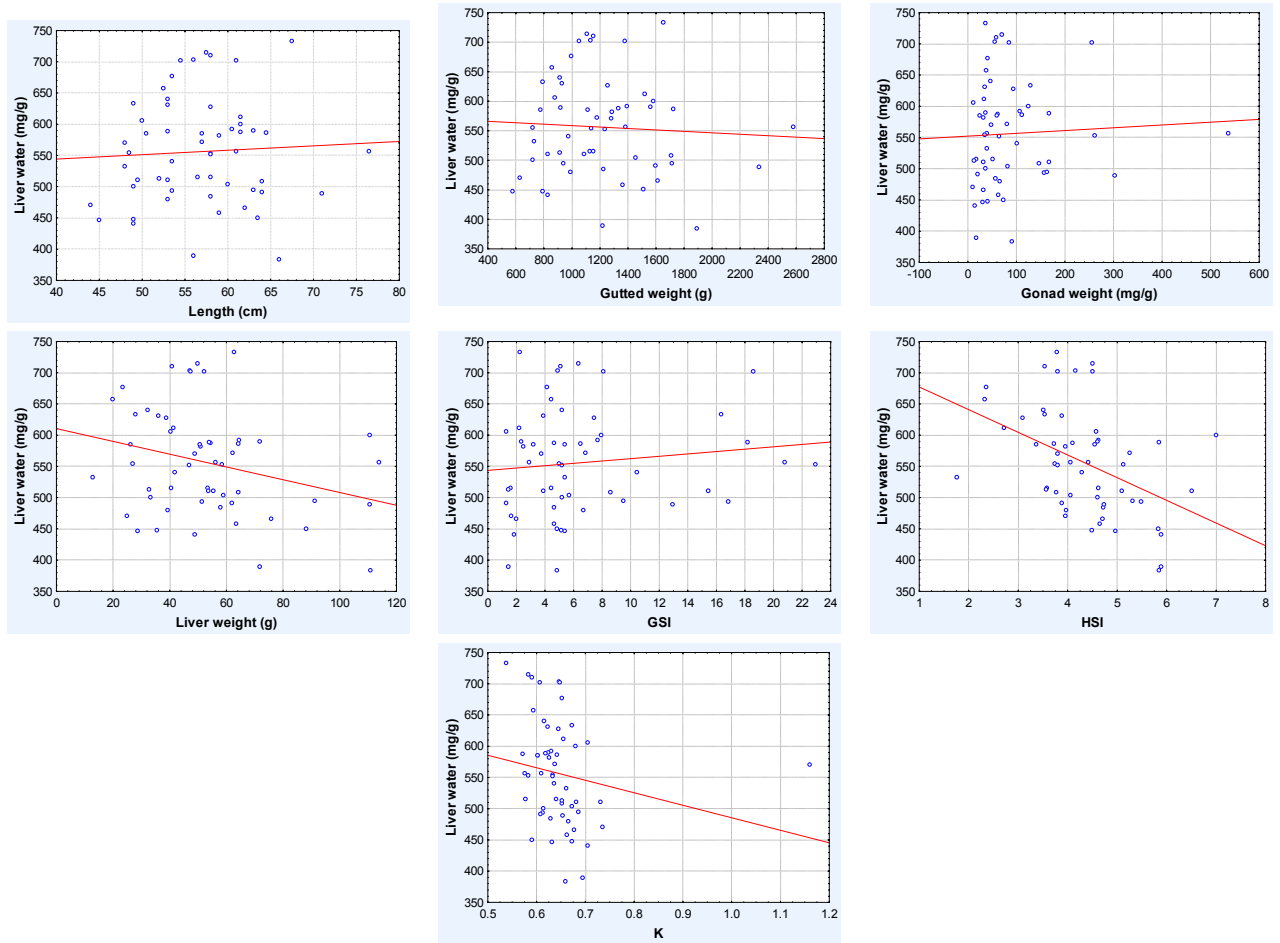*Figure 3.38: Inorganic matter content of gonad (mg/g) versus female attributes and somatic condition indices.*
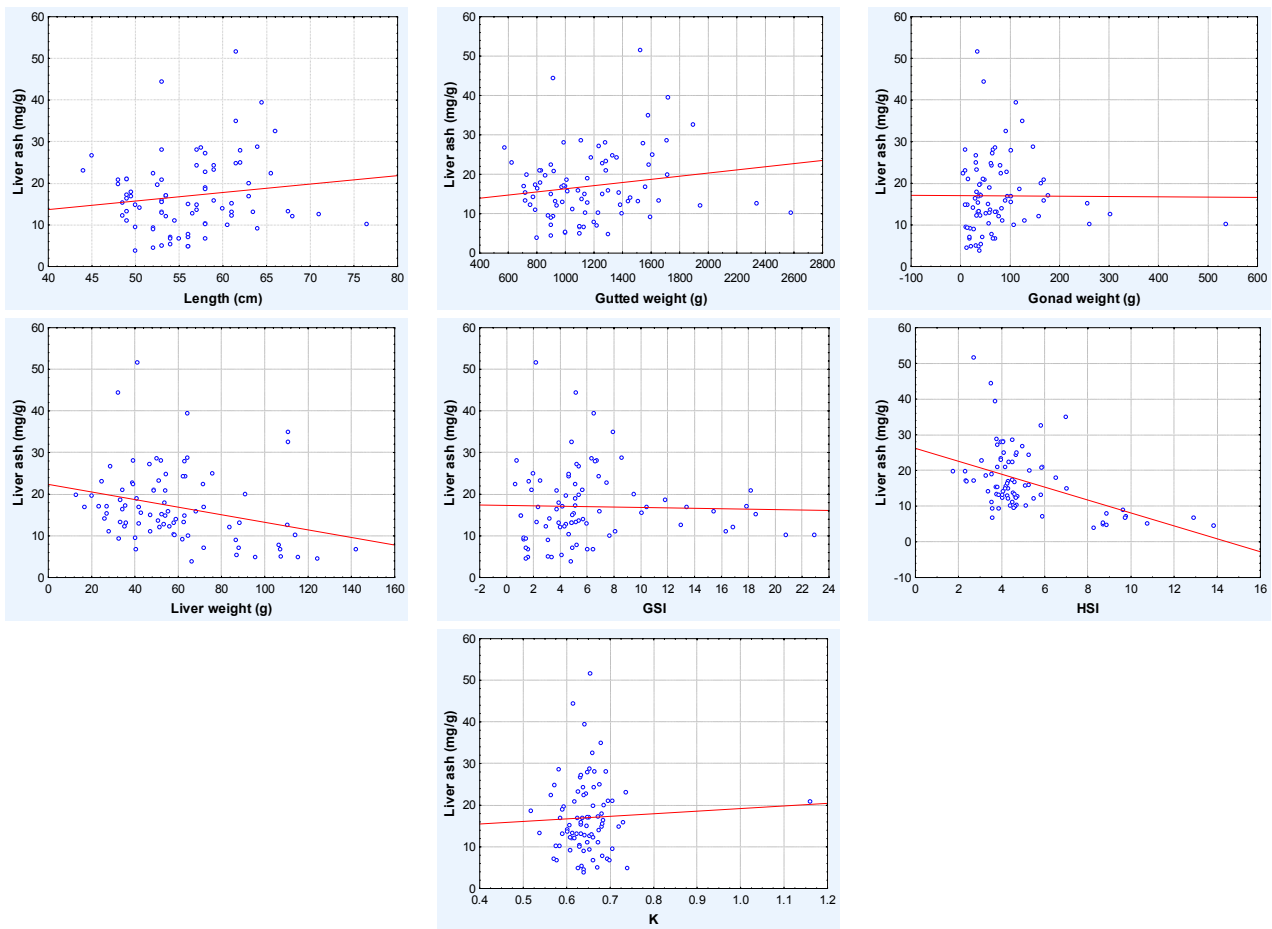
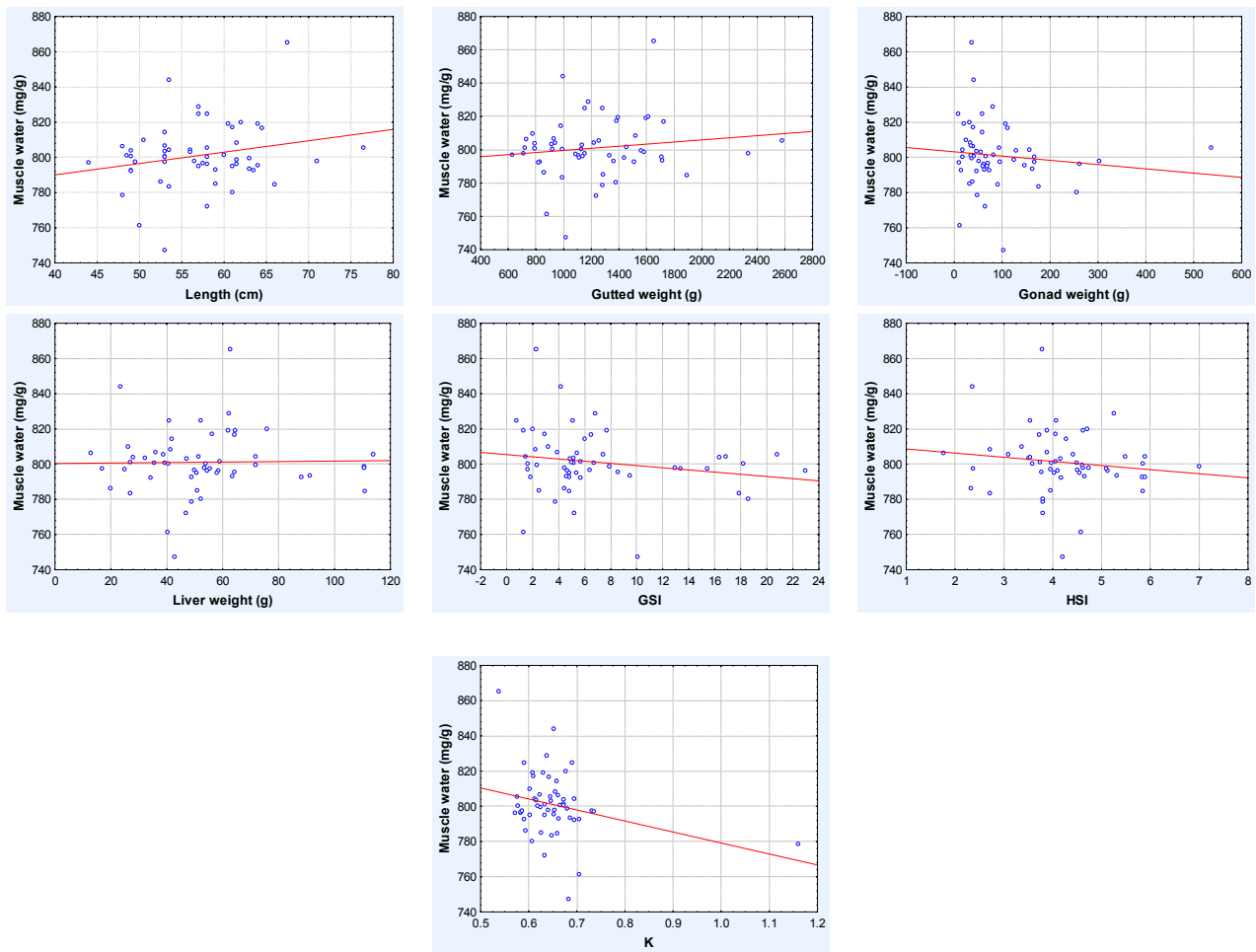*Figure 3.39: Inorganic matter content of liver (mg/g) versus female attributes and somatic condition indices.*
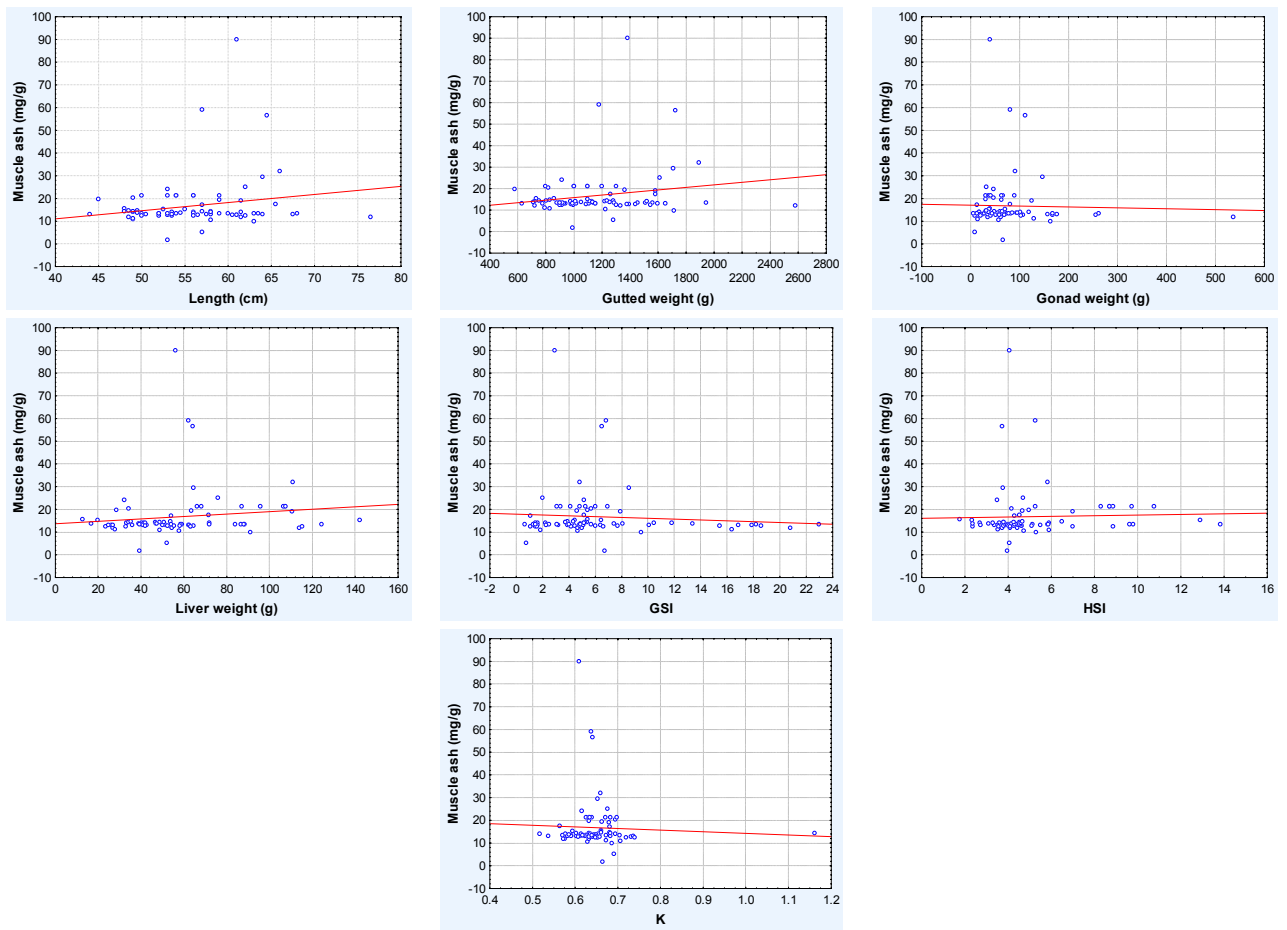
Figure 3.40: Inorganic matter content of muscle (mg/g) versus female attributes and somatic condition indices.
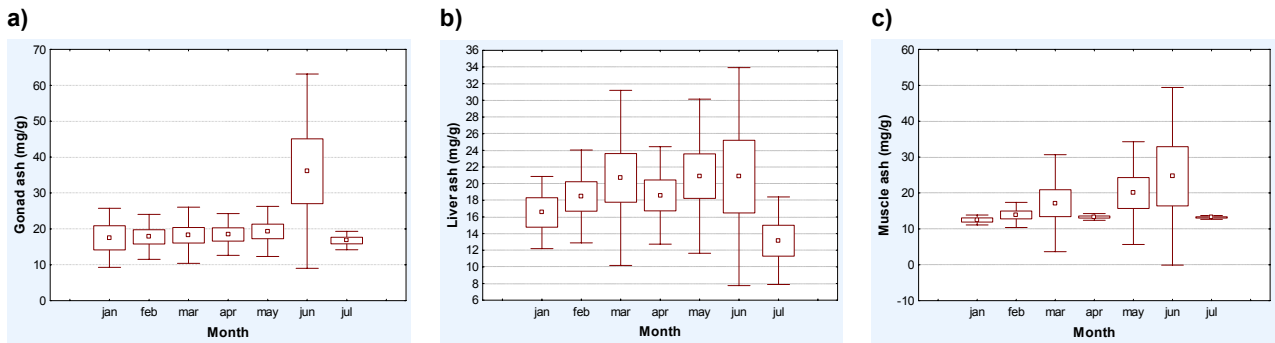
Figure 3.41: Monthly variation of mean inorganic matter content (mid point) in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.
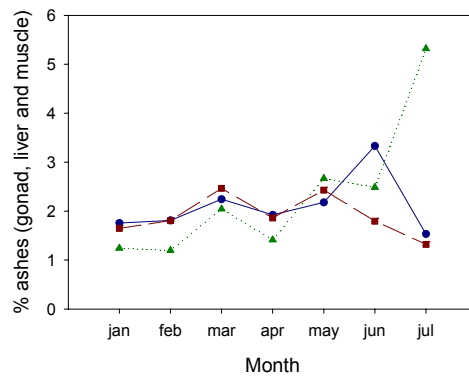
Figure 3.42: Monthly variation of mean inorganic matter content as percentage of total biochemical components in every tissue. Gonad = solid blue line, liver = dash red line and muscle = dotted green line.
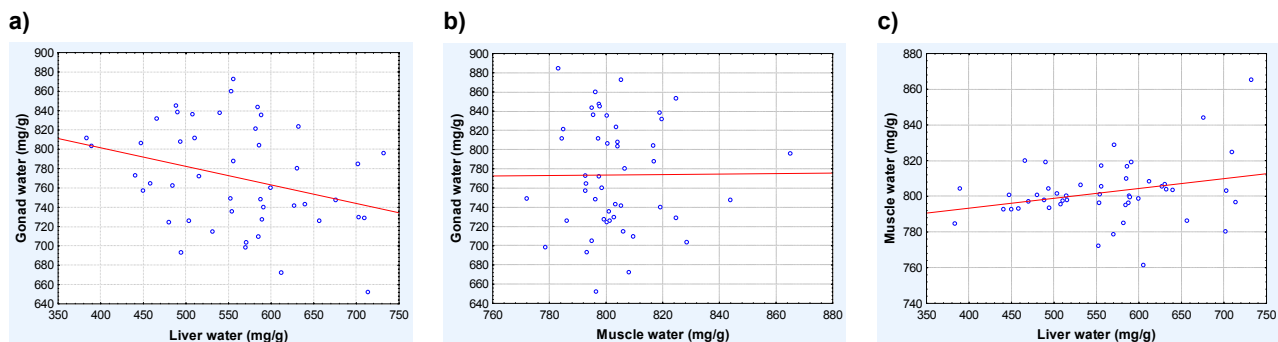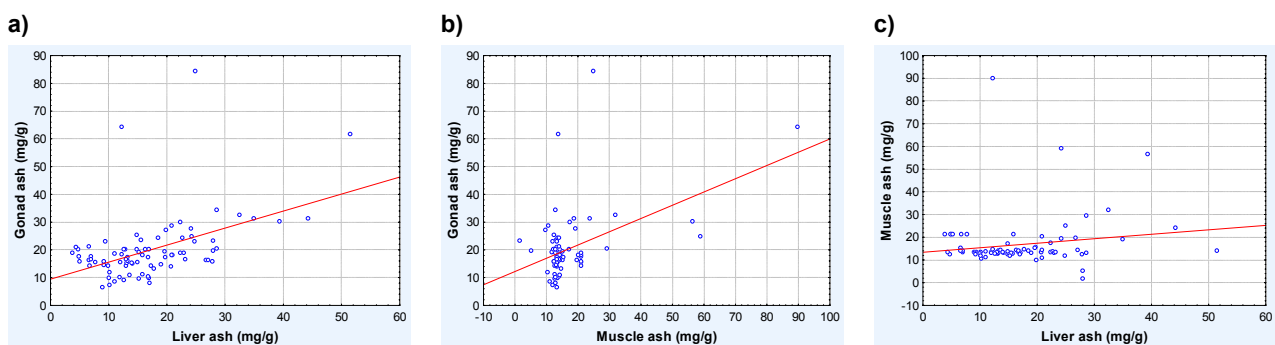

Figure 3.43: Relationship of inorganic matter content among tissues: a) gonad versus liver; b) gonad versus muscle and c) liver versus muscle.

3.4.6 VARIATION OF BIOCHEMICAL COMPOSITION BETWEEN OVARY DEVELOPMENTAL STAGES

Because of the strong asynchrony in ovarian development among females in the population (see section 3.1), it is not feasible to use time (in weeks or months) to analyze changes in biochemical composition during the reproductive cycle. At the same time, to ascertain the stage of the female within the annual reproductive cycle from histology also showed some difficulties as addressed in that section. Nevertheless, the proximate composition in each ovarian development stage (described from histology) was estimated and compared. This analysis has two purposes: firstly, to understand the biochemical shifts among tissues as the reproductive cycle progresses, and secondly to validate the appropriateness of the histological staging made in section 3.1.

Figure 3.44 shows variation of energetic compounds as percentages of total biochemical components in every tissue between ovarian developmental stages. Gonad lipid percentage increased from immature (i; 1.13%) to ripening ovaries (m1; 8.69%) and remained more or less constant until spawning (m2; 8.60%), then began to decrease, reaching the minimum value in recovering ovaries (rc; 2.19%). Liver and muscle lipid percentages followed the opposite trend, decreasing from immature (i; 66.01% and 1.32% respectively) to late spawning ovaries (m3; 26.61% and 0.76% respectively) and then increased progressively to recovering ones (rc; 9.28% and 16.34% respectively; Figure 3.44.a). Protein percentages showed basically the same pattern in the three tissues, except for late spawning ovaries (m3) in which gonad protein percentage increased to its maximum mean value (19.06%), whereas liver and muscle protein percentage decreased (10.11% and 15.26% respectively). In inactive mature ovaries the opposite trend was observed (Figure 3.44.b). Mean values of gonad glycogen percentage increased from immature (i; 1.69%) to ripening ovaries (m1; 1.96%), decreasing abruptly in ovulating-hydrated ones (h; 1.01%); then it increased again until inactive mature ovaries (im; 1.76%) that were followed by a sharp decrease in recovering ones (rc; 0.74%). For liver and muscle, glycogen percentages decreased progressively from immature (9.18% and 0.40% respectively) to recovering ovaries (rc; 2.93% and 0.15% respectively), except in ovulating-hydrated and late spawning (m3) ones in which muscle glycogen percentage increased (0.30% and 0.28% respectively; Figure 3.44.c). Gonad water percentages diminished from immature (i; 79.73%) to recovering ovaries (rc; 69.86%), except ovulating-hydrated ones that presented the highest water percentage (h; 81.28%). In contrast, liver water content tended to increase from immature (i; 10.41%) to late spawning ovaries (m3; 58.58%), decreasing in inactive mature (im; 52.77%) and recovering ones (rc; 35.75%). In muscle, water percentage increased at the beginning (from 81.55% in i to 82.43% in m1) and diminished continuously until inactive mature (im; 79.26%), excepting late spawning and recovering ovaries (81.46% and 79.96% respectively; Figure 3.44.d). Finally, inorganic matter percentages remained practically constant in all ovarian developmental stages between 0.5% and 3% in the three tissues, except in gonad

that sharply increased in recovering ovaries (rc; 7.09%) and in muscle that showed the highest percentage of ash in ovulating-hydrated ones (h; 4.65%; Figure 3.44.e).

Figure 3.45 shows the mean concentration of lipid, protein, glycogen, water and ash in gonad, liver and muscle for each ovarian developmental stage. It is observed that most of the biochemical compounds showed some variation and trends during the reproductive cycle in all tissues, especially in gonad, but also in protein and glycogen in muscle and glycogen in liver. However, the ANOVA performed to compare mean concentrations of each compound revealed little variation (Table 3.11). This is the case of glycogen content that statistically remains unchanged during sexual maturation in the three tissues in spite of the decreasing trend in liver and muscle (Figure 3.45).

In gonad, lipid content changed significantly between ovary developmental stages (F=10.16, p<0.001; Figure 3.45.a). Firstly, lipid content increased to maximum values (90.8±15.31 mg/g), decreased progressively as the spawning season advanced, and reached minimum values in recovering ovaries (38.1±15.1 mg/g). Taking into account that immature and recovering female samples were scarce, these values are only considered indicative. Gonad protein content also increased progressively from 95.5±6.1 mg/g in immature ovaries to 175.3±56.9 mg/g in late spawning (m3) ones, then, decreased to reach 123±27.2 mg/g in recovering ovaries (F=6.43, p<0.001; Figure 3.45.b). Glycogen content of gonad was not statistically significant but tended to increase from immature (i; 18.04 mg/g) to ripening (m1; 22.2±12.0 mg/g) ovaries, subsequently decreased abruptly in ovulating-hydrated (h) to 11.7±4.5 mg/g, and then increased in spawning ones (m2; 18.2±10.2 mg/g) and remained constant in late spawning and inactive mature ovaries; it finally decreased in recovering ones to 8.8 mg/g (Figure 3.45.c). Regarding water content, significant differences between ovary developmental stages were observed and the highest water values were observed in ovulating-hydrated ovaries (h; 831.5±27.3 mg/g) as is logical. Immature and recovering females also showed high values of water content (853.2 and 831.8 mg/g respectively) but we have to take in account that these values are only indicative and without statistical value (Figure 3.45.d). Regarding inorganic matter content, it remained constant in all ovary developmental stages around an average of 20 mg/g, except in inactive mature and recovering females that were significantly different (32.4±21.96 and 37.4±31.43 mg/g respectively; Figure 3.45.e).

Concerning liver, no significant changes in lipid content was detected (F=1.30, p=0.27; Figure 3.45.a), nevertheless a decreasing pattern from immature (560.5±140.9 mg/g) to late spawning ovaries (436±202.1 mg/g) followed by a progressive increase until recovering ovaries (578.7±64.9 mg/g) was observed. In fact, LSD *post hoc* test detected significant differences between recovering ovaries liver lipid content and ripening (m1), ovulating-hydrated (h) and spawning (m2) oocytes.

On the other hand, liver protein content showed significant differences between ovary developmental stages (F=2.56, p<0.05; Figure 3.45.b). Ripening (m1) ovaries were significantly different from the others, except immature and recovering ones. In that ovary developmental stage (m1), protein content of liver reached its minimum values (83.7±38.7 mg/g). Glycogen content of liver did not show differences between ovary stages (F=0.60, p=0.729; Figure 3.45.c) but a decreasing pattern is observed from immature ovaries (63.4±40.3 mg/g) to recovering ones (23.3±21.0 mg/g). Finally, water and inorganic matter content of liver (Figure 3.45.d and e) did not show significant relationships with ovarian developmental stages either, but in this case, in contrast to glycogen, no pattern was observed.

Muscle biochemical composition did not change significantly between ovarian developmental stages, except protein content (Table 3.11). In this case, an initial decline was observed from immature (163.2±0.7 mg/g; Figure 3.45.b) to ripening ovaries (m1; 119.3±40.2 mg/g), then protein content started to increase until spawning (m2; 172.4±36.9 mg/g), then decreased slightly in late spawning ovaries (m3; 156.8±38.8 mg/g), remaining constant in inactive mature and recovering females (165.6±10.8 and 164.1±4.9 mg/g respectively). Significant differences were only observed between m1 females and other stages, except immature and recovering females. However, muscle glycogen content, although it did not change significantly, showed a progressive decrease from immature (5.6±2.2 mg/g) to recovering ovaries (1.7±0.2 mg/g; Figure 3.45.c).

Table 3.11: Number of individuals analysed, F statistic and p-values of ANOVA made to study changes of every biochemical component in each tissue in relation to ovary developmental stages.

	Gonad			Liver			Muscle		
	N	F	p	N	F	p	N	F	p
Lipid	83	10.16	≤0.001	82	1.30	0.266	83	1.09	0.378
Protein	79	6.43	≤0.001	79	2.56	0.027	65	3.74	0.003
Glycogen	57	2.12	0.667	59	0.60	0.729	60	1.38	0.242
Water	52	10.68	≤0.001	55	0.24	0.962	55	1.14	0.356
Ash	82	4.99	≤0.001	83	0.67	0.675	81	1.37	0.239

a)

b)

c)

d)

e)
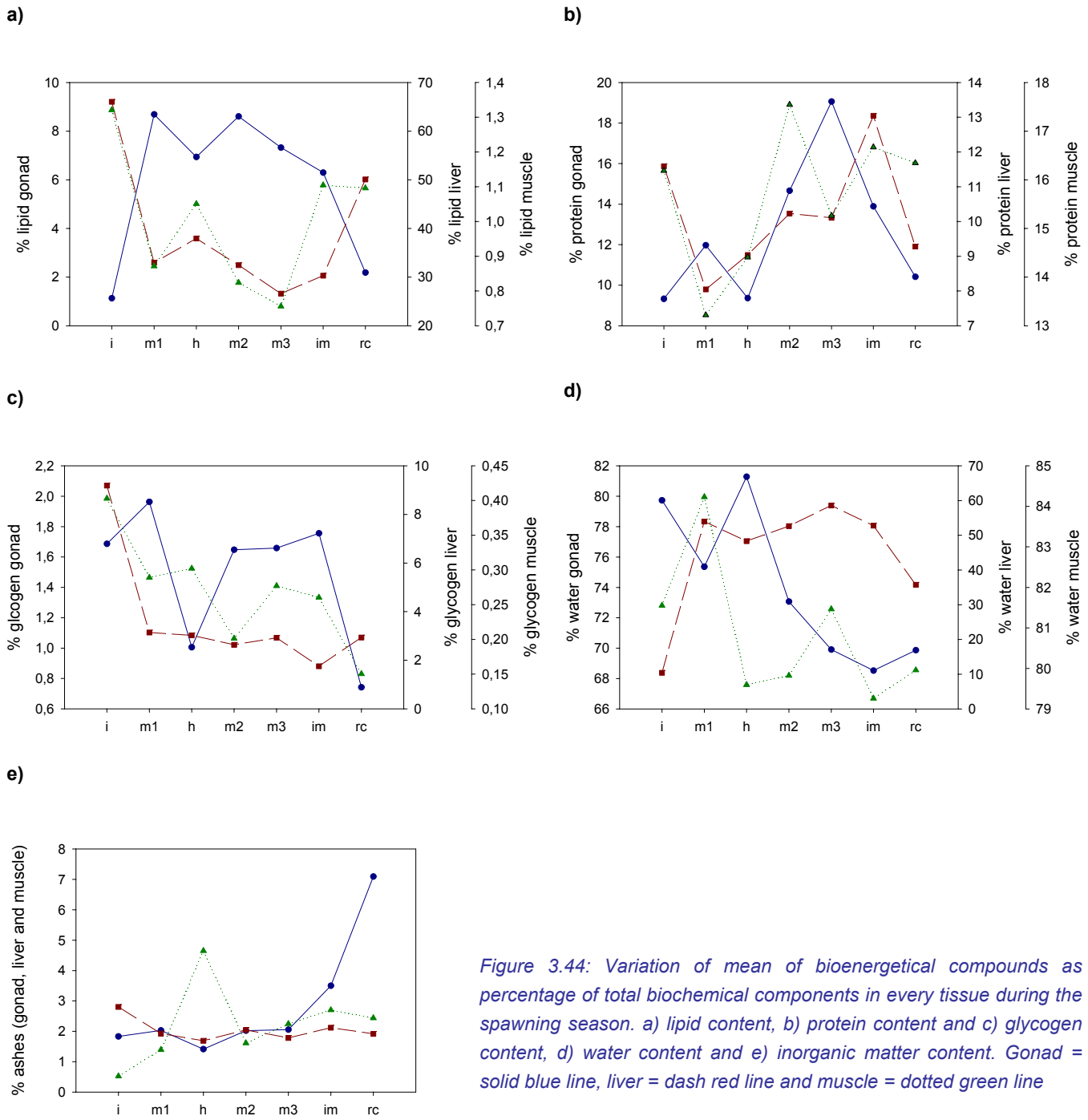
Figure 3.44: Variation of mean of bioenergetical compounds as percentage of total biochemical components in every tissue during the spawning season. a) lipid content, b) protein content and c) glycogen content, d) water content and e) inorganic matter content. Gonad = solid blue line, liver = dash red line and muscle = dotted green line

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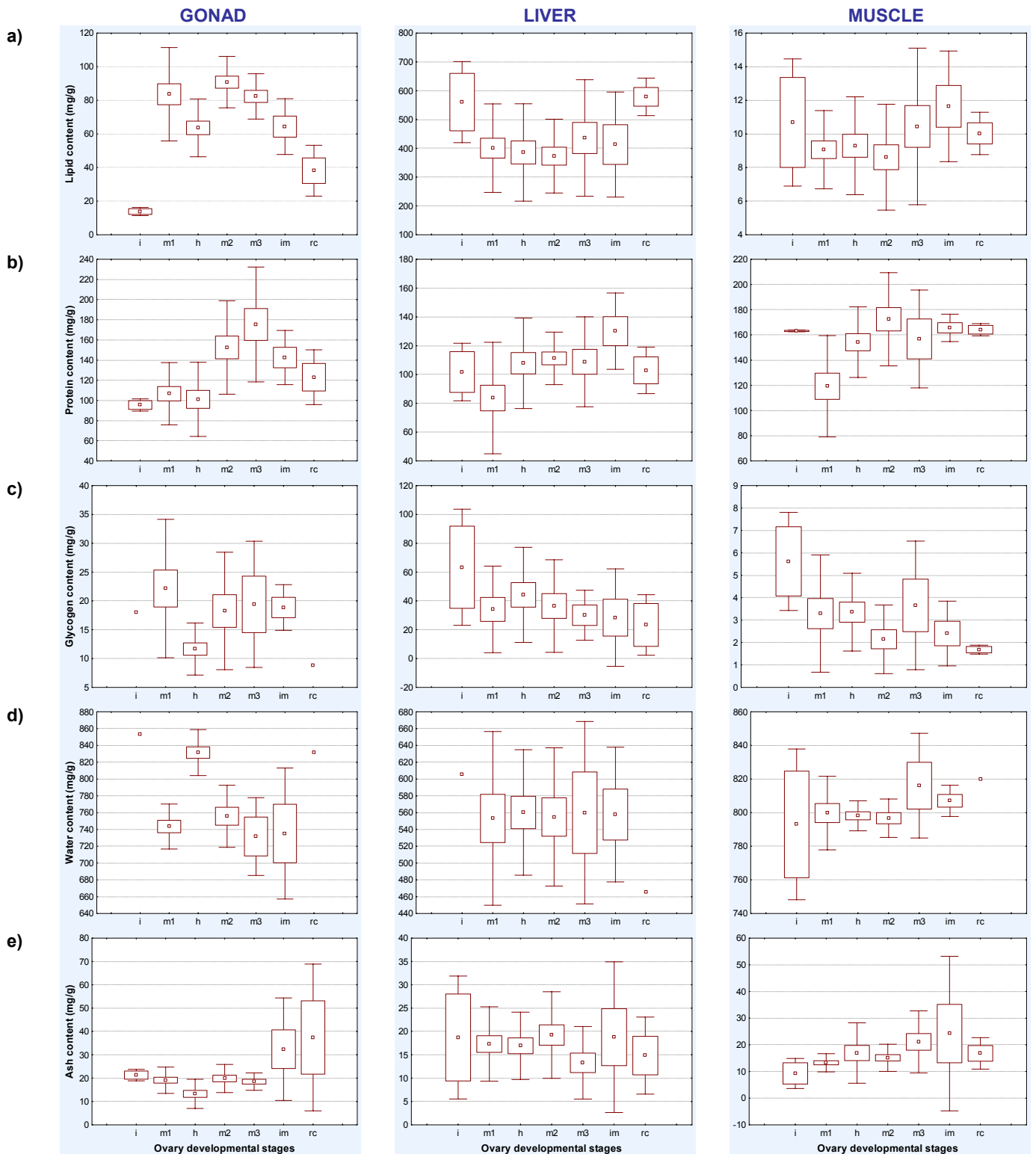
Figure 3.45: Mean concentration (mid point) of a) lipid, b) protein, c)glycogen, d)water and e)ash in gonad, liver and muscle in relation to ovary developmental stages. Standard error (box) and standard deviation (wishker) are also represented.

3.5 VARIATIONS IN ENERGY CONTENT

Variation in energy content was analyzed as energy density, i.e. energy content in kJ per unit mass of tissue (soma, liver or gonad), in order to avoid female size effects. Using the absolute amount of kJ per tissue was not considered appropriate, because larger females have, in general, larger organs and body mass. Simple and multiple regressions were applied, firstly, to investigate the influence of different female individual features (attributes and condition) on tissue energy content (Section 3.5.1). Secondly, another set of multiple regressions were carried out to detect which biochemical component determined mostly the energy density of each tissue and the total energy of the female (Section 3.5.2). Subsequently, temporal trends of energy density in every tissue and total female energy were studied, as well their progression throughout the reproductive cycle (Section 3.5.3). And finally, variations in energy density among tissues was analyzed to ascertain if mobilization of energetic storage took place from one tissue to another and if that depended on ovary developmental stage during the breeding season or sexual maturation (Section 3.5.4). Also, the monthly trend in total fish energy (gonad + liver + muscle total energy) was studied by analysis of variance.

Table 3.12: Coefficient of determination and p-values of linear regression between energy density (kJ/g) and maternal attributes and somatic condition factors.

		kJ/g	
		r^2	p
Gonad	Length	0.03	0.219
	Gut W	0.03	0.225
	Gonad W	≤0.01	0.781
	GSI	≤0.01	0.582
	Liver W	0.03	0.197
	HSI	≤0.01	0.571
	K	0.02	0.367
Liver	Length	≤0.01	0.870
	Gut W	≤0.01	0.694
	Gonad W	0.01	0.394
	GSI	≤0.01	0.513
	Liver W	≤0.01	0.578
	HSI	0.01	0.472
	K	≤0.01	0.879
Muscle	Length	0.09	0.037
	Gut W	0.12	0.016
	Gonad W	≤0.01	0.984
	GSI	0.01	0.422
	Liver W	0.03	0.252
	HSI	0.01	0.481
	K	≤0.01	0.913
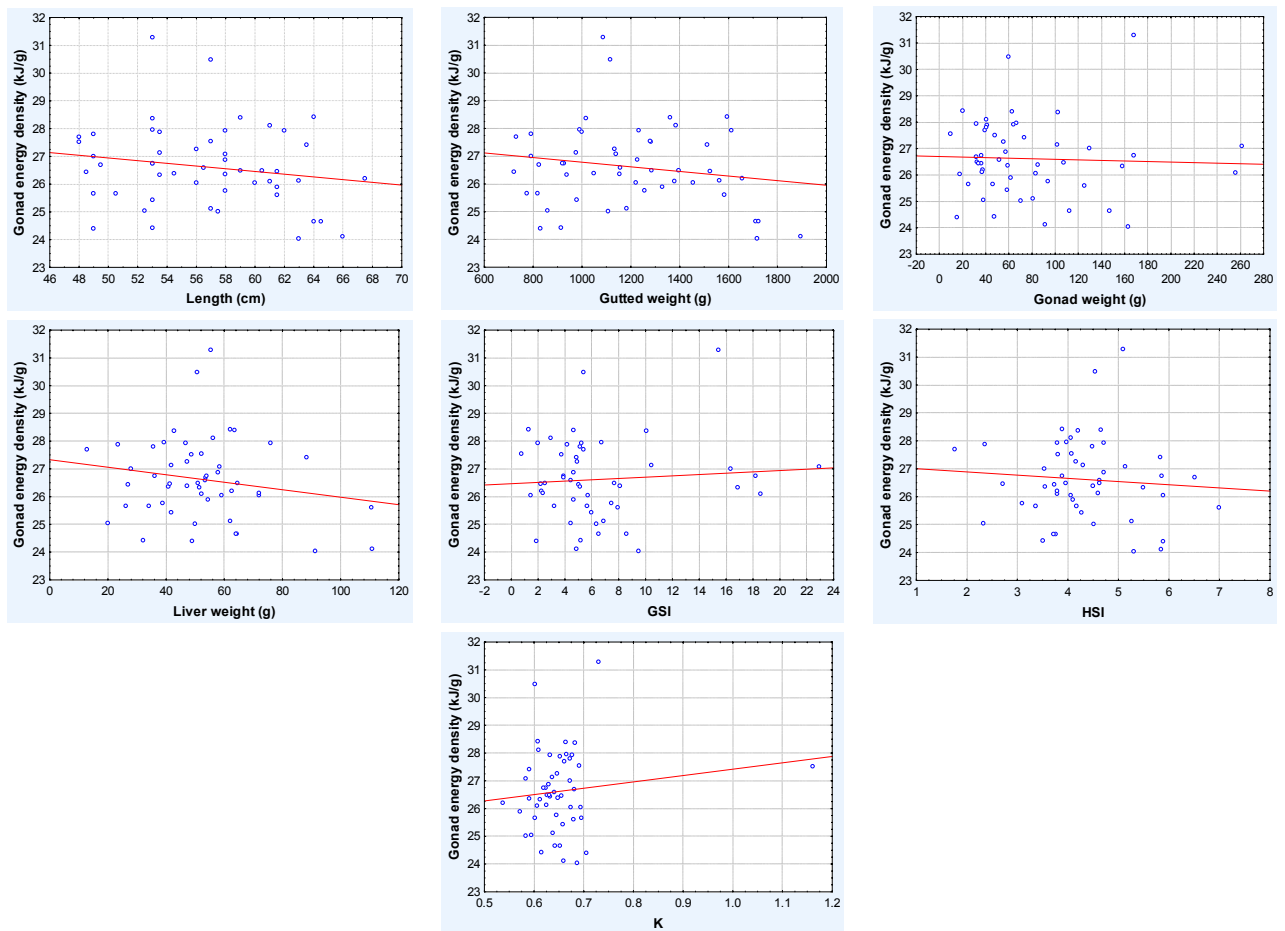
Figure 3.46: Energy density of gonad (kJg/g) versus female attributes and somatic condition indices.
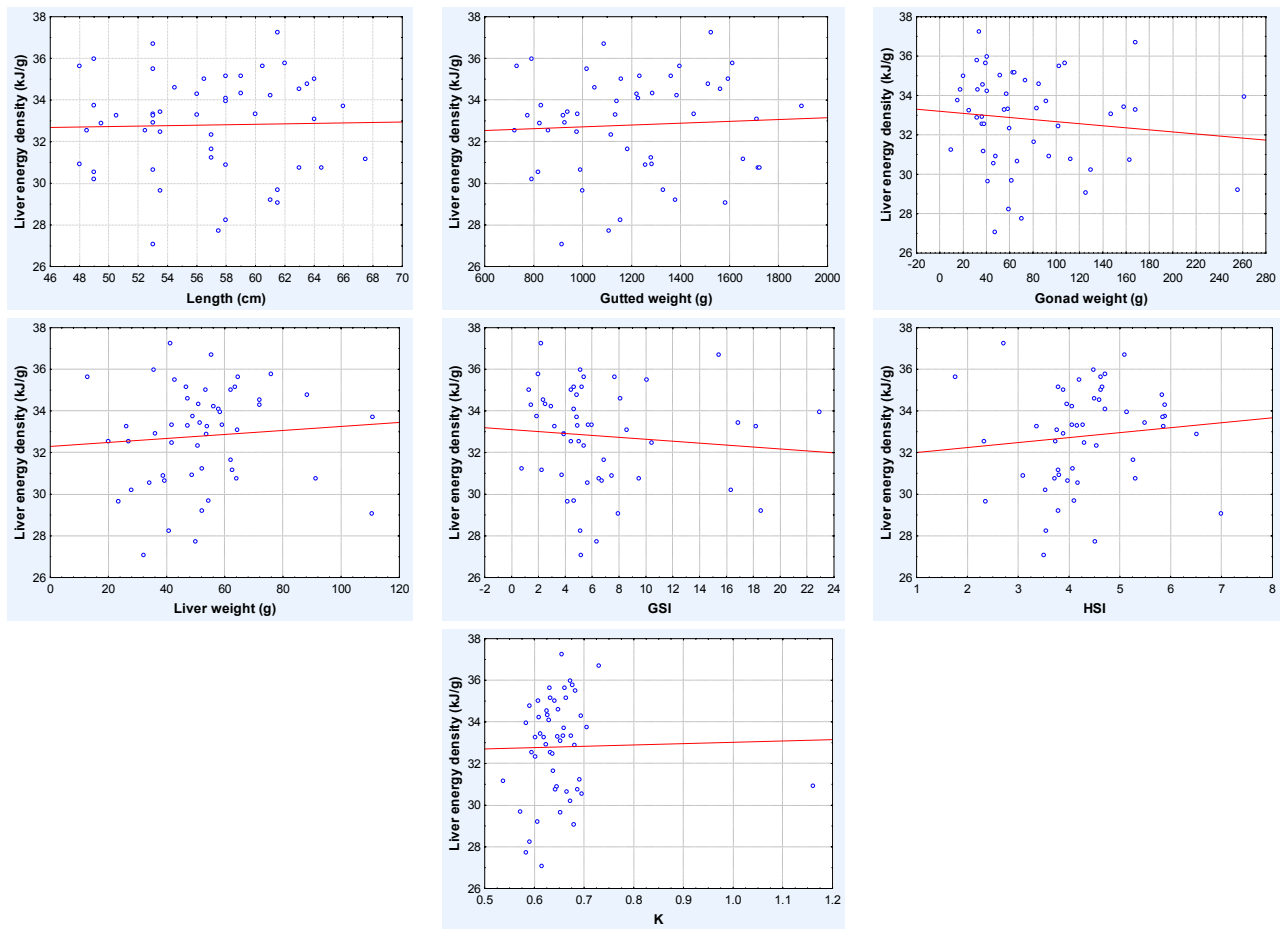
Figure 3.47: Energy density of liver (kJg/g) versus female attributes and somatic condition indices.
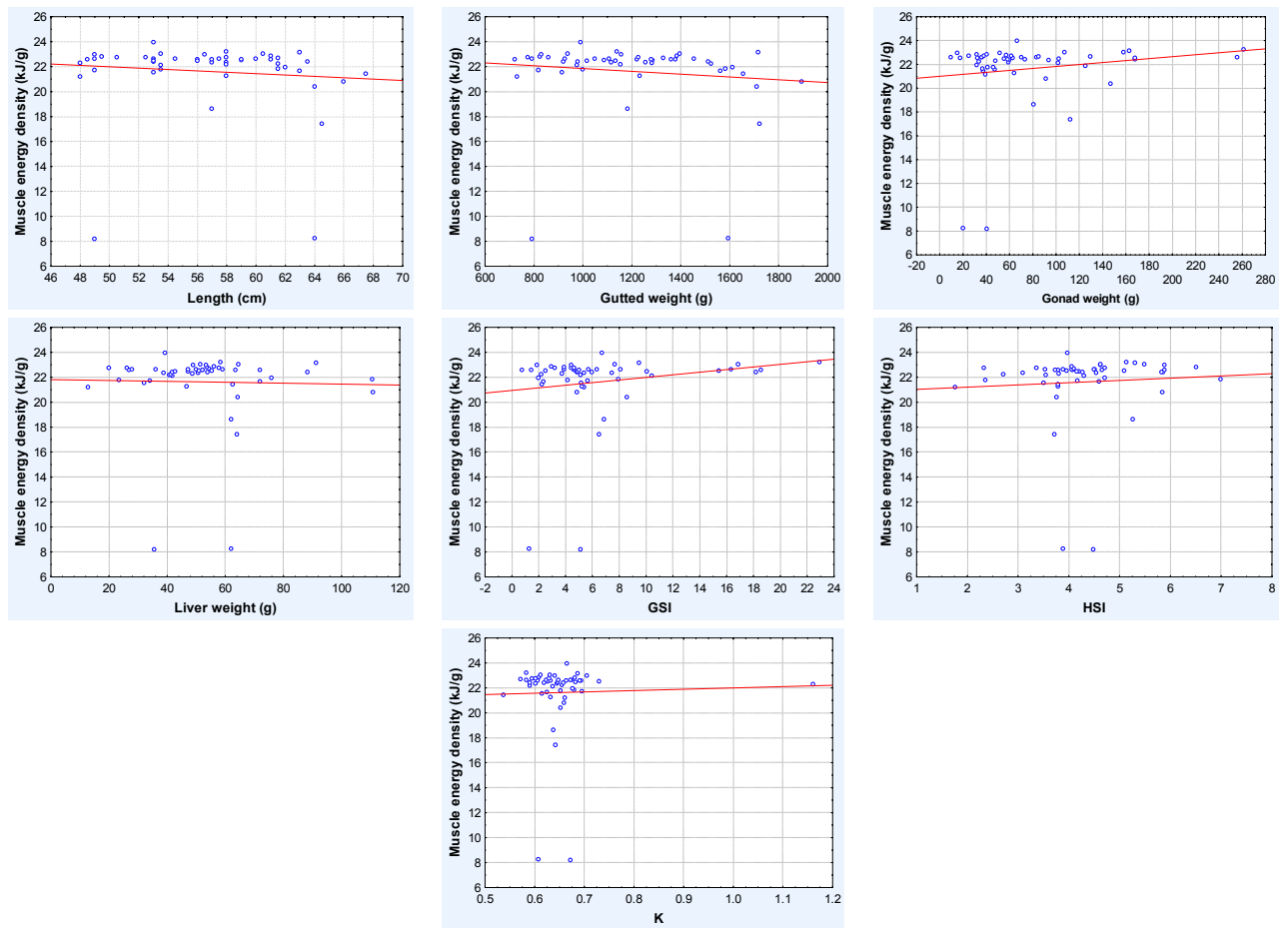
Figure 3.48: Energy density of muscle (kJg/g) versus female attributes and somatic condition indices.

3.5.1 MATERNAL INFLUENCES

Energy density of soma and viscera tissues were not significantly affected by the maternal traits considered (Table 3.12), except the energy density in muscle that were negatively affected by length and gutted weight, although determination coefficients were very low in both cases (r^2=0.09, p<0.05 and r^2=0.12, p<0.05 respectively; Figure 3.46; Figure 3.47 and Figure 3.48).

The stepwise multiple regression carried out to assess the variation in energy density in each tissue as a function of maternal features showed that only liver weight was included in the model of gonad energy density, explaining 3.4% of data variability. Observed and predicted values were significantly related but the correlation coefficient was low (r=0.33, p<0.05; Figure 3.49.a). In the case of liver energy density, no variables were included in the model by the forward stepwise method, and the standard method was carried out; length, gonad and liver weight and condition factor K showed negative Beta coefficients while gutted weight, GSI and HSI had positive ones. All maternal attributes explained 14.6% of liver energy density variation, and predicted and observed values were significantly correlated (r=0.49, p<0.001; Figure 3.50.a). Regarding muscle, GSI was included in the model by the forward stepwise method, and explained 3% of soma energy density variability. Nevertheless, observed and predicted values were not significantly correlated (r=0.25, p=0.065; Figure 3.51.a).
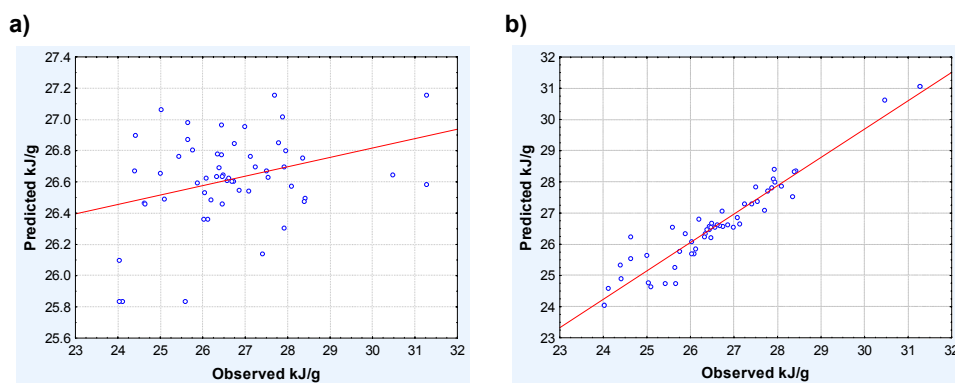
Figure 3.49: Observed versus predicted values of gonad energy density from multiple linear regression with a) maternal attributes (r = 0.33, p<0.05) and b) proxy body composition biochemical compounds (r = 0.96, p<0.001).

When total female energy content was considered as the dependent variable, the stepwise model included three maternal traits that explained 65.3% of total energy variance of a hake female. These variables were, in the same order that were included in the model, liver weight, gonad weight and GSI, but only liver weight and GSI were significantly related to the dependent variable. Proportions of variability explained by each predictor were 58.8%, 2.8% and 3.7% respectively. The correlation between observed and predicted values was positive and significant (r=0.84, p<0.001; Figure 3.52.a).

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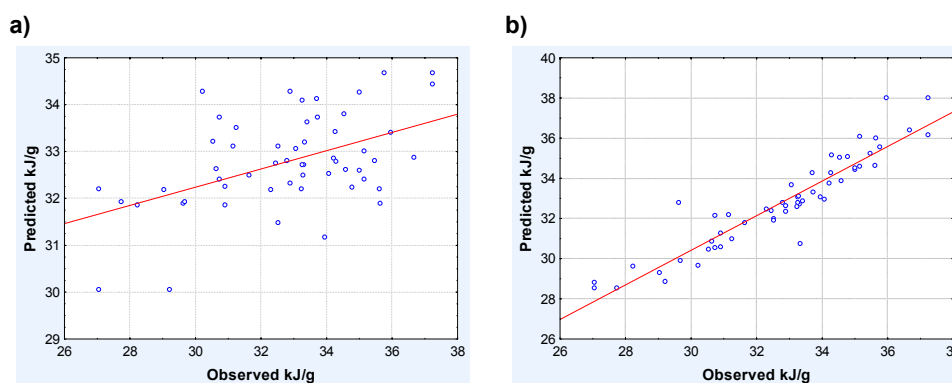
Figure 3.50: Observed versus predicted values of liver energy density from multiple linear regression with a) maternal attributes (r = 0.49, p<0.001) and b) body biochemical compounds composition (r = 0.93, p<0.001).

3.5.2 PROXIMATE COMPOSITION INFLUENCE

The multiple regression performed to assess the variation in energy density in each tissue as function of the concentration of each biochemical component showed that in gonad, 89.5% of energy density variation was explained by lipids, proteins and glycogen: 79% by lipid content, 9.1% by glycogen and the rest by proteins (1.5%). Observed and predicted values were well correlated and showed a correlation coefficient of 0.96 (p<0.001; Figure 3.49.b) In liver, the first variable included in the model of energy density was lipids (25.7%), followed by proteins (37.7%) and glycogen (22%) and they explained a total of 85.5% of energy density variability and good correlation was detected between observed and predicted values (r=0.93, p<0.001; Figure 3.50.b).Finally, in muscle, the protein concentration explained 58.3% of its energy density variability, followed by lipids (3.2%), explaining a total of 61% of the variability. Glycogen content was not included in the model. However, in this case correlation between observed and predicted values was not so good as in gonad and liver (r=0.85, p<0.001; Figure 3.51.b)

For the influence of each biochemical component concentration in total female energy, nine variables were included in the model, in this order: liver and gonad glycogen, gonad protein, liver lipid, muscle and gonad ash, gonad lipid, liver ash and muscle lipids. The model explained 46.5% of variability, and liver and gonad glycogen content on their own 16%. Observed *versus* predicted values correlation was positive and significant (r=0.74, p<0.001; Figure 3.52.b). This indicated that the model would be reasonably adjusted to real energy content of females.
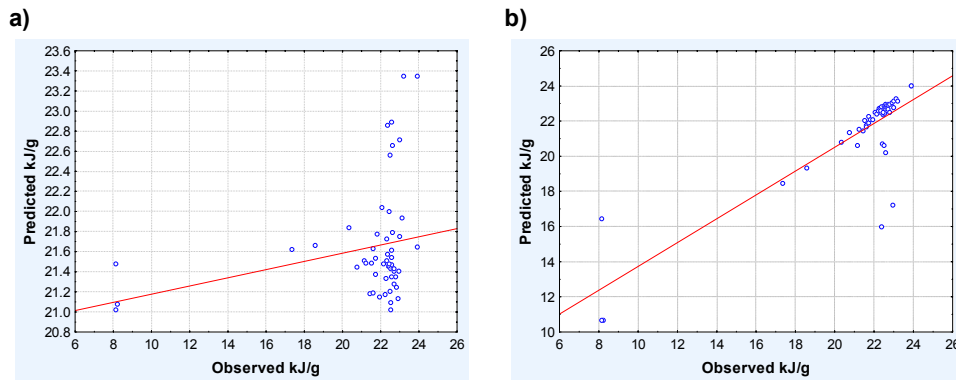
Figure 3.51: Observed versus predicted values of muscle energy density from multiple linear regression with a) maternal attributes (r = 0.25, p = 0.065) and b) body biochemical compounds composition (r = 0.85, p<0.001).
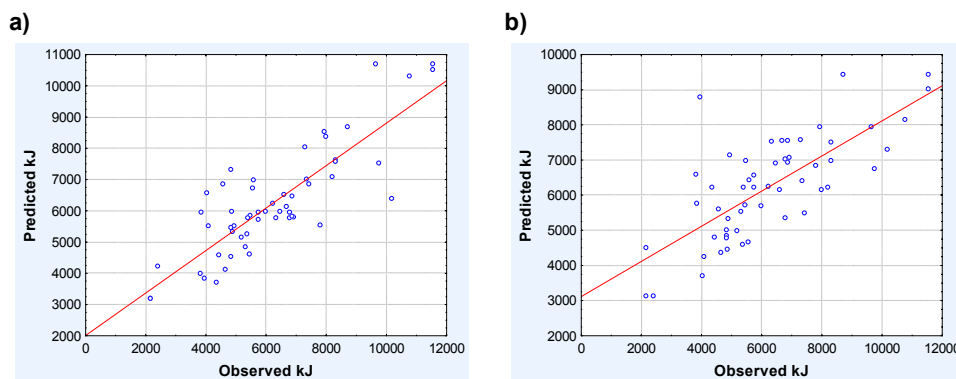
Figure 3.52: Observed versus predicted values of total female energy from multiple linear regression with a) maternal attributes (r = 0.84, p<0.001) and b) body biochemical compounds composition (r = 0.74, p<0.001).

3.5.3   SEASONAL VARIATIONS OF ENERGY CONTENT

Gonad energy densities for hake females ranged from a high of 29 kJ/g dry mass in January and June to a low of 25 kJ/g dry mass in March (Figure 3.53.a). Mean energy density declined by 3.4% from January to February, remaining basically at this level until May, increasing by 4.4% in June and July. However, these variations were not significantly different based on the overall ANOVA (F=0.90, p=0.514) as shown in Table 3.13.

This pattern was similar regarding liver energy density, which ranged between 28.14 kJ/g in March and 36.03 kJ/g in June. Mean energy density declined from 32.83 kJ/g in January to 31.09 kJ/g in March, the lowest value, increasing again to the maximum in July (34.73 kJ/g; Figure 3.53.b). The overall ANOVA (Table 3.13) was not significant (F=2.22, p=0.052), although the *post hoc* test revealed significant differences between the months Feb-May and June-July (p ranging from <0.05 to <0.001 depending of the pair comparison).
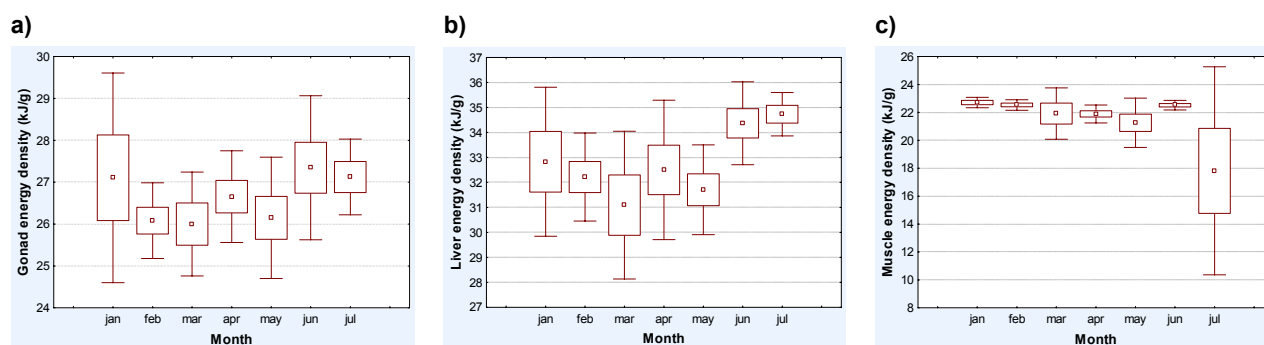
Figure 3.53: Seasonal variation of mean energy density (mid point) in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.

Table 3.13: Results of the ANOVA to compare seasonal variation of energy density (n = 51).

	Gonad		Liver		Muscle	
	F	p	F	p	F	p
kJ/g	0.90	0.514	2.22	0.052	2.11	0.063

Energy density in muscle showed a particular pattern, with a very stable density throughout the seasons around an average of 22 kJ/g, but with a significant lower mean density in July (17.82 kJ/g; Figure 3.53.c), in fact, *pos hoc* analysis showed that this month was significantly different from the rest, although the overall ANOVA was not significant (F=2.11, p=0.063; Table 3.13).

Regarding total energy content of female, its minimum value was reached in April (2,161.77 kJ) and the maximum in May (11,537.15 kJ). Mean total female energy decreased from January (7,252.23 kJ) to April (4,222.74 kJ), excepting March whose mean values increased notably (6,645.39 kJ). Then a recovery of female energy was observed in May, remaining constant until July with mean values between 6,400 and 7,000 kJ (Figure 3.54). Although ANOVA analysis did not detect significant intra-annual differences (F=2.07, p=0.067), LSD *post hoc* test showed that April was significantly different from the other months, except February.

In all cases energy density of each tissue (kJ/g) and total female energy (kJ) showed a high dispersion, practically all year round, with huge standard deviation that indicated the presence of females in several different energetic statuses. This could be due to population asynchrony which means that females in very different phases of their reproductive cycles coincide in time. Because of this, it is necessary to analyse variation of energy reserves with ovary developmental stages determined by histology, to study changes of energetic density and total female energy through the breeding season.
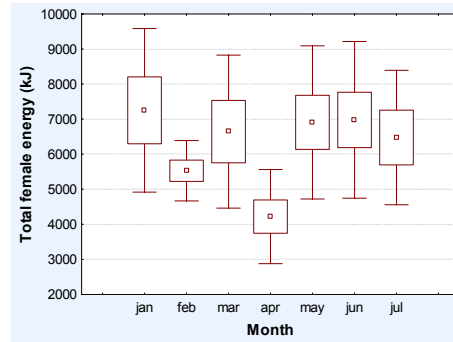
Figure 3.54: Seasonal variation of mean total female energy. F = 2.07, p = 0.067. Standard error (box) and standard deviation (wishker) are also represented.

3.5.4 VARIATIONS OF ENERGY CONTENT WITH OVARY DEVELOPMENTAL STAGES.

Mean energy density of each ovary developmental stage was estimated in gonad, liver and muscle to analyse differences through maturation and reproductive stages (Figure 3.55 and Table 3.14).
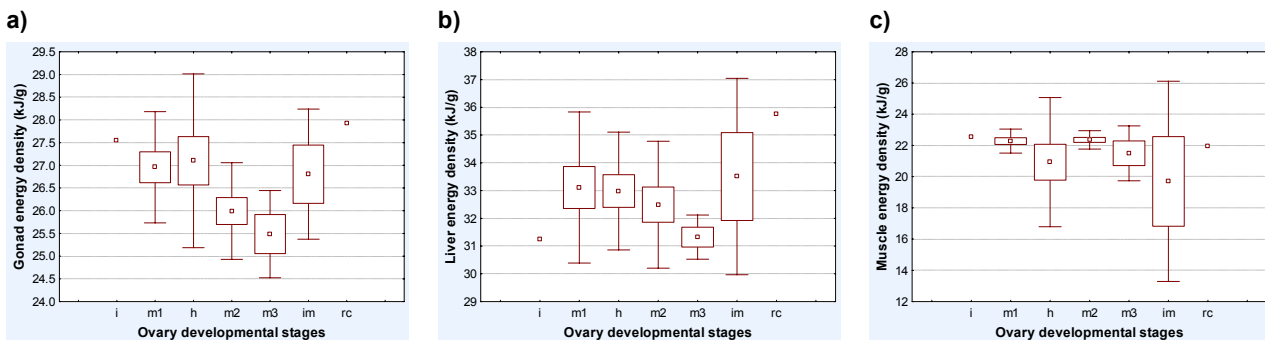
Figure 3.55: Mean energy density (mid point) per ovary developmental stages in a) gonad, b) liver and c) muscle. Standard error (box) and standard deviation (wishker) are also represented.

In gonad, energy density ranged between 24.04 kJ/g of late spawning ovaries and 31.29 kJ/g of ovulating-hydrated ones. Energy density mean values remained constant in the first stages (i-h) around 27 kJ/g, and then a sharp decrease was observed until late spawning ovaries (m3) that had the lowest mean values of energy density (25.48 kJ/g). Subsequently, it rose to 27.93 kJ/g in recovering ovaries (Figure 3.55.a). However, the overall ANOVA was not significant, although *post hoc* test showed that late spawning ovaries (m3) are statistically different from ovulating-hydrated ones (Table 3.14). Similarly, liver mean energy density decreased progressively from ripening (m1; 33.11 kJ/g) to late spawning (m3; 31.32 kJ/g) ovaries, but in this case no significant differences between ovary developmental stages were detected, neither by overall ANOVA nor *post hoc* test (Figure 3.55.b and Table 3.14). On the contrary, muscle energy density did not follow any pattern, and its mean values remained more or less constant in all developmental stages, between 19.7 kJ/g of inactive mature and

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22.56 kJ/g of immature ovaries; nevertheless ovulating-hydrated and especially, inactive mature females presented very high standard deviations: ±4.14 kJ/g and ±6.41 kJ/g respectively which represented 19.8% and 32.5% of their respective mean values (Figure 3.55.c).

Table 3.14: Results of the ANOVA to compare variation of energy density in relation to ovary developmental stages (n = 51).

	Gonad		Liver		Muscle	
	F	p	F	p	F	p
kJ/g	1.57	0.178	0.78	0.588	0.72	0.638

The energy density of the gonad was positively and significantly correlated with density in liver (r=0.42, p<0.01) and muscle (r=0.29, p<0.05), although the correlations coefficients were low in both case due to high variability of data. However, energy density in liver and muscle did not correlate (r=0.12, p=0.402; Figure 3.56 and Table 3.15). All these results will be reanalysed from another perspective in section 3.8.1.
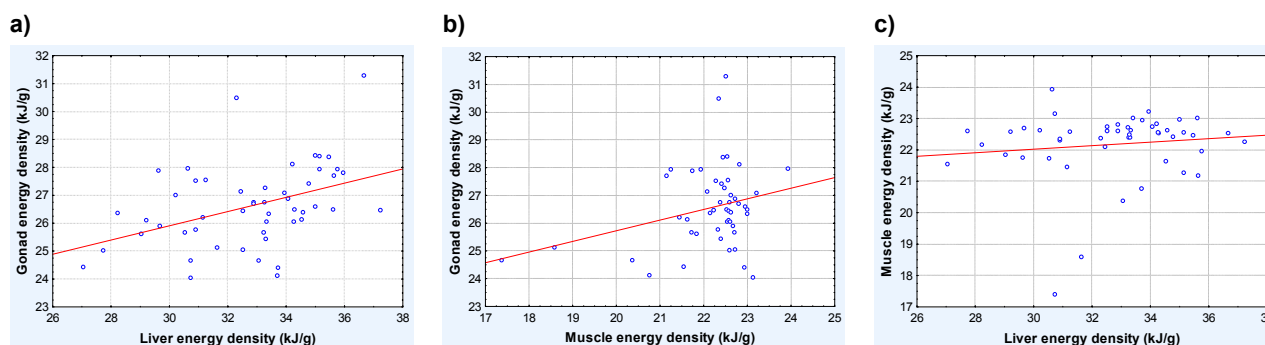
Figure 3.56: Relationship of energy density content between tissues: a) gonad versus liver; b) gonad versus muscle and c) liver versus muscle.

Table 3.15: Correlation coefficients and significance level between energy density of each tissue.

	gonad vs liver		gonad vs muscle		liver vs muscle	
	r	p	r	p	r	p
kJ/g	0.42	0.002	0.29	0.046	0.12	0.402

To analyse the influence of water content in energy of each tissue, relationships between lipid content (mg/g of wet mass), protein content (mg/g of wet mass), glycogen content (mg/g of wet mass) and energy density (kJ/g of wet mass) and the water content (mg/g of wet mass) in gonad, liver and muscle were carried out. Lipids decreased in the three tissues when water content increased, although in gonad and muscle, the relationship adjusted to a quadratic polynomial model whereas in liver the best fit was obtained with a

linear model. On the contrary, proteins showed a negative relationship with water content in gonad and muscle, but not for liver, that fitted better to a quadratic polynomial model. However, this model showed the best fit for glycogen content in the three tissues. Energy density was negative related to water content, and linear regression offered the best model in the three tissues (Figure 3.57). In any case, all relationships of lipid content, protein content, glycogen content and energy density with water content in all studied tissues were significant (Table 3.16).

3.6 FECUNDITY AND EGG ESTIMATION OF *MERLUCCIUS MERLUCCIUS* IN GALICIAN SHELF

To estimate stock reproductive potential, it is necessary to know the egg production of the spawning stock. With this aim, potential, relative, and batch fecundity and density of developing oocytes are usually estimated. In the case of European hake, taking into account that it is indeterminate specie, it is impossible to know potential fecundity exactly because new oocytes are going to be incorporated during the spawning season to the maturing oocyte pool. Consequently the term Number of Developing Oocytes (NDO) has been used to define the number of developing oocytes present in the ovary at a certain moment of the spawning season. To eliminate the effect of female size on oocyte production in order to compare females, the number of developing oocytes per gram of female gutted weight was estimated, which is called relative NDO (NDO_{rel}). Because European hake is a partial spawner, the number of oocytes developed per batch (batch fecundity; BF) is an interesting reproductive parameter for reproductive potential studies. Both, NDO and BF were estimated using different methods. First of all, the accuracy of these methods was analysed (Section 3.6.1). After this, the influence of maternal attributes and somatic condition on NDO, NDO_{rel}, BF and density of developing oocytes in the gonad (number of developing oocytes per gram of tissue) was studied and their temporal variations analysed (from Section 3.6.2 to Section 3.6.5). Finally, realized fecundity was estimated based on batch fecundity, spawning fraction and number of batches per spawning season (Section 3.6.6).

3.6.1 COMPARISON OF METHODS TO ESTIMATE FECUNDITY

Three different methods used to estimate fecundity were compared. To estimate the number of developing oocytes (NDO), results from Image Analysis (IA) and Strereology (Ste) methodologies were compared, while for batch fecundity (BF) an additional method was used, the Manual Gravimetric method (MAN). To avoid female size effects, NDO and BF per gram of ovary were used as dependent variables.
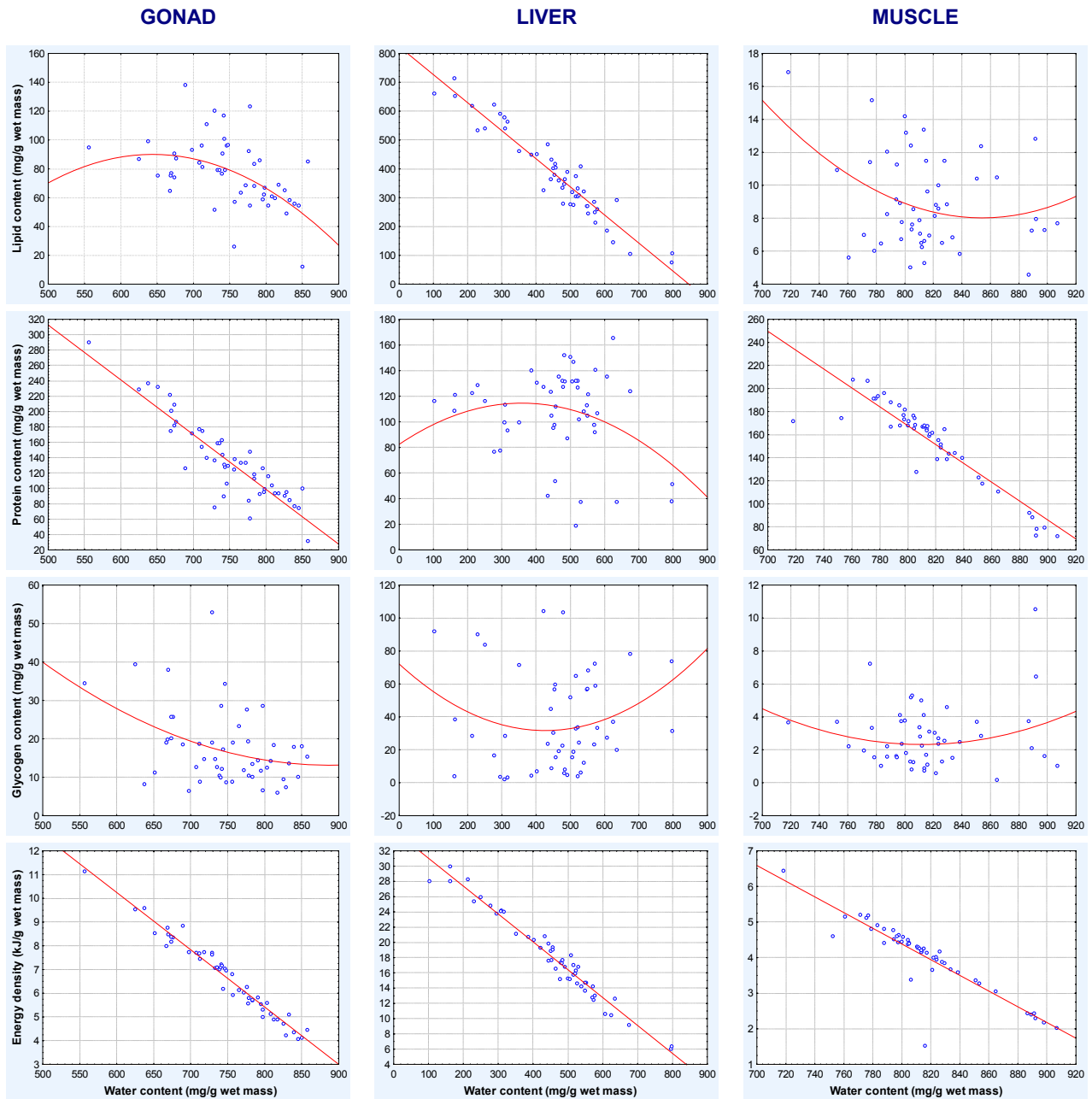
Figure 3.57: Scatterplot of lipid content (mg/g of wet mass), protein content (mg/g of wet mass), glycogen content (mg/g of wet mass) and energy density (kJ/g of wet mass) in gonad, liver and muscle versus water content (mg/g of wet mass) of these tissues.

Stereology showed slightly lower values than Image Analysis (F=5.242; p=0.02, Figure 3.58 and Table 3.17), when estimating NDO. Differences among methods were also detected in batch fecundity, but between Image Analysis and Manual Gravimetric method (F=3.821; p=0.02, Figure 3.58). In batch fecundity cases, data variability was higher when the stereological method was used. On the other hand, the Manual Gravimetric method presented a smaller standard error range.

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Table 3.16: Relationship between lipid content (L; mg/g of wet mass), protein content (P; mg/g of wet mass), glycogen content (G; mg/g of wet mass) and energy density (E; kJ/g of wet mass) in gonad (G), liver (L) and muscle (M) and the water content (W; mg/g of wet mass) in these tissues. Explained variance and significance level *p <0.001 are given.

		Explained Variance	Equation
Gonad	**Lipid (L_G)**	0.266*	$L_G = -307.66 + 1.24W_G - 0.001W_G^2$
	Protein (P_G)	0.804*	$P_G = 668.95 - 0.71\ W_G$
	Glycogen (G_G)	0.151*	$G_G = 153.90 - 0.32\ W_G + 0.0002\ W_G^2$
	Energy density (E_G)	0.962*	$E_G = 24.74 - 0.02\ W_G$
Liver	**Lipid (L_L)**	0.908*	$L_L = 824.01 - 0.97\ W_L$
	Protein (P_L)	0.084*	$P_L = 82.48 + 0.18\ W_L - 0.0003\ W_L^2$
	Glycogen (G_L)	0.058*	$G_L = 72.06 - 0.19\ W_L + 0.0002\ W_L^2$
	Energy density (E_L)	0.962*	$E_L = 34.69 - 0.04\ W_L$
Muscle	**Lipid (L_M)**	0.108*	$L_M = 226.87 - 0.51\ W_M + 0.0003\ W_M^2$
	Protein (P_M)	0.830*	$P_M = 823.02 - 0.82\ W_M$
	Glycogen (G_M)	0.053*	$G_M = 116.40 - 0.28\ W_M + 0.0002\ W_M^2$
	Energy density (E_M)	0.799*	$E_M = 22.02 - 0.22\ W_M$
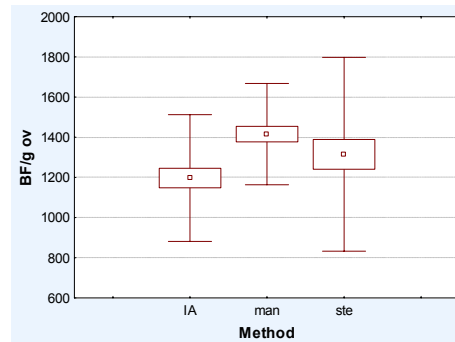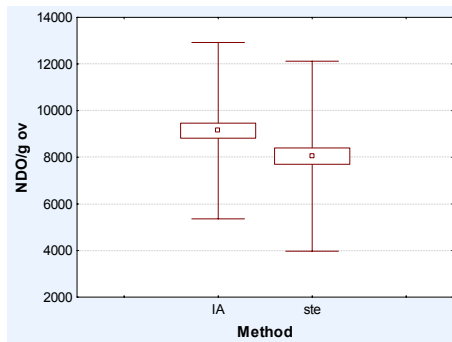
Figure 3.58: Mean values (mid point) of number of developing oocytes (NDO) and batch fecundity (BF) per gram of ovary estimated by each methodology. IA = Image Analysis, Ste = Stereology and man = Manual method. Standard error (box) and Standard deviation (wishker) were also represented.

Table 3.17: Results of the ANOVA to compare methods used to estimate NDO and BF per gram of ovary.

	N	F	p
NDO/g ov	272	5,24	0,023
BF/g ov	126	3,82	0,025

To test the accuracy of the different methods, analysis of covariance of the estimates of NDO and BF relating to length were carried out. Results were:

- Nº of developing oocytes (NDO): As expected, NDO changed significantly with length (p<0.001), regardless of method, although regression fits were not good (r^2=0.48 for image analysis and r^2=0.41 for stereological method). The intercept was not different between the two regression lines (F=0.32, p=0.574), but the slopes were significantly different between methods (F=10.87, p<0.01). Stereological methods tended to underestimate NDO, especially at larger female sizes. This could be a consequence of differences between studied females that were not always the same in both method estimates (Figure 3.59). The general analysis of covariance (ANCOVA) showed significant differences between both methods (F=113.30, p<0.001).

- Batch fecundity (BF): Batch fecundity also increased with length, independently of the method (p<0.01), but, again, goodness of fit was poor (r^2=0.18; r^2=0.32 and r^2=0.29 for image analysis, stereology and manual method respectively). However, in this case at small female sizes, the manual method showed higher values of batch fecundity than the other two methods, but when length increased; it was the stereological method which gave the highest values (Figure 3.59). The general analysis of covariance (ANCOVA) showed significant differences between the three methods (F=14.44, p<0.001)

Figure 3.59: Linear regression between natural logarithm of number of developing oocytes (ln NDO) and natural logarithm of batch fecundity (ln BF) using different methods. IA = Image Analysis; Ste = Stereology and Man = Manual Gravimetric.

3.6.2 NUMBER OF DEVELOPING OOCYTES (NDO)

As mentioned previously, the number of developing oocytes was estimated instead of potential fecundity because European hake is an indeterminate species, and it is impossible to know how many oocytes are going to develop during the spawning season, since new oocytes are added to the developing pool during it. In this section, firstly the influences of maternal attributes and somatic condition factors are analysed and secondly, temporal trends in NDO are studied. A strong variability of NDO was observed between females, mainly because those females that were at the beginning or near the end of their spawning periods had lower NDO values than females that were at the midpoint.

As regards of influence of female attributes and condition factors on NDO, a significant power relationship was established with length, although regression between variables was moderate (r^2=0.461, p<0.001 Figure 3.60 and Table 3.18). Data were more scattered at larger lengths, indicating some chances of heteroscedasticity, or non-normality on fecundity, probably due to the presence of females which had already initiated spawning. A linear relationship with gutted weight was significant too (r^2=0.478, p<0.001; Figure 3.60), and was again moderate. For gonad weight, linear regression established with NDO was significant (r^2=0.544, p<0.001; Figure 3.60), with the highest correlation (0.73) among all the variables analyzed. Relationships between NDO and liver weight, GSI, HSI and K were in all cases significant (Table 3.18 and Figure 3.60), but their coefficients of determination were rather low, especially for HSI and K that showed levels of significance above 0.01.

*Table 3.18: Determination coefficients and significance level of regressions between NDO and BF and maternal attributes. * indicates power regression, otherwise linear.*

	NDO	
	r^2	p
Length*	0.46	≤0.001
Gut W	0.48	≤0.001
Gonad W	0.54	≤0.001
GSI	0.22	≤0.001
Liver W	0.28	≤0.001
HSI	0.01	0.040
K	0.01	0.040

To check which of these somatic and condition variables had greater influence on NDO, a multiple regression was carried out. In the model, five variables were included (gonad weight, gutted weight, liver weight, HSI y K) that explained 65.8% of total NDO variability. However, gonad weight alone explained 55.6% of this variability, which can easily be an artefact considering that to calculate NDO, the number of oocytes in each sub-sample was extrapolated to total ovary weight. When observed values *versus* predicted values were represented, a moderate relationship was established between them (r^2=0.63, p<0.001; Figure 3.61)

As mentioned, the high dispersion of data observed could be the consequence of the females having initiated spawning already. However, the fact that four different years have been included in the previous study may also have had an influence. To compare fecundity among years, two analyses of covariance were carried out, one considering all years and another only 2003 and 2004, the years when more observations were available. The Independent variable employed in this analysis was natural logarithm of length.

NDO increased significantly with length every year (Table 3.19). Besides, the slopes of length-NDO relationships differed significantly between years. At small sizes, females of 1999 showed the highest NDO, and 2000 had the lowest values (Figure 3.62.a). On the contrary, at large sizes, females with the highest NDO were recorded in 2004 and 2000, and the lowest values for the same length females were detected in 1999. The highest regression coefficient was estimated for 2004 (r^2=0.62) and the lowest for 1999 (r^2=0.29). So, it was considered that 1999 and 2000 (n<50) had insufficient observations to obtain a reasonably good regression coefficient. Because of this, analysis was repeated with specimens from only 2003 and 2004 (n>100). In this case, differences between years were also significant (F=147.68; p<0.001; Table 3.19 and Figure 3.62.b).
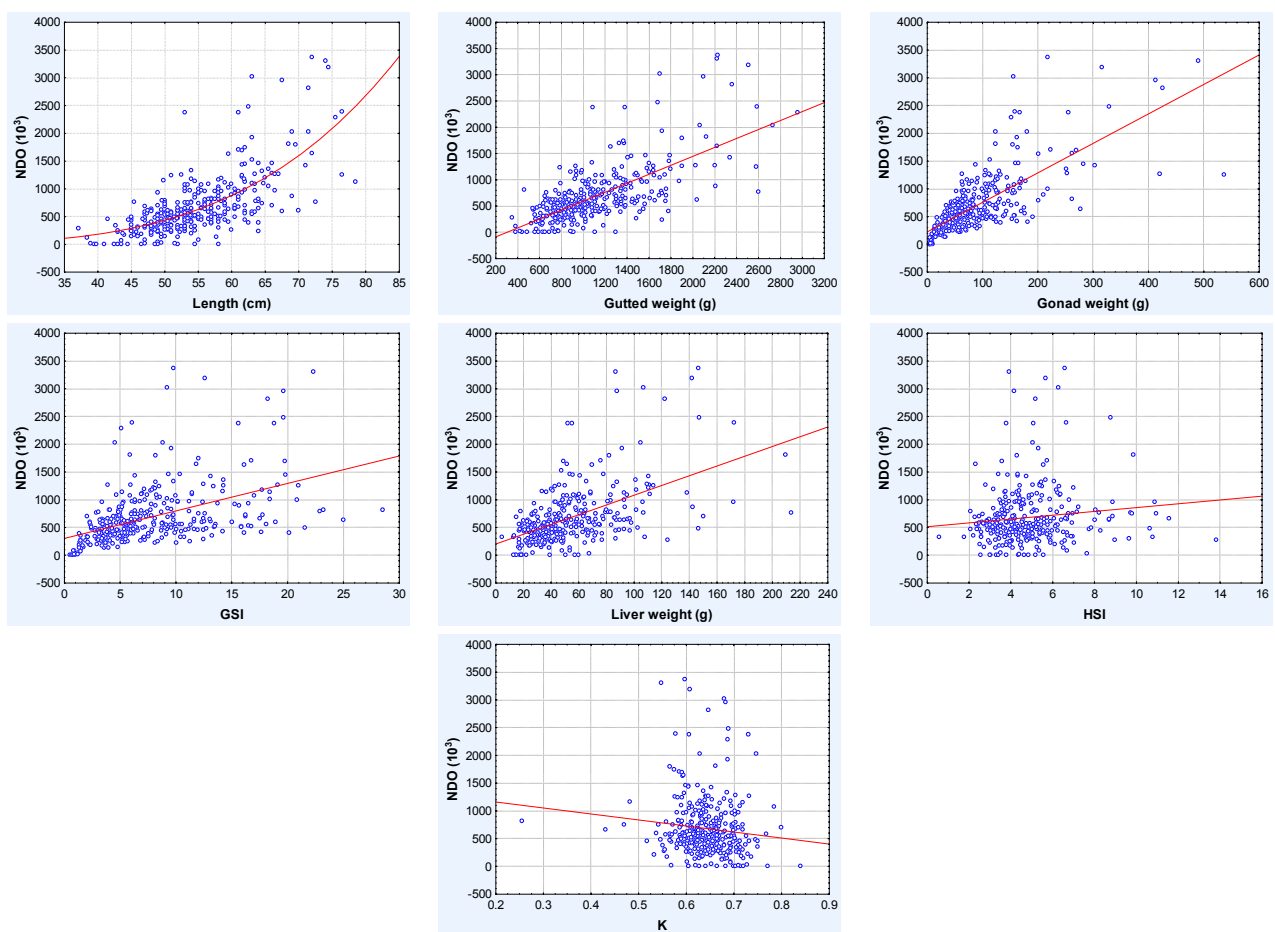
Figure 3.60: Number of developing oocytes (NDO) versus female attributes and somatic condition indices.

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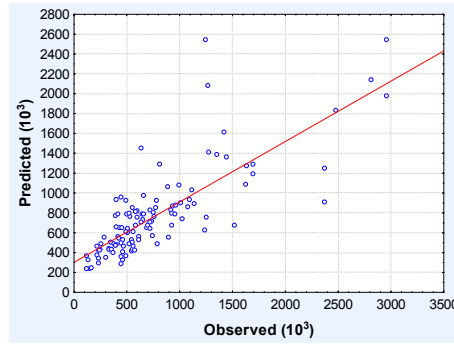
Figure 3.61: Observed versus predicted values of number of developing oocytes (NDO) from multiple linear regression with maternal attributes and somatic condition factors ($r^2 = 0.63$, $p<0.001$).

Table 3.19: Results of the linear regression between NDO and length by year and ANCOVA results of analysis carried out with all years together, only with 2003 and 2004 using length as covariate.

	r^2	p	F
1999	0.29	≤0.001	
2000	0.36	0.001	
2003	0.40	≤0.001	
2004	0.62	≤0.001	
ANCOVA (99-04)		≤0.001	87.18
ANCOVA (03-04)		≤0.001	147.68

a)
b)

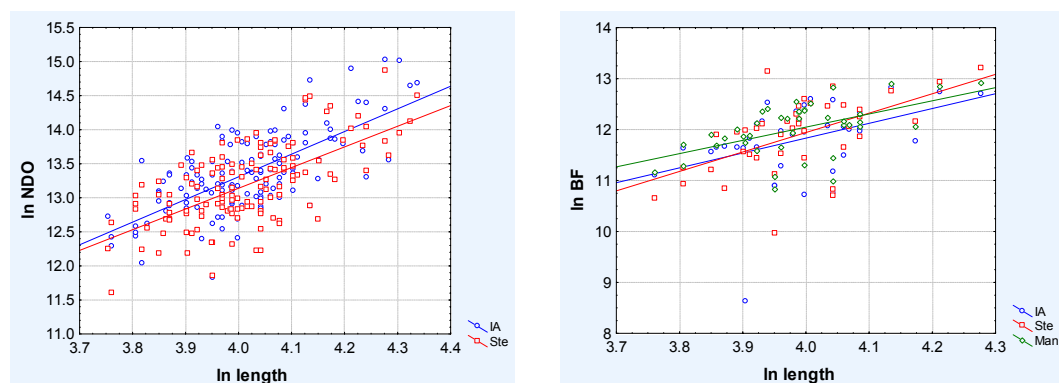Figure 3.62: Linear regression between natural logarithm of number of developing oocytes (ln NDO) and natural logarithm of length (cm) in a) 1999-2000; 2003-2004 periods and b) only 2003-2004 period.

Annual mean NDO increased steadily from 1999 to 2004, with mean NDO lower than 630,000 oocytes during the first three years and increasing to 820,000 in the last one (Figure 3.63), although the range of variation was higher in 2004. The ANOVA performed to compare NDO among years yielded significant differences (F=4.740, p=0.003), but the *post hoc* analysis showed no difference among 1999, 2000 and 2003, and only 2004 was statistically different from the other years.

The monthly mean NDO (all years pooled) showed a diminishing of NDO from January, 1,200,000 oocytes, to April, 500,000 oocytes approximately (Figure 3.63), that may indicate

spawning activity during these months, with the end of the spawning by April-May. After April, NDO stabilized and, although its value oscillated depending on the month considered, it remained at the March-April level. Significant intra-annual differences were detected (F=3.77, p<0.001), with January statistically different from the other months and February different from the April-August period. When the monthly trend of NDO was studied by year, the same significant tendency was observed excepting 1999 and 2000, in which the lack of data do not allow any pattern to be identified (Figure 3.64). In this case, analysis of variance also showed significant differences (F=2.30, p<0.001).
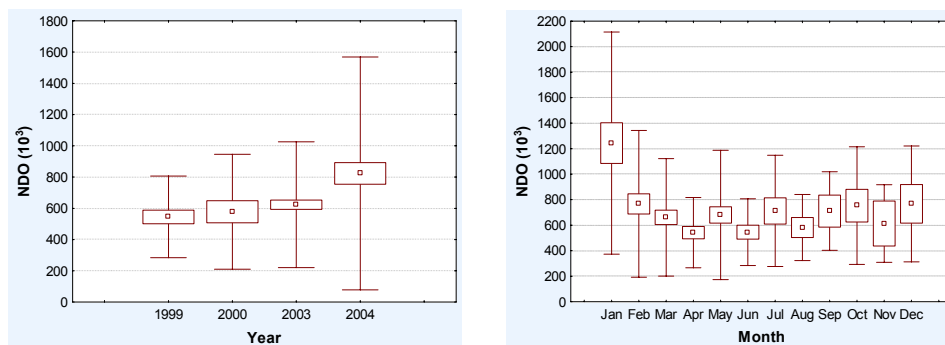
Figure 3.63: Annual and monthly mean (mid point) of number of developing oocytes (NDO). Standard error (box) and standard deviation (wishker) are also represented.

Figure 3.64: Monthly mean NDO by year (mid point). Standard error (box) and standard deviation (wishker) are also represented.

3.6.3 RELATIVE NUMBER OF DEVELOPING OOCYTES (NDO$_{REL}$)

As with NDO, the NDO$_{rel}$ was significantly related with all study variables (Table 3.20), except HSI. NDOrel-GSI relationship was found, however, to best fit a linear regression, as the rest of variables. In spite of the significance of the goodness of fit, most of the relations showed very low determination coefficients, i.e. a high variability of the residuals and a poor prediction power (Figure 3.65). All except gonad weight and GSI showed r^2 below 0.1. For gutted weight, HSI and K, this is a logical result since weight is part of the NDO$_{rel}$ estimation. However, the poor fit with length and liver weight (r^2=0.07 and 0.04 respectively) revealed that NDO$_{rel}$ is independent of female size or growth and energetic reserves in liver. GSI and gonad weight showed the best prediction (r^2=0.40 and 0.33 respectively). However, in these two cases the relationships seems to depart from linearity. Other models were tested and logarithmic transformation of variables was applied because this approach seems to yield better results. Thus, determination coefficient of relationship with gonad weight increased from 0.33 to 0.39 and for NDO$_{rel}$-GSI relationship from 0.40 to 0.43, remaining in both cases significance p-levels below 0.001 (Figure 3.65). In any case, improvements were moderate.

Table 3.20: Results of the regressions between relative NDO and female attributes and somatic condition factors.

	NDO$_{rel}$	
	r^2	p
Length	0.07	≤0.001
Gut W	0.05	≤0.001
Gonad W	0.33	≤0.001
Ln gonad W	0.39	≤0.001
GSI	0.40	≤0.001
Ln GSI	0.43	≤0.001
Liver W	0.04	0.001
HSI	≤0.01	0.430
K	0.05	≤0.001

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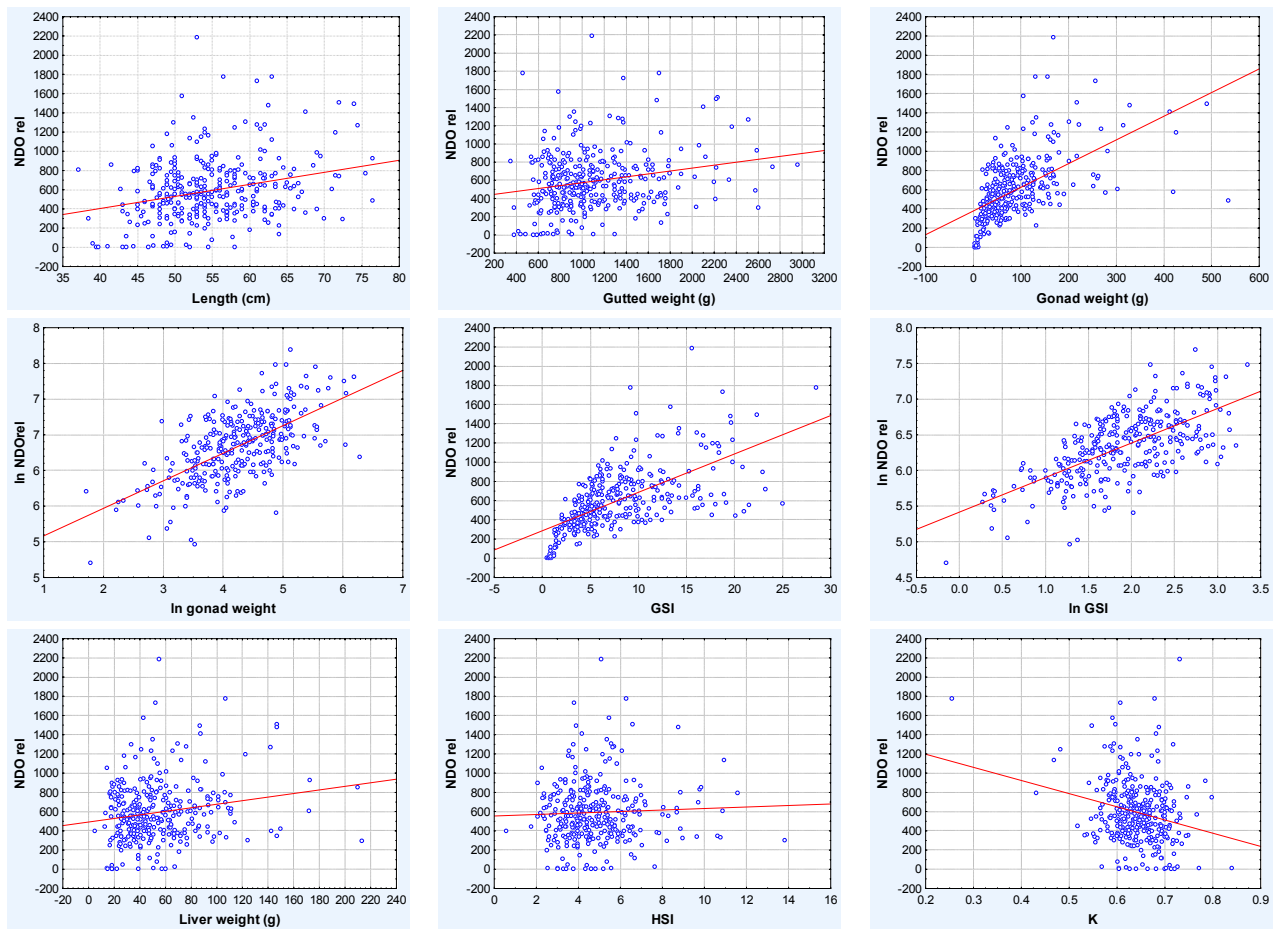
Figure 3.65: Relative number of developing oocytes (NDOrel) versus female attributes and somatic condition indices.
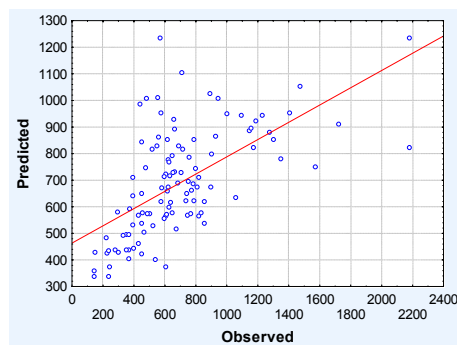
Figure 3.66: Observed versus predicted values of relative number of developing oocytes (NDO$_{rel}$) from multiple linear regression with maternal attributes and somatic condition factors ($r^2 = 0.37$, $p<0.001$).

Despite the large variability observed in the residuals for most of the relationships, a multiple regression model was investigated to ascertain if NDO$_{rel}$ has a relationship with several predictors. Only three variables were included in the model (GSI, liver weight and K)

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that explained 41.1% of relative NDO total variability, GSI being the most important variable, because it explained 37% of this variance (Figure 3.66).

Analysis of covariance between the four years considered and only 2003 and 2004, using natural logarithm of length as the continuous variable, was also carried out this time. It was observed that relative NDO increased with length in a significant way only in 2004, and not in 2000 or 2003; furthermore, in 1999, NDO_{rel} decreased with length although not significantly (Table 3.21 and Figure 3.67.a). When analysis was repeated only with 2003 and 2004 data, differences between years were also significant (F=15.71; p<0.001; Table 3.21) and relative NDO was higher in 2004, especially in larger females (Figure 3.67.b). In any case, the regression coefficient of 2004 was rather low.

Table 3.21: Results of the linear regression between natural logarithm of NDO_{rel} and length by year and ANCOVA results of analysis carried out with all years together, only with 2003 and 2004 using natural logarithm of length as covariate.

	r^2	p	F
1999	0.06	0.208	
2000	0.03	0.422	
2003	0.01	0.124	
2004	0.09	≤0.001	
ANCOVA (99-04)		≤0.001	8.44
ANCOVA (03-04)		≤0.001	15.71

The mean annual relative NDO ranged from 524 oocytes/g female in 1999 to 696 oocytes/g in 2004 (Figure 3.68). The overall ANOVA resulted in significant differences between years (F=6.16, p<0.001). However, the *post hoc* test revealed that only 2004 showed such differences, while relative NDO did not differ statistically between 1999, 2000 and 2003. On the other hand, the monthly average NDOrel showed a similar trend to NDO (F=6.25, p<0.01; Figure 3.68). The highest values were recorded in January (947 oocytes/g) decreasing steadily until April (446 oocytes/g), which indicates also spawning activity. January showed significantly different mean values compared with the rest of the months. In the following months, a clear pattern is not observed. However, it is remarkable that July, September and December showed relatively high values, and August and November smaller ones, indicating probable spawning activity also in summer.

Analysis of variance of NDOrel monthly mean for each year was carried out to detect differences in its temporal evolution between studied years. In the four years considered, NDOrel decreased significantly from the beginning to the end of the year, and differences between years were also significant (F=3.35, p<0.01). In 2003 and 2004 a slightly increase in monthly mean NDOrel was observed in July, this rise was not detected in 1999 and 2000 because of the lack of enough data (Figure 3.69).
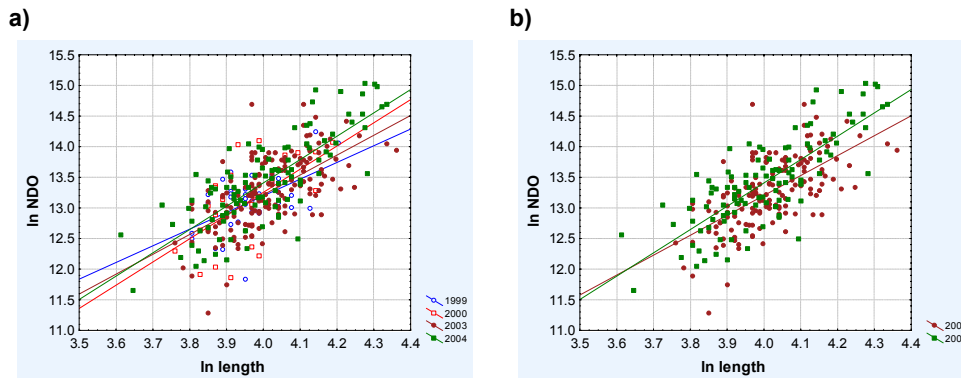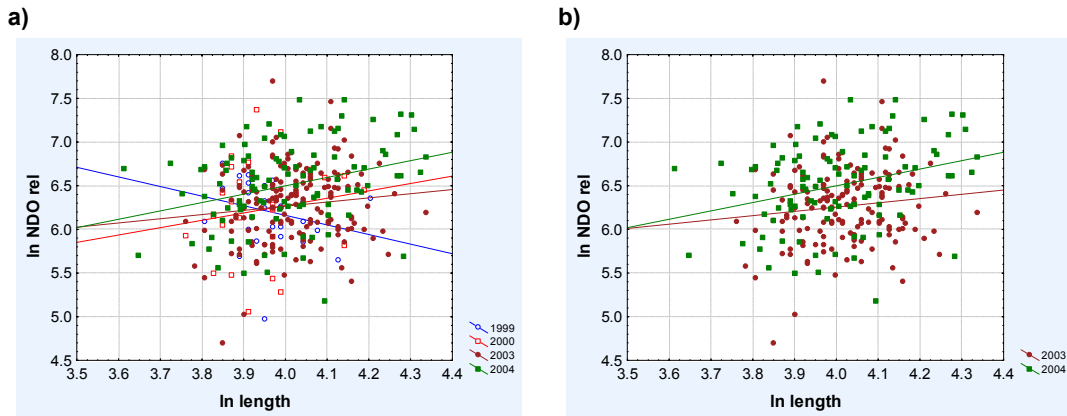
Figure 3.67: Linear regression between natural logarithm of relative number of developing oocytes (ln NDO$_{rel}$) and natural logarithm of length (cm) in a) 1999-2000; 2003-2004 periods and b) only 2003-2004 period.
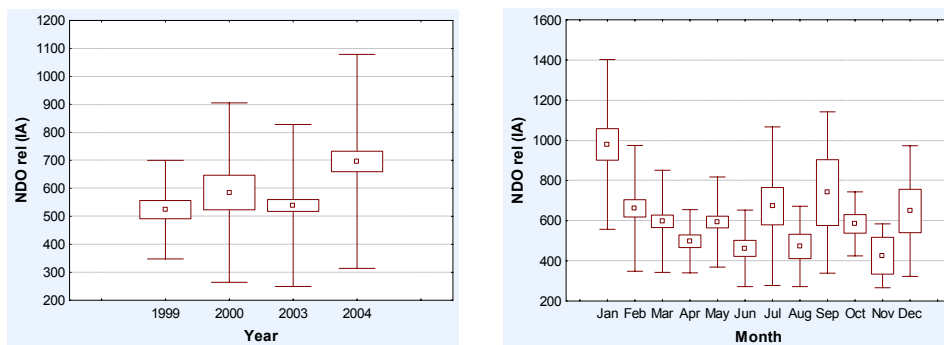
Figure 3.68: Annual and monthly mean NDO$_{rel}$ (mid point). Standard error (box) and standard deviation (wishker) are also represented.
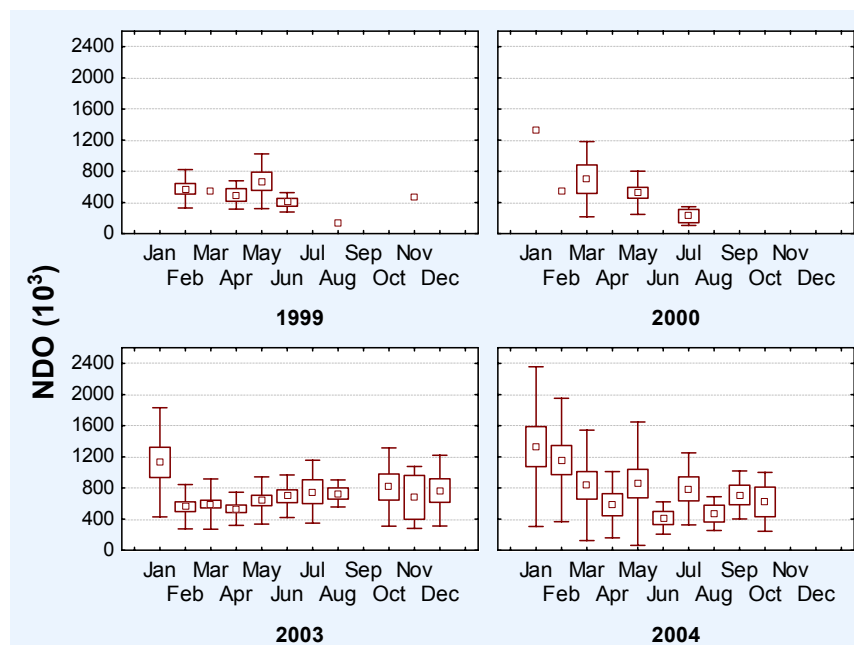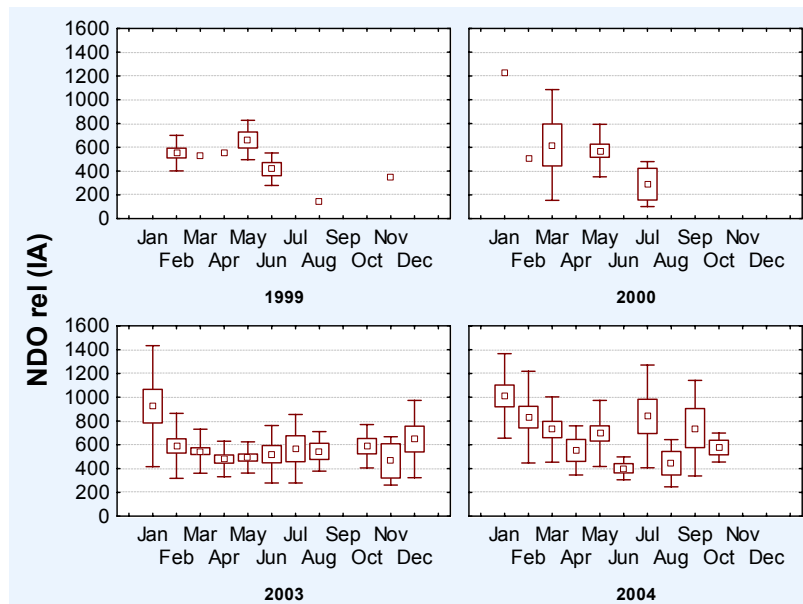

Figure 3.69: Monthly mean NDO$_{rel}$ by year (mid point). Standard error (box) and standard deviation (wishker) are also represented.

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3.6.4 BATCH FECUNDITY (BF)

Attending to the influence of maternal attributes and somatic condition factors, it was observed that a power model was the best predictor for the batch fecundity-length relationship, while for other variables a linear model was the best option (Figure 3.70 and Table 3.22). In the first case, BF increased with length and the relationship between variables was moderate (r^2=0.47, $p<0.001$); data dispersion increased when length increased. Relationship with gutted weight was also positive and significant but linear, but the determination coefficient was relatively low (r^2=0.45, $p<0.001$). Gonad weight was positively related to BF, and the linear regression established between them was significant too, and had the highest determination coefficient (r^2=0.90, $p<0.001$). The liver weight relationship was also positive and significant, but the relation between variables was moderate (r^2=0.30, $p<0.001$). On the contrary, GSI had a higher determination coefficient, though it was not as good as those from gonad weight and batch fecundity relationships (r^2=0.54, $p<0.001$). Neither HSI nor K showed a significant relationship with BF (r^2=0.01, $p=0.270$ and r^2=0.01, $p=0.280$ respectively).

In general, relationships between batch fecundity and maternal attributes and somatic condition factors were better than those between these factors and NDO, especially in relation to gonad weight and GSI. This suggests that increase in gonad weight is derived from the hydration process, those ovaries without hydrated oocytes show lower weight changes.

When multiple regressions was carried out using somatic and condition factors as explanatory variables, it was observed that most of the variability in batch fecundity was explained by ovary weight (89.8%), that was the only variable included in the model by the analysis (Figure 3.71). This is important because the gonad weight, or related indices such as GSI, could be potentially used as batch fecundity indices.

Analysis of covariance was applied to detect if differences between years existed, and it was observed that, on the one hand, batch fecundity increased significantly with length in all years studied and on the other hand, differences between years were also significant (F=39.38, $p<0.001$; Table 3.23). 2000 showed the lowest values of batch fecundity for every observed size followed by 1999. However, 2003 and 2004 show similar values of batch fecundity between them and higher than the other two years when females were small, but when length increased, batch fecundity of 2003 grew at slower rate reaching lower values than large females of 1999. In any case, batch fecundity of females from 2004 was the highest for any size (Figure 3.72.a). If 1999 and 2000 were removed because of lower number of sampled females, analysis showed that significant differences also exist between 2003 and 2004 (F=50.26, $p<0.001$; Table 3.23), mainly at large female sizes, and females of 2004 had higher batch fecundity than 2003 females (Figure 3.72.b).

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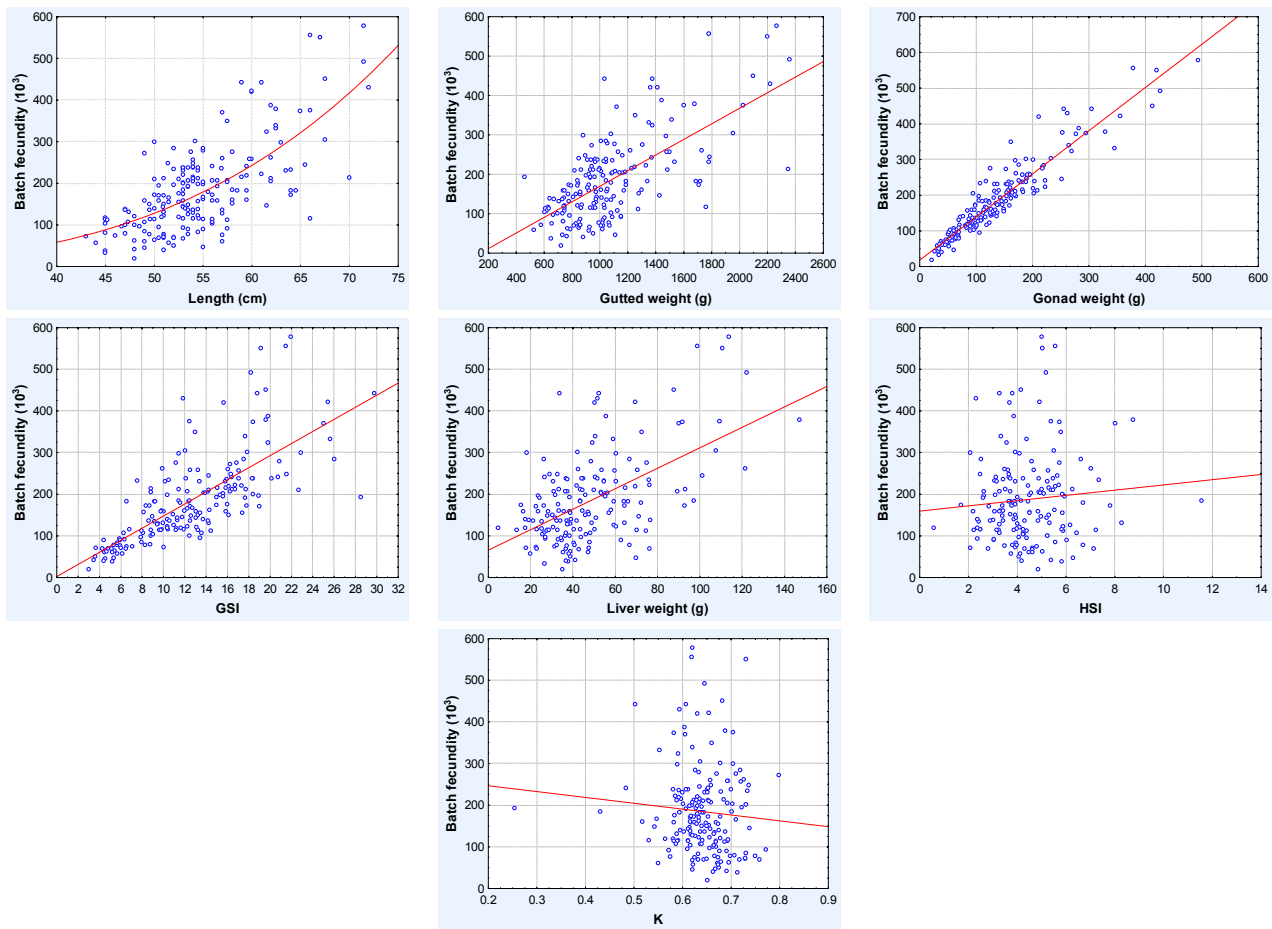
Figure 3.70: Scatterplot of individual Batch fecundity (BF) versus female attributes and somatic condition indices.

Table 3.22: Results of regressions between batch fecundity (BF) and maternal attributes and condition. All regressions are linear except where indicated (*).

	BF	
	r^2	p
Length*	0.47	≤0.001
Gut W	0.45	≤0.001
Gonad W	0.90	≤0.001
GSI	0.54	≤0.001
Liver W	0.30	≤0.001
HSI	0.01	0.270
K	0.01	0.280
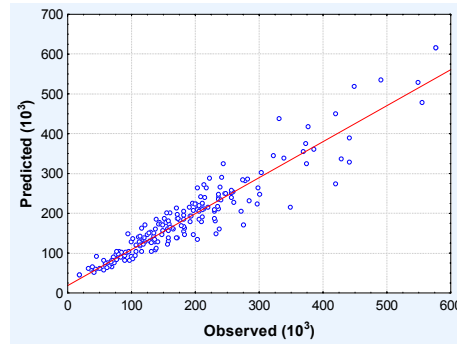
Figure 3.71: Observed versus predicted values of batch fecundity (BF) from multiple linear regression with maternal attributes and somatic condition factors (r^2 = 0.90, p<0.001).

Table 3.23: Results of the linear regression between natural logarithm of batch fecundity (ln BFI and length by year and ANCOVA results of analysis carried out with all years together, only with 2003 and 2004 using natural logarithm of length as covariate.

	r^2	p	F
1999	0.32	0.005	
2000	0.38	0.011	
2003	0.38	≤0.001	
2004	0.42	≤0.001	
ANCOVA (99-04)		≤0.001	39.38
ANCOVA (03-04)		≤0.001	50.26
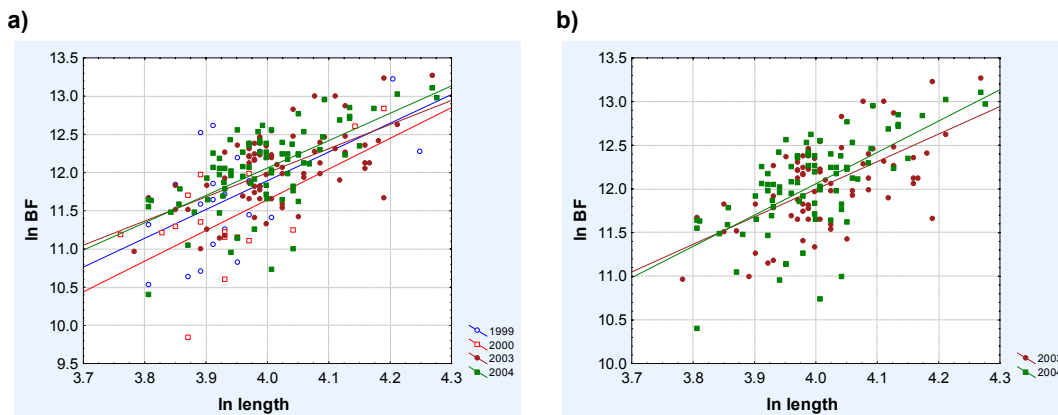
Figure 3.72: Linear regression between natural logarithm of batch fecundity (lnBF) and natural logarithm of length (cm) in a) 1999-2000; 2003-2004 periods and b) only 2003-2004 period.

Batch fecundity decreased slightly from 1999 to 2000 and increased drastically from 114,655 eggs in 2000 to 195,283 eggs in 2003, remaining at the same levels in 2004 (Figure 3.73). Significant annual differences were detected in batch fecundity, *post hoc* analysis showed that 2000 was significantly different of 2003 and 2004 (F=3.816, p<0.05).

Concerning monthly trends, it was observed that batch fecundity decreased from January (222,861 eggs) to April (136,414 eggs), followed by a progressive increase until

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September (194,961 eggs), decreasing again in October and November (144,765 and 114,361 respectively), and rising again in December (241,619 eggs; Figure 3.73). These monthly variations were significantly different (F=2.17, p<0.05). When monthly trends of each year separately were studied, although the general trend was similar to the monthly trend when all years were considered together, it was observed that in 2003 an important increase in batch fecundity took place in May, and in 2004 a peak of batch fecundity was also observed between July and August. An important increase in BF was also observed in 1999 during May. Nothing similar was detected in 2000, probably because sampled females in summer were scarce in this year (Figure 3.74). In any case, analysis of variance showed significant monthly differences by year too (F=2.00, p<0.01).
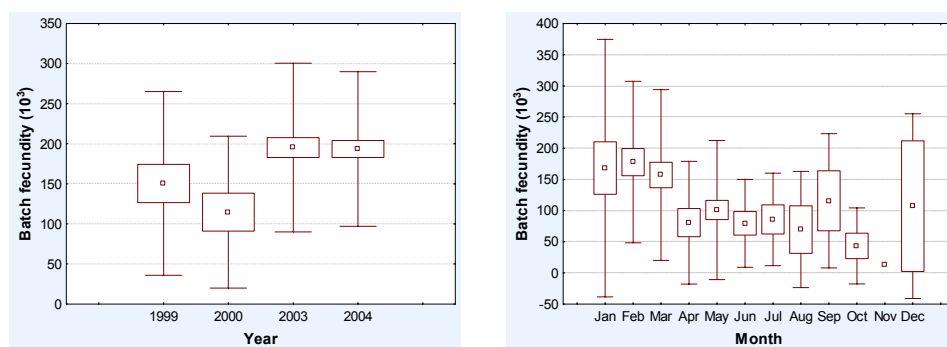
Figure 3.73: Annual and monthly mean (mid point) of batch fecundity (BF). Standard error (box) and standard deviation (wishker) are also represented.
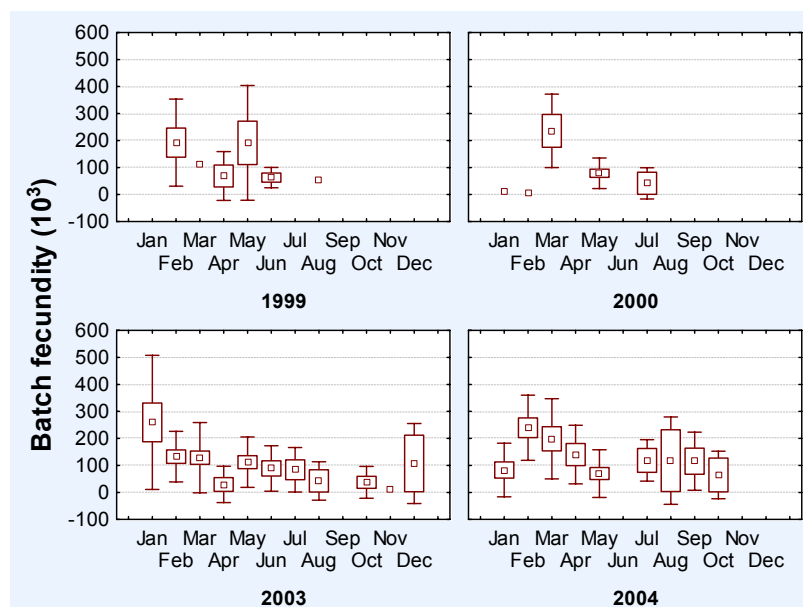
Figure 3.74: Monthly mean batch fecundity (BF) by year (mid point). Standard error (box) and standard deviation (wishker) are also represented

3.6.5 DENSITY OF DEVELOPING OOCYTES IN GONAD (NDO$_{DENS}$)

Density of developing oocytes, defined as number of developing oocytes per gram of gonad, was not related significantly either to length or gutted weight (p>0.3). Gonad weight and GSI showed the highest determination coefficient (r^2=0.31 and r^2=0.46 respectively) and, in contrast to previous occasions, the power model resulted in the best fit for these relationships, that were negative and significant (p<0.001). This suggests that increase of gonad weight is more influenced by increase of egg size than by egg number. Regarding liver weight, the relationship established with NDO$_{dens}$ was linear and significant, although it had a very low determination coefficient (r^2=0.039, p<0.001). About condition index, both HSI and K were significantly and positively related to NDO$_{dens}$ (p<0.001 and p<0.05 respectively), but in both cases determination coefficients were very low again (r^2<0.1; Figure 3.75 and Table 3.24).

The Influence of each variable on NDO$_{dens}$ was analyzed by multiple regression analysis following the forward stepwise method. It was observed that 45.3% of variability was explained by the four variables included in the model (GSI, HSI, gonad weight and length) and 40.4% was explained by GSI alone which could be considered a rather good proxy of NDO$_{dens}$.

Analysis of covariance was made using the natural logarithm of GSI as a continuous predictor because length was not significantly related to NDO$_{dens}$, and GSI showed the best relationship. In all years, density NDO$_{dens}$ decreased in a significant way with GSI, except in 2000. For the three significant relationships, 1999 showed the lowest values of density for every GSI, whereas 2003 was the highest values at lower GSI, though 2004 density values turned in highest values when gonads were larger and GSI higher (Figure 3.76.a). Significant differences were detected between years, both in their intercepts and slopes (F=86.37, p<0.001; Table 3.25). When 1999 and 2000 were removed because of the lower numbers of sampled females during these years, the same differences between 2003 and 2004 values of density were observed, and these differences were significant too (F=148.74, p<0.001; Figure 3.76.b).

Regarding annual variation of NDO$_{dens}$, it increased from 1999 to 2004 reaching a mean value of 10,056 oocytes/g in the last year, 2,388 oocytes/g more than in 1999 (Figure 3.77). 1999 was significantly different from 2003 and 2004, and 2000 was significantly different from 2004 too (F=4.71, p<0.01). Monthly significant differences were also recorded (F=2.86, p<0.01), NDO$_{dens}$ descending from 9,757 oocytes/g in January to 7,922 oocytes/g in March, and subsequently increasing to reach highest values in November-December (13,506 and 13,297 oocytes/g respectively; Figure 3.77). This increase of NDO$_{dens}$ is an indication of the decrease of oocyte diameter, probably because of the release of mature oocytes as eggs and the retention of immature oocytes in the gonad. When each year was considered

separately, although the general trend was similar to that described above, an increase was observed in June 2004, then NDO_{dens} decreased in July and rose progressively until December; this rise was detected two months earlier in 2003. The scarce data of 1999 and 2000 did not reveal any trend (F=2.44, p<0.001; Figure 3.78).
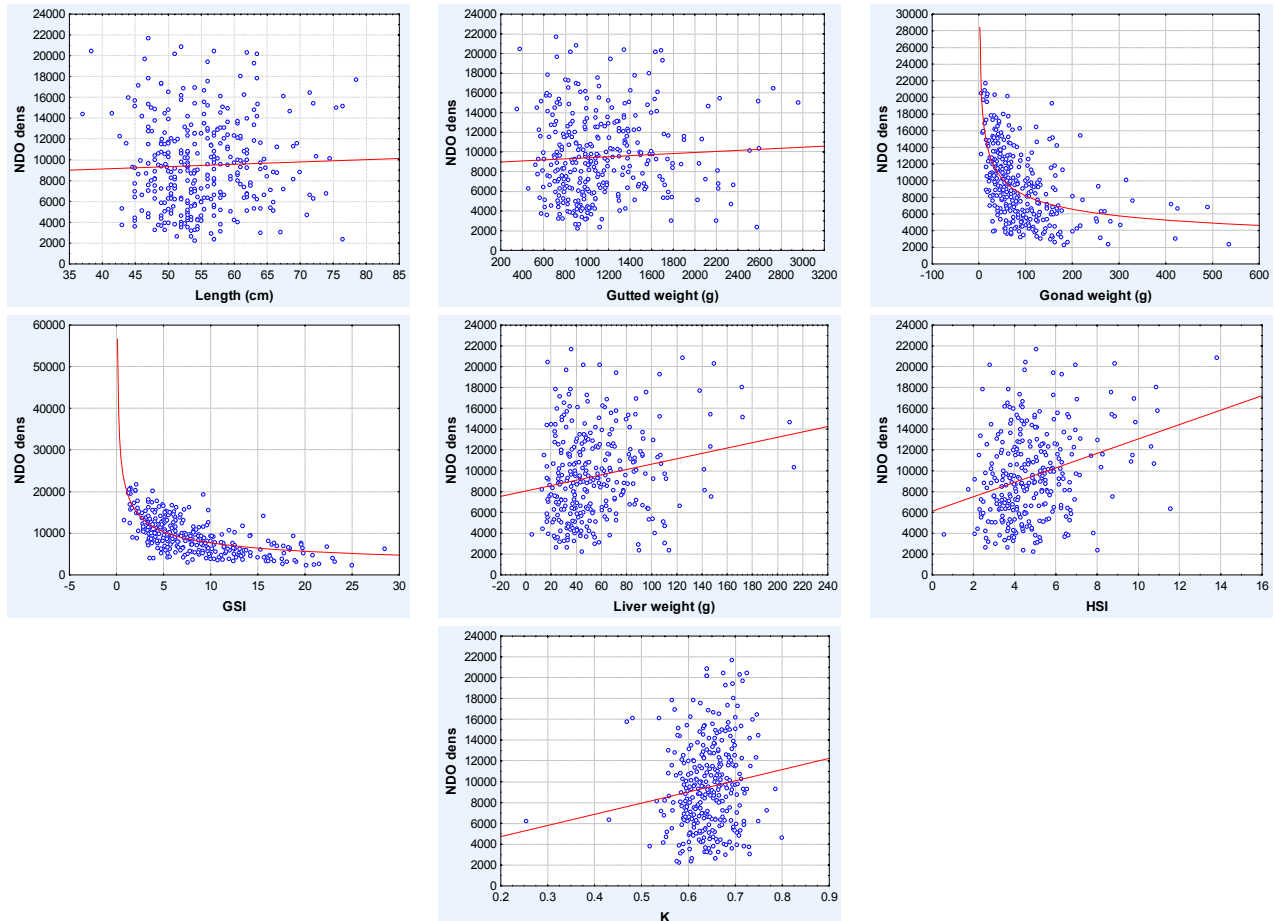
Figure 3.75: Scatterplot of density of developing oocytes (NDO_{dens}: oocytes/g of fresh gonad) versus female attributes and condition indices.

Table 3.24: Results of regressions between density of developing oocytes (NDO_{dens}) and maternal attributes and condition. All regressions are linear except where indicated (*).

	NDO dens	
	r^2	p
Length	≤0.01	0.494
Gut W	≤0.01	0.306
Gonad W*	0.31	≤0.001
GSI*	0.46	≤0.001
Liver W	0.04	≤0.001
HSI	0.09	≤0.001
K	0.029	0.012
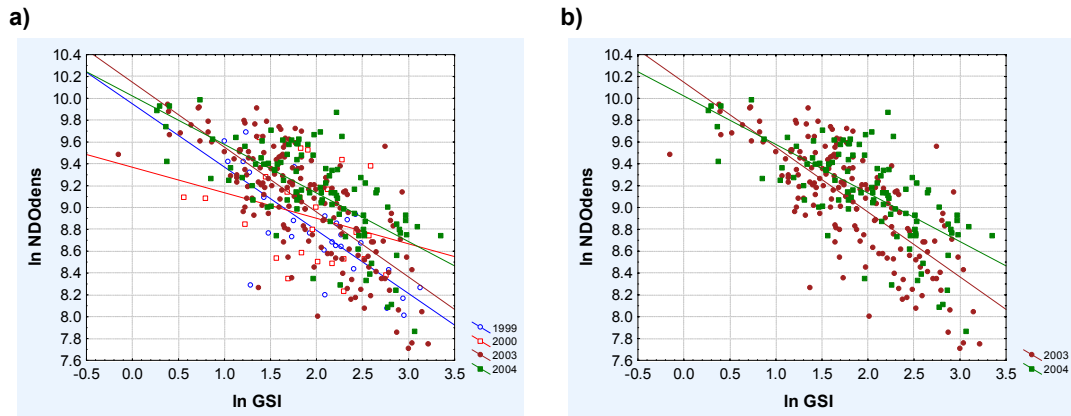
Figure 3.76: Linear regression between natural logarithm of developing oocytes density (ln NDO$_{dens}$) and natural logarithm of GSI (ln GSI) in a) 1999-2000; 2003-2004 periods and b) only 2003-2004 period.

Table 3.25: Results of the linear regression between natural logarithm of developing oocytes density (ln NDO$_{dens}$) and natural logarithm of GSI (ln GSI) by year and ANCOVA results of analysis carried out with all years together, only with 2003 and 2004 using natural logarithm of GSI as covariate.

	r^2	p	F
1999	0.60	≤0.001	
2000	0.09	0.126	
2003	0.55	≤0.001	
2004	0.48	≤0.001	
ANCOVA (99-04)		≤0.001	86.37
ANCOVA (03-04)		≤0.001	148.74
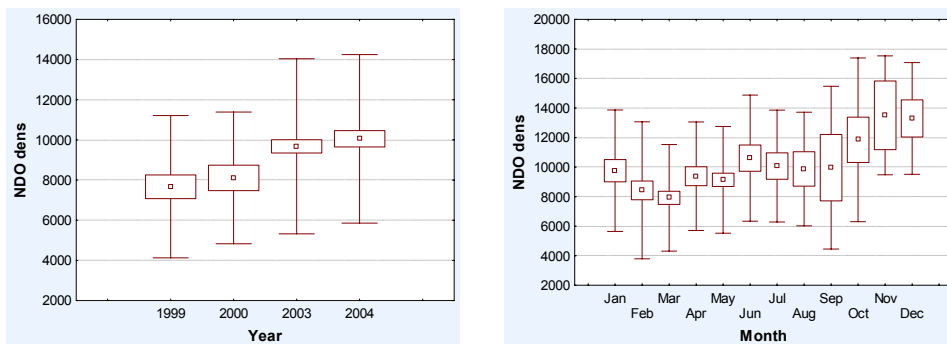
Figure 3.77: Annual and monthly mean (mid point) of developing oocytes density (NDO$_{dens}$). Standard error (box) and standard deviation (wishker) are also represented.

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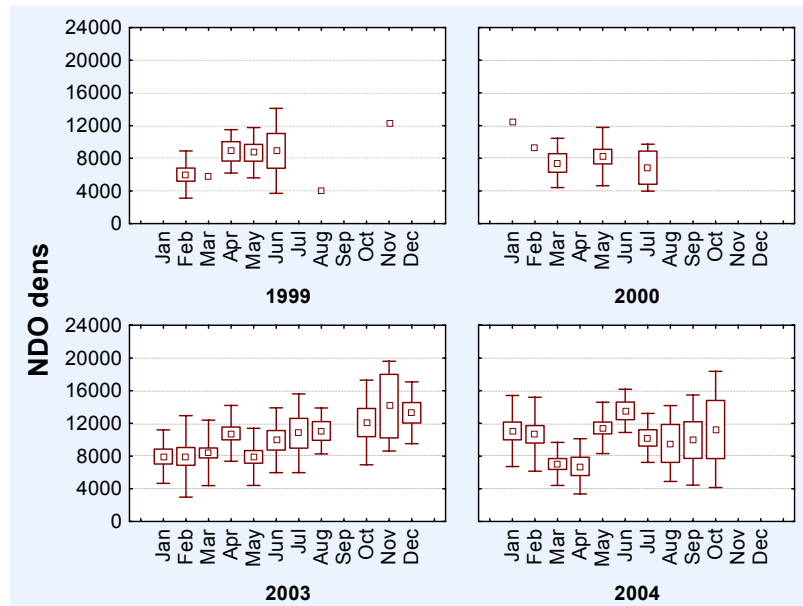
Figure 3.78: Monthly mean developing oocytes density (NDO_{dens}) by year (mid point). Standard error (box) and standard deviation (wishker) are also represented.

3.6.6 REALIZED FECUNDITY AND POPULATION EGG PRODUCTION

Estimates of individual annual fecundity in indeterminate partial spawner species like European hake, should start from batch fecundity (section 3.6.4) and spawning frequency (see section 3.1), and it is also necessary to know the duration of the individual spawning season. One of the objectives of this study was determination of the duration of individual hake spawning season from population samples; however, this has been impossible to estimate because the characteristics of hake reproductive behaviour, the population and individual spawning asynchrony, did not allow definitive conclusions in relation to individual spawning seasons. As the hake population spawns all year round, it could be assumed that the duration of *M. merluccius* individual spawning season, at least, would be of 2 months. To estimate egg production, only 2003 and 2004 samples were considered.

As a compromise solution, a 2-months spawning period was assumed for the present work. In accordance with this, European hake realized daily fecundity in 2003 for those females that spawn during the peak of spawning (January-February) would be 56±18 eggs per gram female gutted mass, when the production of 16 batches was estimated, i.e. one batch each 4 days (S=0.27), and an average batch fecundity of 195,814 eggs. During May-June, with intermediate levels of spawning activity, females produced a batch each 4.4 day, i.e. a total of 14 batches (S=0.23), with a mean batch fecundity of 100,370 eggs, resulting in an daily realized fecundity of 30±16 eggs per gram female gutted mass. Finally, during the less productive months, October-November, around 10±6 eggs per gram female gutted mass were released daily, with a mean of 6 batches (S=0.09) and 25,372 eggs per batch. In 2004, the realized daily fecundity during peak of spawning, in February and March,

was 47±14 eggs per gram female gutted mass, considering a total of about 13 batches (S=0.23) and an average batch fecundity of 218,837 eggs for this period. Daily realised fecundity drops sharply in April-May with around 17±11 eggs per gram female gutted mass, i.e. a mean of 12 batches (S=0.20) and 104,921 eggs of batch fecundity. Finally, around 11±2 eggs per gram female gutted mass were produced in September-October, i.e. an average of 6 batches (S=0.10) and 90,064 eggs per batch.

In general, the spawning fraction was higher in the winter months, and decreased afterwards as the spawning season progressed, though a secondary peak of spawning fraction was observed between May and July 2003 and in April 2004. Regarding relative batch fecundity, it tended to diminish from January to December in 2003, despite some fluctuations in some months. However, in 2004 relative batch fecundity was higher in July and August than in the winter months (Figure 3.79). As said before, both parameters in conjunction with the percentage of active females in the population were used to estimate the daily population egg production, that in general tend to decrease from the beginning to the end of the year, nevertheless a secondary minor peak was observed in daily egg production in May and June 2003 and in July 2004 (Figure 3.80).

Thus, assuming that duration of individual spawning season is 2 months (around 60 days), in 2003 annual egg production of those females that spawn during the peak of spawning (January-February) would range between 2,242 and 4,366 eggs per gram female gutted mass. For those that spawn between May and June it would be between 854 and 2,806 eggs per gram female gutted mass, and finally for those females that spawn at the end of the year (October-November) it would be from 244 to 976 eggs per gram. In 2004, the peak of spawning was observed in February-March, so females spawning during this period would produce annually from 1,947 to 3,599 eggs per gram female gutted mass. In the intermediate period (April-May), annual egg production would range between 366 and 1708 eggs per gram, and finally, those females that spawn at the end of the year (September-October) would produce annually between 549 and 793 eggs per gram female gutted mass.
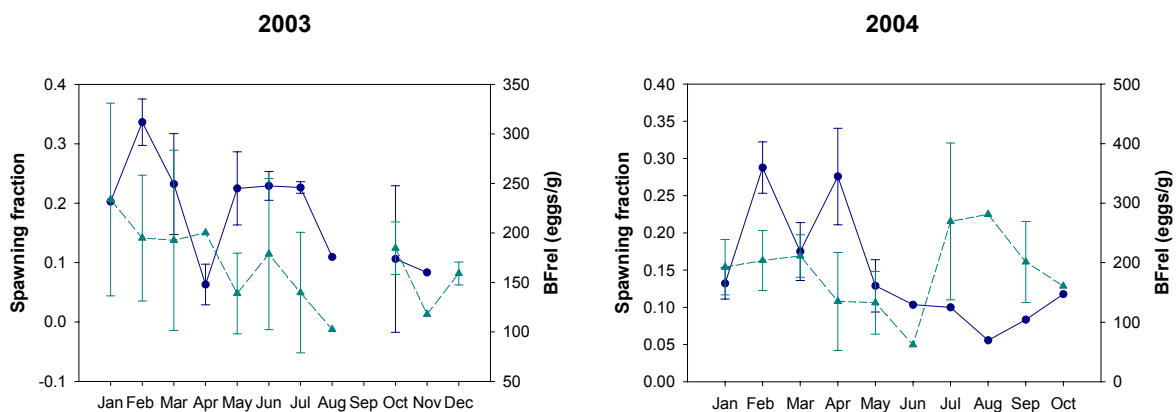
Figure 3.79: Monthly variation of spawning fraction (dark blue circles and solid line) and relative batch fecundity (cyan triangles and dashed line) in 2003 and 2004. Bas represents standard deviation.
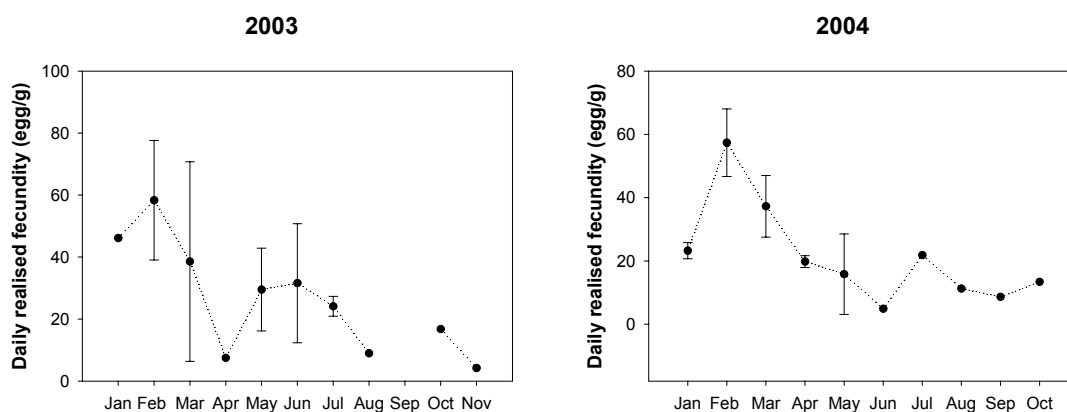
Figure 3.80: Monthly variation of mean daily egg production of European hake in 2003 and 2004. Bar represents standard deviation.

The figures above described of daily egg production are probably overall overestimates of the real figures, particularly in the months with lower spawning activity. The reason for this overestimation is basically derived from the presence of atresia condition and its impact on egg production; this issue is discussed in the following section.

3.7 ATRESIA ESTIMATES OF *MERLUCCIUS MERLUCCIUS* ON GALICIAN SHELF

All the histologically processed females with signs of atresia were further analyzed; it resulted in a total of 209 females. Atresia was determined by stereological method and both intensity and prevalence of atresia were estimated.

First of all, it was studied if atresia intensity was influenced by some female attributes or condition. As shown in Figure 3.81 there were no clear trends with any of the variables. The linear regressions carried out showed a lack of significance for all the relationships (Table 3.26). The analyses of variance comparing annual mean intensity of atresia yielded no significant differences (F=0.01; p=0.998; Table 3.27), and mean values in the four years considered were around 4% as shown in Figure 3.82. Monthly atresia intensity, all years pooled, was relatively constant from January to August (mean values <10%), and showed no significant differences between months except September and October that presented the highest mean values (>20%); the *post hoc* test revealed significant differences with the rest of the months (p<0.001 in both cases).
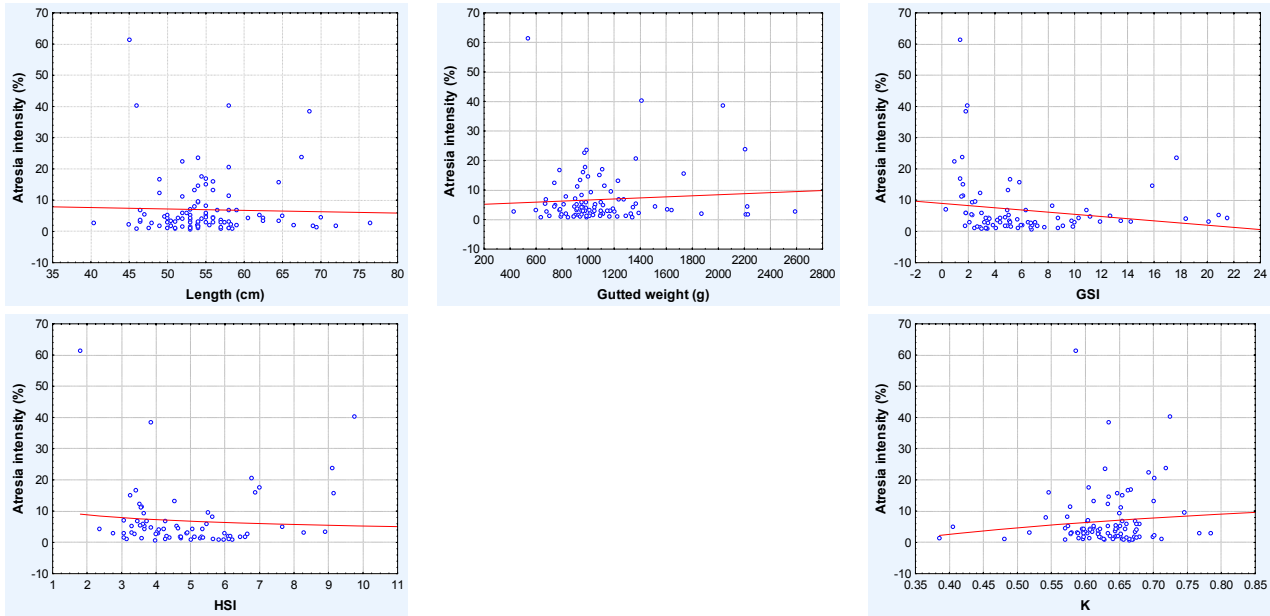
Figure 3.81: Scatterplot of Atresia intensity versus female attributes and condition indices.

When mean monthly values by year were analysed it was observed that in 1999 mean values of atresia intensity were between 0.5% and 13.2%, and it was relatively constant throughout the year excepting May and September; the maximum level of atresia intensity was recorded in May, this month being statistically different from February, April and November. In 2000, monthly mean values of atresia were below 10%, and the maximum level was detected in July (20% approximately), nevertheless no significant differences were observed between months. In 2003, mean values were between 1% and 15.7%; the maximum values of atresia intensity were recorded in the last quarter, reaching 40% in October; from January to August atresia intensity remained more or less constant, with monthly mean values below 10%. October differed significantly from the other months except November which was only statistically different from March and April. Finally in 2004, atresia intensity followed the same trend as in 2003, with constant values below 10% during all the year except September and October that showed maximum values of atresia intensity (>40%); September significantly differed from the rest of months except August and October which were different from May (Figure 3.83). Significant differences were detected by the general ANOVA test (F=1.75, p<0.05, Table 3.27).

Prevalence of atresia was calculated as number of females with signs of atresia regarding the total number of mature females, i.e. females with vitellogenic oocytes. In order to compare mean annual and monthly prevalence, several ANOVAs were performed weighted by number of mature females captured every sampling day.

Annual mean value of atresia prevalence increased slightly from 1999 (9.7±9.4%) to 2000 (8.4±5.7%), increased again in 2003 (10.3±7.1%), and diminished in 2004 (9.3±5.4%; Figure 3.84). *Post hoc* analysis revealed significant differences between 2000 and 1999 and

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2003, and between 2003 and 2004 (F=6.70, p<0.001; Table 3.27). Regarding monthly variation of atresia prevalence (all years pooled), it was around a mean value equal to 11% from January to March, then decreased to 6.2±3.5% in April, and remained constant with some fluctuations until September (13.5±1.2%); the mean value slightly decreased in October (9.9±4.6%) followed by a sharp rise in November and December (25.0±6.0% and 17.6±8.8%; Figure 3.84). Differences between months were statistically significant (F=49.39, p<0.001; Table 3.27).

Table 3.26: Determination coefficients and significance level of linear regression between intensity of atresia and maternal attributes.

	Intensity	
	r^2	p
Length	≤0.01	0.776
Gut W	≤0.01	0.466
GSI	0.03	0.114
HSI	≤0.01	0.584
K	0.11	0.299

Table 3.27: Results of the ANOVA to compare intra- and inter-annual variations in both intensity and prevalence of atresia.

	Intensity		Prevalence	
	F	p	F	p
Year	0,01	0,9984	6,70	≤0,001
Month	3,44	≤0,001	49,39	≤0,001
Year & Month	1,75	<0,05	41,77	≤0,001
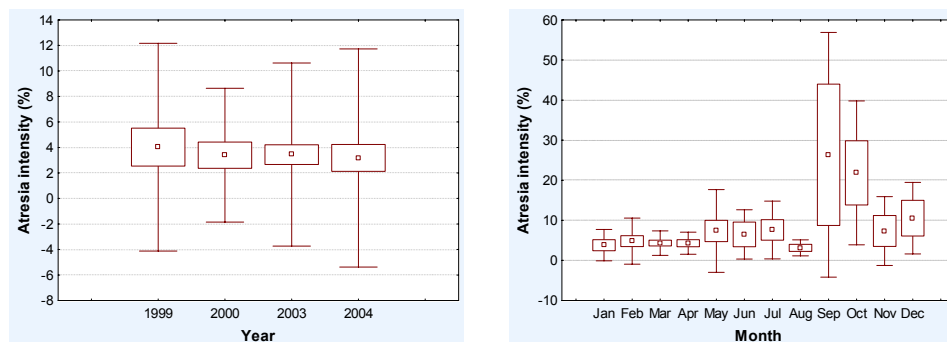
Figure 3.82: Annual and monthly mean atresia intensity (mid point). Standard error (box) and standard deviation (wishker) are also represented.

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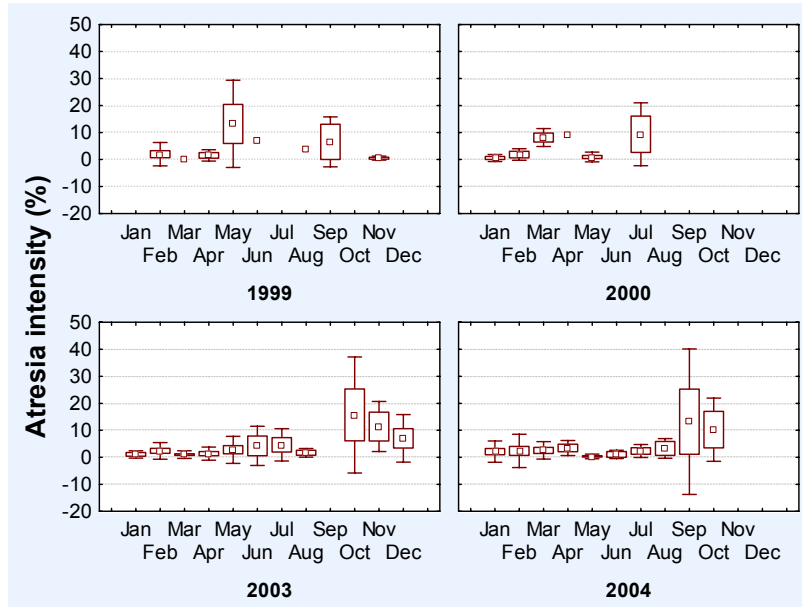
Figure 3.83: Monthly mean atresia intensity by year (mid point). Standard error (box) and standard deviation (wishker) are also represented.
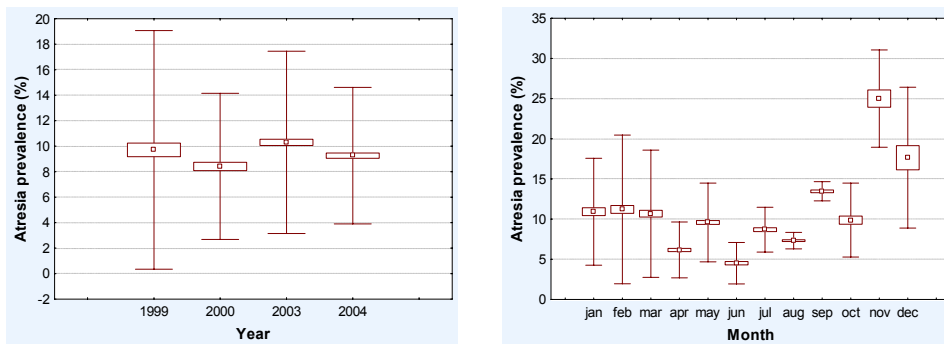
Figure 3.84: Annual and monthly mean atresia prevalence (mid point). Standard error (box) and standard deviation (wishker) are also represented.

When monthly variation of atresia prevalence by year was studied it was observed that the highest values of prevalence appeared at the beginning (January-March) and at the end (October-December) of every year (mean values between 10.4% in 2000 January and 29.4% in November 1999), although in 2004 prevalence was rather constant during all months. In 2000 there were few observations to detect any trend (Figure 3.85). In any case, analysis of variance detected significant differences (F=41.77, p<0.001; Table 3.27).

Mean intensity and prevalence of atresia were negatively related, although with a very low coefficient of determination (r^2=0.01. p<0.001). As shown in Figure 3.86) high levels of atresia occurred only in a few females, while most females of the stock underwent atresia at low intensity throughout the year. Intensity of atresia estimated by the profile method based on stereology was between values near to 0% and maximum values higher than 60%, although these high cases were detected in few specimens. Because the profile method may

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underestimate levels of atresia, the corrected figures to dissector method were used. The new values showed that atresia ranged from 1.59% to 84.92% (Figure 3.87).
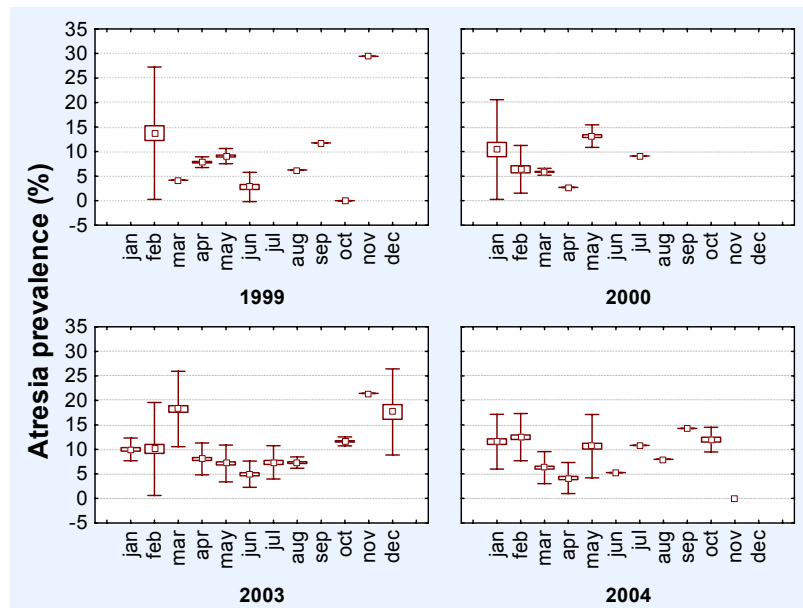
Figure 3.85: Monthlyl variation of atresia prevalence by year (mid point). Standard error (box) and standard deviation (wishker) are also represented.
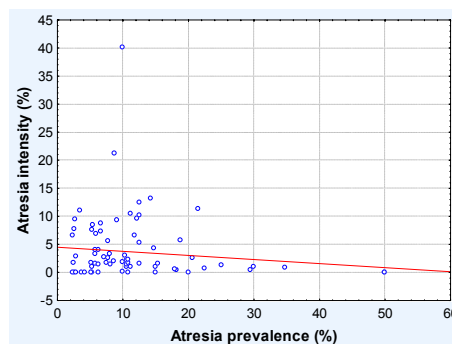
Figure 3.86: Relationship between mean intensity and prevalence of atresia (r = -0.10, p< 0.001).
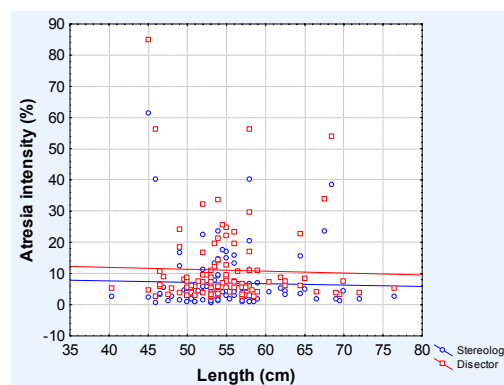
Figure 3.87: Relationship between intensity of atresia and length estimated by stereological method and corrected by dissector method.

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3.8   OVARY DEVELOPMENTAL STAGES

One of the most important handicaps of work with indeterminate and asynchronous species like European hake is the difficulty of differentiating between those females that have started to release eggs and pre-spawning mature ones. Looking for a proxy of ovary developmental stages in hake is one of the objectives of the present work, and with this aim different approaches were analysed. On one hand, analysis of variance was used to study variation of somatic condition factors (GSI, HSI and K) between ovarian developmental stages histologically determined (Section 3.8.1). On the other hand, as it is accepted that biochemical composition of fish changes through the spawning season. Factorial Analysis was used to extract some factors that explained variability of proximate composition during the spawning season. These factors were related to ovary developmental stages determined histologically previously. Firstly, only gonad biochemical composition was considered; secondly, biochemical composition of the three studied tissues (gonad, liver and muscle) was used to take out explanatory factors. Finally, it is known that in determinate species oocyte size distribution changes during the spawning season: based on this, the oocyte size data pool from image analysis was used to study if differences between biochemically determined groups existed. With this aim variation of the mode in relation to the mean was analysed (Section 3.8.2).

3.8.1   ANALYSIS OF CONDITION AND REPRODUCTIVE INDEX

In section 3.4.6 proxy body composition was analyzed according to ovary developmental stages determined by histology (Table 3.11and Figure 3.45). It was observed that proteins varied with ovary developmental stages in all tissues (gonad, liver and muscle), while glycogen did not show significant changes in any of them. Lipids only changed significantly in gonad.

The different condition indices, GSI, HSI and K also showed significant differences in relation to ovary developmental stages (Table 3.28). Mean GSI of ovulating-hydrated ovaries differed significantly from any of the other stages (p<0.001) while m3 stage was different to recovering stage too (p<0.05). The lowest mean GSI values appeared in immature individuals (Figure 3.88) with a mean value of 1.03% increasing to reach 12.96% in ovulating-hydrated females, and diminishing gradually to the stage of recovering females that reach mean values of 1.43%. HSI showed differences between m3 ovaries and ovulating-hydrated, m2 and inactive mature females that presented lower values than the rest of ovarian developmental stages, but no consistent pattern could be identified (Figure 3.88). K, however, showed a   clearer pattern, decreasing from a high value of immature females (0.70) to m2 (0.62), it remained more or less constant in m3 and inactive mature ovaries and increased sharply in recovering females that had the maximum average of K (0.70; Figure 3.88). *Post hoc* tests revealed significant differences between recovering ovaries and the

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other stages except immature ones (p<0.05) that are significantly different from ovulating-hydrated, m2 and inactive mature females (p<0.05). Differences were also detected between m1 and m2 and between m2 and m3 females (p<0.05).

Finally, the relationship between the three condition indices was explored. In GSI-HSI relationship, no significance was observed (r=-0.15, p=0.175), but GSI decreased significantly as K increased (r=-0.22, p<0.05; Figure 3.89), although the coefficient was so low that correlation could be considered practically non existent.

Table 3.28: Results of the ANOVA to compare variations of GSI, HSI and K in relation to ovaries developmental stages.

	F	p
GSI	18.27	≤0.001
HSI	2.23	≤0.05
K	4.09	≤0.01
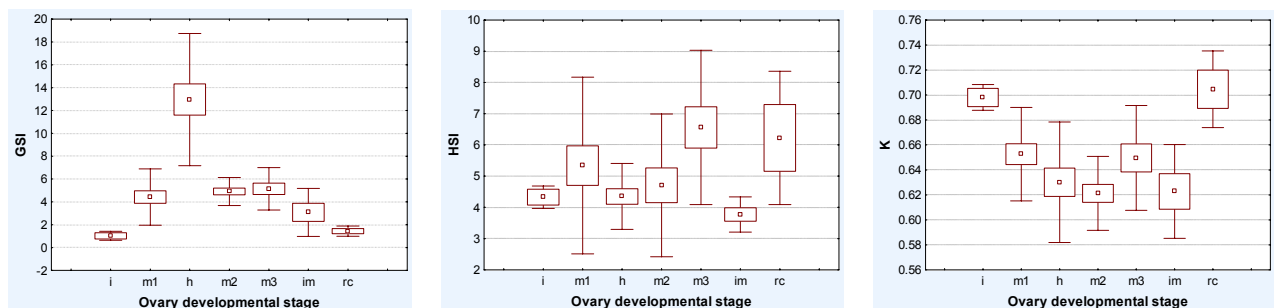
Figure 3.88: Mean GSI, HSI and K by ovary developmental stages (mid point). Standard error (box) and standard deviation (wishker) are also represented.
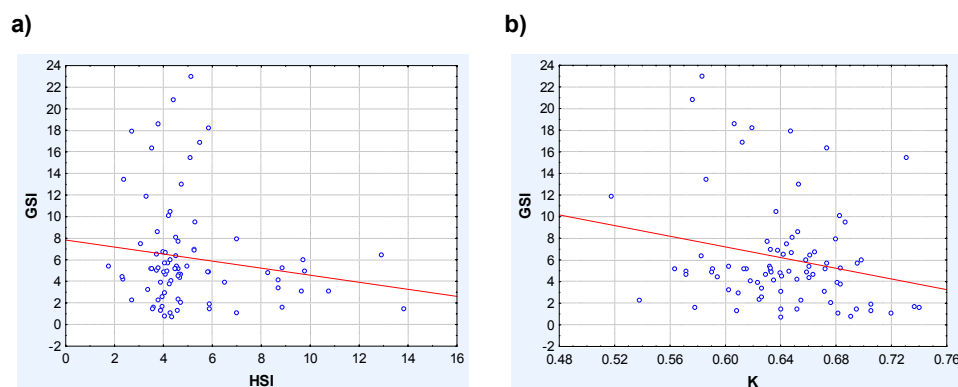
Figure 3.89: Relationship between a) GSI and HSI and b) GSI and K.

3.8.2 FACTORIAL ANALYSIS

Condition factors, in particular K and GSI seem to be good proxies for the ovarian developmental stage. However, both parameters showed high variability at each stage, overlapping very much. Thus GSI can be used only to identify ovulating-hydrated females, while K overlaps considerably among the critical stages, i.e. m1 to m3. As a next step, factorial analyses were applied to find new factors that may allow discrimination among stages, summarizing the variability of data from two data sets: biochemical variables and distribution of oocytes size.

BIOCHEMICAL FACTORS

The first factorial analysis conducted involved all the biochemical variables that had been analyzed previously in gonad, liver and muscle of selected specimens. This method involved maximizing variance of factors by minimizing number of variables with high loadings in one factor.

Factorial analysis was performed considering only the proximate composition of the gonad. Two factors were extracted (F_g1 and F_g2) explaining a total of 72.7% of data variability, 48.03% by F_g1 and 24.7% by F_g2. Variables which had the highest weight in the first factor (F_g1) were lipid, protein and water while in the second one (F_g2) were glycogen and inorganic matter content (Table 3.29).

Table 3.29: Factor loadings obtained by Principal Components Analysis using biochemical composition of gonad.

	F_g1	F_g2
Inorganic matter	0.215	0.767
Water	0.720	0.103
Lipid	0.949	-0.058
Glycogen	-0.066	0.823
Protein	-0.930	-0.154

The analysis of variance showed significant differences in mean scores of F_g1 between developmental stages (F=3.85; p<0.01; Figure 3.90). Stages m2 and m3 showed significant differences with m1, h and rc (*post hoc* test, p<0.01), but not between them. The mean score decreased steadily from immature (0.77) to m3 ovaries (-1.10±1.1) and increased in inactive mature (-0.06±0.6) and recovering females (1.10). The scores of the second factor (F_g2) did not show significant differences between stages (F=0.40; p=0.875; Figure 3.90).

Analysis of variance was repeated using all biochemical variables of the three studied tissues (gonad, liver and muscle) and this time three factors were extracted. The variables

which carried the most weight in the first factor (F1) were lipid content of gonad and muscle and protein content of gonad and liver. In the second (F2), liver lipid content and muscle proteins, water and ashes were the variables with most weight. Finally, in the third factor (F3), inorganic matter content of gonad and liver were the most important variables (Table 3.30). These three factors explained 55.7% of total variability of data, F1 explaining 23.8%, F2 19.4% and F3 12.5%. The individual factor scores were used as dependent variables in an analysis of variance to compare the ovarian developmental stages. The factor scores from F2 and F3 showed no significant differences between developmental stages (F=0.92, p=0.488 and F=0.77, p=0.595 respectively; Figure 3.91).
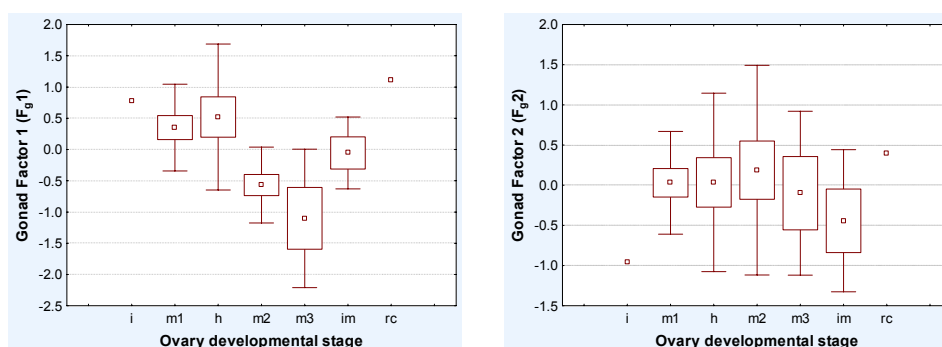
Figure 3.90: Mean factor scores by ovary developmental stages (mid point) for the two factors extracted by factorial analysis using gonad biochemical variables. Standard error (box) and standard deviation (wishker) are also represented.

Table 3.30: Factor loadings obtained by Principal Components Analysis using biochemical composition of gonad, liver and muscle.

		F1	F2	F3
Gonad	Lipid	0.849	-0.148	-0.059
	Protein	-0.924	0.037	-0.089
	Glycogen	0.165	-0.081	0.080
	Inorganic matter	0.359	0.145	0.639
	Water	0.637	-0.002	0.028
Liver	Lipid	0.050	-0.837	0.038
	Protein	-0.555	-0.396	0.437
	Glycogen	-0.246	0.090	-0.073
	Inorganic matter	-0.089	-0.146	0.910
	Water	-0.166	0.477	0.568
Muscle	Lipid	0.733	0.017	-0.217
	Protein	-0.307	0.702	-0.108
	Glycogen	0.373	-0.271	-0.245
	Inorganic matter	0.076	-0.824	0.068
	Water	0.452	0.709	0.136
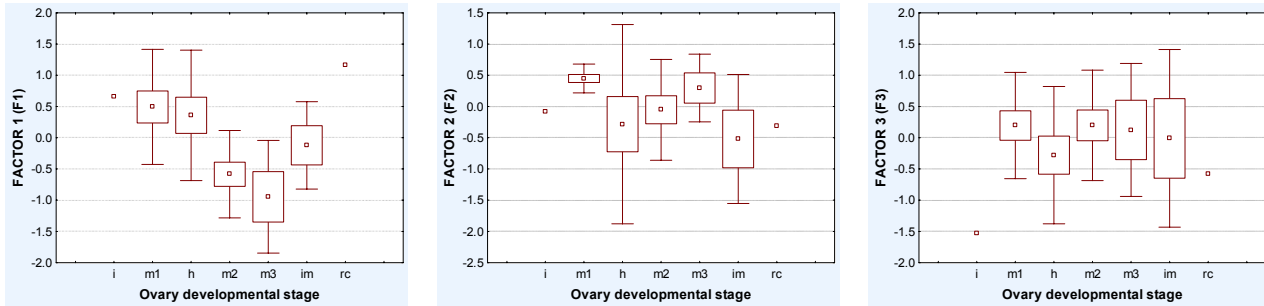
Figure 3.91: Mean factor scores by ovary developmental stages (mid point) for the three factors extracted by factorial analysis using all biochemical variables of gonad, liver and muscle. Standard error (box) and standard deviation (wishker) are also represented.

That means that no relation could be established between the information contained in F2 or F3 and ovary development. Nevertheless, F1 scores showed significant differences among stages (F=3.33; p<0.01; Figure 3.91). The *post hoc* test showed that m2 differed significantly from m1 and ovulating-hydrated females (p<0.01) while m3 showed significant differences with m1, ovulating-hydrated and recovering (p<0.005). In spite of the overlap among stages, especially m2 and m3, a clear trend was appreciated, with mean F1 scores decreasing as the spawning season advance, increasing again as the spawning season concluded (inactive mature and recovering females), which indicated that lipid content in gonad and muscle increased as the breeding season progressed while protein content of gonad and liver diminished.

OOCYTE SIZE DISTRIBUTION

Among those females where the number of developing oocytes was determined, the oocyte size distribution was estimated in 344. Figure 3.92 shows the mean oocyte diameter, the deviation, and the distribution range for each individual. The range is expressed as 0.9 times the minimum and maximum oocyte value, to avoid outliers that may distort the analysis. In most of the individuals the smallest oocyte present in the ovary was always around 150 µm, though both the mean, standard deviation and especially the maximum oocyte diameter recorded varied substantially from fish to fish. Mean oocyte diameter did not change with either female size, gutted weight, or the sampling month in any of the years analyzed (Figure 3.93.a, b and d). However, the oocyte diameter seems to be smaller at low numbers of developing oocytes (Figure 3.93.c), increasing steadily with NDO but remaining basically constant at most of the NDO values.
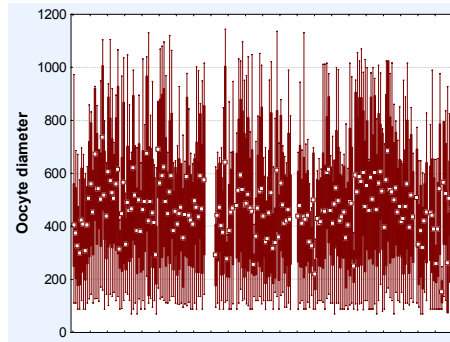
Figure 3.92: Mean (mid point), standard deviation (box) and 0.9 * Min-Max (whisker) of oocytes diameter measured in each specimen.

Mode is the second more intuitive measure of the distribution to be used in this context. Mode was estimated for each female however in 58 of them a single mode could not be detected. The scatterplot of individual female mode *versus* its mean diameter is shown in Figure 3.94, this relationship allowed the definition of five groups: i) females with low mode and mean (the mode being lower than 395µm and mean diameter lower than 400µm), ii) females with low mode (<395µm) and medium-high mean (>400µm), iii) females whose mode was multiple, iv) females with high mode and mean (higher than 800µm and 450µm respectively) and v) females with medium-high mode (540-800µm) and mean (>350µm). In the second, third and fifth groups, all ovary developmental stages are present, while in the first group non-hydrated females dominate and in the fourth group most of the specimens are ovulating-hydrated females.

Figure 3.95 shows histograms of oocyte diameter distributions that were represented for every group. It is observed that mode and mean progressed probably due to spawning season advance. Females from the first group presented ovaries with small oocytes, with low means and modes. In this group would be included those ovaries with many oocytes in primary growth and cortical alveoli stage. Vitellogenic or hydrated oocytes would be scarce or nonexistent. So it could be hypothesized that these females were at the beginning of spawning season. In the second group, means showed intermediate values but modes remained low which could indicate that, although most oocytes were small, the number of vitellogenic oocytes had started to increase. These females would be in a slightly more advanced stage than previous ones. The third group would correspond to those ovaries that presented multiple modes and a wide range of mean values. This could indicate that all oocytes stages were present in the same proportions, more or less. These females would be approximately half-way through the spawning season. The fourth group presented high means and modes, and corresponded mainly to ovulating-hydrated ovaries. These females would be close to release a batch of eggs. Finally, the fifth group showed intermediate values for both mean and mode. In these ovaries, probably oocytes in cortical alveoli or hydrated stages did not exist. More or less advanced vitellogenic oocytes would be present ready to be hydrated and released in the last batches, or to be resorbed by atresia, but new oocytes

would not be being incorporated into the mature oocytes pool. So it could be hypothesized that these females were at the end of spawning season.
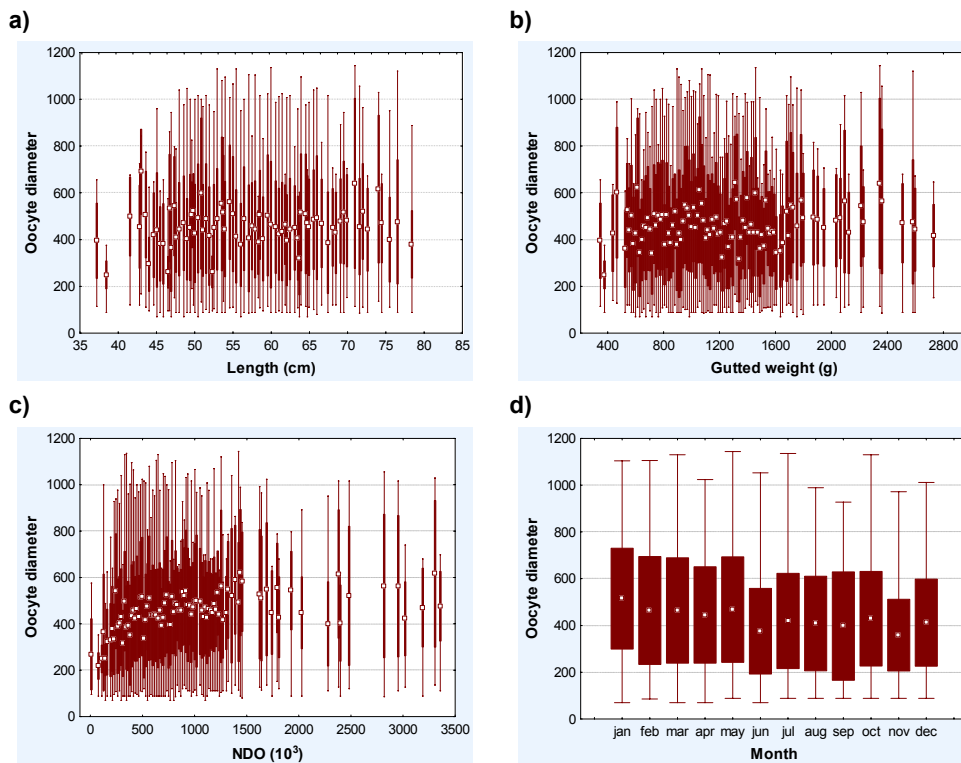
*Figure 3.93: Mean (mid point), standard deviation (box) and 0.9 * Min-Max (wishker) of oocytes diameter versus a) female size, b) female gutted weight, c) number of developing oocytes (NDO) and d) month.*
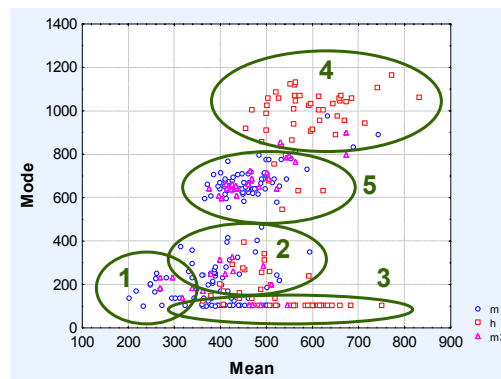
Figure 3.94: Mode versus mean of oocytes diameter of each female categorized by ovary developmental stage. Note that females with multiple modes are represented with 0 values. See text for the definition of the groups.

The mean energy density content of every tissue in each of these five groups was compared through ANOVA. There were no significant results among groups in any tissue (Figure 3.96 and Table 3.31). However, a clear trend seems to exist in gonad and liver. In gonad, energy density content increased progressively from group 1 (25.48±3.1 kJ/g) to

group 3 (27.97±1.6 kJ/g), remained constant in group 4 and then decreased to 26.30±1 kJ/g in group 5.

In liver, energy density content increased from 33.19±3.3 kJ/g in group 1 to 35.87±0.7 kJ/g in group 3, diminishing to the minimum value in group 5 (32.58±2.7 kJ/g). As energy density content of every tissue is affected by tissue weight, a second ANOVA was performed comparing the proportion of energy in every tissue in relation to the total energy of female (gonad + liver + muscle energy). Now the gonad and muscle proportions of total energy were significantly different between groups, determined from the oocytes size distribution (Table 3.31). In gonads, the proportion of energy increased from group 1 (3.43%) to group 4 (14.21%) and subsequently decreased abruptly in group 5 (7.12%; Figure 3.97). The opposite trend was observed for muscle, decreasing from group 1 (90.86%) to group 4 (78.39%) and increasing in group 5 (87.31%; Figure 3.97). In fact, when proportion of energy in every tissue was plotted *versus* percentage of energy in the other tissues, a strong negative correlation was observed between gonad and muscle (r=-0.92, p<0.001; Figure 3.98), while the regression with liver was poor (r=0.06, p=0.701). Correlation between muscle and liver energy proportion was significant, but the correlation coefficient was moderate (r=-0.43, p<0.01). These results are not coincident with those observed when energy density content of each tissue was considered instead of proportions (see Figure 3.56 in section 3.5.4).

The mean number of developing oocytes by group was determined and compared (Figure 3.99). ANOVA showed significant differences among groups (F=2.90, p<0.05), and the *post hoc* test showed differences between group 1 and group 3 and group 4. NDO increase from a lower value in group 1 (534,073 developing oocytes) to the maximum in group 3 (1,250,294 developing oocytes), then diminished progressively to 750,719 developing oocyte in group 5. This could indicate that European hake incorporates new oocytes into the developmental pool until approximately half-way through the spawning season; when the end of spawning season is approaching, it would stop incorporating new oocytes into the pool, and would hydrate the rest of the mature oocytes in the ovary to release them, and/or would resorb surplus by atresia; this would lower NDO in the latter groups rather than in the former.
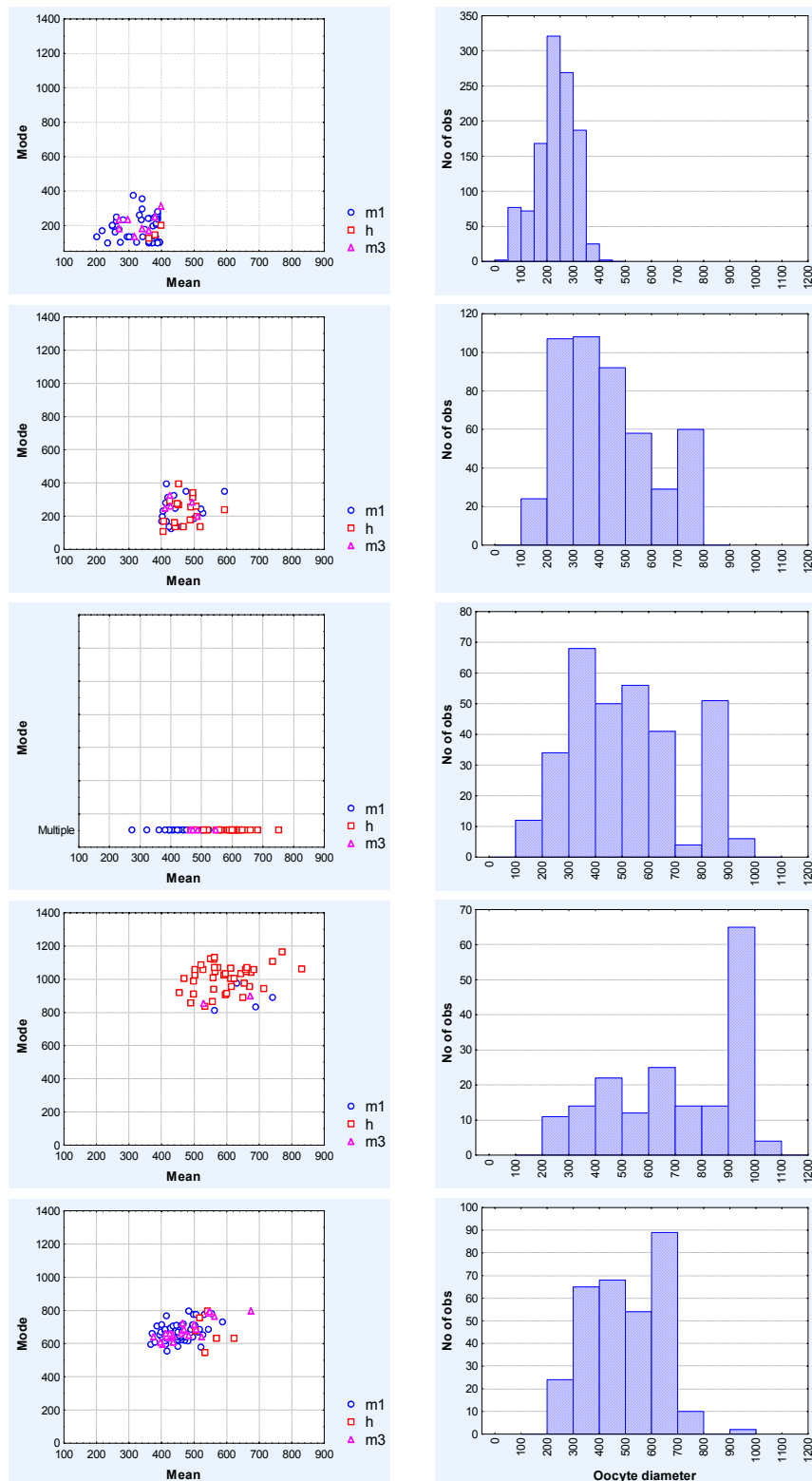
Figure 3.95: Plot of every group obtained when mode is represented versus mean with the oocyte size distribution histogram of an archetypal specimen of each group.
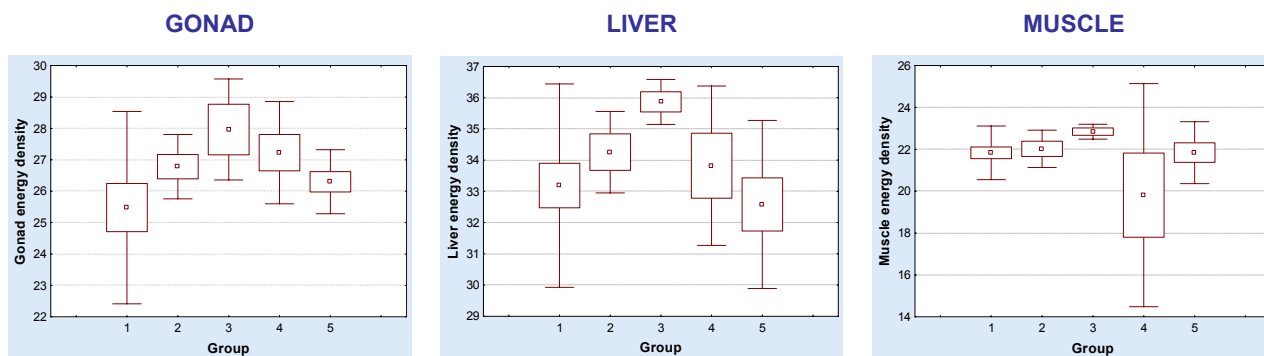
Figure 3.96: Differences in mean energy density (kJ/g) content of gonad, liver and muscle (mid point) according to groups established from mode and mean oocyte diameter relationship. Standard error (box) and standard deviation (wishker) are also represented.

Table 3.31: ANOVA results to compare energy density content variations in relation to groups determined from mode and mean oocyte diameter.

	N	F	P
Gonad kJ/g	45	1,64	0,183
Liver kJ/g	47	1,38	0,257
Muscle kJ/g	48	1,48	0,224
% Gonad kJ	39	15,62	≤0,001
% Liver kJ	39	0,78	0,545
% Muscle kJ	39	17,56	≤0,001
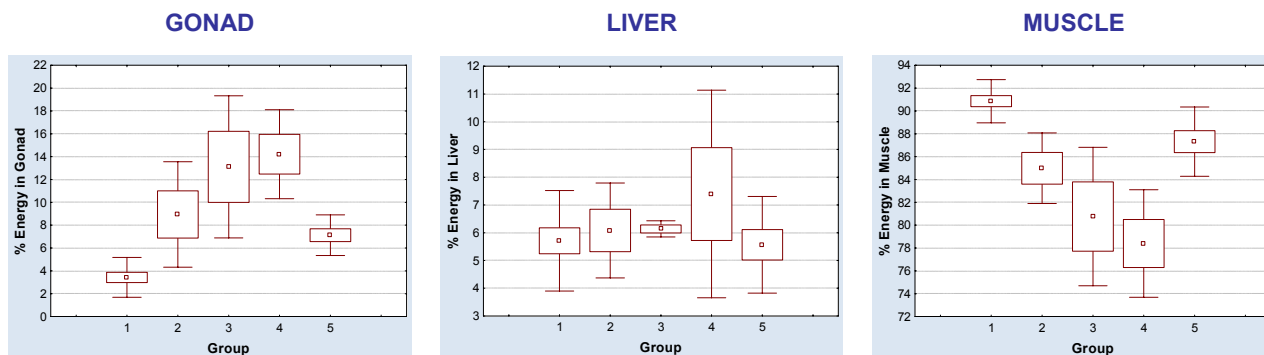
Figure 3.97: Differences in percentage of energy content of gonad, liver and muscle from the total fish energy according to groups established from mode and mean oocyte diameter relationship. (Mid point = mean, box = standard error and wishker = standard deviation).

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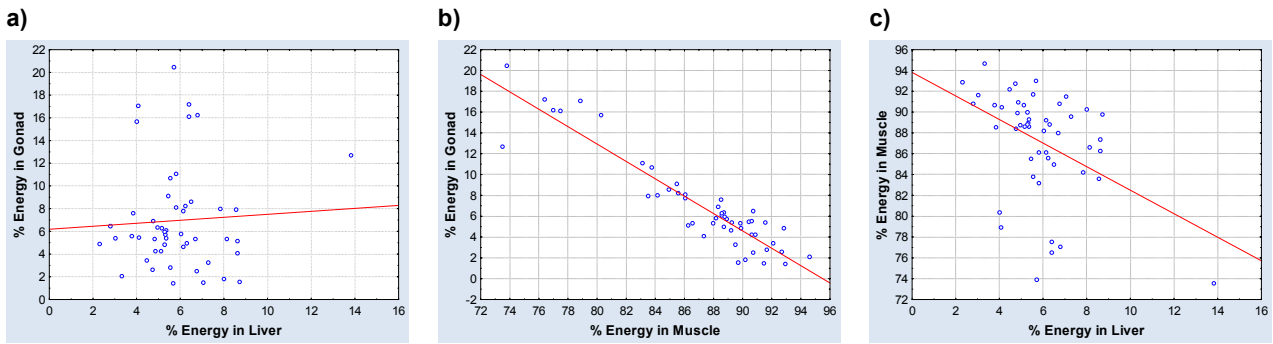
Figure 3.98: Relationship of energy percentage between tissues: a) gonad versus liver (r=0.06, p=0.701); b) gonad versus muscle (r=-0.92, p<0.001) and c) liver versus muscle (r=-0.43, p<0.01).
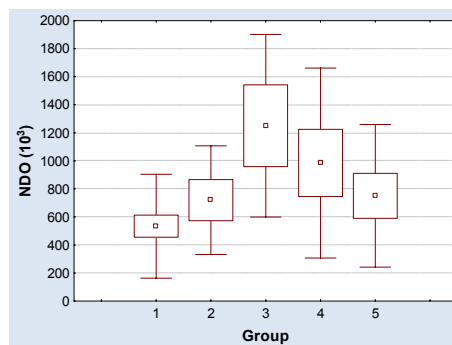
Figure 3.99: Variation of number of developing oocytes (NDO) according to groups established from mode and mean oocyte diameter relationship; F=2.90, p<0.05. (Mid point = mean, box = standard error and wishker = standard deviation).
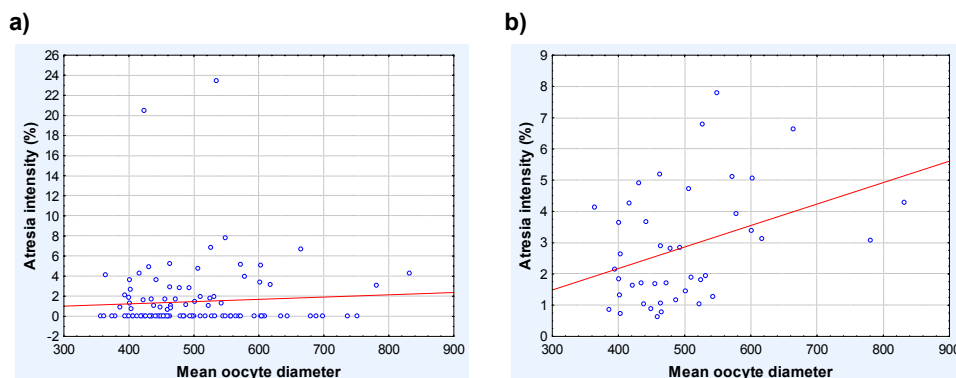
Figure 3.100: Relationship between intensity of atresia estimated by stereological method and mean oocyte diameter. a) all specimens (r=0.12; p=0.421) and b) without outliers (r=0.37; p<0.05).

Finally, if we assumed that oocyte diameter is an estimator of ovary developmental stage, atresia should be related to the mean oocyte diameter of each individual, so that specimens at the beginning of their spawning season (low mean and mode) should show less intensity of atresia that those at the end of it (medium-high mean and mode). To check this hypothesis, atresia intensity was regressed with mean oocyte diameter (taking into

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account that oocyte diameter was also estimated in females that had no post-ovulatory follicles; Figure 3.100). This regression was not significant (r^2=0.016; p=0.421), although the existence of two specimens with high levels of atresia may be acting as outliers and affecting the regression. Perhaps these females were inactive mature and therefore lacked vitellogenic oocytes, or all of them were atretic and their diameters were not correctly measured by image analysis, so that the mean diameter of those females could be lower. These two females were removed and regression estimated again to obtain a significant result (r^2=0.139; p=0.015; Figure 3.100); atresia intensity increased when mean egg diameter increased, although residual variability was high.

Many other size distribution parameters, apart from mean and mode, can be estimated, and indicate the developmental stage of the ovary. A total of 23 size distribution parameters were investigated, as described in Material and Methods chapter (see Section 3.2.8).

Principal component analysis was chosen to examine any further variation in the data for the 23 descriptors of oocyte size distribution. Figure 3.101 shows the scatterplot of the individual scores for the three extracted factors performed with the 23 variables. These scatter plots do not allow the definition of clusters or groups of individuals. In order to ascertain if the factor scores can be a good proxy for developmental stage, the scores of the first two factors were plotted against month of collection and the number of developing oocytes (NDO) in the ovary. The results did not show any pattern that allowed us to differentiate between maturity groups.

A final approach analysed oocyte density, i.e. the number of oocytes per female body unit weight (g). Figure 3.102 shows the relationship between oocyte density and mean diameter. As density decreased, the mean oocyte diameter increased, as well as the standard deviation and maximum oocyte size. Nevertheless, the minimum oocyte size remained always around 150µm, indicating that there is a progressive skewness with decreasing density. Figure 3.103 shows the mean oocyte density by month and year. Roughly, in 2003 the lowest values were observed in winter (January-March) and mainly in May, and the highest in autumn (November), decreasing steadily until spring 2004, when the lowest recorded values are found. Following oocyte density increased until June, decreasing again in July and August and finally, increased in September and October with the highest values of 2004. This result is in accordance with the proposed spawning peak described in section 3.1, indicating that oocyte density may be a good proxy for ovarian developmental stage.
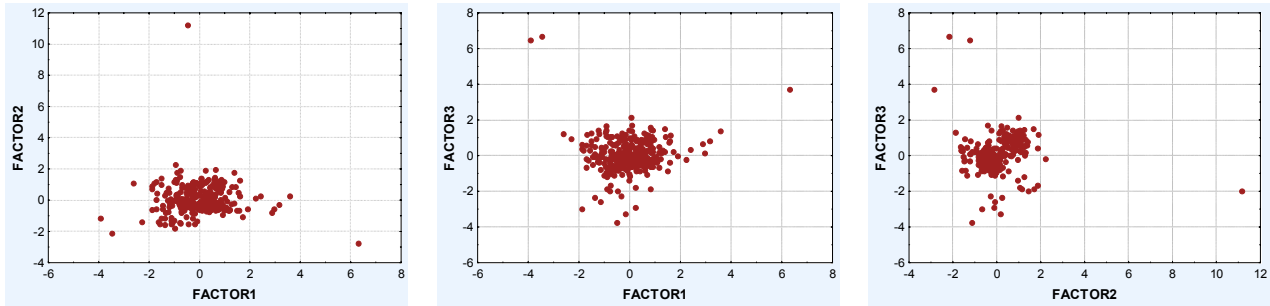
Figure 3.101: Plot of factor scores for the three extracted factors performed with 23 oocyte size distribution parameters
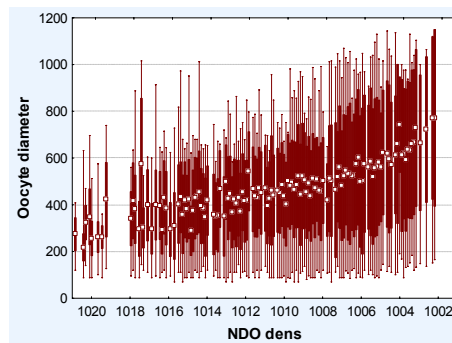
*Figure 3.102: Mean diameter versus number of developing oocytes density. (Mid point= mean, box = standard deviation and wishker = 0.9 * Min-Max).*

3.9 ESTIMATES OF REPRODUCTIVE POTENTIAL OF *MERLUCCIUS MERLUCCIUS* ON GALICIAN SHELF

The expression *reproductive potential* embraces not only the number of oocytes that a female is developing (potential fecundity), but also the combination of factors that determine the real number of eggs that female finally releases (realized fecundity) and the quality and viability of those eggs. Because these features depend very much on female condition, the atretic process, environmental factors, and all those aspects determining fish egg production, all these concepts are involved in the definition of reproductive potential.
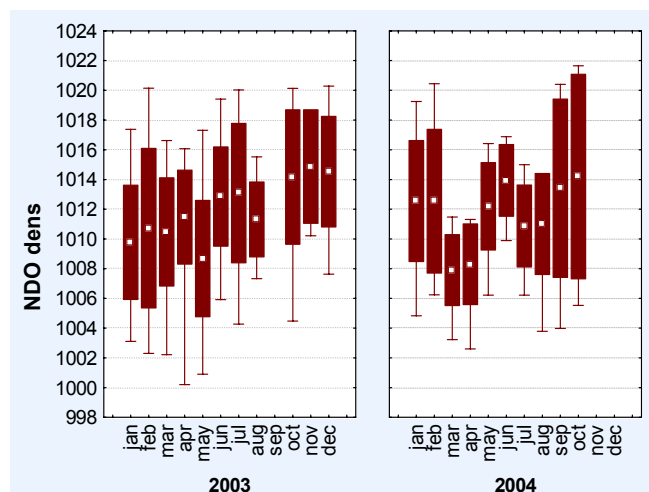
Figure 3.103: Monthly mean of number of developing oocytes density by year. (Mid point= mean, box = standard deviation and wishker = non-outlier range).

To study the influence of energy, water and ash content on reproductive potential, a series of linear and multiple regression analyses were carried out. Both total and energy density of each tissue were included in the model because they integrate all information about biochemical composition (lipids, proteins and glycogen) and the effects of studied tissue weights (gonad, liver and gutted weight).

A set of simple linear regressions were carried out between every biochemical and energetic compound and NDO, relative NDO and diameter. Results are presented in Table 3.32. NDO was significantly and positively related (Figure 3.104) to total energy of the three studied tissues (r^2=0.11, p<0.05 for gonad, r^2=0.13, p<0.01 for liver and r^2=0.13, p<0.05 for muscle). Gonad lipid and muscle glycogen content (Figure 3.104) were also significantly related to NDO (r=0.06, p<0.05 and r^2=0.07, p<0.05 respectively). However, relative NDO was significantly related only with lipids, proteins and total energy content of gonad (Table 3.32), but not with any other tissue components (Figure 3.105). Similarly, oocytes diameter was significantly related with gonad water, inorganic matter, and total energy content as well liver lipid (Table 3.32 and Figure 3.106). Except for the gonad total energy content and oocyte diameter relationship that yielded a relatively high determination coefficient (r^2=0.56), the rest of the relationships showed poor correlations, even when significant.

Total and relative number of developing oocytes and mean oocyte diameter were regressed with total energy of females, but only the first relationship was significant (Table 3.33), so when total energy of fish increased, number of developing oocytes also did (r^2=0.16, p<0.01; Figure 3.104).

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Table 3.32: Determination coefficients and significance level of linear regression between biochemical and energetic components of each tissue and number of developing oocytes (NDO), relative number of developing oocytes (NDO rel) and oocyte mean diameter.

		NDO		NDO rel		Diameter	
		r^2	p	r^2	p	r^2	p
Gonad	**Lipid**	0.06	0.0295	0.10	0.003	0.02	0.224
	Protein	0.05	0.055	0.09	0.007	≤0.01	0.766
	Glycogen	≤0.01	0.924	≤0.01	0.636	0.02	0.339
	Water	≤0.01	0.639	0.02	0.372	0.12	0.011
	Ashes	≤0.01	0.772	≤0.01	0.580	0.05	0.036
	kJ/g	0.03	0.219	0.01	0.481	≤0.01	0.739
	Total kJ	0.11	0.020	0.11	0.019	0.56	≤0.001
Liver	**Lipid**	0.01	0.296	0.04	0.072	0.07	0.015
	Protein	≤0.01	0.100	≤0.01	0.424	0.01	0.298
	Glycogen	0.03	0.166	≤0.01	0.589	0.01	0.429
	Water	≤0.01	0.810	≤0.01	0.573	0.02	0.318
	Ashes	0.04	0.069	0.02	0.179	≤0.01	0.436
	kJ/g	0.03	0.235	0.05	0.138	0.02	0.367
	Total kJ	0.13	0.010	≤0.01	0.601	≤0.01	0.629
Muscle	**Lipid**	0.01	0.301	0.04	0.076	≤0.01	0.394
	Protein	0.01	0.364	0.04	0.116	≤0.01	0.572
	Glycogen	0.07	0.038	0.05	0.076	0.06	0.056
	Water	0.02	0.301	0.02	0.348	0.03	0.190
	Ashes	≤0.01	0.893	≤0.01	0.446	≤0.01	0.785
	kJ/g	0.01	0.470	0.03	0.224	0.02	0.341
	Total kJ	0.13	0.010	0.01	0.480	≤0.01	0.930

Table 3.33: Determination coefficients and significance level of linear regression between total fish energy and number of developing oocytes (NDO), relative number of developing oocytes (NDO rel) and oocytes mean diameter.

	NDO		NDO rel		Diameter	
	r^2	p	r^2	p	r^2	p
Total fish kJ	0.16	0.005	0.01	0.792	0.02	0,708

Three parameters were selected as estimators of reproductive potential: i) number of developing oocytes, NDO, ii) relative NDO, and iii) mean hydrated oocyte diameter that was the best proxy to egg diameter; multiple regressions were performed to assess the influence of energetic features on them. For NDO and NDO$_{rel}$ energy density was used instead total energy, because it includes the effects of lipid, protein and glycogen content, while total energy reflects the effects of tissue weight, inorganic matter and water content that were not considered as energetic components. For hydrated egg diameter, several combinations of biochemical and energetic features were used as predictors to get larger sample size because the number of ovulating-hydrated females with all biochemical analysis carried out in order to estimate energetic composition (kJ/g and total kJ) was scarce (n=9).
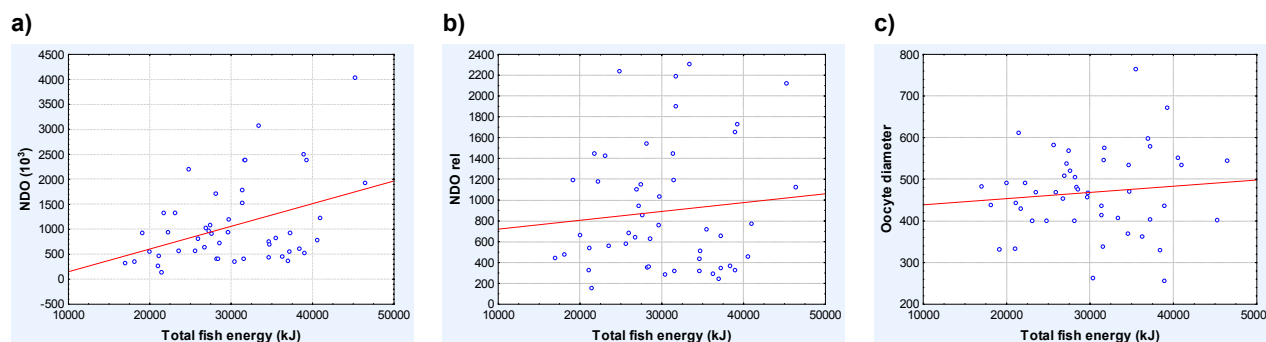
Figure 3.104: Relationship between total fish energy and a) number of developing oocytes (NDO); b) relative number of developing oocytes (NDO rel) and c) mean oocyte diameter.

In the first case, 30.3% of variability of NDO was explained by three biochemical variables included in the model in this order: total energy of liver, energy density of liver, and muscle water content, the first variable alone explaining 15.3% of this variability. Of these variables, total liver energy and muscle water content were significantly related to NDO. Observed and model values (Figure 3.105) showed significant correlation (r=0.61, p<0.001). In the case of relative NDO, also three variables were included in the model and they explained 21.3% of total variability of the dependent variable: total gonad energy alone explained 8.97%; when muscle water content was included in the model, explained variability increased by 5.8%, and if energy density of liver was included, the proportion of variability explained increased to 6.6%. None was significantly related to relative NDO. Correlation between predicted and observed values (Figure 3.105) was, however, significant (r=0.52, p<0.001; Table 3.34).
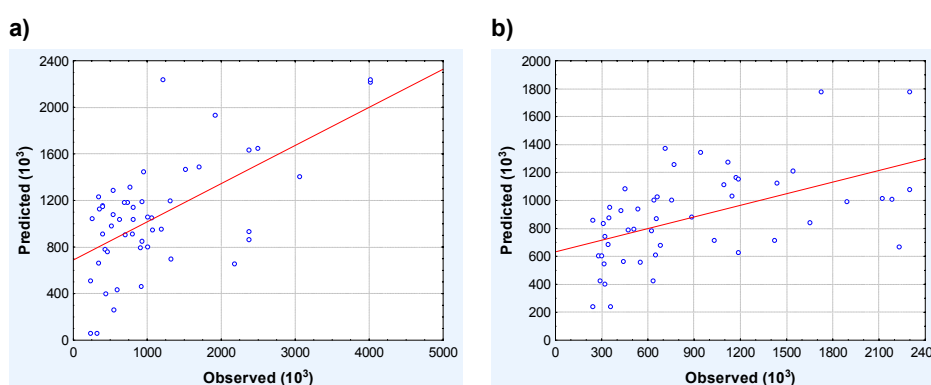
Figure 3.105: Observed versus predicted values obtained from the multiple regression of a) Number of developing oocytes (NDO), b) relative number of developing oocytes (NDO rel) using total energy, energy density, inorganic matter content and water content of every tissue as predictors.

In total, six multiple regressions with different combinations of predictor factors were carried out. The first one, when multiple regression was carried out for hydrated oocyte

diameter using total energy, energy density and inorganic matter and water content as predictors, the model obtained explained 100% of variability, which did not make it a reliable model. The same happened when all biochemical variables were used (lipid, protein, glycogen, inorganic matter and water content). However, when only energy density, inorganic matter content and water content were used as predictors (n=9), the model for hydrated oocyte diameter explained 99.8% of its total variability, though the two first explained 95.8% of this variability by themselves. Variables included in the model were, in this order, muscle water content, liver energy density, gonad water content and finally, inorganic matter content of muscle; from these, only liver energy density and gonad water content were significantly related to the dependent variable (p<0.01), and all of them showed negative beta coefficients. These beta coefficients allow us to compare the relative contribution of each independent variable in the prediction of the dependent variable, and to evaluate the relative contribution of each predictor to the overall prediction of it; their interpretation is similar to that of partial correlations. Observed and predicted values were highly related (r^2=0.999, p<0.001).

Table 3.34: Multiple regression analysis results for number of developing oocytes (NDO) and relative number of developing oocytes (NDO$_{rel}$). Total kJ = total energy; kJ/g = energy density and ash = inorganic matter content.

		Beta	Multiple R^2	R^2 changes	F to enter	p-level
NDO	**Liver total kJ**	0.443	0.15	0.15	7.05	0.011
	Liver kJ/g	-0.315	0.22	0.07	3.34	0.076
	Muscle water	-0.291	0.30	0.08	4.34	0.044
NDO$_{rel}$	**Gonad total kJ**	0.250	0.09	0.09	3.84	0.057
	Muscle water	-0.289	0.15	0.06	2.56	0.118
	Liver kJ/g	-0.261	0.21	0.07	3.12	0.086

The second approach took into account only lipid, protein and inorganic matter content of the three studied tissues (n=15), because it was considered that glycogen content had little effect on tissue energy. These variables explained 73% of hydrated oocyte diameter variability. Predictors included in the model were lipid content of gonad and muscle, protein content of liver, and muscle inorganic matter content, but only the two first were significantly related to the dependent variable. In this case, beta coefficients were also negative except for muscle lipid content. The relationship between observed and predicted values was significant, and showed a high determination coefficient (r^2=0.82. p<0.001). The following multiple regression was done using protein and water content of gonad, liver and muscle as predictor variables (n=10). This time 92.57% of variability of hydrated oocytes diameter was explained by gonad water content, liver and gonad protein content, and water content in liver and muscle that were included in the model in this order. Only the first one showed a significant relationship with the dependent variable, and explained by itself 52% of its variability. Their beta coefficients were positive, except for liver protein and muscle water

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content. The relationship between observed and predicted values was positive and significant (r^2=0.94, p<0.001). If only gonad biochemical composition was used as predictor (n=15), explained variability reached 64.31%; including water and protein content in the model gave a positive beta coefficient in the first case and a negative one in the second. Observed and predicted values were positive and significantly related (r^2=0.74, p<0.001). Finally, when only lipid and water content of gonad was considered (n=11), 65.53% of hydrated oocytes diameter variability was explained, although only water content was included in the model with a positive beta coefficient. A significant relationship was established between observed and predicted values too (r^2=0.74, p<0.001). Figure 3.106 summarize results of linear regression between observed values and values predicted by each model. Table 3.35 shows beta coefficient of every predictor in each model. In any case, scarce number of analysed females obliged us to be cautious with interpretations, although results suggest that it is necessary to make more analyses on these lines for future studies of the influence of proximate composition on egg size.
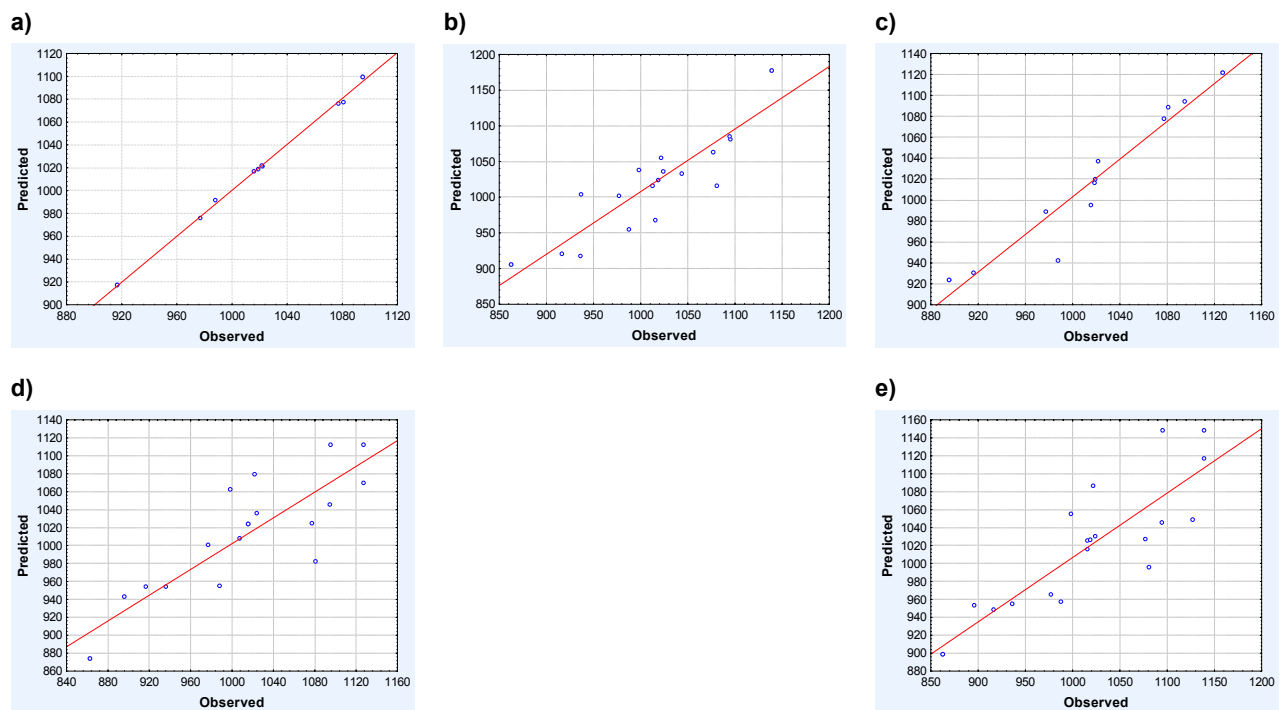
Figure 3.106: Observed versus predicted values obtained from the multiple regression of hydrated oocytes diameter using as predictors: a) energy density, inorganic matter content and water content of each tissue (r^2 = 0.999); b) lipid, protein and inorganic matter content of each tissue (r^2 = 0.824); c) protein and water content of each tissue (r^2 = 0.943); d) gonad biochemical composition (r^2 = 0.742) and e) gonad lipid and water content (r^2 = 0.740) In all cases p-level was lower than 0.001.

Table 3.35: Multiple regression analysis results for hydrated oocyte diameter using different combinations of predictor variables. kJ/g = energy density; ash = inorganic matter content and gonad composition = lipid + protein + glycogen + inorganic matter + water content of gonad.

		Beta	Multiple R^2	R^2 changes	F to enter	p-level
kJ/g+ash+water	**Muscle water**	-1.413	0.492	0.492	5.801	0.053
	Liver kJ/g	-0.962	0.958	0.467	55.757	<0.001
	Gonad water	-0.308	0.997	0.039	51.045	<0.01
	Muscle ash	-0.048	0.998	0.001	1.607	0.294
Lipid+protein+ash	**Gonad lipid**	-0.887	0.339	0.339	6.661	<0.05
	Muscle lipid	0.443	0.553	0.214	5.733	<0.05
	Liver protein	-0.381	0.634	0.081	2.436	0.147
	Muscle ash	-0.319	0.730	0.096	3.566	0.088
Protein+water	**Gonad water**	0.669	0.520	0.520	8.665	0.019
	Liver protein	-1.248	0.649	0.129	2.580	0.152
	Gonad protein	0.938	0.791	0.141	4.062	0.090
	Liver water	0.474	0.886	0.095	4.182	0.096
	Muscle water	-0.317	0.926	0.040	2.131	0.218
Gonad composition	**Gonad water**	0.710	0.599	0.599	19.433	<0.001
	Gonad protein	-0.219	0.643	0.044	1.476	0.248
Gonad lipid+water	**Gonad water**	0.809	0.655	0.655	26.611	<0.001

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CHAPTER 4

DISCUSSION

Sentado en estas rocas, mar, te escucho.
No entiendo tus palabras pero adivino a ciegas
que algo quieres decirme mas no puedes llevarme
adonde yo quisiera, ¡oh inmensidad sin centro!

(Gabriel Celaya)

4.1 SITUATION OF HAKE FISHERIES, WHY WE NEED TO STUDY REPRODUCTIVE POTENTIAL IN HAKE

Alheit and Pitcher (1995) date the earliest documentation of the European hake fisheries to the 18th century, and suggest that the introduction of otter trawls during the mid 1800s as the factor that led to decrease in abundance of small hake. For assessment purposes, the International Council for the Exploration of the Sea (ICES) considers two assessment areas for European hake, one in the North-east Atlantic and one in the Mediterranean Sea. The separation of North-east Atlantic hake into two stocks is based on two main criteria; the first is the presence of a geographical barrier, Cape Breton Canyon, which separates Spanish waters from France in the extreme corner of the Bay of Biscay. The second reason is the observation of two spawning areas, one located from the French coast to the Celtic Sea and the other located on the northwest coast of the Iberian Peninsula. Separation of the Mediterranean stock is based on the existence of a geographical barrier represented by the Strait of Gibraltar that may prevent genetic flow between Mediterranean and Atlantic waters. However, recent studies based on genetic and biological parameters and incidence of identified parasites used as tags for stock identification suggest that only two stocks are really differentiated: the North-east Atlantic stock and the Mediterranean stock; probably also, two sub-populations in the Northeast Atlantic exist and are separated by Cape Breton Canyon (Balado *et al*., 2003; Lo Brutto *et al*., 2004; Mattiucci *et al*., 2004; Castillo *et al*., 2005). These results are relevant for management purpose and the boundary between these stocks should be reconsidered based on biological evidence.

In 1977, López Veiga *et al.* already emphasized a critical stock size of hake on the Galician Coast and recommended an increased mesh size and reduction of fishing effort to palliate the situation. European hake landings have diminished considerably in recent decades in both Northern and Southern stocks, since reduced reproductive capacity puts this resource in danger of being unsustainable. Furthermore, no signs of recovery have been observed in spite of measures taken by the Governments of countries that exploit this resource, and nowadays, both stocks are in a critical state (ICES, 2005). However, despite the commercial importance of European hake, and the harmful situation of its populations, studies on reproductive potential and reproductive strategy of hake are scarce (Murua *et al*., 2006). Estimates of spawning stock biomass of European hake lack accuracy, partly because maturity ogives have not been properly estimated or validated. And partly because hake age determination still remains under debate (Norbis *et al.*, 1999; Piñeiro and Saínza, 2003; Morales-Nin and Moranta, 2004). Management of most commercial fish species follows an age-based analytical assessment. Accurate age estimation techniques are of crucial importance if the data collected are to support sustainable management policies. For many commercial species, the techniques are well established, and there is good agreement in age estimation. For other species, reliable aging techniques have not yet been

established. European hake in particular, age estimation is difficult because otolith annuli are not easily recognisable. A large amount of literature on the age and growth of European hake in different areas exists (López Veiga *et al*., 1974; Iglesias and Dery, 1981; ICSEAF, 1983; Goñi and Piñeiro, 1988; Morales-Nin and Aldebert, 1997; Morales-Nin *et al*., 1998; Lombarte *et al*., 2003; Piñeiro and Saínza, 2003; Torres *et al*., in press; Arneri and Moreales-Nin, 2000). Despite all these studies and other unpublished studies carried out within ICES, there is still no internationally agreed method of aging European hake from their bony structures. All authors agree that the ring structure of European hake otoliths is difficult to interpret. Factors such as the definition of the otolith nucleus, formation of annual or intermediary rings and the extended spawning period, all contribute to the discrepancies between different otolith readers. More recently, preliminary results of tagging experiments show that hake probably grow twice as fast as previously recorded (de Pontual *et al.*, in press). The use of microstructure has been suggested as a robust method to estimate fish age (Morales-Nin and Aldebert, 1997; Gordoa *et al*., 2001; Morales-Nin and Moranta, 2004; Álvarez and Cotano, 2005).

A lot of effort has been devoted to hake distribution, growth and feeding (Pereiro and Fernández, 1983; Bowman, 1984; Fariña and Fernández, 1986; Calvo, 1989; Guichet, 1995; Velasco and Olaso, 1998; Molina, 1999) but not many studies have focused on European hake reproductive potential or simply on reproductive features, probably because indeterminacy and the asynchronous spawning cycle of this species make it very difficult differentiate pre-spawners, estimate fecundity, duration of spawning season, and other reproductive parameters such as spawning fraction and frequency. Nevertheless, some attempts to know more about reproduction of European hake have been made, e.g. Andreu (1956) analysed oocyte size distribution during the spawning season. Monteiro and Limas-Días (1966) also studied gonad development on the Portuguese coast and suggested a peak of spawning in spring-summer months. Pérez and Pereiro (1981) studied the spawning distribution and suggested that European hake spawn in deep waters. This is coincident with the observations of McFarlane and Saunders (1997) for *M. productus* and of Pájaro *et al*. (2005) for *M. hubbsi*, but contrary to other authors who consider that hake moves to shallower waters to spawn (Poulard, 2001; Rikhter *et al*., 2001 among others),. Murua *et al.* (1996; 1998a; 1998b) as well as Lucio *et al.* (1998; 2000; 2002) describe reproductive modality, analyse changes in reproductive parameters, and estimate fecundity and egg production in the Bay of Biscay.

4.2 REPRODUCTIVE POTENTIAL IN THE CONTEXT OF FISHERIES. WHY IT IS IMPORTANT AND HOW IT CAN IMPROVE STOCK ASSESSMENT

Traditionally, it has been assumed that spawning stock biomass (SSB) can be used as a proxy for stock production; this implies that the survival rates of offspring are

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independent of parental age, body size, or condition, and that total relative fecundity and thus annual egg production per unit weight of adult stock is invariable over time (Marshall *et al*., 2003, Murua *et al*., 2006; Yoneda and Wright, 2004). However, there is increasing evidence indicating that direct proportionality between spawning stock biomass and viable offspring production may not exist (Marshall *et al*., 1998; Scott *et al*., 1999; Marteinsdottir and Begg, 2002; Marshall *et al*., 1999; O´Brien, 2003; Tripple, 1997). In 1999, Trippel defined the Stock Reproductive Potential as "the ability of a fish stock to produce viable offspring that may recruit to the adult population or fishery". It is known that the reproductive system of fish reacts to any changes in life conditions, consequently variations in growth and reproduction dynamics of fish populations substantially affects not only quantitatively but also qualitatively fish production (Godø and Haug, 1999; Oven, 2004; Saborido-Rey and Kjesbu, in press). In other words, the influence of parental effects (mainly maternal effects) on the reproductive potential, like those of age/size composition and condition of spawning stock, is likely to be an important component of the total production of viable offspring by the stock. Maternal characteristics are associated with the quality and viability of eggs and larvae, these maternal effects are expressed in the offspring by differences in size, yolk volume and dry weight of eggs and total length of larvae or their DNA quantity. The relative production of large, good quality offspring by females of different sizes and condition is likely to be an important component in the total production of future recruits to the stock (Trippel *et al.*, 1997; Heyer, 2001). Macchi *et al*. (2005) observed an increase in survival rates of Argentine hake (*Merluccius hubbsi*) eggs and larvae from 1997 to 2001 associated with changes in size composition of spawning stock caused by restrictions on fishing activities in the study area. Egg and larval size affect their own growth rates, first feeding time or air bladder development among others offspring features, influencing survival rates (Marteinsdottir and Steirnarsson, 1998; O´Brien, 1999). In this sense, maternal size also affects the proportion of eggs which hatch, e.g. in Norwegian inshore cod, eclosion success of first time spawners is 40% while in repeated spawners it increases to 70% (Solemdal *et al*., 1995). Hatching success is related not only to female size, but also to offspring buoyancy that depends on its density and consequently on its proximate composition (Wiegand *et al*., 2004). Egg and larvae positions in the water column determine the environmental conditions at which larvae have to survive, depending on the quality of these conditions (low turbulence, suitable water flows, temperature, salinity, availability of food, etc.). Clear differences in buoyancy between larvae of first and repeat spawners have been observed in Arctic cod (Solemdal, 1997; Saborido *et al.*, 2003).

Although maturity depends partly on genetic factors, it is strongly influenced by environmental changes, variations in age/size structure of the spawning stock, growth strategies, density-dependent phenomena, nutritional status, hydrography, photoperiod, disease, and pollution. Fecundity is also affected by food availability, environmental conditions, female condition, spawning stock abundance and female size (Bromley *et al*., 2000; Fedorov, 1971; Hislop, 1988; Lambert *et al*., 2003; Morgan, 2004; Morgan and Lilly,

2004; Óskarsson *et al*., 2002; Solemdal *et al*., 1992b). Large females in better condition produce larger higher quality eggs that at the same time give rise to larger and stronger larvae. In the words of Trippel and Neil (2004), "Egg production and their size, viability, and seasonal pattern of release are therefore important to understanding several key processes that shape early life growth, survival and recruitment of marine fish species."

Reproductive success depends on the reproductive strategy that fish adopt, including when and where reproduction takes place, and how available energy resources are distributed. Stock density, age composition, interactions with prey species and environmental conditions are, among others, some of the components that determine not only the maturation and reproductive processes of fish, but also the strength of year classes descending from them (Helser and Almeida, 1997; Lambert, 2000; Tereshchenko, 2002). When a fish is sexually immature, all assimilated energy is used for metabolism and growth, but when maturation begins, part of the energy is allocated to gamete production and reproductive behaviour (Figure 4.1). Subsequently, a trade off is necessary between survival, reproduction and growth costs during the lifetime of the fish. This balance or energy distribution can change depending on the life history and reproductive strategies and tactics of each species and stock, e.g. iteroparous, semelparous, parental care, etc. (van Winkle *et al*., 1997; Saborido-Rey and Kjesbu, in press) and can be modified by environmental conditions (Buckley *et al*., 1990; Roff, 1992; Chambers, 1997).

Interactions between offspring and the environment should also affect the level of recruitment (RASER, 2006). These topics would include male characteristic, however they have largely been ignored in reproductive potential studies. Trippel (2003) and Trippel and Neil (2004) suggest the importance of male condition on quantity and quality of sperm and consequently on success of fertilization. On the contrary, Bromley *et al*.'s (2000) studies on turbot revealed that feeding regime did not affect sperm production; but they did not account for characteristics like mobility, fertilization rates, and life expectancy. Lambert *et al*. (2003) observed that male condition did not affect fertilization rate, although they suggested that it may have an impact on the motility and shape of spermatozoids. Lloret and Planes (2003) detected that reproductive potential of males *Diplodus sargus* decreased with depth, suggesting the importance of environmental factors on their reproductive success. Shearer and Swanson (2000) corroborate the effect of condition of male Chinook salmon on the probability of being mature, whereas Morgan (2004) did not observe any relation between condition factor and the maturation process of cod male. In the present work, only characteristics of females have been taken into account in SRP estimates, because, although male condition plays an important role in fertilization success and development rates of embryos, egg production is the limiting factor in stock productivity.
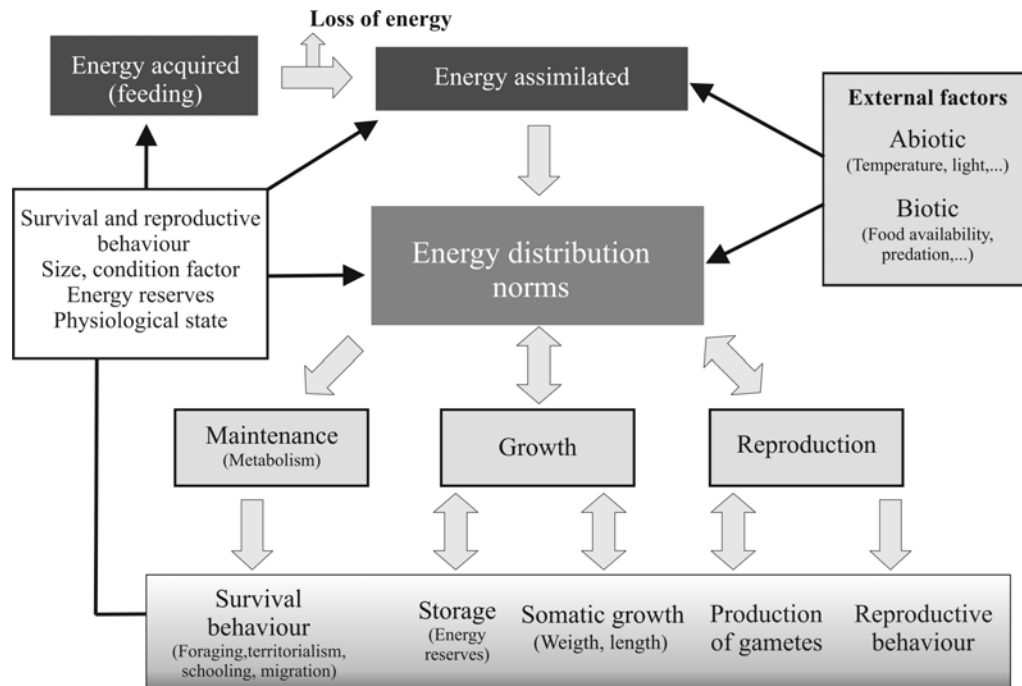
Figure 4.1: Schematic representation of energy flow allocation norms. Open arrows represent energy routes while solid arrows represent factors affecting energy allocation. Energy allocated for maintenance, growth and reproduction is partitioned between survival (including survival behaviour), storage of energy reserves, somatic growth, production of gametes and reproductive activity, all of which may affect energy acquisition, assimilation and distribution through feedback regulating systems. External factors influence the level of energy intake and allocation patterns. Energy invested in growth and reproduction can be reallocated to other locations (by e.g. reduction in reserves, loss of somatic weight, yolk absorption). (From Saborido-Rey and Kjesbu, in press).

Many stocks are managed on the general assumption that there is a relationship between the stock and the number of recruits that are generated from that stock. Based on this assumption, a large number of stock-recruitment models has been developed by (for example) Beverton and Holt (1957), Ricker (1954) and Shepherd (1982). Whilst these models were originally developed using fecundity to recruitment (Rothschild and Fogarty 1989, Koslow 1992), the term Spawning Stock Biomass (SSB) has generally been used instead of fecundity or egg production. The stock-recruitment relationship forms a cornerstone of fisheries assessment and management via projected stock development and through biological reference points. Management advice generally has the objective to ensure that spawning stock biomass (SSB) and fishing mortality (F) remain above and below threshold values (B_{lim} and F_{lim} respectively). Precautionary reference points (B_{pa} and F_{pa}) are used to take into account uncertainty in assessment and management. Management is implemented as a stock-specific total allowable catch (TAC) corresponding to a fishing mortality level that will ensure that SSB remains above or recovers to the precautionary biomass level (B_{pa}). In many cases, the reference points are fixed, explicitly assuming that no change in productivity occurs. However, biological productivity and the underlying processes, including maturity have fluctuated over a large range of spatial and temporal scales independently of human exploitation, and in response to environmental changes. These

reference points are, in some other cases, almost explicitly defined on the basis of SSB as a proxy for productivity.

SSB used in most of the Stock-recruitment relationships assumes that fish of all ages contribute equally to spawning success; i.e. reproductive potential is directly proportional to total egg production. However, as discussed above there is evidence that these assumptions are not correct. The alternative to SSB, the Stock Reproductive Potential (SRP) coined by Trippel (1999) is clearly defined as a concept. However, it is not so clear how it can be estimated, as the features affecting it may change from stock to stock. For this reason, it is important to understand the mechanisms influencing SRP in each particular stock, and to include the information related to SRP in assessment models, and account for stock composition, structure and quality of the spawning stock. Identifying the intrinsic and environmental factors which influence fish reproductive dynamics is essential also for stock assessment methodology and management of wild fish populations (Faahraeus-Van Ree and Spurrell, 2003; Godø and Haug, 1999; Heder, 2001; Jørgensen *et al*., 2006; Marteinsdottir and Steinarsson, 1998; Solemdal, 1997). Marteinsdottir and Thorarinsson (1998) observed that inclusion of a stock age diversity index in Icelandic cod assessment improved the stock-recruitment relationship, and variation of recruitment explained by the model increased from 15% to 31% when age composition was considered. Furthermore, Saborido-Rey *et al*. (2004) not only analysed effects of including age composition of spawner stock in stock-recruitment models, but also considered sex ratio and presence of skip spawners that directly affected estimates of SSB; it was observed that recruitment predictions improved when these variables were included in the model. Marshall *et al*. (1998) suggested that survey-based indices of total abundance and reproductive potential should be incorporated into the analytical assessment of stock status to indicate the quantity and quality of old spawners, because models based on SSB-recruitment relationship are not sensitive to the true variation in reproductive potential of the stock. According to Lambert *et al*. (2003), relationships between biological and environmental factors, and reproductive characteristics could potentially be used to predict the reproductive potential of a stock or some of its components. Smith *et al*. (2001) observations support this idea, and suggest that high recruitment rates require high egg production and high embryonic and larval survival, which depend directly on maternal status, i.e. in good condition to face adverse environmental conditions. However, they pointed out that although these features are required, they are not sufficient to guarantee high recruitment, because of dependence of reproductive success on ocean physics that has been underestimated. Katsukawa *et al*. (2002) proposed the use of Total Fisher's Reproductive Value of the standing stock as a robust tool for evaluation of long-term stock productivity. Other authors defend the view that it is necessary to simplify assessment models, e.g. Kelly and Codling (2006) consider that mathematical models on which most analytical methods are based, like VPA or Bayesian approaches, are deterministic, make many important assumptions and/or require a large amount of data, and require a high level of expertise to interpret them; they suggest using

theory from management process to assess and manage fisheries resources, because this approach provides a simple way to use and interpret indicators by "eyeballing" the available data and making subjective judgments based on prior knowledge and experience. Obviously, fishing pressure also plays an important role in regulation of reproductive potential, acting as a predator on the fish stock, and removing from the spawning stock the larger and older individuals with the consequent effect that this has on stock reproductive potential. Consequently, fishing pressure has to be considered as another factor to take into account in fisheries management. At the same time, economical fisheries success depends on sustainability and diminution of recruitment failure.

Morgan and Brattey (2005) observed that inclusion of improved biological and fecundity information changed perception of SSB absolute levels, although general patterns in SSB were comparable between approaches, those in which models were implemented with biological and fecundity information and those in which they were not. However, these differences are not always due to the inclusion of fecundity; frequently they are due to improved information on variation of maturity ogives, particularly where working groups have used constant ogives over time. If assessment models consider ogives constant, underestimation of SSB can result; but if only recent maturity ogives variations are considered, historical SSB might be overestimated. Thus, it is necessary to provide historical maturity data. Since management is largely based upon relative values of spawning stock estimates and limit/precautionary biomass reference points, further investigation is necessary. Taking this into account, differences in the reproductive potential of a stock could result in very different recovery expectations, dependent on the recruitment rate used in stock projections. The main disadvantage of these improved models is that a number of assumptions have been made in relation to missing data or the way data was compiled, which further underline the caution required at the time of interpreting the results and developing conclusions (Kelly and Codling, 2006).

Improvements in stock assessments are required for several reasons. Under exploitation, stocks generally decline in size and at present many stocks are in some form of depletion, ranging from light to a collapsed state. In the EU, the Common Fisheries Policy (CFP) is explicit in the requirement that the Council adopts as a priority, recovery plans for exploited stocks which are outside safe biological limits (2371/2002). Furthermore, the EU is now committed to the recommendations of the Johannesburg World Summit on Sustainable Development Plan of Implementation, including targets to restore depleted fish stocks by 2015. Management entities are managing at the edge for many species, and therefore require the most accurate and precise stock assessments possible; for example, most European marine fish resources are outside safe biological limits, i.e. overexploited, in spite of the various technical measures undertaken to recover the stocks. Following the Precautionary Approach to Fisheries (FAO, 1995), overfishing is no longer permissible; and there are currently increased demands for adopting not only a precautionary approach but

also incorporating ecosystem considerations into stock assessments and fisheries management. Inclusion of new parameters that are more or less easily measure, such as condition index, age composition of spawning stock, incidence of skip spawners, or environmental parameters, is necessary to increase prediction capacity of assessment models, and would guarantee protection of fish stock and provide more sustainable fisheries systems (Mace *et al*., 2001). However, a trade-off between accuracy and costs must be reached to maximize efficiency of fisheries research, assessment and management.

The lack of correlation between spawning stock biomass and reproductive potential could be even more pronounced in the case of species with indeterminate fecundity, where the egg production per unit of spawning stock biomass may vary substantially between years, depending upon environmental conditions (temperature, food availability, etc.) during the spawning season (Murua *et al*., 2006). This is the case for European hake (*Merluccius merluccius*). In contrast to most gadoids and demersal species, hake exhibit a high growth rate, indeterminate fecundity, and a protracted spawning season, so that environmental and biological factors may lead to large inter-annual variation in egg production per unit of spawning stock biomass.

Until now, hake assessment has been carried out based on stock-recruitment models where SSB has been estimated using maturity ogives based on macroscopic observations of gonads from research surveys and landings. This may produce unreliable maturity ogives as it has been shown (Domínguez *et al*., 2005) that errors are often made, especially between immature and spent/resting specimens. The impact of these errors on SSB estimation has not been properly evaluated, and is a desirable exercise. But it has also strongly recommended the use of histology to determine maturity stage and/or to calibrate macroscopic staging (Murua *et al*., 2003). A further problem in hake assessment is that maturity ogives are sex combined. Study of the SRP of *Merluccius merluccius*. Is hampered by the lack of proper maturity ogives, together with the asynchronous behaviour of individuals in the populations characteristic of the hake reproductive cycle, the impossibility of maintaining specimens in captivity, the difficulty of calculating age, and the uncertainty concerning how many different stocks of this species exists in the East Atlantic and Mediterranean Sea.

It is obvious that current assessment models are not able to realistically predict stock fluctuations, and progressive decline of hake populations is taking place in spite of preventive measures. Changes in hake population structure and biological parameters have occured and are not being taken into account in forecasting models.

4.3   REPRODUCTIVE CYCLE OF *M. MERLUCCIUS* FROM GALICIAN SHELF.

The scientific literature is replete with articles describing the reproductive biology and oocyte development dynamics of fish. Differences among spawning strategies of different species have produced descriptions using numerous terms and classification systems for the various stages of oocyte development. This excess of terminology results in confusion and makes communication between fisheries biology researchers difficult. Consequently it is necessary to establish a unified terminology that responds to two basic questions: when and how often will the fish spawn? In the present work a specific classification system was made for the particular case of *Merluccius merluccius* (see Results) based on microscopic observations.

Results of this study indicate that the southern stock of hake shows asynchronous ovarian development, indeterminate fecundity, and that the species is a partial spawner, confirming the findings of other authors for other stocks (Murua et al., 1996; Macchi *et al*., 2004; Murua and Motos, 2006). Hake ovaries in the migratory nucleus and hydration stages, as well as with different POF stages, were found all year round. The prevalence of these spawning stages indicates also a protracted spawning season, which may well extend to the whole year, at the population level. However, the duration of individual spawning seasons still remains unknown, and it has not been possible to estimate this efficiently in this work, partially due to the long population spawning season which shows clear asynchrony at the population level, i.e. basically all developmental stages are found at any time of the year. Nor have individual spawning seasons been properly estimated elsewhere either.

A lengthened population spawning season has been also observed in the same species in the Bay of Biscay (Lucio *et al*., 1998) and in the Adriatic Sea (Ungaro *et al*., 2001), as well as in *M. hubbsi* that has two main breeding periods, one in autumn-winter on the continental platform of Buenos Aires, and another in summer in Isla Escondida area, despite a spring spawning period in small southern areas (Louge, 1996). Osborne *et al*. (1999) also detected spawning females during the whole year for *M. capensis*. Both, protracted and asynchronous spawning seasons has been observed in other species. Rideout *et al*. (1999) observed that oogenesis in Greenland halibut from the North-west Atlantic is not synchronous between individuals of the same population, suggesting that the spawning season is not well defined; fish in spawning condition are detected all year round, indicating an uncommonly protracted spawning season (Junquera and Zamarro, 1994; Junquera and Saborido-Rey, 1995). Something similar is observed in, bigeye grenadier, another gadoid, that presents all oocyte developmental stages and sizes in mature ovaries, which suggests that they undergo either a prolonged spawning season that is typical of cold water species with low growth rates, and/or that they may be multiple batch spawners, although there is a main spawning peak (Morely *et al*., 2004). Allain (2001) also observed very long spawning seasons (from February to November) in other indeterminate, asynchronous species like

Coryphaenoides rupestris and *Alepocephalus bairdii*, and related the increase of reproductive period duration and the decrease in synchronicity of maturation with the increase of depth, suggesting that long spawning periods and asynchrony might be partially explained by the constancy of environmental factors. In contrast, Garvey *et al.* (2002) suggest that protracted reproduction at the population level may increase expected recruitment in variable environments, influencing growth and survival across multiple life stages. In the case of the Galician Coast, hake fry survival is very dependent on annual upwelling events that occur in sporadic episodes during a long period of the year (Wooster *et al.*, 1976; Fraga, 1981 and Castro *et al.*, 1994). Thus, asynchrony and protracted spawning season would be in accordance with above hypothesis. In the Mediterranean Sea, a very long spawning season has also been observed (Orsi-Relini *et al.*, 2002; Abella *et al.*, 2005) which could be considered a strategy to reduce temporal variance in offspring survival, in accordance with observations on Atlantic cod made by Hutchings and Myers (1993). This reproductive structuring in some fish populations makes it very difficult to follow the progress of gamete development and spawning activity throughout the year, and prevents making distinctions between pre-spawning and spawning females, because practically no differences in ovarian structure between them are detected.

The spawning fraction was highest between January and March, with a second smaller peak observed, especially in June-July in some years. In 2003, however, the situation reversed, and the highest spawning fraction was detected in early summer. More than one peak of spawning has also been observed by other authors in Mediterranean populations, e.g. Biagi *et al.* (1995) detected one peak of spawning activity in February-March and another one in September in the Tyrrhenian Sea, while Orsi-Relini *et al.* (2002) suggests the existence of three peaks of spawning in hake from the Tunisian Shelf, in summer, winter and spring. The existence of two or more peaks of spawning may be explained by the presence of several sub-populations leading to a temporal difference in spawning period; nevertheless sampling surveys carried out in this work were made in the same area, so it is not a probable cause, although it cannot be discarded. The existence of different cohorts could also explain the existence of different peaks of spawning, although more studies are needed to corroborate this hypothesis, because our analyses do not cover these aspects. Some authors suggest that this could be an effect of differences in the duration of spawning between small and large females (Alarcón *et al.*, 2004) which may be a more plausible explanation. Macchi *et al.* (2004) suggested that smaller and younger females of Argentine hake start to spawn before larger and older ones. Different spawning periods depending on size and/or age have been described for a number of species (Mertz and Myers, 1994; Bleil and Oeberst, 1998; Scott *et al.*, 2005; Simønsen and Gundersen, 2005). According to our results, nevertheless, a peak of batch fecundity was observed between January and April; corroborating the hypothesis that the principal spawning period lies between these months although there was spawning activity present at generally lower levels the rest of the year.

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The existence of a prolonged spawning season but with a main spawning peak is corroborated by evolution of GSI during the year which, although it showed relatively high values from January to October, reached the highest values in January, February and March. These results coincide with data published by Lucio *et al*. (1998) on Bay of Biscay hake, and with studies of Álvarez *et al*. (2001) that detected a peak of eggs and larvae production of this species in late winter-early spring in the Bay of Biscay. Murua *et al*. (1996) described peak of spawning in April 1994 with 26.8% of hydrated females from the total mature stock. In the Mediterranean Sea, some differences in spawning activity have been observed depending on the area considered. In the eastern Mediterranean, peak spawning occurs in Februray-May (Lleonart, 2001), whereas in the western area it is earlier, in Autumn-Winter (Recasens *et al*., 1998; Morales-Nin and Moranta, 2004). According to Alheit and Pitcher (1995), the spawning peak of hake is earlier in southern areas than in northern ones, probably because environmental conditions favour reaching optimal conditions for reproduction earlier (Piñeiro and Saínza, 2003). In Portugal, the spawning season of European hake takes place in February-March, on the Galician Shelf and in the Cantabrian Sea and Bay of Biscay, from January to May (Pérez and Pereiro, 1985; Martin, 1991; Sánchez and Gil, 2000), in the Celtic Sea between April and June (Horstman, 1988); according to O´Brien (1986) the breeding season on the Western Irish Coast lasts from April to June, and in Western Scotland Shelf from May to August; finally,on the West Coast of Norway, the hake breeding season occurs in August (Kvenseth *et al*., 1996). Results of these authors corroborate Alheit's hypothesis; nevertheless this hypothesis; does not always find support. For example, for *Zeus faber* from the Eastern China Sea, individuals from the northern population mature before than those with a southerly distribution, probably because this species shows ontogenetic dietary shifts in relation to the onset of sexual maturity, and larger individuals are expected to exploit a wider range of the water column to gain more energy compared with smaller fish. The warmer temperatures of southerly distribution areas may lead to additional energetic costs for obtaining food that may increase metabolic costs, affecting the energy investment into the onset maturation (Yoneda *et al*., 2006). Differences in the spawning season have also been described for hake species. In the northern hemisphere, Pacific hake (*M. productus*) starts its spawning period in winter (Averson and Larkins, 1969; Benson *et al*., 2002); silver hake (*M. bilinearis*) spawns from April to May in the Southern areas (Helser and Almeida, 1997) and later in the Northern areas, e.g. on the Nova Scotia shelf spawning season starts in June and finishes in September, with a peak of reproductive activity in July-August (Rikhter *et al*., 2001).

In the southern hemisphere, Argentine hake (*M. hubbsi*) has its main spawning period from November to April with a peak of spawning in January, i.e. in summer (Macchi *et al*., 2004; Pájaro *et al*., 2005); common hake (*M. gayi*) has the peak of spawning in late summer (March) in Chilean Shelf waters (Cerna and Oyarzún, 1998). Louge (1996), Macchi *et al*. (2005) and Pájaro *et al*. (2005) considered that one of the main factors that determined

temporal variability of spawning is bottom water temperature that for *M. hubbsi* must be higher than 9ºC; in addition, there are factors such as upwelling indices and other oceanographic processes that determine retention of eggs and larvae and availability of food. In other species, the effects of other environmental factors such as the lunar cycle have been observed to affect spawning behaviour, e.g., *Sparus aurata* showed a peak of egg production during Full Moon (Saavedra and Pousão-Ferreira, 2006).

As mentioned above, the durations of individual spawning seasons were not accurately estimated due to population asynchrony. While it is not possible to estimate individual spawning duration in the field, an average value can be estimated from the interval between the time when 50% of the mature females are in pre-spawning condition and when 50% of them are post-spawning. In this study this approach was not carried out, on one hand, because it was not possible to differentiate pre-spawning females, and on the other, because the proportion of post-spawning females never reached 50% in any of the years studied. Some authors assumed that the hake spawning season lasted three months (Sarano, 1984; Murua *et al.*, 1996), although the limits of the reproductive period at an individual level are difficult to define (Martin, 1991). Kjesbu (1989), based on potential fecundity, batch fecundity and batch interval, estimated the spawning duration of Northeast Arctic cod in captivity between 47 and 60 days; however, assuming that potential fecundity of wild cod is 2.5 times lower than that of captive specimens, he suggested that the spawning duration of wild cod may be between 17-25 days. Thorsteinsson and Marteinsdottir (1998) calculated the spawning duration of wild Icelandic cod by estimating the time individuals spend on the spawning grounds, and calculated a spawning duration equal to 17-31 days with an average of 22.5 days. On the other hand, Hutchings and Myers (1993) recorded the duration of the individual spawning season for wild Northwest Atlantic cod as 25.2±8.4 days. In summary, all these estimates are around one month. Hislop (1984) observed that female whiting in captivity were able to spawn during 13 weeks, i.e. three months, approximately.

In the present work, a 2 month individual spawning season has been chosen as an average from other studies for the southern stock of hake for three reasons: i) No exact knowledge of individual spawning duration is available for hake, although some authors have assumed it to be three months (Sarano, 1984; Murua *et al.*, 1996); ii) Whiting showed a three month spawning season in captivity, which could be a little bit shorter in wild specimens as Kjesbu (1989) suggested and iii) cod spawning duration has been empirically recorded around one month. Thus, a two month spawning duration is a proxy based on other studies of other gadoids in which annual egg production has been estimated. More studies are necessary to accurately estimate the duration of individual spawning seasons, but this is not an easy goal in European hake. Until now, attempts to maintain hake in captivity have not led to reproduction, because species of this genus are very sensitive to handling-related damage (Bjelland and Skiftesvik, 2006). On the other hand, some capture-recapture experiments have been carried out, but no good quality information has been obtained due to the fragility

of these species (Lucio *et al*., 2000; de Pontual *et al*., 2003). It is necessary to improve the methodology of hake culture to reach a better knowledge of its reproductive behaviour. It is also necessary investigate the effects of environmental factors, age, size and condition on spawning time and duration, as shifts in spawning activity may change stock reproductive potential. Some authors have observed that old experienced females start spawning before and finish later than young and inexperienced females, have shorter intervals between batches, produce more eggs, and thus ensure that some batches hatch coinciding with good environmental and food conditions (Hutchings and Myers, 1993; Chambers and Waiwood, 1996; Thorsteinsson and Marteinsdottir, 1998). Others suggest the opposite situation, that younger specimens have a more protracted spawning season, and start to spawn earlier than older ones (Morgan 2003). Furthermore, egg condition is not the same throughout the spawning season (Solemdal *et al*., 1992a; 1992b). All these factors can affect reproductive and recruitment success and the consequent effects on assessment.

Spawning frequency is an individual concept defined as the number of times a single female spawns during a spawning season, expressed in times per day. For example, a female that spawns every second day has a spawning frequency of 0.5. The batch interval is the number of days between two batches released by a female and is, therefore, the inverse of spawning frequency, also an individual concept. At the population level, spawning frequency may well be expressed as the average of individual spawning frequencies. The most accurate way to estimate spawning frequency, i.e. batch interval, is to follow batch production sequences of an individual fish in tank experiments (Hunter and Goldberg, 1980; Kjesbu *et al*., 1991). However, this approach often cannot be used, and, the spawning fraction is estimated as a proxy for spawning frequency, (Hunter and Goldberg, 1980; Hunter and Macewicz, 1985). The spawning fraction is defined as the proportion in a given period of the total number of females releasing eggs from the total of mature active females, although there are some discrepancies among authors on the use of spent or regressed females as active (see Stratoudakis *et al*., 2006). The terms spawning frequency and spawning fraction are too often confused and misused. If a population spawns basically in the same time period, the estimate of spawning fraction made during peak spawning is near to the spawning frequency. However, in species or populations where spawning extends over a long period with high asynchrony in individual spawning, such as hake in Galicia, the use of spawning fraction estimated during peak spawning may overestimate egg production, while if estimated during months of low activity, egg production may be underestimated. Thus a regular estimate of spawning fraction is required.

The inverse of the spawning fraction estimated for each sample is considered a proxy of batch interval (Fitzhugh *et al*., 1993; Hunter and Goldberg, 1980; Lasker, 1985; Macchi and Acha, 2000). However, to repeat, while batch interval refers to the interval between two batches in a single female, the inverse of spawning fraction is a population concept. To avoid confusion, it is preferable to introduce a new term, Population Batch Frequency (PBF). PBF

is a population concept because it not only depends on individual batch intervals but also on the number of females that release-eggs in a given period; e.g. if a female hake releases a batch every 5 days at any time of year, PBF estimated during peak spawning (February) would be near this value because almost all females from the stock are releasing eggs at that particular moment; however, if PBF is estimated 7 months later (September), it will be higher, for example 10 days; this does not mean that females in September release a batch every 10 days, simply that fewer females are releasing eggs, so that the PBF monthly average is higher than in February. Batch interval may change during the individual spawning season, but only slightly.

For hake in Galician shelf waters waters, the highest values of spawning fraction are around 0.2 in January and June of 1999, May of 2000, February and July of 2003 and February 2004, but in 1999, in contrast to the other years, the summer peak of spawning fraction was higher than in February. Based upon these results, it was estimated that PBF was between 5-7 days during January and March and, subsequently, activity decreased, PBF being 10 days or more during the remaining months. Murua *et al*. (1996) and Murua and Motos (2006) estimate PBF for European hake in the Bay of Biscay during peak spawning at around 5 days, increasing afterwards to 10 days or even more. For Argentine hake (*M. hubbsi*) something similar was observed, Macchi *et al.*, 2004 suggest that it spawned once every 7 days during the peak of spawning and every 10 days at the end of the spawning season. On the contrary, for this species, Pájaro *et al*. (2005) estimated spawning frequency as 10 days (S = 0.10) during the peak of spawning.

Concerning the peak spawning fraction observed during June-July 2003, it may have been the consequence of a good environmental situation. In November 2002, the oil tanker *Prestige* sank after breaking into two halves 150 miles off the Galician coast, releasing 39,700t of heavy fuel to the marine environment. Large patches of oil were washed onshore, blackening more than 550 km of the coast. Fisheries remained closed to different degrees and for different periods depending on area and fleet, but in general were closed from December 2002 to April 2003. According to Benson *et al*. (2002), environmental variations and decrease of stress induced by fishing could modify the behaviour of a stock, and could change the timing, location, and condition of spawning: this could in turn determine the survival rates of eggs and larvae and consequently affect recruitment in subsequent years. In fact, in 2005, the highest catches of hake in recent years were recorded in Galician coastal waters (Cerviño, personal communication). However, late in the year, a peak was also observed in 1999 (June), so other environmental conditions may be responsible for these changes, e.g. upwelling which varies in intensity during the year in response to variations in the wind regime (Castro *et al*., 1994).

The ovarian stages used to estimate spawning fraction differ in the literature. In principle, any ovarian stage associated with spawning can be used, providing its duration is

known (Priede and Watson, 1993). The hydrated oocyte method (Hunter and Macewicz, 1985) is preferred, and has been used in this study. However, PBF changes depending on the method used to estimate it, for example Murua *et al.* (1998) estimated the PBF of hake from hydrated females prevalence as 5.25 days during peak spawning, but if estimates were based on presence of hydrated and migratory nucleus stages (as suggested by Priede and Watson, 1993), then PBF increased to 8.3 days. Nevertheless, Murua and Motos (2006) and Macchi *et al.* (2004) estimate the spawning fraction based upon the percentage of POF day-0 and POF day-1, obtaining PBF values of 5 and 7 days respectively during peak spawning, whereas Wright (1992) observed in *Encrasicholina heteroloba* that PBF changed from 9.5 to 16.7 days when the average between hydrated oocytes and POF day-0 or POF day-1 respectively were considered. In this study, PBF has been estimated from the mean prevalence of hydrated oocytes and POF day-0, which results in a mean PBF during peak spawning between 3 and 5.1 days in Galician shelf waters waters. This method has been also used by Pájaro *et al.* (2005) in Patagonian hake (PBF = 10 days during peak spawning). The reason to use the average between hydrated oocytes and POF day-0 was that the prevalence of POF day-1 in the Galician Shelf samples was extremely low compared with POF day-0 and day-2, which gives low confidence to spawning fraction estimates based upon day-1 POFs. Besides, hydrated oocytes and POF day-0 are more easily identified than other POF stages, even for a non-experienced reader, improving the accuracy of estimates. However, for some species, such as *Engraulidae* and *Clupeidae*, estimators based on the day of spawning provide higher estimates of spawning fraction than estimators based on the days immediately after spawning. Those species show clear evidence of daily spawning synchronicity towards dusk or early night (Hunter and Goldberg 1980; Picquelle and Stauffer 1985; Murayama *et al.* 1994; Lo *et al.* 1996; Macewicz *et al.* 1996; Motos 1996; Zwolinski *et al.* 2001; Ganias *et al.* 2003). As a result, the hydrated oocyte method provides biased estimates of spawning fraction, so estimation has to be based on the POF method (Hunter and Goldberg 1980; Hunter and Macewicz 1985). However, part of this bias is due to the gear used for those species, purse seines, which often target such aggregations. There are few studies of the daily spawning activity of hake, but if spawning females are more available to the gear used in this study (gillnet), spawning fraction is clearly overestimated, although figures obtained were similar to those obtained with bottom trawls on the Northern stock by other authors (Murua *et al.*, 1998; Murua and Motos, 2006).

In any case, as the aging of different oocytes and follicle stages is not yet exact, the results of spawning fraction should be considered with caution. The duration of different oocyte stages used to estimate spawning fraction are not sufficiently known, therefore partly hampering estimates of spawning fraction; consequently, further work is needed to estimate the duration of the spawning stages or to develop an alternative method to determine spawning fraction (Claramunt and Roa, 2001).

One important conclusion brought up by this study is the population asynchrony in spawning, as well as the extensive spawning season spanning almost the entire year for European hake; this feature is also detected in other areas like the Adriatic Sea (Ungaro *et al.*, 2001) and Bay of Biscay (Lucio *et al.*, 1998), and for other species of this genus like *M. capensis* (Osborne *et al.*, 1999). This result is also important because one would expect a different pattern of batch production from the hypotheses of a critical first-feeding period (Hjort, 1914) and larval match mismatch theory (Cushing, 1975; Solemdal *et al.*, 1992b; Bromley *et al.*, 2000); which stated that stocks adapted their spawning season to the peak of plankton production of the area to avoid starvation during the critical first-feeding period. Nevertheless, Bailey (1981) suggested that for Pacific hake (*M. productus*), the "first-feeding" period may not be as important as for other fish species due to their longer period to locate food based on (1) slow growth, slow metabolic rates and low daily ration of Pacific hake larvae, (2) large mouth of first-feeding hake larvae and, therefore, larger food items in the diet and (3) the relatively longer starvation time for larvae. In the case of European hake, Álvarez and Cotano (2005) found that hake larvae exhibited slow growth, low daily rations, and large mouth at first feeding, and relatively high starvation endurance, in agreement with the findings of Bailey (1981). Although certain endogenous mechanisms of reproductive timing control exist, environmental factors, mainly photoperiod and salinity can also affect the beginning and duration of the spawning season. Most fish species have reproductive cycles that try to guarantee propagule survival. Constant environmental characteristics of the fish habitat can provoke extended spawning seasons, with individuals releasing eggs at different times during the year, although there are regular periods of more intense spawning activity (Bye, 1984). This is the case of Greenland halibut from Canada (Junquera and Saborido-Rey, 1995). Fish that live in habitats with pronounced seasonal environmental changes have breeding seasons which are almost invariably confined to a brief and specific period of the year, e.g. polar species. These are the species on which Hjort's and Cushing's hypotheses are based, because these species have to match their breeding seasons with seasonal variations in availability of food.

The characteristics of reproductive strategy of hake described above make it difficult to deal with studies, at different levels, on reproduction of this species. Consequently, the present work tries to analyse several aspects of reproduction (condition effects, bioenergetics, fecundity, atresia, etc.) to tackle different viewpoints directed towards improving knowledge of the reproductive cycle of this species.

4.4 MATURITY OGIVES OF *M. MERLUCCIUS* ON GALICIAN SHELF.

Estimates of maturity in this work are based on size but not on age, because nowadays age determinations of hake are questioned and under debate (see previous discussion).

Size at maturity (L_{50}) of females was estimated at 44.45 cm in 2003 and 42.97 cm in 2004 in this study. These values are slightly higher than those observed in the Bay of Biscay (Northern Stock) for the same period, estimated at 41.70 cm and 41.40 cm respectively (RASER, 2006). Similarly these values are higher compared with those estimated in Mediterranean Sea. Female size at maturity was estimated at 31 cm in the Southeast Iberian Peninsula in 1991-1993 (García-Rodríguez and Esteban, 1995), 26 to 33 cm in the Adriatic Sea in 1985-1997 (Ungaro *et al.*, 2001), and 30 to 39 cm in the Western Mediterranean (average value of diverse authors; Alheit and Pitcher, 1995). More similar is, however, is the L_{50} of Piñeiro and Saínza (2003) for the Iberian Peninsula (45.4 ± 6.2 cm), and of Lucio *et al.* (2002) who observed a decrease in L_{50} from 50.2 cm in the 1987-1990 period to 46.6 cm in 1999-2001 in the Bay of Biscay. Domínguez *et al.* (2005; in press) reported significant changes of length at first sexual maturity of European hake from the Bay of Biscay and from the Galician Coast. In the Bay of Biscay, L_{50} has progressively decreased from 56 cm in 1987 to 41 cm in 2004, while on the Galician Shelf L_{50} diminished from 56 cm in 1980 to 39 cm in 1988, increasing again to 53 cm in 1998. From 1999 to 2004, it has remained rather stable at 47 cm. These shifts in size at maturity probably reflect changes in growth rate (Domínguez et al., in press). In other species of *Merluccius*, some differences have been also detected. For Pacific hake (*M. productus*), Averson and Larkins (1969) estimated size at first maturity at 52 cm during the 1960s. For Argentine hake (*M. hubbsi*), Pájaro *et al.* (2005) calculated L_{50} equal to 31.8-33.8 cm in January and March 2001 respectively. In Chilean hake (*M. gayii*), Alarcón and Arancibia (1993) described size at first maturity at 37.9 cm and Cerna and Oyarzún (1998) at 38.9 cm in 1992.

References to age at first maturity (A_{50}) in the literature show similar differences among studies, partially reflecting the inherent difficulties in age determination as mentioned, but partially revealing also differences in methods of estimating maturity. Thus, Lucio *et al.* (2000; 2002) estimated A_{50} for the years 1987 to 1990 and 1996-1997, while Piñeiro (2003) observed an age at maturity of 4.4 years for 1996-1997 for the same area, Iberian Atlantic waters. Averson and Larkins (1969) for Pacific hake and Cerna and Oyarzún (1998) for Chilean common hake obtained similar results. On the other hand, Helser and Almeida (1997) and Rikhter *et al.* (2001) for silver hake (*M. bilinearis*) reported an A_{50} equals to 2 years.

Differences in maturity between stocks and species may result from genetic characteristics, as well as from differences in environment, such as hydrographical conditions (i.e. water temperature) and biotic factors, such as food supply (Helser and Almeida, 1997; Helser and Brodziak, 1998; Saborido-Rey and Kjesbu, in press). Environmental conditions are not only important during the pre-spawning/spawning period, but conditions at earlier stages might also determine differences in age and size at maturity, as initiation of maturation may to some extent depend on the attainment of a critical size (Le

Bail, 1988; Roff, 1991), and hence be affected by growth rates. For example, Martell *et al.* (2006) observed in haddock (*Melanogrammus aeglefinus*) that high egg incubation temperatures increase embryonic developmental rates and provoke earlier hatching, so that exogenous feeding starts earlier; in addition, faster growth of larvae results in early maturation. This is corroborated by Ojaveer (2006) in his study of the NE Kamchatka herring (*Clupea pallasi*) in which he concluded that variations in temperature and other environmental conditions during the early life history have a substantial influence upon the abundance of year-classes, and affect reproductive parameters of the spawning stock. O´Brien (1999) observed for cod from the Georges Bank and the Gulf of Maine that differences in size and age at maturity were influenced by stock biomass and bottom temperature during the juvenile stage. Saborido-Rey and Kjesbu (in press) proposed four different ways that exploitation, and other factors, may influence the individual growth of fish, both in the short and long term, and the subsequent attainment of maturity: 1) by reducing population abundance (giving rise to density-dependent responses), 2) by altering the density of different prey (incl. switch to non-optimal food) 3) by diminishing the abundance of predators/competitors, and 4) by systematic, selective removal of individuals growing at rates deviating from the average for the original population as a whole.

Stress episodes, including fishing pressure that extracts older and larger individuals from the spawning stock, can provoke changes in age and size at maturity. In this situation, intra-specific competition diminished and smaller fish have more available food, grow at higher rates and reach maturity younger. Morgan and Brattey (2005) propose two possible explanations; on the one hand, they consider that this may be the response to a stock biomass reduction, and on the other, suggest it could be a genetic response to increased mortality. The compensatory response and effects of environmental factors have been recorded by several authors in different species. Haug and Tjemsland (1986),e.g., in Atlantic halibut from Norwegian waters, but in this case, it was observed that while age at maturity decreased, size at maturity remained more or less constant. Junquera *et al.* (1999) described similar results for size at maturity of Greenland halibut from the Northwest Atlantic, and suggest that this species is relatively resilient to environmental changes, and that its reproductive parameters were remain stable despite unfavourable conditions, although age at maturity was not considered in their study. Engelhard and Heino (2004a) observed in Norwegian herring that during collapse of the population, age at maturity diminished, whereas size at maturity increased slightly, probably as a compensatory response. Marteinsdottir and Begg (2002) described for Atlantic cod a decrease in size and age at maturity, not only between years due to overexploitation, but also between Northern and Southern stocks, probably as a consequence of changes in temperature. Some stressing factors, like fishing, exert a biased selection of individuals, and if they continue for along time, can fix genetic changes that are reflected in variations of reaction norm (Jørgensen, 1990; Engelhard and Heino, 2004b; Ernande *et al.*, 2004; Barot *et al.*, 2005). This seems to be the case of Flemish Cap cod whose size and age at maturity have decreased, and show no

signs of recovery despite the moratorium (Saborido-Rey and Junquera, 1999). Bowering (1989) detected in witch flounder a decrease in size at maturity but an increase of age at maturity; the former could be explained by overexploitation but not the latter. Something similar was observed in striped bass (*Morone saxatilis*) in coastal Rhode Island waters during 1985-1987, that showed a decrease in size at maturity accompanied by a slight increase in age at maturity. In contrast, *Catostomus commersoni* from acidified lakes exhibited late age and large size at sexual maturity despite declines in population size (Trippel, 1995). Although a certain phenotypic plasticity exists, Helser and Brodziak (1998) emphasizes that from determined fishing mortality (F=0.5-0.6), a stock compensatory response disappears or is very slow.

All these changes in maturity parameters affect stock reproductive potential, because smaller and younger females have different reproductive attributes than larger and older individuals (Solemdal, 1997; Trippel *et al.*, 1997). Maternal physiological status, spawning experience (recruit or repeat spawners) or food rations during gametogenesis are all known to alter fecundity, egg and larval quality, as well as duration of the spawning season (Hislop *et al.*, 1978; Kjesbu *et al.*, 1991; Trippel, 1999; Marteinsdottir and Begg, 2002). Fecundity and relative fecundity generally increase with body size (Wootton, 1979; Kjesbu *et al.*, 1998). Consequently, at the population level, the annual population fecundity (total egg production) will, in addition to the number of mature fish, depend not only on size (age) structure, but also on fish condition (Marshall *et al.*, 1998). Change in stock structure entails a compensatory response of age/size at maturity because depletion of large fish can be compensated by increased egg production by young fish (Trippel, 1995). If this compensatory response does not occur a stock could reach a critical stage. Consequently, to improve fisheries assessment, it is necessary to account for compensatory mechanisms of stocks and the variability of maturation parameters.

Body growth and accumulation of energy stores are likely to act as early triggering signals for determining the onset of puberty (Saborido-Rey and Kjesbu, in press and references therein). Condition factor is a function of relative densities of prey and predator, and has been revealed as an important factor affecting maturation, ripening and fecundity.

4.5 CONDITION.

Somatic condition indices are important factors to be considered in stock reproductive potential together with energy reserves and proximate composition of females.

The analyses performed in this work on hepatosomatic index (HSI) and condition factor (K) showed that HSI values ranged from 2.7% to almost 11% (mean=4-5% depending on year considered). Not many data on the HSI or condition index of hake are published. In

M. australis, HSI has been estimated at 4.2±5% (Balbontín and Bravo, 1993) and for *M. merluccius* from the Northern Tyrrhenian Sea, monthly mean HSI was between 1.5-5% (Biagi *et al.*, 1995). In other species high variations in HSI have been observed depending on distribution, e.g. for cod, HSI in Greenland waters ranges between 1-16% (Lloret y Rätz, 2000) whereas Dutil *et al.* (2003b) for Northwest Atlantic cod suggest an HSI between 1.98 and 4.53%, which are values lower than those observed in Newfoundland, 2-9%, and in Iceland, 1.7 to 18.9% (Mello and Rose, 2005; Marteinsdottir and Begg, 2002). Differences are also notable between wild and captive specimens; the latter had HSI values between 0.5-9% in (Lambert and Dutil, 1997a), and wild females from St. Lawrence Gulf had values between 2 and 6% (Lambert and Dutil, 1997b). In Greenland halibut, HSI have been recorded between 1% and 4% (Gundersen *et al.*, 2001); in American plaice HSI values ranged from 0.33 to 8.05% (Maddock and Burton, 1999) and in yellowtail flounder HSI averaged 0.97±0.08% in wild fish, being higher (1.85±0.22) than in cultured specimens (Faahraeus-Van Ree and Spurrell, 2003). For haddock from the Scotian Shelf, HSI has been estimated between 1 and 7% (Blanchard *et al.*, 2003) and for white seabream in the Mediterranean Sea, between 0.68 and 2.94% (Lloret and Planes, 2003). Regarding condition factor, results from this study yield K values between 0.57 and 0.74 (mean=0.64-0.68 depending on year considered). For other species of this genus, similar values have been reported, e.g. *M. hubbsi* K values range between 0.6 and 0.8 (Montecchia *et al.*, 1990; Méndez and González, 1997). As with HSI, condition factor in cod changes depending on its distribution and whether wild or captive specimens are considered. For Northwest Atlantic wild cod, condition factor values have been reported between 0.64 and 0.85 (Krohn *et al.*, 1997; Lambert and Dutil, 1997b, 2000; Dutil *et al.*, 2003; Mello and Rose, 2005), although range was larger for captivity females (0.4-1.1; Lambert and Dutil, 1997a). Higher values were observed for Greenland, Barents Sea and Icelandic cod that range between 0.82 and 0.95, 0.7-1.2 and 0.46 and 1.59 respectively (Lloret and Rätz, 2000; Marteinsdottir and Begg, 2002; Jørgenses and Fiksen, 2006). Other species like Flemish Cap Greenland halibut, Scotian Shelf haddock and Mediterranean white seabream show higher condition factors than *M. merluccius* in Galician shelf waters (Junquera *et al.*, 1999; Blanchard *et al.*, 2003; Lloret and Planes, 2003). These geographical differences could be due to environmental conditions, prey availability and composition, spawning and feeding behaviour or genetic factors. However, since condition changes during the year and depends very much of the reproductive strategy of the species, comparisons among species should be carefully considered.

In this study, hake HSI was positive and significantly related to length and gutted weight, which suggests that larger females may have larger livers. The same was observed for Atlantic cod (Lloret and Rätz, 2000) whose HSI was significantly related to size. On the contrary, the same author in 2003 (Lloret and Planes, 2003) found that K of *Diplodus sargus* is independent of size. HSI of hake also increased significantly with liver weight, probably because of a direct relation between them. K, for its part, decreased slightly when length

increased, but not when gutted weight did. This is not seen with Atlantic cod from the Gulf of St. Lawrence, where condition is independent of female size (Lambert *et al*., 2003). K also diminished significantly as gonad weight increased, probably as a result of consumption of somatic energy reserves due to reproductive activity, and this is reflected in a diminution of female condition. Thus, as GSI depends directly on gonad weight, K also decreases significantly with GSI.,In contrast, K and liver weight are positively related, probably because large females, in good condition with high energy reserves have larger and heavier livers, the main store of lipids. In any case, regression coefficients were very low (r^2<0.2), except for the relation between HSI and liver weight (r^2=0.522). Thus, although there is a clear correlation between variables, the prediction power of K on GSI or gonad activity is very low.

However, the importance of liver and soma as energetic reserves for European hake reproduction is not clearly reflected in their monthly variability. HSI has minimum values in March, and then progressively increase until the last quarter of the year, reaching the maximum in October. Something similar happens with K, but this time minimum values are observed in January and then progressively increase to reach the maximum in November. This could be interpreted as a diminishing of condition index through the spawning season, because minimum values are recorded during or just before the spawning peak occurs, but hake population asynchrony and the protracted spawning season mask this trend; nevertheless when K is made independent of temporal variations and is analysed using ovary developmental stages as grouping factor, significant and clear patterns are recognized through the spawning season; maximum values are reached in immature and recovering females, and remain constant at lower values during the breeding season. Less clear results are observed for liver. Returning to the temporal variation of condition indices, both HSI and K changes significantly during the year, but deviations within each month are so high that differences between months are not so evident in *post hoc* analysis. In species with higher synchronicity in spawning like Atlantic cod, maximum values of HSI and K were recorded in summer-autumn during the post-spawning period, and minimum values in spring during the spawning peak (Lambert and Dutil, 1997b; Dutil *et al*., 2003a; Mello, 2005), the opposite pattern from the gonadosomatic index (GSI). On the other hand, in this study, no relation between HSI and GSI was detected for hake. Similar results were found in sardine (Somarakis *et al*., 2004), probably because these species do not stop feeding during the spawning season as species like cod do, so the role of the liver as energetic reservoir is, somehow, less clearcut. However, K of European hake showed a small but significant decrease when GSI increased. Dependence of reproductive success on female condition and of this on the environmental factors has been reported in several species. Protein and lipid reserves used for metabolism, gonad development and spawning behaviour of fish co-vary in response to the abundance of food (Lambert and Dutil, 1997b). Annual mean HSI of Northeast Arctic cod was positively related to the abundance of capelin, a lipid-rich prey species common in arcto-boreal ecosystems (Yagarina and Marshall, 2000). In a similar way, Marshall *et al*. (1999) established a significant relationship between total egg production of

Atlantic cod and total lipid energy of females that is strongly affected by capelin biomass in the area, revealing the importance of quantity and quality of food on the Stock Reproductive Potential. There is a need to evaluate the degree to which depleted food resources and in general environmental conditions, reduce the energy reserves and consequently condition of the spawning stock and constrain recruitment in fish stocks.

With regards to inter-annual variability, significant changes were detected in both indices (HSI and K), of hake from the Galician Coast, although no clear trend was observed for HSI, which may be in accordance with the lack, or low, importance of the liver index on reproductive events. Nevertheless, annual mean K showed a progressive decline from mean values around 0.68 in 1999 to average around 0.63 in 2004, despite of high dispersion of data, and the fact that the number of females sampled in 1999 and 2000 was lower than in 2003 and 2004, and sampling was concentrated in the first half of each year. However, K is lower at the beginning of the year, coinciding with peak spawning, and increases progressively until the end of year. So sampling limitations would not be the cause of the K decrease, because those years with sampling concentrated in the first half might be expected to present lower values of K than those years with a homogeneously distributed sampling programme. Therefore hake in 1999 and 2000 were in better condition than in 2003-2004. This might be due to variations in environmental conditions, changes in food availability and quality, or increased fishing pressure that removed the females in best condition Stevens *et al*. (2006) observed that food consumption rates in *Platichthys flesus* are influenced by water temperature and growth and are limited not only by food consumption but also the quality of that food. According to Krohn *et al*. (1997), changes in bottom temperature and capellin biomass explained 52% of growth rate and condition variability for cod. Nevertheless, condition and growth rate were not correlated. It is necessary to take also into account that energy reserves are used not only for reproduction, but also to maintain activity and metabolism, so lack of resources could be a critical factor in fish survival. For example, food may be scarce during winter and environmental conditions adverse; if fish condition at the beginning of winter is not good enough, probably fish will not be able to spawn successfully, moreover, it might affect metabolic activities, increasing fish vulnerability, and even natural mortality (Eckmann, 2004; Lloret and Rätz, 2000). In some scenarios, these effects might be more evident in larger individuals that need more energy to maintain higher metabolic costs with the subsequent effect that this would have on the reproductive potential of the population. Furthermore, females in worse condition invest less energy in maturation, but proportionally waste more energy than those females that are in good condition, increasing mortality risk, even though they do not reach the egg production levels of females with better condition (Lambert and Dutil, 2000; Dutil *et al*.; 2003b). In Galician shelf waters waters, hake mainly prey on blue whiting, horse mackerel, and clupeids (Velasco and Olaso, 1998; ICES, 2005b), specifically on juveniles and smaller individuals of these species (Bruno *et al*., 2006). When abundance of these species in Galician waters during the study period was analysed, it was observed that blue whiting abundance

decreased from 1999 to 2004, together with total consumption estimated from simple bioenergetic models (Saborido-Rey, F., personal communication), coinciding with a decrease of hake condition factor, which indicates the dependence of hake on this resource. However, the HSI annual pattern did not follow any recognizable trend. In the next section, changes in proximate composition will be discussed in detail; nevertheless, bearing the results in mind, it can be postulated that European hake reproduction does not depend on energy reserves, but on daily feeding. Consequently, the lack of any trend in HSI would be explained, and variations in K could be assumed to depend on variations in growth rate rather than changes in female condition, i.e., if egg production depends only on daily food intake, when reproductive energy costs are covered, energy surplus, if it exists, is devoted to growth. This will be discussed later.

4.6 PROXIMATE COMPOSITION AND ENERGY STORAGE ON *M. MERLUCCIUS* IN GALICIAN SHELF WATERS.

Knowledge of the structures and energy reserves of commercially important fish species is important in understanding metabolic processes and in assessing the impact of potential environmental physical and chemical stressors on fish stocks (Faahraeus-Van Ree and Spurrell, 2003). In hake, as seen in the previous section, condition indices (HSI and K) are not good proxies of stock reproductive potential. Consequently, it is necessary study other aspects that provide better indicators of it. Because of this, in the present work, proximate composition of hake was studied in order to evaluate the specific weight of each biochemical component on total energy stores, and how they affect metabolism and reproductive processes. Not many studies of the proximate composition of hake exist, and, in general, the proximate composition of different species of *Merluccius* has been studied because of its importance in human diet, so all these studies are more focused on muscle (edible portion) than on other tissues (Dill, 1925; Gordon and Roberts, 1977; Pérez-Villareal and Howgate, 1987; Montecchia *et al*., 1990; Méndez and González, 1997; Soriguer *et al*., 1997; Pagano *et al*., 2001; Roldán *et al*., 2005). There are hardly any earlier results the proximate composition of liver and gonad in hake, and comparative analyses are only possible with other species. Additionally, most of the comparisons should be taken with caution as variations during the year on biochemical components and methodological differences among the different works mentioned (such as temporal differences in sampling) may hide species or stock differences.

It is known that lipids play an important role as energy reserves (especially during non-feeding and reproductive periods) and as a regulators of body density, cellular metabolism, detoxification, behaviour and reproduction (Love, 1980; Chellapa *et al*., 1989; Finn *et al*., 1995; Lucas, 1996; Jonsson *et al*., 1997; Henderson and Wong, 1998; Silverstein *et al*., 1998; Andrew and Ole, 1999; Marshall *et al*., 1999; Robards *et al*., 1999; Eckmann,

2004; Richoux *et al*., 2004; Blanchard *et al*., 2005). Lipids are a principal energy reserve in teleosts and are often the first components to be mobilized during periods of stress. As energy reserves strongly affect spawning and egg quality of many fish species, such as cod, herring, salmon, sardine, walleye pollock or trout among others, a deficiency negatively affects gonad development, fecundity, fertilization and hatching rates (Andrew and Ole, 1999; Shearer and Swanson, 2000; Hendry *et al*., 2002; Kurita *et al*., 2003; Lambert *et al*., 2003; Ohkubo *et al*., 2003; Riveiro *et al*., 2004; Wiegand *et al*., 2004). Inadequate lipid reserves have been implicated in the reduced reproductive potential of several fish species through reduced fecundity and quality of eggs and larvae. Low lipid reserves may also lower the chances of survival of fish, leading to an increase of natural mortality (Lloret *et al*. 2005). The present study estimated lipid content of hake gonad between 12 and 138 mg/g of fresh tissue, liver fatness was the highest observed in any tissue with a minimum value of 56.4 mg/g and maximum of 729.6 mg/g. In contrast, hake muscle is a lean tissue, with lipid content ranging between 2.4 and 17.5 mg/g, rather lower than other fat species like salmon, whose mean value of lipids in somatic tissues is twice that of hake, between 9 and 88 mg/g of fresh muscle, though gonad lipid content is not so high, ranging from 17 to 75 mg/g (Jonsson and Jonsson, 2003). The results found here in muscle are also lower then those of Roldán *et al*. (2005) for *M. hubbsi*, that ranged between 10.1 mg/g in pre-spawning females and 39.3 mg/g in post-spawners, but similar to *M. merluccius* from Atlantic and Mediterranean waters of South Spain with an average of 12.6 mg/g (Soriguer *et al*., 1997) and for *M. productus*, 14.4 mg/g (Gordon and Roberts, 1977). Differences could be due to environmental factors, e.g. it has been demonstrated that temperature affects the lipid content and fatty acid composition of fish (Greene and Selinovchick, 1987). Refered to wet weight (i.e. including water and inorganic matter content), lipid ranged between 0.5 and 1.5% of muscle, between 8.5 and 56% of liver and between 2 and 13% of gonad in this study. Pérez-Villareal and Howgate (1987) estimated the mean percentage of lipids in European hake muscle at 2.24%, slightly higher than observed in this study. *M. hubbsi* and *M australis* showed values of total body lipid percentage between 3.65% and 4.48% respectively (Eder and Lewis, 2005). More similar, although slightly lower, is the composition observed in cod (*Gadus morhua*) where 1% of muscle composition or even less corresponds to lipids (Lambert and Dutil, 1997a) and 66% of liver (Black and Love, 1986). Holdway and Beamish (1984), also for Atlantic cod, estimated lipid composition of gonad, muscle and liver between 1.29-2.53%, 0.80-1.81% and 35.69-71.17% of wet weight respectively. In *Perca fluviatilis* from Meuse River (France), lipid content estimated by Blanchard *et al*. (2005) is significantly lower in gonad (0.37-1.03%), muscle (0.45-0.69%) and liver (0.03-0.06%) than found in this study, though Eckmann (2004) presents notably higher total body lipid values (25-36%) for the same specie in April in Lake Constance (Germany). However, it is important to consider that freshwater fish are able to synthesize their own fatty acids independently of their availability in food resources (Blanchard *et al*., 2005).

Proteins are the main component of muscular tissue and are not only the principal energy source to active metabolism of fish, but also gonad growth takes place at the expense of body proteins (Love, 1980; Tyler and Colow, 1985; Black and Love, 1986). The protein content of European hake in Galician shelf waters measured in this study ranged from 50 to 240 mg/g in gonad (3-25% of gonad wet weight), from 20 to 165 mg/g in liver (1.8-15% of liver wet weight), and from 60 to 215 mg/g in muscle (7.5-20% of muscle wet weight). Muscle values are slightly lower than those reported for hake in Atlantic and Mediterranean waters of South Spain i.e. 232 mg/g of wet muscle mass (Soriguer *et al.*, 1997) but higher than estimated from total nitrogen content of muscle, 2.96%, in waters off Scotland (Pérez-Villareal and Howgate, 1987). However, protein in muscle yields similar values for *M. hubbsi* (14.65%), *M. australis* (15.66%) and *M. productus* (157 mg/g; Eder and Lewis, 2005; Gordon and Roberts, 1977). In the case of Monterey Bay hake, protein in muscle was determined at 16% (Dill, 1925), although only one specimen was analyzed in this case. Muscle protein composition observed in Gulf of St Lawrence cod was between 10-14% of muscle wet weight whereas for liver, protein composition was reported between 5 and 15% (Lambert and Dutil, 1997a), which is similar to results from this study in hake. Similarly also, cod in Passamoquoddy Bay and Bay of Fundy showed protein content between 11.14 and 14.39% in gonad, 14.94 and 18.61% in muscle and from 6.76 to 10.24% in liver, all expressed in terms of wet weight (Holdway and Beamish, 1984), and 105 mg/g to 175 mg/g of protein in wet muscle mass for cod in Newfoundland (Martínez *et al.*, 1999). In *Salmo salar* protein ranged from 171 mg/g to 219 mg/g in muscle, and between 161 and 163 mg/g in liver (Jonsson and Jonsson, 2003) which were in the range observed here for hake, although average values were slightly higher. This may be due to the semelparous reproductive strategy of salmon that is that makes long migrations to reproduce; this reproductive strategy is energetically costly, and depends on body reserves.

Traditionally, glycogen has been considered an insignificant biochemical component in fish despite the indisputable function that this component carries out in other marine organism like molluscs, which use glycogen as the main source of energy in the absence of oxygen (Wilbur and Hochachka, 1983; de Zwaan and Mathieu, 1992). Not many data on fish is available. However, Love already described in 1970 the important role that glycogen plays, not only in reproduction, but also in general metabolic processes, and mainly in stress situations when a high proportion of muscle glycogen is mobilized. Liver and muscle glycogen is the first energy store used by fish, prior to lipids, which suggests that it could be an immediate energy substrate easily mobilized (Chellappa *et al.*, 1989). In spite of reluctance of some authors to include glycogen in proximate composition analyses of fish (Andrew and Ole, 1999), other authors have started to take into consideration the importance of glycogen in fish (Bouleckbache, 1981; Tyler and Colow, 1985; Black and Love, 1986; Chellappa *et al.*, 1989; Lambert and Dutil, 1997a; Silverstein *et al.*;, 1998; Gisbert *et al.*, 1999; Rosa *et al.*, 2004; Blanchard *et al.*, 2005). Moroever, Faahraeus-Van Ree and Spurrell (2003) stated that some species of pleuronectids can be divided into groups depending on

their liver-storing type, that could be based on lipid- or glycogen-storage,, which suggests the importance of this compound for some fish is higher than previously reported. In this study, glycogen content varied in the three analysed tissues, although neither in gonad nor muscle, differences are so marked as in liver, in which glycogen content ranges between 2 and 10%. A progressive decrease in liver and muscle glycogen content was observed from immature females (63 mg/g and 6 mg/g in liver and muscle respectively) to recovering ones (23 mg/g and 2 mg/g in liver and muscle respectively). In gonad, glycogen content remains more or less constant during the oocyte maturation process, so no relation between its decrease in liver and muscle and oocyte development could be established. Black and Love (1986) reported rather low values for Atlantic cod in a starvation-refeeding experiment; changes of liver glycogen content were between 0.005 mg/g and 0.1 mg/g, and from 0.05 to 2 mg/g in muscle, being lower in dark muscle than in white muscle. Lambert and Dutil (1997a), also for Atlantic cod, suggest that glycogen represents between 0.1 and 0.4% of the muscle composition, and between 1 and 5% of liver; these values are in the range of those found here. For Chinook salmon, Silverstein *et al*. (1998) observed liver glycogen values between 5 and 60 mg/g.

In general, water content of the different tissues analyzed normally follows the opposite trend than organic components (i.e. lipids, proteins and glycogens) because water proportion increases when tissues energy is being consuming, and sometimes is considered to be a proxy of condition (Lambert and Dutil, 2000; Dutil *et al*., 2003b). In this study, water content in gonad was between 660 and 880 mg/g (61-85% of wet mass), in liver it ranged from 380 to 740 mg/g (10-60% of wet liver mass), and in muscle between 770 and 865 mg/g (79-85% of wet muscle mass), in accordance with values estimated in muscle for hake in waters off Scotland, in 79.04% (Pérez-Villareal and Howgate, 1987). In other species of hake, results are very similar to those found here, although only water in muscle has been reported. Thus Pacific hake reaches 833 mg/g (Gordon and Roberts, 1977), 76.1% for *M. australis* and 77.27% for *M. hubbsi* (Eder and Lewis, 2005). In other species like Atlantic cod, some authors found similar values of water content for muscle and liver but higher values in gonad (Holdway and Beamish, 1984; Black and Love, 1986; Martínez *et al*., 1999), but others detected higher values of water content in muscle and liver than in hake, e.g. Lambert and Dutil (1997a; 1997b; 2000) recorded gonad, liver and muscle water content ranged between 78 and 84%, 15 and 85% and 78 and 92% respectively. In *Salmo salar*, gonad water content changes between 59 and 66% depending on maturity stage, and between 70 and 84% in muscle (Jonsson and Jonsson, 2003). In Nile *Tilapia*, a freshwater species, water content of muscle is between 68 and 79% while in gonad it is between 55 and 58.3%, and these values change with diet composition (Al Hafedh *et al*., 1999). In both cases, values were lower than recorded in this work, which may be because these are freshwater species, and lay eggs in nests, so no strong hydration is present; as shown by the fact that gonad water content in hake was significantly related to gonad weight and GSI because the larger, heavier ovaries are the ovulating-hydrated ones.

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Inorganic matter content can be considered as a proxy of organic matter present in tissue. Normally it is an insignificant proportion compared with the other components and few published data on ash content of gonad, liver or muscle have been found. Inorganic matter content in European hake from Galician Shelf waters was between 1-7% in gonad, 1.5-3% in liver and 0.5 and 5% in muscle. Dill (1925) estimated inorganic matter content of *M. productus* muscle at 1.21% and Gordon and Roberts (1977) at 1%, values slightly lower than those reported in the present work, although within the same range. Similar values of inorganic matter content, around 1%, have been reported for cod (Holdway and Beamish, 1984).

All variability of proximate composition can be, nevertheless, summarized as variation of tissue energy content. In this study, energy density ranged from 24 to 31.5 kJ/g in gonad dry weight; between 27 and 38 kJ/g in liver and from 17 to 24 kJ/g in muscle; similar energy content values of liver and muscle are found in cod where maximum values were 22 and 36 kJ/g respectively (Lambert and Dutil, 1997a). The highest values of energy density in hake gonad were recorded in ovulating females with hydrated eggs, because, just prior to release, these eggs contain maximum yolk amount. However, energy density was not significantly related to either maternal attributes or somatic condition factors in gonad or liver; although in muscle it was negatively related with both female length and gutted weight, meaning larger females have more energy because of their size, but accumulate less reserves in each gram of tissue; but the predictive power of this regression is rather low. Consequently, the increase of total energy of large females is due to the existence of more cells in each gram of tissue (Black and Love, 1986). In gonad, the bioenergetic components that explained the highest variability of energy density were lipids and glycogen. In liver, contrary to expectations, biochemical compounds that explained the highest variability of energy density were proteins and finally, in muscle, proteins were the principal component followed by lipids and glycogen. In cod, it has been observed that when the energetic content of liver is low, lipids and proteins contribute in the same proportion, but when energy content increases, lipids become the main energy source (Lambert and Dutil, 1997a).

The proximate compositions of gonad, liver and muscle were unrelated with either maternal attributes (length, gutted weight, gonad and liver weight) or somatic condition factors (GSI, HSI and K). Other studies reveal similar results for lipid content of different species of *Pagellus, Mullus, Diplodus* and *Sardina* from the Mediterranean Sea (Somarakis *et al*., 2004; Lloret *et al*., 2005). These results suggest that biochemical relative composition (mg per gram of wet mass) of hake females is independent of their size. On the contrary, in other species, proximate composition is related to condition indices; in pollock (*Theragra chalcogramma*), liver lipid and water content and muscle water content are significantly related to HSI, in cod muscle and liver composition are exponentially related to K and HSI respectively, and in plaice (*Pleuronectes platessa*) K may be a good proxy of proximate

composition (Lambert and Dutil, 1997a). In the case of hake in Galician shelf waters somatic condition factors (HSI and K) are practically unrelated to any of the biochemical components of any tissue. This suggests that in fast growing species, population mean HSI and K are more related to growth rates than to condition. In hake from Galician Shelf waters, liver and muscle proximate composition show similar trends, probably because of the energy dynamic of this species. When food surplus exists, energy is accumulated in liver and muscle, independently of female size. If a female is in a spawning period, it will give part of this energy over to oocyte development, if not it will be entirely set aside for metabolic reserves. In some fish, it has been observed that when an individual is depleted of energy, water replaces organic components (energetic reserves) of tissue when these are consumed to threshold values (Black and Love, 1986; Miglavs and Jobling, 1989). This negative relationship between lipid, protein, glycogen and energy content and water content of liver and muscle has also been observed in this study. Water could be considered an alternative proxy of fish condition.

Changes in biochemical composition and condition are not only due to reproductive energy waste but also could be a consequence of scarce food supply or negative energetic balance between energy intake and energy waste (Black and Love, 1986; Lambert *et al.*, 2003; Weber *et al.*, 2003; Eckmann, 2004). Environmental factors may also affect condition and provoke not only monthly variations but also inter-annual differences, as has been reported in cod by Krohn *et al*. (1997), Lambert and Dutil (1997b) or Lloret and Rätz (2000), in Winter flounder by Buckley *et al*. (1990), in *Mysis mixta* by Richoux *et al*. (2004), in Mediterranean species of *Pagellus* and *Mullus* whose lipid composition varied depending on their habitat characteristics (Lloret *et al*., 2005), or in freshwater species like *Salmo trutta* from a temperate zone lake (Berg *et al*., 2000). Furthermore, inter-annual differences in condition may affect even reproductive success; this is the case of Norwegian spring-spawning herring that can skip spawning if size, condition or environmental factors do not guarantee its future reproduction, growth and survival (Engelhard and Heino, 2006); this will be discussed later. Reinitz *et al*. (1979) considers that the nutritional composition of prey is the factor with most influence on proximate composition of fish. Black and Love (1986) observed in cod that when they were fed with squid, glycogen content of muscle increased, and when they were fed with herring, liver lipid content increase. In summer flounder, no significant changes in total lipid content were observed during a 1-year period, but fatty acid composition varied seasonally, whereas in Atlantic croakers and striped mullet, total lipid content increased in late summer; in the latter two species, high food availability linked to preparation for the spawning season seems to be a primary determinant, but for other fish like summer flounder, changes of fatty acids composition in response to temperature variations are more important (Gallagher *et al*., 1991). According to Love (1970) and Bouleckbache (1981), many substrates are accumulated in gonad during fish maturation, glycogen and lipids being the most energetic substrates; this does not seem to be the case of European hake in which no relation between glycogen content of gonad and ovary

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developmental stages was observed. In *Perca fluviatilis*, neither liver, nor muscle lipid content change with progression of spawning season (Blanchard *et al*., 2005) because this species uses peri-visceral fat as an energy substrate for vitellogenesis. But European hake has no peri-visceral fat storage. There are other species with special energy storage organs that support gonad activity during spawning, e.g. surgeonfishes (Fishelson *et al*., 1985). Many other factors can affect the proximate composition such as presence/absence of predator species (Hayes and Taylor, 1994), variations in prey composition (Aksnes *et al*., 1996; Elangovan and Shim, 2000) and food availability (Gerking, 1995). Even more, ecosystem organization can vary depending on the proximate composition of the community (Litzow *et al*., 2006).

Interestingly, proximate composition of gonad did not follow a pattern during the year or among years, lipid and protein content of liver and muscle showed significant monthly variations, both shifting in parallel, but with no defined pattern. This is an unexpected result as changes in proximate composition or energetic dynamics have been widely reported during the reproductive cycle, both in marine and freshwater species. For example, species that migrate for reproduction spend a lot of energy which alters their proximate composition, e.g. *Salmo salar* (Jonsson *et al*., 1997) or *Clupea harengus* (Iles, 1984; Slotte, 1999). This pattern has also been described for freshwater species such as *Barbus scaleri* which reach maximum energy reserve levels just prior to spawning, reserves decreasing during and after the reproductive season (Encina and Granado-Lorencio, 1997), or, as in *Perca fluviatilis* in which two marked declines in perivisceral fat take place coinciding with the beginning of exogenous vitellogenensis and the final accumulation of yolk, just before spawning; besides, liver lipid content decreases during ovarian development whereas lipid stored in ovary increases significantly (Craig, 1977; Blanchard *et al*., 2005). Energetic reserves of cod are almost completely depleted during the reproduction cycle (Lambert and Dutil, 2000; Lambert *et al*., 2000), and can be reflected in weight losses (Kjesbu, 1998). Nevertheless, lack of temporal variations in proximate composition has been observed in *Merluccius hubbsi* and *Merluccius australis* (Eder and Lewis, 2005). Martínez *et al*. (1999) suggested that reproductive maturity in cod has few effects on biochemical parameters, and no differences have been detected in relative proximate content between maturing and immature cod (Holdway and Beamish, 1984). In stable environments, the need for large energy reserves is less important, in contrast with environments where food resources vary seasonally (Stickney and Torres, 1989), and seems to be the case for deep-sea habitats where homogeneous proximate composition has been observed (Koslow *et al*., 2000).

However, it can be hypothesized that the strong asynchronicity at population level in spawning activity in hake discussed earlier may prevent the detection of patterns in proximate composition in relation with reproduction cycle. In fact, the proximate composition of gonad changes significantly between ovary developmental stages excepting glycogen content, although in liver and muscle significant variations were only observed in protein

composition. In gonad, lipid, protein and water content increased from the first maturity stage, reaching maximum values in spawning and late-spawning females, and decreased in inactive and recovering females; on the other hand, inorganic matter content was lowest in ovulating females with hydrated eggs, and reached the highest values in inactive and recovering ones. This shows a clear energetic dynamic in gonad as the reproductive cycle progresses. But this dynamic in gonad does not seem to have a counterpart in liver and muscle. Proteins in liver and muscle decrease in ripening females, and progressively increase towards the last ovary developmental stages, and despite no statistical significance, both lipids and glycogen content of liver and muscle showed certain patterns, following lipids the opposite trend than in gonad and decreasing glycogen progressively from immature females to recovering ones. Also, when proximate composition was analyzed as the relative proportion of each component, it was observed that lipids followed opposite trends in gonad that in liver and muscle, i.e. when peaks of protein in gonad took place, low values of protein were observed in liver and muscle. These results would suggest that certain exchanges of lipid and protein from liver and muscle to gonad exist, in order to supply raw material to gonad for oocyte development. But the decrease of some components results in an artificial increase of others, as expressed as percentage of the total (Donelly *et al.*, 1990). The dynamic in liver and muscle is, anyway, less clear than in gonad, or in comparison with other species. For example, Pagano *et al.* (2001) found an important influence of gonadal stage of *M. hubbsi* on biochemical muscle properties. Unlike other fish species in which energy tends to be maximum just prior spawning season, and decreases progressively as it advances (Lambert and Dutil, 1997b; Lloret and Rätz, 2000; Jonsson and Jonsson, 2003; Richoux *et al.*, 2004; Blanchard *et al.*, 2005); in this study, no significant differences of energy density were observed either between months or ovary developmental stages. However, Pérez-Villarreal and Howgate (1987) detected a decrease in condition of European hake during spring-summer, citing the lack of food and gonad development as causes of these variations.

The lack of a relation between liver and muscle energy and the gonad or reproductive cycle may be due to the fact that these tissues, or their reserves, contribute to gonad composition for only short periods, and are thus not critical for ovary maturation. In other words, the fact that relative energy remains constant through the spawning season supports the idea that European hake does not stop to feed during reproduction, and suggests that when total energy increases in a determined organ, it is not due to an increase of cell storage capacity, but of an increase in the number of cells that make up the organ. This seems to be supported by the fact that when relationships between the biochemical composition of each tissue was analyzed, it was found that protein and lipid content increase simultaneously in the three tissues. This suggests that hake reproduction does not completely depend on energy reserves. In general, immature fish expend assimilated energy on survival and growth, but once they are mature, part of this energy is used for gamete production and reproductive behaviour. Trade-offs in energy budget distribution must exist which affect growth and reproductive dynamics (Iles, 1984; Saborido-Rey and Kjesbu, in press). For

example, haddock is a long-live species that generally use surplus energy to reproduce, but if energetic resources are scarce, it continues reproductive activity, using somatic reserves, but assuring survival by reducing offspring production (Ware, 1984).

Generally speaking, fish can be divided into two groups depending on their reproductive strategies: A strategists are species that breed once they have obtained enough energy to afford reproduction successfully and independently of size and age, while B strategists need to reach a threshold value of length or age to mature. Younger groups of mature A strategist fish invest proportionally more surplus energy in growth than in reproduction, reducing mortality (Ware, 1984; Larrañeta, 1996). Roughly, these two strategies can be related with food availability, and hence, somehow, with latitude. In cold waters, primary production cycles and environmental conditions force fish to spawn in late winter/spring and develop gonads during winter, experiencing periods of food depletion. The strategy in these cases is to store part of the assimilated energy for later use when food supply is limited (Bagenal, 1969). The cyclic pattern in weight for such species needs to be considered as part of the growth dynamics as the energy reserves are of special importance for the onset of maturation as well as the seasonal variation in gonad development (Eliassen and Vahl, 1982; Rowe *et al.*, 1991; van Winkle *et al.*, 1997); in these species there is a trade-off between growth and reproduction throughout the entire life, and fecundity is determinate. In temperate waters, in contrast, environmental conditions allow the existence of protracted spawning seasons and there are less marked periods of food depletion. Oocyte development is at the expense of food availability during gonad development, rather than from reserves, which allows egg production to be adjusted to food surplus (indeterminate fecundity). These species have faster growth before maturation. Present results seems to support in a rather convincing way the idea that hake reproduction is not at the expense of liver and muscle energy stores, but that egg production depends on immediate energy intake, although both, liver and muscle, can provide certain compounds to gonad development, and liver may act as an intermediate in biochemical translocations. Nevertheless, the energy content of organs may vary depending on environmental conditions (Dutil *et al.*, 2003a), and both maternal energy reserves and biochemical composition may affect fertilization rates and catabolism, and energy reserves of eggs (Buckley *et al.*, 1990; Tamaru *et al.*, 1992; Finn *et al.*, 1995). This may be so, even in species with the strategy assumed for hake, with the subsequent effect on the reproductive potential of the stock. In the words of Fisher (in Jørgensen and Fiksen, 2006), "energy allocation, how available energy is diverted towards alternative uses, is the mechanism that integrates the trade-offs through shaping the individual's growth trajectory. This trade-off is the essential core of life history theory." Subsequently, more investigations on the energy budget of European hake are necessary to improve our knowledge on stock dynamics and environmental effects on it.

4.7 FECUNDITY AND EGG ESTIMATION OF *M. MERLUCCIUS* IN GALICIAN SHELF WATERS.

The energetic dynamics and the reproductive strategy of hake is, as discussed above, closely related with egg production dynamics and hence fecundity. Although there have been some contradictions concerning the fecundity type of hake and some authors have considered its fecundity as determinate (Sarano, 1984; Tsimendis and Papaconstantinou, 1985; McFarlane and Saunders, 1997; Biagi *et al*., 1998), nowadays most researchers consider all species of *Merluccius* as indeterminate (Murua *et al*., 1996; 1998; Osborne *et al*., 1999; Macchi *et al*., 2004; Murua and Motos, 2006), and they simply estimate population annual egg production from batch fecundity, spawning fraction, spawning duration and number of batches (Balbontín and Bravo, 1993; Smith, 1995; Cerna and Oyarzún, 1998; Murua *et al*., 2006), because this is the only useful proxy for fecundity measurement for multiple spawning fish with indeterminate fecundity (Ganias *et al*., 2004). Our results also corroborate the hypothesis of indeterminate fecundity in hake. Ecologically, both reproductive strategies (determinate vs indeterminate) are adaptations to environmental conditions, food availability and energy trade-off between reproduction, growth and survival (Nikolsky, 1963), as discussed earlier. A fish must spawn at a period where lifetime production of offspring is maximum, and egg hatching ought to take place in an adequate environment that supplies larvae enough food, protects them from predators, and offers good abiotic conditions for survival (Bye, 1990; Wootton, 1990; Räsänen *et al*., 2005).

Determinate fecundity may be the best adaptation to environments with pronounced seasonal differences, where periods with high food availability are followed by scarcity. In species that live in these environments, individuals are forced to spawn in a relative short period in order allow egg hatching to coincide with good conditions for larvae; thus these species have a synchronized breeding season. In these species, fecundity depends on somatic energy reserves accumulated during feeding season; subsequently a compromise between reproduction and survival has to be established. In contrast, species displaying indeterminate fecundity accommodate egg production to the energy intake during oocyte formation and egg production. These species live in more stable environments than determinate ones, and normally the need to spawn in a short period does not exist, leading to a prolonged population spawning season and important asynchrony of the breeding season, although individual spawning duration may be shorter. In general, differences between reproductive strategies are related to the ecology of species (Hislop, 1984; Lambert and Ware, 1984), and energy availability through the year may be the main factor deciding which fecundity type is more adequate. In any case, a balance of energy allocation must exist that will be reflected in growth rates and reproduction dynamics (Saborido and Kjesbu, in press), affecting not only fecundity, but also spawning time, number of batches/spawning seasons (total *versus* partial spawners and iteroparity *versus* semelparity), age effects on

offspring production, etc. (Bagenal, 1967; Roff, 1981; Lambert and Ware, 1984; Wootton, 1990).

The research approach differs depending on fecundity type, thus total egg production per female in determinate species can be estimated based on potential annual fecundity after correcting for atretic losses, whereas for indeterminate species annual fecundity has to be estimated from numbers of eggs spawned in every batch, the spawning fraction, and the duration of the spawning season (Murua and Saborido-Rey, 2003). Unfortunately, in species with indeterminate fecundity, since it is not possible to estimate potential fecundity, and only population annual fecundity is affordable, individual variations in egg production cannot be studied, and hence its relation with different mechanisms, such as maternal effects, energetic dynamics or environmental factors. This is the case of European hake, where additionally the population asynchrony of spawning season adds other problems, e.g. it is difficult or impossible to know if estimated batch fecundity belongs to the first, intermediate or last batches. Variation in batch fecundity and egg size during the spawning season has been demonstrated in other species (Wootton, 1990), like cod (Kjesbu *et al.*, 1991; Chambers and Walwood, 1996; Solemdal, 1997), haddock (Trippel and Neil, 2004) or Atlantic mackerel (Greer Walker *et al.*, 1994). In the Bay of Biscay, hake intra-annual variation in batch fecundity was not observed (Murua *et al.*, 1996, 1998); but in *M. hubbsi* fecundity was higher at the beginning of the spawning season than at the end (Macchi *et al.*, 2004). Lucio *et al.* (1998) suggested that hake release larger eggs at the beginning of the spawning season, although their suggestion is based on monthly variation in egg size, that may be related with the size, age or experience of the female spawning fraction, food availability, cohort strength, or environmental conditions, more than with the number of batches (Bagenal, 1967; Trippel, 1998). All these factors make it difficult to know the origin of batch fecundity variations. In spite of this, batch fecundity is the only proxy that can be used in European hake to estimate egg production, so in the present work, relations with maternal attributes (length, weight, gonad weight, etc.) and condition factors (HSI and K) were analyzed.

In the present study, observed batch fecundity (BF) ranged between 18,710 and 577,302 hydrated oocytes (mean value=182,537 oocytes), and average relative batch fecundity between 140-170 eggs/g. For 60 cm females, it was 243,000 oocytes per batch. Similar values were found in the Bay of Biscay in 1994-1995, with 220,000 hydrated oocytes for a female of 60 cm (Murua *et al.*, 1998); however in 1997, a slightly decrease was observed, and 60 cm female BF was estimated to be around 170,000 eggs (Murua *et al.*, 2006). Batch Fecundity values reported for other species of the same genus show a wide range, for example, for *M. capensis* BF was estimated at 417,205±64,568 oocytes and for *M. paradoxus* at 374,375±45,562 hydrated oocytes (Osborne *et al.*, 1999), although the mean BF of females with length between 50 and 75 cm and these results are hardly comparable; the BF of *M. gayi* equals 142,031±70.44 oocytes (Cerna and Oyarzún, 1998), similar mean values to those found by Alarcón and Arancibia (1993) and Cerna and Oyarzún (1998) for

the same species. For *M. hubbsi*, estimated relative BF was 670±234 hydrated oocytes/g of female weight which is close to 1,000,000 eggs (Louge, 1996), and Macchi *et al.* (2004) reported maximum values equal to 2,300,000 hydrated oocytes, much higher than values recorded here. However, similar high values have been reported for Icelandic cod, with BF ranging from 14,000 to 2,325,000 oocytes, with high variability within size classes (Marteinsdottir and Begg, 2002). In haddock, mean BF was estimated at 60,000 oocytes per female (Trippel and Neil, 2004). Temperate pelagic species such as mackerel shows similar relative BF (Yamada *et al.*, 1998), but sardines are reported to have larger relative BF, between 200-500 eggs/g (see Zwolinski *et al.*, 2001).

Batch Fecundity of European hake from Galician Shelf waters was related to length and weight. Although the relationships were significant, the total length and gutted weight explained only around the 45% of the variability on BF, which is considered low. However, previous works published on European hake and other species show that length and gutted weight are good predictors of BF variability (Kjesbu, 1998; Murua *et al.*, 1998; 2006). This may be due to the existence of different productivity periods in relation to BF by season; however, because of the low sample size, it was not possible to construct relationships by month. In *M. hubbsi*, BF is positively related to length and weight (Macchi *et al.*, 2004), but such relationships were not found in other studies of the same species (Cerna and Oyarzún, 1998). For *M. capensis*, most variability of BF was explained by length, while for *M. paradoxus*, the variable that mainly explained BF variability was gutted weight, despite the fact that BF values were not significantly different between the two species (Osborne *et al.*, 1999). Also in cod, BF showed a good relation with female size (Bleil and Oeberst, 1998; Trippel, 1998). In the Netherlands, mackerel BF was not related to length but to condition factor (Lambert *et al.*, 2003); and for Mediterranean sardine, there was no relation between BF and length or weight (Ganias *et al.*, 2004). Wootton (1990) comments that although in general BF and length are well related, females of the same size can show different values of BF, and even the BF of a single female may vary through the spawning season and/or between years.

Among the different female attributes analyzed, only gonad weight was clearly related to BF, explaining 89% of its variability. The same relationships were found for Bay of Biscay hake in 1996, 1997 and 1998 (Murua *et al.*, 2006). Although, one would expect a straightforward relationship for BF and gonad weight, since it is estimated as a combination of hydrated oocyte density and gonad weight, this is not always the case. For example, Jons and Miranda (1997) in gizzard shad (*Dorosoma cepedianum*, Lesueur) found that gonad weight only accounted for 50% of the variability in BF. This is important, because as such the gonad weight, and related indices such as gonadosomatic index, could be used as an index of BF. In this sense, the relationship between BF and gonadosomatic index was positively related, and this explained about 54% of variability. Marteinsdottir and Begg (2002) found in

cod that HSI had no effect on BF, but K had. Nevertheless, no relation with any of the condition factors has been detected for European hake in Galician waters.

Batch fecundity varied among the years analyzed, especially when comparing the regression lines between BF and the different maternal attributes studied, e.g. length. The relationship in 2004 had the highest determination coefficient comparing those of 2003, 1999 and 2000. However, these two last years might not be considered representative, as the sample size was small. In other species, differences of BF between years are due to nutritional condition, different environmental conditions, or a combination of both (Kjesbu *et al.*, 1998; Kraus *et al.*, 2000; Wang and Houde, 1994; Milton *et al.*, 1994; Somarakis *et al.*, 2005). However, in this study, condition indices seem to have no influence on BF, so inter-annual variations in BF might be related mainly to food availability, environmental conditions, stress factors, differences in cohort strength, or stock structure (Wootton, 1990). However, differences among years are not always observed, as in North-East Arctic cod (Marshall *et al.*, 2000).

In the present work, BF decreased from the beginning of the year towards its end. Eventually, in some years, a secondary late spring-summer peak was observed. Murua *et al.* (1996; 1998; 2006) observed in the Bay of Biscay that BF might change between months depending on the year considered. These results agree with those of Macchi *et al.* (2004) in Argentine hake, and, in other gadoids like Atlantic cod, it has been suggested that BF decreases as the spawning season advances (Kjesbu *et al.*, 1991). In general, based on the data gathered in this study, it can be concluded that, although spawning females can be detected all year round, the BF is highest between January and April, and remains at a lower level during the rest of the year.

Although potential fecundity cannot be estimated in hake, the number of developing oocytes (NDO) has been analyzed during the spawning season. NDO dynamics may allow to know if it is directly related to egg production and to elucidate reproductive dynamic and strategy of European hake in Galician shelf waters. Also may help to understand the oocyte recruitment process.

In Galician shelf waters, NDO was estimated for a female of 60 cm at 881,000 eggs, ranging between 311 and 3,366,352 oocytes (mean value=748,907 oocytes). Similar NDO values have been found for the same size females previously, both in Galician and Cantabrian Shelf waters, with NDO of 908,803 for oocytes larger than 250 µm (Pérez and Pereiro, 1985); in the Bay of Biscay, 610,057 oocytes larger than 160 µm (Sarano, 1984) and 730,732 oocytes larger than 100 µm in the Tyrrhenian Sea (Biagi *et al.*, 1995). In Greek waters, NDO ranged between 10,000 and 540,000 oocytes (Tsimendis, 1985), although considering only oocytes larger than 500µm. However, NDO estimated in the Bay of Biscay in 1994-1995 was 1,266,000 eggs for a female of 60 cm (Murua *et al.*, 1998), and even

higher in 1997, with 1,450,000 oocytes (Murua *et al*., 2006). Differences between these values may be due to differences in sampling time (closer to or further from the peak of spawning), considered oocyte size thresholds, variations in female and environmental conditions, or between stocks. These methodological factors may also prevent a proper comparison of NDO with other species of the same genus, as in *M. gayi*, NDO between 1,000,000 and 1,500,000, and *M. productus* between 165,700 and 1,800,000 (Balbontín and Fisher, 1981; Mason, 1986). Later studies reported lower NDO for these species, i.e. 700,000, 200,000 and 79,000 oocytes for *M. bilinearis, M. gayi* and *M. productus* respectively (Mertz and Myers, 1996), although in these cases, female size is not specified. For other gadoids like cod, fecundity, which is determinate, presents large variations depending on condition; for example, Lambert and Dutil (2000) estimated a mean potential fecundity of 726,000 eggs for a wild female of 60 cm, whereas Kjesbu (1998) for captive cod found females of 70 cm with fecundity equal to 6,000,000 eggs and condition factor 1.5-2.5 times higher than wild specimens, although Kjesbu *et al*. (1998) suggest that high proportion of vitellogenic oocytes of reared cod are resorbed by atresia. In *Solea solea*, an asynchronous species, fecundity ranges between 39,745 and 303,169 oocytes (Witthames, 2003), and in Greenland halibut from 32,500 to 277,100 oocytes (Gundersen *et al*., 1998), much lower than hake values.

A literature review indicates that in most of cases, authors made the mistake of failing to differentiate between NDO and potential fecundity. Some species are determinate *sensu stricto*, and it is possible to know exactly how many eggs are going to be released several months before the spawning season by counting developing oocytes and correcting for atretic losses; in these species, such as cod, NDO and potential fecundity are equal. In other species, such as hake, it is impossible to get this information because oocyte recruitment continues after spawning starts; only NDO can be estimated, which is lower than potential fecundity. Nevertheless, in some species with asynchronic oocyte development but determinate fecundity, the exact number of oocytes that are going to be laid can only be calculated just prior to the beginning of the spawning season but not previously. A review of the reproductive strategies of Atlantic species is found in Murua and Saborido-Rey (2003). Actually, the terms determinate and indeterminate are concepts that refer more to the capacity of researchers to know accurately the number of eggs that are going to be shed by a single female, rather than to a real reproductive strategy of fish. Females always modulate their own egg production, depending on a number of factors by different mechanisms, such as atresia or recruiting more or less oocytes. In cases where potential fecundity cannot be estimated, the use of the NDO concept is strongly recommended.

Results indicate that the number of developing oocytes (NDO) was significantly related to all considered maternal attributes and somatic condition factors in Galician Shelf waters. The variable that best explains NDO variation is gonad weight; nevertheless, although one would expect a straightforward relationship between NDO and gonad weight,

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since NDO is estimated as a combination of oocyte density and gonad weight is one of the parameter need in this estimate, this is not always the case. The NDO was also positively related to the liver weight, which is an important result because it can be used as an index of female condition. However, when NDO were correlated with HSI or K, although significant relationships could be established, correlations were very low ($r^2 < 0.3$), and relationships were positive for HSI but, strangely, negative for K. Lambert and Dutil (2000) observed in cod that females with poorer condition at the beginning of the spawning season had significantly lower potential fecundity, and Yoneda and Wright (2004) revealed the importance of liver condition in the same species. On the other hand, a weak correlation between condition factor and fecundity has also been reported for Greenland halibut (D´yakov, 1982). But maternal length and condition significantly and positively affect potential fecundity in Norwegian spring-spawning herring (Óskarsson *et al.*, 2002).

Relationships with length and gutted weight were significant but explained only around 40% of the variability of NDO. In most, fish, potential fecundity increases with female size, showing best fit to potential models, though sometimes linear regression is more suitable (Boehlert *et al.*, 1982) and generally, but not always, fecundity increases with maternal weight too. In this work it was observed that European hake NDO increased exponentially with length, meaning that larger females mobilize more oocytes than smaller ones, so it can be hypothesized that their contribution to total stock production is higher than that of smaller, inexperienced females. This phenomenon has been observed in other species, and varies depending on species; for example, the stock-recruitment relationship of haddock improves if younger spawners are removed from the model, but this does not happen in sole (Wigley, 1999). Experiments carried out by Wroblewski and Hiscock (2002) with Atlantic cod, both wild and in captivity, corroborate these results. For Yellow perch, Heyer *et al.* (2001) observed that older and larger females have higher fecundity, and the same was reported in cod by Bleil and Oeberst (1998). In *Sebastes mentella*, more than 75% variability of fecundity was explained by both female length and weight (Drevetnyak and Gusev, 1996). According to Kjesbu *et al.* (1998) total weight is the most powerful predictor of potential fecundity of cod, as well as in haddock (Trippel and Neil, 2004); but this is not the only factor affecting fecundity, for example food depletion may lead to a decrease of fecundity (Solemdal, 1997; Kjesbu, 1998) .

The observed low predictive power of maternal attributes and/or somatic condition indices could be due to i) oocyte recruitment rate varies along spawning season, i.e. NDO is not completely proportional to potential fecundity, and ii) the inter- and intra-annual variation in the relationships between NDO and these variables. In this sense, the comparison of the relationship between NDO and length/weight by year showed that they were significantly different. Different months, and thus seasons, are grouped in a single year, probably indicating the existence of different productivity periods in relation to NDO by season, as reflected by changes in batch fecundity during the year. However, due to the low sample

size, it was not possible to construct relationships by month. Besides, fecundity-length relationships may change between stocks (Bowering, 1980; McFarlane and Saunders, 1997), with female condition (Marteinsdottir and Begg, 2002), female age (Bagenal, 1967; Abdoli *et al*., 2005) and between or within years. The predictive power of regression models can improve when condition factor is included with length or weight (Blanchard *et al*., 2003; Koops *et al*., 2004). Several authors have observed a rise in fecundity-length relationships when a stock is overexploited (Bleil and Oeberst, 2005).

NDO-length relationships differ among the years analyzed. 2004 showed highest NDO values. However, in Galician shelf waters, 1999 and 2000 were not considered to be representative as the sample size was small, and smallest and largest females were underestimated, probably because of the sampling design. The difference on monthly NDO average by year is more difficult to interpret due to the asynchrony of hake population spawning, i.e. in the analyses, fishes in different maturity stages (ripening, spawning and inactive mature) and fishes in different batch number are probably analysed together. Nevertheless, NDO variation during the year showed a clear pattern, probably related to the progression of spawning season. Independently of fluctuation detected between February and December, NDO showed a clear decrease during the year, reaching its maximum value in January, prior to the beginning of the spawning season: this indicates that, although European hake is an indeterminate spawner, the rate of recruitment of previtellogenic oocytes to the vitellogenic stock was not constant, being higher when the spawning season started but decreasing progressively in the following months. Other factors that could also affect NDO are physiological/nutritional condition of the fish and environmental conditions as previously mentioned (Wang and Houde, 1994; Milton *et al*., 1994; Somarakis *et al*., 2005), because it is known that in the case of indeterminate species, energy intake during the breeding season affects mobilisation rate from the previtellogenic stock to the vitellogenic one. Murua *et al*. (1998) did not observe variation of Bay of Biscay hake NDO during the spawning season in 1994 and 1995, but in 1997 it decreased progressively from January to June (Murua and Motos, 2006); as in the current analyses, this suggests the strong influence of punctual local environmental conditions and energy intake on NDO. In Atlantic cod, as the spawning season advanced, released eggs were smaller but more numerous (Chambers and Waiwood, 1996). In the Barents Sea plaice population, fecundity is related to the number and age composition of the spawning stock (Shvagzhdis, 1989). Inter-annual changes observed in cod between years in North Sea and Scottish West Coast waters seems to be a consequence of variations in energy allocation (Yoneda and Wright, 2004), and temperature can also affect fecundity as well as fish population density (Abdoli *et al*., 2005). No variations were reported for American plaice by Zamarro (1992).

In general, fecundity is affected by food availability, environmental conditions (temperature), female status (K and HSI), SSB and female size. Parasitism may also affect potential fecundity. In indeterminate species, probably NDO and oocyte recruitment rates

change during the breeding season influenced by these factors too. Although in short-lived species like cupleids fecundity is more influenced by environmental factors, in long-lived ones it is more affected by female condition that reflects energy reserves (Lambert *et al*., 2003). In conclusion there is no unique factor that enables accurate prediction of fecundity. Furthermore, the predictive capacity of different factors varies between species, stocks and geographic areas (within the same stock) because "the fecundity of fishes is an adaptation which ensures the survival of the species under the conditions in which it originated" (Nikolsky, 1963)

Relative NDO (number of developing oocytes per gram of female gutted weight; NDO_{rel}) observed in the current analyses was between 1 and 2,302 oocytes/g (Mean value=693 oocytes/g), probably the lower values being at the beginning and end of the breeding season. For the same species in the Bay of Biscay, NDO_{rel} has been estimated between 298 and 1606 oocytes/g (mean value=957 oocytes/g); although the Bay of Biscay hake NDO_{rel} range is narrower than in Galician shelf waters, the mean value is higher, which is logical because NDO was rather higher in specimens from Bay of Biscay than those from Galician Shelf waters. Lambert and Dutil (2000) recorded for cod a realized relative fecundity equal to 4,179±230 eggs/g, that varied depending on female condition; this value is considerably higher than relative fecundity estimated by Marteinsdottir and Begg (2002), who found values between 51 and 1327 oocytes/g which were affected by female condition too. Lower values (998 oocytes/g) were observed in first-time spawning turbot just prior to spawning (Bromley *et al*., 2000). In long-lived species with reproductive strategies closer to K strategists, total and relative fecundity is much lower; for example, *Sebastes mentella* from the Norwegian-Barents Sea population produced 87.5 eggs/g at the most (Drevetnyak and Gusev, 1996).

A significant linear relationship was detected between NDO_{rel} and all considered female variables, excepting HSI, as in other species like Greenland halibut (Gundersen *et al*., 2001). All relationships were positive, except with condition factor, K. In general, correlations were low ($r^2 < 0.3$) and only gonad weight and GSI were highly related to NDO_{rel} (r=0.63 and 0.57 respectively). On the contrary, no changes of NDO_{rel} neither with length nor gonad weight were detected in *M. hubbsi* (Christiansen and Louge, 1993). Chambers and Waiwood (1996) reported that in captive Atlantic cod, relative fecundity was independent of female size, whereas for wild Arcto-Norwegian cod Kjesbu *et al*. (1998) observed that larger females had higher relative fecundity. Probably, larger wild specimens have some advantages in predation eficiency and improve their energetic reserves; besides, energy consumed in maintenance of older and larger females in reproduction is lower than in younger and smaller ones (Love, 1980; Slotte, 1999). As cited before, Lambert and Dutil (2000) and Marteinsdottir and Begg (2002) observed a dependence of relative fecundity on female condition, but this was not observed in cod from northern North Sea and Scottish west coast waters by Yoneda and Wright (2004). These results suggest that changes in relative fecundity have a genetic

component, and are not completely explained by feeding or environmental factors, although more studies are required in this field before reaching definitve conclusions. In haddock, relative fecundity increased from 274 oocytes/g at age 2 to 493 oocytes/g in older specimens (Hislop, 1988).Some cases with complex relative fecundity dynamics have been reported, e.g. in Greenland halibut, relative fecundity decreases with length in younger individuals, but when length and age increase, relative fecundity also does (D´yakov, 1982).

NDO$_{rel}$ was higher in 2004, like NDO and batch fecundity, varied significantly between months, and followed the same trend as NDO with a peak in January, and diminishing in subsequent months, although not straightforwardly. Higher values in January may be related to the beginning of the spawning season of the bulk of the spawning stock; consequently, monthly NDO$_{rel}$ patterns would support the idea that productivity of females at the beginning of spawning season is higher than when the spawning season is advanced, because hake females produced more oocytes per gram of body mass. On the other hand, increased NDO$_{rel}$ in the first month of the year may be due the presence of good food and environmental conditions. Murua *et al*. (2006) also report NDOrel changes in hake from the Bay of Biscay, and consider that it is a consequence of variations in maternal condition during the spawning season. For Greenland halibut, relative fecundity remains constant between areas and years (Junquera *et al*., 1999). In traditional spawning grounds off Greenland, cod relative fecundity decreased from 1988 to 1996, recovering to earlier values in 2000, whereas in new offshore spawning areas, relative fecundity increased progressively after 1997 due to a changes in size composition of the spawning stock (increase of larger spawners), stability of environmental conditions, and cessation of fishing activities in both areas (Marshall *et al*., 2000).

It has to be taken into account that fish weight changes within and between years, so differences in relative NDO or fecundity can be caused not only by variations in oocyte mobilisation or egg production, but by changes in female somatic mass. Accordingly, relative NDO or fecundity would not then be a good index to compare fecundity between months, years or stocks (Bagenal, 1967). Besides, as pointed out previously, fecundity is affected by several factors, and relationships with maternal attributes or somatic condition factors may change between stocks and species; it is then difficult to make comparisons between them, and necessary to consider the particularities of each stock.

In summary, according to these results, larger, and/or experienced females of European hake in better condition from Galician Shelf waters not only might produce higher number of oocytes because have larger gonads, but also they may produce more oocytes per body mass unit than smaller ones. Furthermore, in the present work females sampled in 1999 and 2000 were smaller than those sampled in 2003 and 2004, and it was observed that NDO$_{rel}$ increased from 1999 to 2004. When females from 2003 and 2004 were compared, it was observed that NDO was larger in 2004. This increase of fecundity in 2004 females might

be due to the influence of environmental factors or to a cohort effect. It is necessary to consider that fishing activity was banned in Galician shelf waters during most of the first semester of 2003 as a consequence of the *Prestige* fuel-oil spill, This may have had effects on the spawning stock; on one hand, the lack of fishing pressure might have had a positive effect on the reproductive potential of small specimens in the next spawning season (2004), such as increasing growth rate, and on the other hand, prey of large hake could have migrated as a consequence of adverse environmental conditions derived from the oil-spill, affecting the condition of large individuals and consequently their egg production in 2003. Besides, hormonal alterations can take place in spawners and this might alter their reproductive processes. For example, in juvenile turbot (*Scophthalmus maximus*), it has been observed that acute exposure to the *Prestige* fuel oil elicits alterations in some hepatic biotransformation enzymes with different sensitivities, and leads to decreased levels of testosterone and estradiol in plasma (Martin-Skilton *et al.*, 2005); in adult rainbow trout (*Oncorhynchus mykiss*), levels of reproductive steroid hormones displayed significant changes after naphthalene exposure (Tintos *et al.*, 2005), suggesting an alteration in the gonad capacity for synthesizing and releasing sexual hormones, with the subsequent effect that this has on reproductive success. Furthermore, an increase in atresia rates in hake after the oil spill was reported (Domínguez and Saborido-Rey, 2005). Another mechanism that could explain observed changes is the density-dependent response that some stocks experience when the number of large female is reduced. It has been experimentally proved that reduced survival of large adults favours selection of earlier maturity ages and an increase of reproductive effort (high NDO_{rel} values) to compensate low fecundity of smaller females (Yoneda and Wright, 2004)

The relation between batch fecundity and NDO obtained in this study indicates that in the case of having determinate fecundity, hake would produce between 3 and 6 batches, which are rather unreliable. However, Louge (1996) proposed that *M hubbsi* only released 3-4 batches in each spawning season and Sarano (1984), for *M. merluccius* from theBay of Biscay, considered that the total number of batches is six; Biagi *et al*. (1995) suggest between 3 and 5 batches in the Tyrrhenian Sea. Murua *et al*. (1996) assumed a three month spawning period for European hake, and suggested the production of 5-21 batches. In other gadoids like cod, the number of batches has been estimated between 3 and 16 (Tripple, 2004). Bye (1990) said that temperate marine fish can spawn from a single batch on one day (total spawners) to as many as 20 batches spread over several weeks, depending on the species. Bearing this in mind, it is unlikely that hake females will spawn only 3-6 batches since during the peak of spawning, the spawning frequency is high (a batch interval of about 5 days), and this would imply a very short individual spawning period. In addition, the literature on other multiple spawning fish shows that females are capable of spawning many more batches (Hunter *et al*., 1985; Kjesbu *et al*., 1989; Tripple, 2004). Thus, the ratio between NDO and batch fecundity in European hake would support the hypothesis that this species is an indeterminate spawner, as suggested before (Murua *et al*., 1998). Since

oocytes are eventually incorporated into the mature oocyte pool during the spawning season, estimates of numbers of batches obtained by dividing NDO by batch fecundity are wrong, and it is concluded that the use of the potential number of developing oocytes (NDO) as a proxy for annual realized fecundity is rather inaccurate. In fact, if Greer-Walker *et al*. (1994) criteria to differentiate between determinate and indeterminate species are considered, European hake of Galician Shelf waters fit the indeterminate category: no gap in oocyte size distribution is observed between previtellogenic and advanced vitellogenic oocytes, and high levels of atresia are observed at the end of spawning season, although not all authors agree with this criterion (Sarano, 1984; Tsimendis and Papaconstantinou, 1985; McFarlane and Saunders, 1997; Biagi *et al*., 1998).

Concerning oocyte density in gonads (i.e. number of developing oocytes per gram of ovary) it is observed that density decreases from the beginning to the peak of spawning, increasing progressively thereafter. This indicates that the diameters are higher during peak spawning than at the beginning or end of the spawning season. Similar results have been described in Northeast Arctic and Northwest Atlantic cod (Kjesbu, 1991; Chambers and Waiwood, 1996), whereas in Icelandic cod oocyte density remains constant during the spawning season (Marteinsdottir and Begg, 2002). Oocyte density has also risen in 2003 and 2004 with respect to previous years. This may be related with changes in NDO_{rel} derived from environmental effects, or be a compensatory response of the spawning stock; but increases of oocyte density not always provoke an increase of NDO_{rel} due to growth of gonad (Yoneda and Wright, 2004).

Consequently, we conclude that it is more accurate to estimate the fecundity of European hake from the Egg Production Method based on batch fecundity and spawning fraction, coinciding with Ganias *et al.* (2004) who propose batch fecundity as the only useful and reliable measure of fecundity for multiple spawning fish species with indeterminate annual fecundity. However, for that purpose, one needs to know the duration of individual spawning seasons, which is still not known. In the case of European hake, the duration of different oocyte stages used to estimate spawning fraction are not sufficiently known, therefore partly hampering estimates of annual realized fecundity; consequently, further work is needed to estimate the duration of the spawning stages, or to develop an alternative method to determine spawning fraction and duration of individual breeding seasons (Claramunt and Roa, 2001).

Daily Egg Production (DEP) estimated in the period 2003-2004 ranged between 4.2 and 91 eggs/g of female gutted weight with a mean value of 29.4 eggs/g of female gutted weight. In February, a peak of DEP is observed in both years (2003 and 2004), coinciding with a peak of spawning fraction; then DEP progressively decreased towards the end of the year, although a secondary minor peak was detected in June of 2003 and in July of 2004. In 2003, the DEP upturn in June coincides with an increase in spawning fraction, whereas

those in July 2004 match a peak of relative BF. Differences in spawning fraction could be caused by differences in environmental conditions, while differences in relative BF are due to differences in maternal attributes, possibly related to different spawning cohorts, though environmental conditions may also have an affect.

Annual Egg Production (AEP) based on DEP estimates varies depending on the spawning period considered. But a 2 months spawning season has been assumed yielding an AEP highest in individuals that spawn during winter-early spring months (January-March) and lowest in those that spawn during autumn months (September-November). Depending on this, AEP ranged between 1,947 and 4,366 eggs/g of female gutted weight at the beginning of the year, between 366 and 2,806 eggs/g of female gutted weight in June-July, and finally, between 549 and 793 eggs/g of female gutted weight during the last quarter of the year. These values are higher than those reported in the Bay of Biscay between January and March, i.e. 985 eggs/g of female gutted weight Murua et al. (2006), nevertheless, these authors estimate mean AEP in the period April-October at 445 eggs/g female gutted weight, more similar to values observed in this study. In *M. hubbsi*, reported mean AEP values are much lower, being 1000 egg/g of female gutted weight in January and 150 egg/g of female gutted weight in March.

The impact the number of inactive or atresic eggs would have on population relative daily egg production has not been considered in this study. However, it is known from the literature that the proportion of inactive mature female or females in mass atresia condition may represent an important fraction of the spawning stock (Rideout *et al.*, 2000; 2005; Engelhard and Heino, 2006; Jørgensen *et al.*, 2006; Murua and Motos, 2006; Rideout and Rose, 2006; Rideout *et al.*, 2006), and it will be discussed below. Therefore, the seasonal pattern of relative population daily egg production shown here could be overestimated, mainly during months outside peak spawning. In other words, the decrease in daily egg production would have been much more pronounced if the percentage of inactive females or atretic females had been considered. The results of adult daily egg production are in agreement with ichthyoplankton data, which show that the peak of egg abundance occurs in February-March in the Bay of Biscay (Álvarez *et al.*, 2001; 2004).

Indeterminate spawners, like hake, are characterized by a broad vitellogenic oocyte size distribution with no size gap to the previtellogenic oocytes. In this investigation, both vitellogenic and cortical alveoli oocytes up to 150 μm were measured and counted in hake. Determinate spawners usually have a dome-shape size distribution of vitellogenic oocytes that is rather narrow compared to that found among indeterminate spawners like hake. For determinate spawners, previtellogenic oocytes are usually not considered to be a part of the annual fecundity, but for indeterminate species it is necessary to consider them. It makes the oocyte counting process rather complicated and tedious. In this study, several methodological processes have been implemented to improve fecundity and NDO

estimation.NDO estimates were obtained by two methods: Image Analysis and Stereological Method, whereas for BF three methods were used: Image Analysis, Stereological Method and Manual Counting. But the three methods yielded significant differences, both in NDO and BF. In the first case, the Stereological Method showed lower values than Image Analysis. Regarding BF estimates, the Image Analysis method was significantly different from the Manual Counting method, while the Stereological Method did not differ from the other two methods, probably because data variability and standard error ranges were higher than in the other methods. On the one hand, extrapolation from points to areas and from these to volumes is inherent in stereology, and correction factors that must be introduced to its application in fecundity estimates are a source of error. On the other hand, degradation and disorganization of ovary and oocytes due to the mechanical process involved in histological processing (fixation, embedding, sectioning and staining of the ovary) sometimes cause the structures that are going to be studied to lose their shape. The advantage of this method is that identification of those oocytes that have started the maturation process is more exact because of direct cellular observation. The Manual Gravimetric method presented the smallest standard error range and without doubt, this method is the most accurate estimator of BF, because hydrated oocytes are directly observed. But it is important to take an adequate sample size to be representative of the whole gonad, and it is a time consuming method. Image Analysis yielded the smallest mean values of BF, which may be a consequence of an intrinsic characteristic of this method, in which hydrated oocytes are selected based on a size threshold (750 µm) predetermined by histological observations; these oocytes suffer shrinkage because of formaldehyde fixation and consequently, can be ignored, thus leading to underestimates of BF. In contrast, hydrated oocytes are selected *de visu* in the other methods. Nevertheless, for NDO estimates, Image Analysis is considered the most suitable method, and is quick and simple. This method not only allows us to count oocytes but also to measure them, estimate their area and their roundness, and to eliminate those that are damaged or broken, automatically through a computer routine. The disadvantage of this method is that NDO as BF estimates are based on diameter discriminations: oocytes larger than 150 µm in diameter are considered to be in the cortical alveoli phase, and those larger than 750 µm as hydrated. This assumption is not always correct in all females, and also changes from one species to another, although in general is a good proxy. A previous histological study of diameter distribution could improve results. In any case, in comparison with other authors' results, the Manual Method for BF and Image Analysis for NDO estimates are considered the most appropriate methods because of their simplicity, their rapidity, and the good information obtained.

4.8   LEVELS OF ATRESIA IN *M. MERLUCCIUS* IN GALICIAN SHELF WATERS.

For many authors, there is no correlation between variability of fecundity and recruitment, for three major reasons discussed already in this chapter. On one hand, the egg

and larval stages in most of species of commercial interest undergo high rates of mortality (McGurk, 1986; Myers and Cadigan, 1993; Mertz and Myers, 1996; Bradford and Cabana, 1997; Houde, 1997; Rickman *et al.*, 2000), which is beyond the concept of reproductive potential. Secondly, as shown before, spawning biomass is not a good index of fecundity, as larger, and/or experienced females in better condition, at least of hake, can produce higher number of oocytes per body mass. But thirdly, fecundity estimates may not correspond to realized fecundity due to the effect of atresia (Lasker, 1985; Bromage *et al.*, 1990; Billard, 1992; Kjesbu *et al.*, 1998) that may be more or less intense (Scott, 1962; Zamarro, 1992). Follicular atresia is an involutive process widespread in ovaries of fish and other vertebrates. Study of the prevalence and intensity of histological stages of atretic follicles provides the chief criteria for identifying regressing ovaries, and thus predicting the cessation of spawning in fish populations (Ganias *et al.*, 2003). In species with determinate fecundity, egg production is estimated from potential fecundity, and thus it is important to account for atretic oocyte losses during the breeding period (Kjesbu *et al.*, 1998; Bromley *et al.*, 2000). Nevertheless, for indeterminate species egg production is estimated based on batch fecundity, therefore atretic losses are inherently considered in the method. However, the definition of atretic states and subsequent assignment of females to different states (active, inactive/immature) are of great importance for spawning biomass estimates through the Daily Egg Production Method. The study of atresia mechanisms and the factors affecting atresia rates have a great impact on understanding the whole reproductive ecology of the species. Finally, assessment of the atretic condition of the ovary contributes to the estimation of crucial variables of fish populations such as length/age at first maturity.

The mortality of early stages depends mostly on environmental factors, both abiotic and biotic, but their survival, or viability, is determined to a great extent by egg and larval quality, especially size and energy storage (yolk sac), but also on other related features like buoyancy or growth rates. Egg size and yolk stores are basically determined by both maternal attributes and condition (Kjesbu *et al.*, 1991; Hardardóttir *et al.*, 2003; Kurita and Kjesbu, 2003), and by the energetic dynamics of the production. To face shortage or environmentally adverse situations, a female may reduce size and viability of its eggs (quality) or may reduce fecundity (quantity), the number of eggs to be released. The second is the most ecologically acceptable option, and normally is achieved by mobilizing fewer oocytes to the secondary growth stage, or by atresia once they have being mobilised to the maturing stock. It has been demonstrated that atresia incidence is affected by maternal status (Ganias *et al.*, 2003; Rideout *et al.*, 2006), food availability (Solemdal, 1997; Bromley *et al.*, 2000), parasitic affections (Clarke *et al.*, 2006), environmental conditions like temperature (Linares-Casenave *et al.*, 2002; Privalikhin, 2003), pH or presence of pollutants (Wootton, 1990), increase of stock abundance (dense-dependent effect; Privalikhin, 2003), or the imbalance of population sex-ratios (Rideout *et al.*, 2000). In highly stressed habitats like the Black Sea, it has been reported that periods of high atresia coincide with high anthropogenic pressure (Oven, 2004). Atresia is not exclusive to teleost fishes, and has also

been observed in other vertebrates such as mammals and birds (Hsueh *et al*., 2006). In cartilaginous fish like sharks that have low fecundity compared with teleosts, and which have internal fertilisation, fecundity regulation by atresia is replaced by sterile egg production as an adaptative mechanism to environmental conditions (Fahy, 1989).

There is no doubt that atresia impacts the reproductive potential of fish, but the strength of this influence varies depending on species, stock and environmental characteristics. In North Sea cod, atresia has a low or negligible effect on potential fecundity (Yoneda and Wright, 2004), while in Atlantic cod up to 33% of fecundity reduction may occur depending on the nutritional status of females (Kjesbu *et al*., 1991). Zamarro (1992) reported low values of atresia in American plaice (*Hippoglossoides platessoides*), suggesting that it is not an important regulatory mechanism in this species. In other species, atresia can be very variable, e.g. the mean annual oocyte resorption rate was 56% in Atlantic herring (Kurita *et al*., 2003), while in Norwegian spring-spawning herring, the highest value of atresia intensity was 4% (Óskarsson *et al*., 2002), changing with latitude. On the other hand, in Mediterranean sardine, some specimens have more than 70% of their oocytes in the atretic stage during the spawning season (Ganias *et al*., 2003). Simønsen and Gundersen (2005) reported for Greenland halibut a global atresia level lower than 10%, although some specimens exceeded 50%. In the present study on hake, it was observed that atresia intensity was relatively low in most months, and in general did not exceed 10%; however, values exceeded 40% at the end of the year, probably coinciding with a decrease of reproductive activity, while the highest values of atresia were recorded in the second half of the year. These results corroborate the view that European hake is an indeterminate spawner, with higher levels of atresia at the end of the spawning period, and more or less low levels the rest of the year (Greer Walker *et al*., 1994). Energy recovered from oocytes by atresia can lead to increased female survival and probability of future spawning. It therefore does not make sense in semelparous species; its importance in iteroparous species can change depending on the reproductive strategy of each stock.

Atresia may occur at any time during the spawning season, and can even be massive before spawning (Marshall *et al*., 2003; Murua *et al*., 2003). This has been observed in several species, both in females and males, e.g. turbot (Bromley *et al*., 2000), three-spined stickleback (Chellappa *et al*., 1989), cod (Rideout *et al*., 2000; Jørgensen, 2006), orange roughy (Bell *et al*., 1992), Greenland halibut (Fedorov, 1971), herring (Óskarsson *et al*., 2002; Engelhard and Heino, 2006), and other species. Several terms have been used to describe this phenomenon; reproductive failure, skip spawning, and mass atresia, are among the most used. The last one is the most correct when simply defining the event, i.e. most or all of the oocytes in the ovary are undergoing atresia. However, after the conclusion of the spawning season, especially in species with indeterminate fecundity, but also in some with determinate fecundity, the residual oocytes are in this stage. On the other hand, mass atresia may happen once the spawning has started, so technically the female has not skipped the

spawning, since it has effectively spawned eggs, although the season has been interrupted. Reproductive failure is the best choice, since it clearly indicates alteration of the normal run of spawning. In hake, nevertheless, it is not easy to differentiate between females in post-spawning condition and reproductive failure. The main factor is due to asynchronous spawning of the population, provoking a protracted breeding season, as may happen, in general, in iteroparous species (Rideout et al., 2005) .Reproductive failure seems to be more frequent and regular than expected, being more usual in early mature life. Between 8.4 and 55.6% of potentially spawning cod females from 1999 to 2004 were "skipped spawning" during their first reproductive attempt, although it was also relatively common in older females (Rideout and Rose, 2006). Arctic cod tend to skip a spawning season when survivorship is at risk because of energetic cost of reproduction increasing an average of 36% its fecundity in the subsequent year (Jørgensen et al., 2006). If fecundity losses by reproduction suppression are not accounted, annual egg production and consequently recruitment, can be overestimated, with consequences for stock assessment and fisheries management. Suppression of gamete production for the same species changes between areas, which indicates that environment plays an important role on the incidence of reproductive failure (Rideout et al., 2006). Results of this study show that the proportion of hake females with mass atresia was generally not very high, around 10%. Mass atresia in European hake from Galician Shelf waters is observed during the last quarter of the year, and can be associated with normal cessation of spawning activity, i.e. post-spawning females. Mass atresia in post-spawning individuals in hake has also been observed by other authors (Sarano, 1984; Murua and Motos, 2006). Fedorov (1971) reported relatively frequent mass atresia in mature females of Greenland halibut as a consequence of environmental conditions, and Oven (2004) who recorded high levels of atresia in fish populations from the Black Sea during the period in which anthropogenic disturbance was highest, as mentioned previously. In other species, it seems to be female condition which sparks off episodes of high intensity of atresia (Kjesbu, 1991; Kurita et al., 2003). Atresia may be even more marked if individuals have to carry out energy consuming activities prior to reproduction like migrations, or when the spawning stock is excessively abundant (Privalikhin, 2003). Some females may "decide" to skip a spawning season in one or more consecutive years as a function of their condition and energy reserves, resorbing mature oocytes (Fedorov, 1971; Silverstein et al., 1998; Bromley et al., 2000; Óskarsson et al., 2002; Morgan and Lilly, 2004). Millner et al. (1991) explain skip spawning for long-lived species like sole by suggesting that the strategy of these species is get enough food to grow and improve condition instead of increasing reproductive potential. Growth increments indirectly result in higher egg production. This strategy maximizes reproductive effort, being more important for long-lived species which spawn regularly during their lives rather than invest great energy in a single year at the expense of growth and condition. Jørgensen et al. (2006) approach skip spawning from two points of view: ecological (skip spawning is due to phenotypic plasticity of fish life strategies as a response to physiolgical and ecological factors), and evolutive (how

ecological changes can modify fish life strategies and what consequences these have on stock reproductive plans).

In species with indeterminate fecundity, like European hake, oocyte recruitment is adjusted, to some extent, to available ambient energy, as hypothesised earlier in this chapter; females will produce more or less eggs depending on their capacity to progressively incorporate energy into the reproductive process. According to this hypothesis, some authors are of the opinion that species of this type never reach reproductive failure (Larrañeta, 1996). This may not be true, however. Hake, as other species with similar strategies, may fail to produce the number of eggs that would have been produced in normal conditions. Shifts in egg production from year to year should be considered as normal, but early cessation of spawning season should be considered as reproductive failure. Skip spawning may occur in these species too, although this has not been investigated properly. Probably environmental factors are not so determinant as in northern latitudes, and hence the described reproductive strategy of hake, but drastic changes in stock structure towards early maturation, and global climate change, may have profound impacts on hake reproductive dynamics that should be further investigated. However, the intensity of atresia of European hake in Galician waters was not related to length, weight, GSI, HSI nor K, contrary to what occurs in other species; for example, small cod females present higher atresia rates than larger ones (Rideout *et al.*, 2000; Kraus, 2002), and atresia in Norwegian spring-spawning herring is related to maternal condition (Óskarsson *et al.*, 2002). In contrast, in halibut (Simønsen and Gundersen, 2005) and in Mediterranean sardine (Somarakis *et al.*, 2004), the intensity of atresia is related to GSI, but there is no relationship between atresia intensity and fish length or condition. As in other indeterminate species, it seems that in hake atresia intensity is low during individual spawning season, taking place a mass atresia process at the end of individual breeding period. Even more, seasonal variation of atresia intensity is inversely related to spawning fraction (Murua and Motos, 2006). This pattern has been reported for other indeterminate species, and has been related to post-spawning nutritional or physiological condition (Hunter and Leong, 1981; Rinchard and Kestenmont, 2003).

The highest values of atresia prevalence (around 30%) were detected in 2003, in February and December; this could be linked to a cohort effect, to availability of food, or to environmental factors. Considering again that in November 2002, the oil tanker *Prestige* sank, it may be that the oil spill indirectly affected egg production (Domínguez and Saborido-Rey, 2005), i.e. fishing was banned, increasing intra-specific competition because larger and more competitive females were not removed from the population. On the other hand, primary production was strongly affected by the spill, which contaminated inshore waters with extensive patches. All these factors could have caused the pelagic species on which hake prey, that depend on primary production, to move to cleaner areas in search of food. Consequently, if we add the increase of intra-specific competition caused by lack of food, and incorporate hormonal effects that the oil spill might have exerted on females, egg

production could be have been affected by low fecundity at size (as described above) and by an increase of atresia. This hypothesis agrees with that of Bromley *et al.* (2000), who suggested for *Scophthalmus maximus*, which does not stop feeding during the spawning season, that the critical factors in optimizing sexual maturation are a combination of adequate feeding, leading to the build up of body reserves inmediately prior to the start of vitellogenesis, coupled with high rations to provide nutrients to sustain the production of vitellogenin during vitellogenesis. *M hubbsi* shows the maximum incidence of atresia (45%) at the end of spawning season, in March (Pájaro *et al.*, 2005). In indeterminate species like *Engraulis mordax*, atretic processes take place during the spawning season, but prevalence was higher at the end of breeding period (Ganias *et al.*, 2004). Something similar was observed in Bay of Biscay hake (Murua *et al.*, 1996; 1998), in *M. hubbsi* (Macchi *et al.*, 2004; Pájaro *et al.*, 2005) and in herring (Kurita *et al.*, 2003), in which the incidence of atresia increased at the end of the spawning season, and was minimal during the spawning peak. So atresia can be consider indicative of the end of spawning, and allows differentiation between immature and inactive mature females (Lasker, 1985).

4.9 OVARY DEVELOPMENTAL STAGES. IS PROXIMATE COMPOSITION A GOOD PROXY OF OVARY DEVELOPMENT STAGES IN INDETERMINATE SPAWNERS?

It has been mentioned several times during this study that the high asynchrony in spawning activity observed among females prevents the study of hake reproduction using standard sampling routines and protocols. Immature, ovulating-hydrated, inactive mature and recovering females are rather accurately identified, but it is practically impossible to differentiate between mature pre-spawning (first egg batch still not released) and females in spawning condition (one or more batches already laid). In other species, especially in cold waters, spawning activity is usually concentrated in a particular period of the year, so that, although it may still be difficult to identify pre-spawning and spawning females histologically, the time scale may be used as a good proxy. In addition, POF in those species, due to seawater temperature, remain in the ovary longer (Fitzhugh and Hettler, 1995; Wood and Van der Kraak, 2001), increasing the chances of properly identifying a spawning female. In hake, at least in Galician waters and in the Bay of Biscay, any ovarian developmental stage can be found at virtually any time of the year, and POF degenerate at high rates, long before the production of the next batch (Murua *et al.*, 1998; 2006). Various species of *Merluccius* have been catalogued as determinate species (Tsimendis and Papaconstantinou, 1985; Biagi *et al.*, 1995; McFarlane, 1997), but probably not all are. Also some authors, despite considering hake an indeterminate species with a protracted spawning season, distinguish between pre-spawning and spawning females (Cerna and Oyarzún, 1998; Osborne *et al.*, 1999; Macchi *et al.*, 2005), but probably using incorrect criteria. Difficulties in maturity staging have also been detected in other species with prolonged and asynchronous spawning seasons (Arocha, 2002; Morely *et al.*, 2004; Rideout *et al.*, 2005). Stable environmental

conditions and cold water habitats favour the spawning season extension and asynchronous maturity, although there are exceptions (Allain, 1998; Morely *et al*., 2004). In some species, the period of embryo release is relatively short, while other species showed a wide range of maturity stages which may be indicative of a single protracted or multiple spawning respectively (Gunderson *et al*., 1980). Even more, Somarakis *et al*. (2004) established 18 maturity stages for Mediterranean sardine based on histological analysis.

Macroscopic, *de visu*, staging is a quick and common method, but is limited because it provides scarce information and is very subjective, which may induce errors (West, 1990; Kjesbu, 1994; Domínguez *et al*., 2005); but for some species it has been shown that macroscopic classification can be rather accurate (Gerritsen and McGrath, 2006). The usefulness of macroscopic maturity staging depends on information requirements, and the reproductive strategy of the species; but it is highly recommendable to calibrate the results with microscopic analyses. For this reason, in the present study, it was decided to develop new techniques to determine ovary developmental stages in European hake as accurately as possible. The results of these studies can be applied in the future to other species with similar problems. The methods developed and assessed are i) temporal changes of somatic condition indices (GSI, HSI and K), ii) variations in biochemical composition and iii) evolution of oocyte size distribution compared with variations in tissues energy density.

The variations of the general condition indices, HSI and K, were analysed in relation to ovary developmental stages, determined by histology. Although both indices showed significant differences, a clear pattern was only identified in K, which tends to decrease from immature to inactive mature females, and increase in recovering individuals. This suggests that energetic loss occurs during the spawning season, probably linked to metabolic costs derived from the formation of yolk, and probably associated with other activities like reproductive behaviour. Regression between GSI and K corroborates these observations; K decreases significantly when GSI increases, that is as the spawning season advances. If energetic reserves change through thespawning season due to the metabolic costs of reproduction (Love, 1970; 1980; Lucas, 1996), as has been demonstrated in other species (Trippel, 1999; Trippel and Neil, 2004; Lambert and Dutil, 2000; Blanchard *et al*., 2003; Marshall *et al.* 2003), then biochemical composition might be used as a tool to classify the exact stage of the breeding season in which a female is. The multivariate analyses performed with the different biochemicals resulted in three factors, but only one varied significantly with ovary stage as determined by histology, and its trend was similar to that of K. The most relevant variables were gonad and muscle lipids and gonad and liver proteins. That lipids and proteins of gonad change during the spawning season is logical, because gonad incorporates many biochemical compounds as yolk during this time. However, it is surprising that liver protein and muscle lipid significantly vary with maturation, since it is usually considered that liver provides lipid to gonad and muscle is the protein source (Love, 1970; 1980; Marshall *et al*., 2000). Probably, lipids and proteins are so abundant in liver and

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muscle respectively that changes related with reproduction are minor; nevertheless, as lipids in muscle are scarce, the small changes that take place are evident and detected by the analysis. The same might be so with liver proteins. This suggests that these two components can be good indicators of ovary developmental stage. If only gonad components are considered, lipid, protein and water content were the most important variables related with ovary developmental stage. However, variation in water content probably is driven by hydration, more than the simply progression of the spawning season. These results also indicate that the main ovary developmental stages based on histological observations and described in this study (i.e. m1 to m3) classify with relative accuracy the stage of the breeding season in which a female was.

Oocyte size (diameter) was independent of female length and gutted weight. There was also high variability, i.e. large females with small oocytes and *vice versa*, and there were no intra-annual differences in oocyte size, as in the Bay of Biscay (Murua and Motos, 2006). Analysis of modal sizes of oocyte distributions in *M. capensis* and *M. paradoxus*, to identify progression of the spawning seasons, revealed that bi-modal distributions are only observed when hydration take place, and are multi-modal the rest of spawning seasons (Osborne *et al*., 1999). Nevertheless, progression of mode was reported in earlier studies on hake (Andreu, 1956), although high overlaps between groups were detected except for the hydrated oocyte group. But in the case of *M. merluccius*, mode alone was not fully explanatory. In this study, after analysis of several descriptors of size distribution (mean, standard deviation, minimum, maximum, quartiles, geometric mean, harmonic mean, mode, median, etc.), mode and mean were selected to try to classify ovary developmental stages, resulting in the definition of five groups. In the second, third and fifth groups, all ovarian developmental stages are present (m1, m3 and h), while in the first group mature non hydrated females dominate, and in the fourth most of specimens are hydrated females. These groups represent progression of the spawning season very, effectively, from the beginning (group 1) to the advanced stage (group 5).

In synchronous or group-synchronous ovaries, all yolked oocytes develop in unison; there is no incorporation of earlier stages, and oocyte size mode thus progresses with oocyte development. In asynchronous ovaries, there is no gap in size distribution between unyolked and yolked oocytes, except when hydration occurs (Hunter and Macewicz, 1985; West, 1990). For species with short and well defined spawning seasons, all females with mature oocytes in the gonad just prior to the identified spawning season can be considered pre-spawning females (Myers *et al*., 1993; Morgan and Brattey, 1996). In these species, mean or modal oocyte size allows different spawning stages of females to be identified, as in cod (Solemdal *et al*., 1992) or Norwegian spring-spawning herring, in which mean oocyte diameter is considered a good proxy of maturity status of females, although interpretation of results must be made with caution when comparisons among years are made (Óskarsson *et al*., 2002). According to Murua *et al*. (1996), in European hake larger oocytes form a

homogeneous group, whereas smaller oocytes are usually distributed in 2 or 3 modes. Something similar was suggested by Sarano (1984), although he established a maturity stage classification for European hake, differentiating pre-spawning females with continuous oocyte size distribution (until 650-950 μm), spawning females with 1000 μm mode (i.e. hydrated oocytes or egg release stage), and post-spawning females with 650 μm mode but few oocytes and important signs of atresia. This is a simple classification that has been improved in the current study with a spawning (m2) category which includes the presence of POF. Sarano's classification does not distinguish between those females that have released one or more batches (spawning) from those that have not released any (pre-spawning), since he used the term spawning to refer exclusively to females in egg release stage. McFarlane and Saunders (1997) made a similar classification for Pacific hake, and distinguished two pre-spawning maturity stages that showed bi-modal oocyte size distributions, with one peak at 100 μm, another one between 500-700 μm, and post-spawning females in which most of the oocytes ranged between 150 and 280 μm; nevertheless, these authors considered Pacific hake a determinate species; and Helser and Almeida (1997) agree because they consider that all spring females of Silver hake are pre-spawning. This does not occur in European hake, as shown in this thesis, and corroborated by other authors (Louge, 1996; Murua *et al*., 1998). In other species like Greenland halibut that is considered indeterminate (Rideout *et al*., 1999), with a bi-modal oocyte size distribution, maturity staging has been based on proportions of different sized oocytes (Simønsen and Gundersen, 2005).

The validity of the five groups established using mean and mode of oocyte distribution was assessed through relative variation in energy content (kJ/g) of gonad, liver and muscle, resulting in a clear trend in gonad and liver. In both, relative energy increases from group 1 (mode<395 μm and mean<400 μm) to group 3 (Multiple mode) in which maximum values are reached, then progressively decreases until group 5 (mode between 540 and 800 μm and mean>350 μm). In gonad, the result is reasonable, because oocytes accumulate energy progressively until just prior to release, while as the spawning season advances, the rate of incorporation of previtellogenic oocytes to the vitellogenic stock is lower than the rate of egg release, until females stop spawning. During this period, energy progressively decreases in the gonad. However, interpretation of liver results is more confusing. Perhaps the increase in relative liver energy reflects the increase of metabolic activity in the liver to create vitellogenin. When the proportion of energy in each organ from the total energy of fish was studied, results improved, probably better reflecting the energetic dynamics of the whole process. The proportion of energy in gonad increases as the breeding season progresses, but the maximum is reached in group 4 (mode>800 μm and mean>450 μm), decreasing sharply thereafter. On contrast, the new approach shows that the proportion of energy in muscle follows the opposite pattern, diminishing significantly with gonad progression (r^2=0.85). This suggests that the most important effect of reproduction falls on muscle energy reserves; proportionally muscle loses more energy than liver during reproduction, and this is

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reflected also by K, which decreases when GSI increases, as shown before. Results suggest that at the beginning of the spawning season, the relative rate of incorporation of previtellogenic oocytes to the vitellogenic stock is higher, decreasing as the spawning season advances. The relative rate changes not only because of the number of unyolked oocytes mobilizing to the yolked stock, but depending also on the egg release rate, i.e. batch production or frequency. This result is in accordance also with those achieved with NDO discussed earlier. In any case, this is a promising but preliminary study, that needs further attention and research.

In summary, hake is an indeterminate spawner, that with highly asynchronous spawning activity, making it difficult to classify ovary developmental stages. This handicap can be solved using two proxies, the condition/biochemical composition or oocytes size distribution. The first one offers more information of bioenergetic process and dynamics during reproduction in fish, but due to high variability of biochemical composition and condition within females at the same maturity stage, results are rather inexact. Estimates of condition indices are quick and cheap but not really accurate in indeterminate species, because egg production depends directly on energy intake during the breeding season, and consequently condition indices do not reflect properly spawning ovary developmental stages. Something similar happens with biochemical composition, adding the high cost and time consumption of this type of analysis. The oocyte size distribution approach combines both, mean and mode, seems to be an encouraging method, is relative rapid, and when fecundity studies need to be carried out, this information can be obtained automatically, since it is easily estimated aided by image analyses. It could be applied to other indeterminate species, and no special knowledge of fish reproduction is necessary. In any case, more studies focusing on females with post-ovulatory follicles and spent females have to be carried out. Regarding oocyte size distribution techniques, new investigations should to be focused, on one hand, on applying the method for determinate species to validate it, and on the other hand, on improving it in order to get a better separation of maturity stages in indeterminate species.

4.10 Reproductive potential of *M. merluccius* in Galician Shelf waters: Influence of atresia and condition on real fecundity.

Stock Reproductive Potential needs to be determined in each stock and on a regular basis for several reasons. First, because it depends on population factors like genetic pool, size/age at maturity, or size/age structure of the stock, that are known to change with time. Scott *et al.* (2005) studied the effects of population size/age structure, condition and temporal dynamics of spawning on reproductive output in Atlantic cod (*Gadus morhua*), and results showed that even when a population was in stable balance and spawning stock biomass remained constant, differences in size/age structure of the stock combined with an increase

of fishing mortality provoked a substantial decrease in stock reproductive potential, because not only was egg production affected, but also the duration of the spawning season; depending on egg quality, the reproductive potential of the stock might increase from 48% to 74% with respect to unfished populations. Secondly, because SRP depends also on individual characteristics like proximate composition, condition, atresia incidence, and fecundity, all of which also fluctuate. Not all females contribute in the same proportion to offspring production; it is known that larger females produce larger eggs, which develop into larger and more competitive larvae with better survival probabilities (O´Brien, 2003). Moreover, females in worse condition at the beginning of the spawning season spend proportionally more energy than those in good condition, and the subsequent risk of mortality is higher. If such a situation coincides with long periods of adverse environmental circumstances, the spawning stock may collapse and not recover even if fishing pressure stops. This is probably the case of Atlantic cod from the Gulf of St Lawrence (Lambert *et al*., 2000) and from Flemish Cap (Saborido-Rey *et al*., 2004).

Stock reproductive potential is not always exclusively related to the condition of females. Sometimes, variability of offspring production or even changes in genotypic characteristics of stocks is due to long periods with unfavourable environmental conditions or high rates of selective mortality (Yoneda and Wright, 2004; Morgan and Brattey, 2005). However, not all fish species are equally affected by external pressures; some stocks show surprising resilience to ecosystem changes (Helser and Brodziak, 1998; Junquera *et al*., 1999). Furthermore, Blanchard *et al*. (2003) observed that inclusion of condition factor in Total Egg Production models (TEP) did not notably improve the stock-recruitment relationship in haddock, although it generated a new viewpoint of stock dynamics. It seems that strong year-classes are independent of parental effects, but when they are not so strong, a maternal influence on recruitment gains relevance (Tereschenko, 2002). Furthermore, the proximate composition of fish may reflect habitat quality and lack of stress factors (Lloret *et al*., 2005). Another question to be considered is the effect of external and inherent factors experienced by fish during their early life history on the future reproductive potential of individuals. O´Brien (1999) suggested that maturation of Atlantic cod from the Georges Bank and the Gulf of Maine depends on stock biomass and bottom temperature during the juvenile stage, and Helser and Almeida (1997) observed in silver hake from U.S. that sexual maturity may be mediated through competition and growth during the first and second years of life.

The precautionary approach to fisheries already forces these factors to be considered during the assessment process, but it has been strengthened with the Ecosystem Approach to fisheries (FAO, 2001). Inclusion of stock reproductive indices has notably improved stock-recruitment relationships (Marteinsdottir and Begg, 2002; Marshall *et al.,* 2003; Saborido-Rey *et al*., 2004; Burton, 1999). However, the factors with the most impact on SRP may change with species, stocks, and even between years (Tomkiecvicz *et al*. 2003). In European hake from Galician waters, maturation, condition, energy dynamics, fecundity, and atresia, have

been discussed above separately, but is the conjunction of all these parameters which may improve our understanding of stock reproductive potential of hake. The number of developing oocytes (NDO) is affected by biochemical conditions. NDO increases with female energetic content, well expressed in total terms by the energy reserves in liver and muscle, less so by those in gonad. To what extent these reserves reflect the short-term energy reserves of the body has been discussed earlier, but probably it means that females with higher energy reserves are able to mobilize more oocytes more rapidly, hence increase their chances of producing better batches in better condition. This is partly confirmed by the fact that NDO_{rel} and oocyte size are not related to energy density except in gonad. Oocyte size as described here does not reflect final egg size, but the progress of oocyte development. Egg size is an important determinant of egg survival, and hence of larval survival too (Hislop, 1988). Saborido-Rey *et al*. (2003) observed in Atlantic cod that differences in the size and specific gravity of eggs affect their buoyancy, and consequently may affect the probability of survival. At the same time, the size of oocytes depends on their composition, which is function of female condition (Marteinsdottir and Begg, 2002). On the other hand, both NDO and batch fecundity increases with female length and weight. However, hydration process has low energetic requirements, especially compared with vitellogenesis. This suggests that the NDO mobilized in each period by a female may depends on its short-term energy reserves, but the number of oocytes released in each batch may depends more on female size. In some species, like cod, females store energy during the main feeding period, which occurs well before the spawning season, and vitellogenesis relies basically on the strored energy. On the contrary, hake continues feeding during the breeding season, and thus accumulates energy reserves on short time scales which may determine the NDO to be mobilized periodically. The number of eggs from those developing oocytes that are released in each batch depends on female size. If this is correct, and if potential fecundity does not change with female size, larger females should produce less batches than smaller females. However, the most likely situation is that larger females have larger potential fecundity, thus recruiting small oocytes to form yolked oocytes more times during their spawning season, although this trend must be modulated by their capacity to capture external energy. In other words, larger females can release similar values of NDO faster, which allows them to recruit more oocytes to the NDO which results in higher total egg production. It can also be hypothesised that the oocyte development rates depend on female size or short-term energy reserves (feeding capacity), but more studies are needed to clarify relationships between maternal condition and attributes like oocyte growth dynamics and egg production in this species.

In general, this work corroborates the importance of proximate composition as a short-term energy reserve for egg production of European hake in Galician shelf waters, although the energy dynamic associated with egg production are different from those observed in cold water species. However, as oocyte development in hake almost coincides with spawning activity, and follows an asynchronous pattern, classification of female maturity

stages has proved to be a hard task. Lack of proper staging of ovarian development also makes it difficult to reveal the connection between body reserves and gonad formation.

However, due to high population asynchrony and the lack of analysis of complete temporal series, results are not as enlightening as expected. It is important to establish a more precise criterion to determine the precise stage of a female within the breeding season. More studies should be made to relate atresia to female condition, over longer time periods and larger areas. Biochemical analyses should be conducted in larger numbers of females, but to achieve this, low cost and faster methods need to be used. It is also necessary to study how external factors, like environmental conditions or fishing pressure, affects the stock reproductive potential of European hake. More careful analyses of population structure as well as the annual dynamics of condition factors and exploration of functional relationships between female characteristics and egg quality are needed to assess and accurately predict survival and the probability of recruitment levels. After the identification of the key reproductive parameters affecting SRP on hake, the next logical step must be the identification of current stock assessment procedures that potentially can incorporate stock reproductive potential, as well as the development of new techniques and statistical tools to incorporate reproductive potential and maturation-growth interactions into stock assessment procedures.

Past and present join together to act over the future, in words of Ortega y Gasset "I am myself and my circumstances" and the same idea could be applied to fish stocks.

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CHAPTER 5

CONCLUSSIONS

Quisiera beber agua salada
para sentirme un pez libre en mi vida de hombre.
(Felipe Campuzano)

European hake is one of the most important commercial species in Spain, particularly in Galicia. Nowadays its stock biomass has undergone a sharp decline in both stocks and spawning stock biomass has decreased below the precautionary approach and no signs of recovery have been observed in spite of measures taken by the Government. Not many studies on reproductive potential of European hake has been carried out.

1. European hake (*Merluccius merluccius*) is a batch spawner with indeterminate fecundity. Reproductive modality of this species is highly asynchronous both at individual, i.e. oocyte development, and at population level, i.e. spawning pattern.

2. *Merluccius merluccius* presents a protracted spawning season that covers the whole year, but basically from January to July with a main peak of spawning between January and March and an eventual secondary peak in June-July depending on year.

3. Potential fecundity can not be estimated in indeterminate species like hake, so the number of developing oocytes (NDO) and batch fecundity are the only estimation possible in these species.

4. NDO ranged between 78,873 and 3,366,352 oocytes with mean value equals to 721,821 oocytes. Batch fecundity ranged between 470 and 835,230 oocytes with mean value equals to 124,663 oocytes. For a 60 cm female NDO is 881,000 eggs and its batch fecundity is 243,000 eggs per batch.

5. Spawning fraction of European hake in the period 1999-2000 and 2003-2004 in Galician Shelf was between 4% at the beginning and the end of spawning season and 25% during the peak of spawning. Population Batch Frequency of this species was between 3 and 10 days.

6. Daily egg production (DEP) and Annual egg production (AEP) varied along the spawning season. For the whole year, DEP ranged between 4.2 and 91 eggs/g of female weight, and AEP between 366 and 4,366 eggs/g of female weight, what results in a realised egg production for a 1 kg female between 549,000 and 4,366,000 eggs.

7. Female size at sexual maturity was estimated in 44.45 cm in 2003 and 42.97 cm in 2004.

8. No temporal trends are observed in bioenergetic reserves along the spawning season, probably because egg production depends on environmental

conditions and food availability during the breeding season. However, population asynchrony on spawning may also mask patterns of energy reserves variations.

9. Gonad development does not depend on liver and muscle reserves, although both of them may act as intermediary compartments during translocation of energy, especially liver.

10. Female size and weight influence both batch fecundity and NDO, while long term energetic reserves and condition factor has low influence. NDO apparently depends more on energy short-term reserves, i.e. the energy assimilation during the oocyte recruitment period.

11. It is hypothesized that larger females have larger realized fecundity mobilizing more oocytes along the spawning season but modulated by their capacity to capture external energy.

12. Oocyte losses by atresia covers from 1.59 to 84.92%, although mean intensity of atresia is 6% and it is no related with length, weight, HSI or K of females. Atresia incidence is observed at low levels during all the spawning season increasing during the post-spawning period.

13. Proximate composition could be a rather good proxy of ovary developmental stages on this species, although it is necessary to establish more precise criteria to differentiate them.

14. Image analysis is a quick, automatic and accurate method to estimate NDO, batch fecundity and oocyte size distribution in fish and could be applied directly on histological section pictures in the near future.

15. It is important to include information on stock composition and account for size and condition features of the spawning stock on the assessment and management of both European hake stocks.

16. More careful analysis of proximate composition of European hake and its relation with atresia and fecundity are necessary to accurate predictions of offspring survival and recruitment levels as well as studies on the influence that environmental factors and population structure have on them.

CHAPTER 7
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¡Hombre libre, tu siempre preferirás el mar!
La mar es el espejo en que tu alma se mira,
en su onda infinita eternamente gira,
y tu espíritu sabe lo amargo saborear.
(Charles Baudelaire)

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ANEX I
RESUMEN Y CONCLUSIONES

Mar antiguo, madre salvaje
en tus orillas, de rodillas
me paré.
Tierra absurda,
que me hizo absurdo.
Nostalgia de un futuro azul
en el que anclar.
(Manolo García)

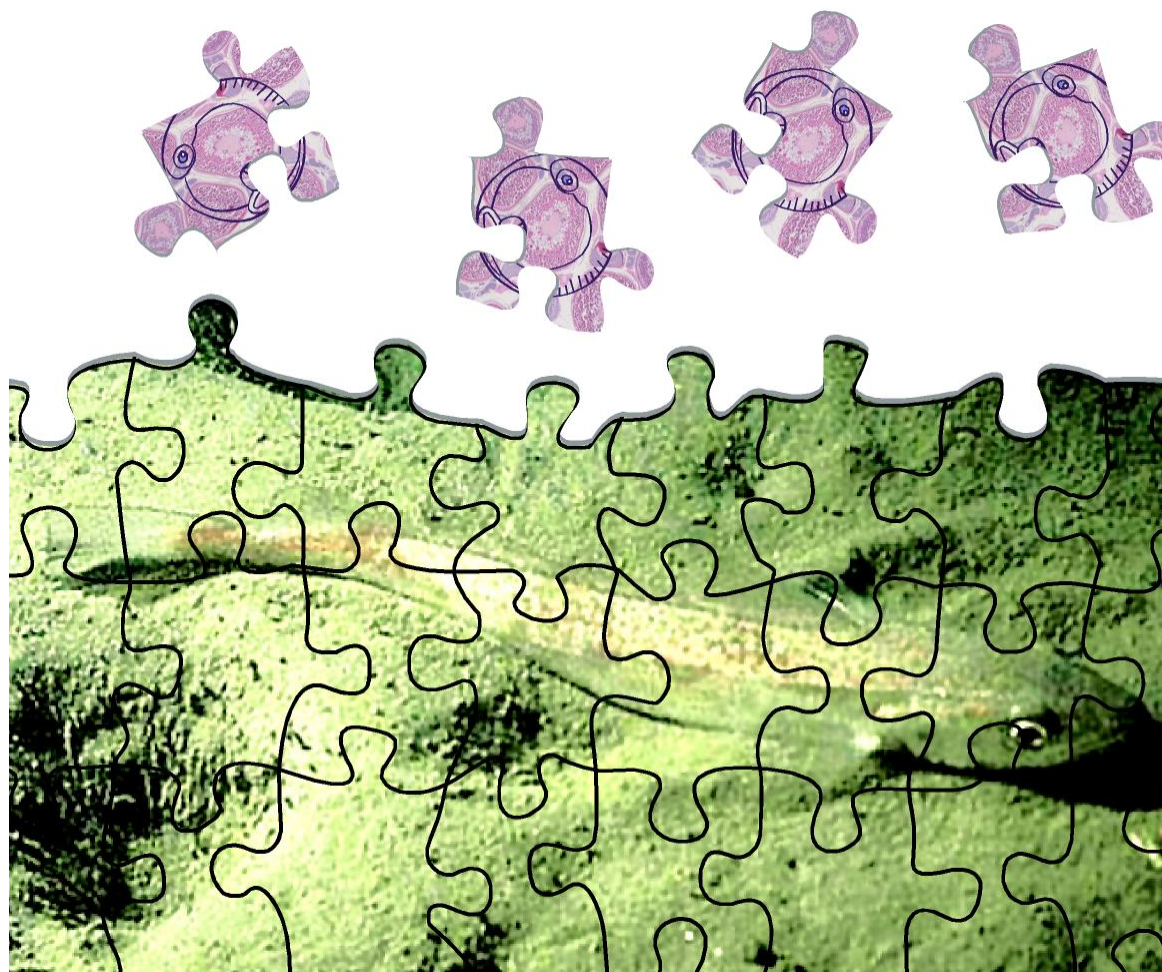Universidade de Vigo
Departamento de Ecoloxía e Bioloxía Animal

Instituto de Investigacións Mariñas

Estudio del Potencial Reproductivo de *Merluccius merluccius* en la Costa Gallega.

Resumen de Tesis Doctoral

Rosario Domínguez Petit

INTRODUCCIÓN

La mayoría de los recursos pesqueros marinos de Europa se encuentran fuera de los límites de seguridad biológica, es decir, sobreexplotados. Diversas medidas técnicas han sido tomadas por las diferentes Administraciones para la recuperación de los stocks, pero la mayoría han fallado. Uno de los objetivos básicos de la gestión de las pesquerías es conservar un potencial reproductivo de la población suficiente para permitir la explotación sostenible del recurso. Con este fin, se han desarrollado e implementado numerosas reglas de control de capturas dentro del llamado Enfoque de Precaución de la gestión de pesquerías. Desde la aplicación de dicho Enfoque de Precaución se ha dirigido una mayor atención a la capacidad real de las poblaciones para producir huevos y larvas viables en un año determinado (Potencial Reproductivo).

Muchos stocks son gestionados basándose en la asunción general de que existe una relación entre la población y el número de reclutas generados por dicha población. A partir de esta asunción se han desarrollado numerosos modelos tradicionalmente usados en la gestión pesquera, ej: Beverton y Holt (1957), Ricker (1954) y Shepherd (1982). Aunque estos modelos fueron desarrollados originalmente usando la relación fecundidad-reclutamiento (Rothschild and Fogarty 1989, Koslow 1992), el término Biomasa del Stock de Reproductores (SSB) ha sido más ampliamente usado en lugar de fecundidad o producción de huevos. Esto ha llevado a la asunción de que una determinada biomasa de individuos reproductores en una población dará lugar a una producción de huevos y un reclutamiento fijos. Esta hipótesis no tiene en consideración la estructura de edades o tallas del stock de reproductores ni la condición de los individuos dentro de la población en puesta. Sin embargo hay evidencias de que el poder de predicción de los modelos tradicionales stock-reclutamiento es débil y existen crecientes evidencias de que la producción de huevos y el reclutamiento no sólo depende de la SSB (Marshall *et al*., 1998; Scott *et al*., 1999; Marteinsdottir and Begg, 2002; Macchi *et al*., 2004). El término Potencial Reproductivo del Stock (SRP) fue acuñado por Trippel (1999) como alternativa al SSB. El SRP representa la capacidad del stock para producir descendencia viable que pueda ser reclutada a la población adulta o a la pesquería. El SRP incluye la contribución de los diferentes atributos maternos y paternos (edad, talla, reservas energéticas, condición, etc.) a la relación stock-reclutamiento ya que pueden determinar no sólo la producción potencial de huevos sino la real, regulada por la atresia (reabsorción de ovocitos en la gónada). Además de las características parentales también es importante añadir la influencia del medio ambiente en la en la producción de descendientes y su supervivencia, ej: efecto de la temperatura, salinidad, disponibilidad de alimento, etc.

Con frecuencia se han observado cambios en la productividad de las poblaciones como consecuencia de los cambios en el ecosistema a diferentes niveles que a su vez

afectan la historia vital de los peces que conforman la población. La necesidad de un enfoque ecosistémico en la gestión de los recursos marinos ha sido claramente identificada por diferentes cuerpos gubernamentales internacionales (FAO, UE, ICES o NAFO). Identificar las causas y consecuencias de las variaciones del SRP y mejorar la evaluación de los recursos pesqueros mediante la consideración de este índice proporcionará herramientas para comprender los efectos de las pesquerías en los ecosistemas y como regularlas para conseguir una explotación sostenible de los recursos.

La merluza europea (*Merluccius merluccius*) es uno de los recursos económicos más importantes para la pesca en España, no sólo por el volumen de capturas sino también por los precios alcanzados en el mercado y la elevada demanda existente por parte de los consumidores. La descarga total de merluza europea en España ha disminuido drásticamente en las últimas décadas, desde 17,300 t en 1972 a 3,950 t en 2002 que es el valor mínimo registrado en la serie histórica. Además existen los descartes que principalmente afectan a los individuos más jóvenes que no alcanzan la talla legal o tienen bajo precio en el mercado y que ha sido estimado para la flota española entre 400 t y 900 t lo que representa entre un 45 y un 70% del total de capturas de merluza. A pesar de la importancia económica de la merluza europea y de la dramática situación en que se encuentran sus poblaciones, hasta el día de hoy no se han realizado muchos estudios sobre esta especie. Existen algunos trabajos de investigación sobre su biología, ecología y crecimiento, aunque referidos principalmente a morfología, taxonomía, alimentación y distribución geográfica (Bozzano *et al*, 1997; Velasco, 1998; Álvarez *et al*., 2001; Ungaro *et al*., 2001; Garza-Gil, 2003; Maynou *et al*., 2003; Olivar *et al*., 2003; Carpintieri, 2005; Kacher and Amara, 2005). En los últimos años muchos investigadores han centrado su trabajo en la determinación de la edad y en el crecimiento de *Merluccius merluccius* (Uçkun *et al*., 2000; Lombarte, 2003; Piñeiro, 2003 and Morales-Nin and Moranta, 2004). En cuanto a reproducción, algunos autores han descrito el ciclo reproductivo de diferentes especies de este género (Murua *et al*., 1996; 1998; Lucio *et al*., 1998; Osborne *et al*., 1999 and Pájaro *et al*., 2005), la maduración (Helser and Almeida, 1997; Helser and Brodziak, 1998) y las variaciones espacio-temporales del proceso reproductivo de la merluza (Louge, 1996 and Macchi *et al*., 2004). Sin embargo, apenas se han realizado estudios comparativos con otras especies de interés comercial (gádidos o no) como el bacalao, el egelfino, la caballa o los peces planos cuya estrategia reproductiva ha sido más ampliamente estudiada. Probablemente esto sea una consecuencia de la complejidad de la estrategia reproductiva de la merluza. Para cuantificar el potencial reproductivo de una especie o población es necesario conocer su biología reproductiva en profundidad. Se cree que la merluza tiene reproducción parcial indeterminada con un desarrollo asincrónico de los ovocitos, lo que significa que existe un *conitnuum* en la distribución de tallas de los mismos en la gónada a lo largo de toda la estación reproductiva. Además, se ha observado cierta asincronía poblacional que hace que el periodo de puesta se extienda prácticamente a lo largo de todo

el año. Todos estos factores junto con la imposibilidad de mantener esta especie en cautividad dificultan tremendamente el estudio de su potencial reproductivo.

El objetivo de esta Tesis fue estudiar en profundidad la estrategia reproductiva de la merluza europea en aguas de Galicia y, por primera vez, se ha tratado de encontrar índices alternativos que permitan estimar el potencial reproductivo de esta población de forma sencilla y que puedan ser aplicados a la evaluación y gestión de ésta y otras especies con estrategias reproductivas similares con el fin de preservar los recursos pesqueros. Con este fin, se emplearon diversos enfoques (histológico y bioquímico) para analizar el ciclo reproductivo de la merluza en aguas gallegas e identificar la condición de las hembras reproductoras durante el periodo de estudio (1999-2000; 2003-2004), se calculó el nº de ovocitos en desarrollo (NDO), que es el único cálculo aproximado que se puede hacer de la fecundidad en especies con reproducción indeterminada, se estudió su variación intra- e interanual y su relación con la condición energética de la hembra. También se estimó la fecundidad parcial y la producción de huevos de la especie en aguas de Galicia. Por otro lado, los análisis tradicionales de cálculo de la fecundidad en peces han sido implementados mediante nuevas técnicas de análisis de imagen, y se han analizado las diferencias entre las diversas técnicas. Además, por primera vez el ciclo reproductivo de la merluza europea ha sido estudiado desde diferentes puntos de vista, integrando diferentes conceptos como NDO, composición bioquímica, atresia y producción de huevos en la estimación del potencial reproductivo de esta especie.

MATERIAL Y MÉTODOS

La elaboración de esta tesis se encuadra dentro del proyecto RASER del V Programa Marco de la Unión Europea (QLRT-2001-01825), aunque también se han utilizado muestras y datos procedentes del Proyecto XUGA-40201B98 de la Xunta de Galicia.

Las zonas de muestreo cubrieron lacosta gallega, aunque se muestreó con mayor intensidad en la Costa da Morte y Ribeira (Figura 1). Para la captura de ejemplares adultos se contó con la colaboración de la flota artesanal de volanteiros de Laxe, Cedeira y Celeiro, mientras que para el muestreo de juveniles se acudió a la flota arrastrera de Ribeira. En ninguno de los casos transcurrieron más de 24 horas desde la toma de muestras hasta su conservación en laboratorio, realizándose el muestreo de adultos principalmente a bordo.
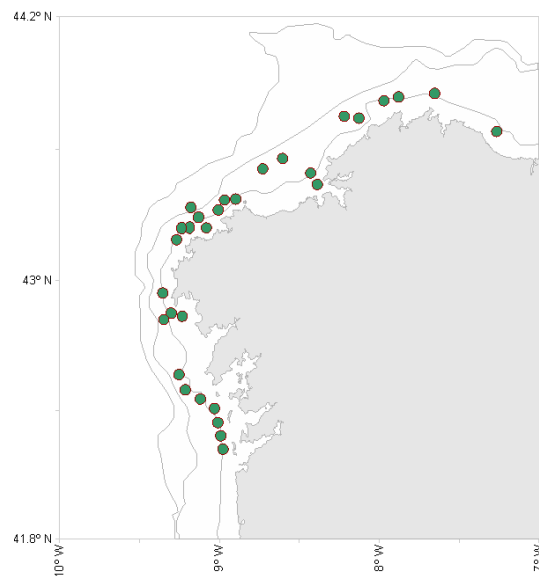
Figura 1: Areas de la costa de Galicia muestreadas en los periodos 1999-2000 y 2002-2004.

En total, 3.416 merluzas fueron muestreadas, 865 en los años 1999 y 2000 y 2,551 desde 2002 a 2004. Del total de capturas 2,779 fueron hembras, 689 del primer periodo de muestreo y 2,090 del segundo (Tabla 1 and Tabla 2). En cada individuo se tomó la talla (cm), peso total y eviscerado, el sexo, el estado de madurez macroscópico, el peso del hígado y el peso de la gónada.

Tabla 1: Hembras muestreadas mensualmente para cada rango de tallas en 1999- 2000.

Talla (cm)	1999									2000					Total
	feb	mar	abr	may	jun	ago	sep	oct	nov	ene	feb	mar	abr	may	
35-39									1						1
40-44	7	1	1	4					10	1	1	1	1	2	29
45-49	28	9	6	19	11	1	3	2	4	14	10	13	10	20	150
50-54	60	18	24	15	16	11	10	6	6	10	29	29	14	35	283
55-60	29	12	16	14	7	7	4	4	6	15	9	15	12	16	166
>60	9	4	6	5	3				1	11		13	3	5	60
Total	133	44	53	57	37	19	17	13	27	51	49	71	40	78	689

Tabla 2: Hembras muestreadas mensualmente para cada rango de tallas en 2002- 2004.

Talla (cm)	2002		2003											Total
	nov	dic	ene	feb	mar	abr	may	jun	jul	ago	oct	nov	dic	
<35			2	8		1	15	20	4	1	14	21	3	89
35-40		2	11	5	3	15	7	11	20	6	5	3	10	98
40-45		1	6	3	10	14	5	2	1	7	3	2	11	65
45-50		1	1	16	20	11	9		2	1	5	1	12	79
50-55	9	1	13	63	50	45	51	13	11	5	11	5	8	285
55-60	5		14	26	31	40	71	35	33	21	19	6	9	310
60-65			11	4	25	17	22	26	23	14	8	3	9	162
>65					6	5	5	9	3	1	4	1	1	35
Total	14	5	58	125	145	148	185	116	97	56	69	42	63	1123

Talla (cm)	2004											Total
	ene	feb	mar	abr	may	jun	jul	ago	sep	oct	nov	
<35	15	3	11	20	17	21	19			6	4	116
35-40		1	5		16	11	10			5	15	69
40-45			9	2	11	5	2		1	6	5	41
45-50	8	7	10	14	35	8	9	6	10	6	5	118
50-55	29	39	37	36	40	16	16	7	12	13	12	257
55-60	34	33	42	27	24	10	10	7	12	6	19	227
60-65	18	23	24	10	12	1	3	3	1	2	3	90
>65	8	11	7	7	6	2	1	2	1	2	1	48
Total	112	117	145	116	161	74	70	25	37	46	64	967

Algunas hembras del periodo 2002-2004 fueron seleccionadas en función de su talla y estado de madurez para realizar análisis bioenergéticos. Se extrajeron las gónadas de todas las hembras. Un ovario se fijó en formol tamponado al 4% para determinar la madurez microscópicamente y calcular la fecundidad y el otro ovario se congeló para la realización de los análisis bioquímicos, junto con una muestra suficiente de hígado y músculo.

Se calcularon tres índices generales de condición, el índice gonado-somático (GSI), el hepato-somático (HSI) y el factor de condición (K), cuyas fórmulas son:

$$GSI = \frac{gonadW}{guttedW} \cdot 100 \qquad HSI = \frac{liverW}{guttedW} \cdot 100 \qquad Fulton's K = \frac{guttedW}{length^3}$$

Basándose en los criterios histológicos los ovocitos fueron clasificados en diferentes estadios de desarrollo que en función de su aparición en la gónada y su proporción en la misma permitieron establecer una clasificación miscroscópica del estado de desarrollo del ovario. De este modo se distinguieron los siguientes (Figura 2):

- **Inmaduros** (**i**): Individuos juveniles que sólo presentan oogonias u ovocitos en crecimiento primario.
- **En maduración 1** (**m1**): Aparecen los primeros ovocitos en estadíos más o menos avanzados de desarrollo pero no hay signos de puestas anteriores (folículos post-ovulatorios).
- **En puesta** (**m2**): Igual al anterior pero se observan signos de puestas anteriores.
- **Final de la puesta** (**m3**): Aunque no se observan las estructuras propias de puestas anteriores aparecen ciertos rasgos que indican que el individuo se encuentra hacia el final del periodo de puesta (alta vascularización, engrosamiento de la pared ovárica, laxitud entre células, cierto grado de atresia, etc.)
- **En ovulación-hidratadas** (**h**): La mayor parte de los ovocitos de la gónada están hidratados, listos para ser ovulados en cualquier momento.
- **Maduros inactivos** (**im**): Se observan estructuras que indican que no va a tener lugar ninguna puesta (batch) más en la presente estación reproductiva (atresia masiva, alta vascularización, escaso número de ovocitos en desarrollo, etc.)
- **Recuperación** (**rc**): no aparecen ovocitos maduros, la pared del ovario es relativamente gruesa y la estrucutra del ovario no es tan compacta como en inmaduros. La vascularización puede ser más o menos abundante. Estas estructuras indican que la hembra a puesto en la estación reproductiva anterior y se está preparando para la siguiente.

Se describió el ciclo reproductivo de la merluza en aguas de Galicia y se estimaron las ojivas de maduración (proporción de hembras maduras para cada talla) que a su vez permitieron calcular la talla de primera maduración (L_{50}, talla a la que el 50% de las hembras de una población son sexualmente maduras) de la merluza en aguas de Galicia en el 2003 y 2004.

Por otro lado, se estimó la fracción de hembras en puesta (S; proporción de hembras que están ovulando respecto del total de hembras activas para un momento dado), así como la frecuencia de puesta (tiempo transcurrido entre batches teniendo en cuenta la fracción de

hembras en puesta de la población en un momento dado). La fracción de hembras en puesta se calcula a partir de la fórmula:

$$\bar{S} = \frac{\sum_{i=1}^{n} m_i * y_i}{\sum_{i=1}^{n} m_i}$$

Donde y_i es el porcentaje medio de hembras que presentan signos de ovulación inmediata o muy reciente en el área de muestreo; m_i nº de hembras sexualmente maduras muestreadas en dicha área de muestreo y n nº total de áreas de muestreo.

La frecuencia de puesta es el inverso de S.
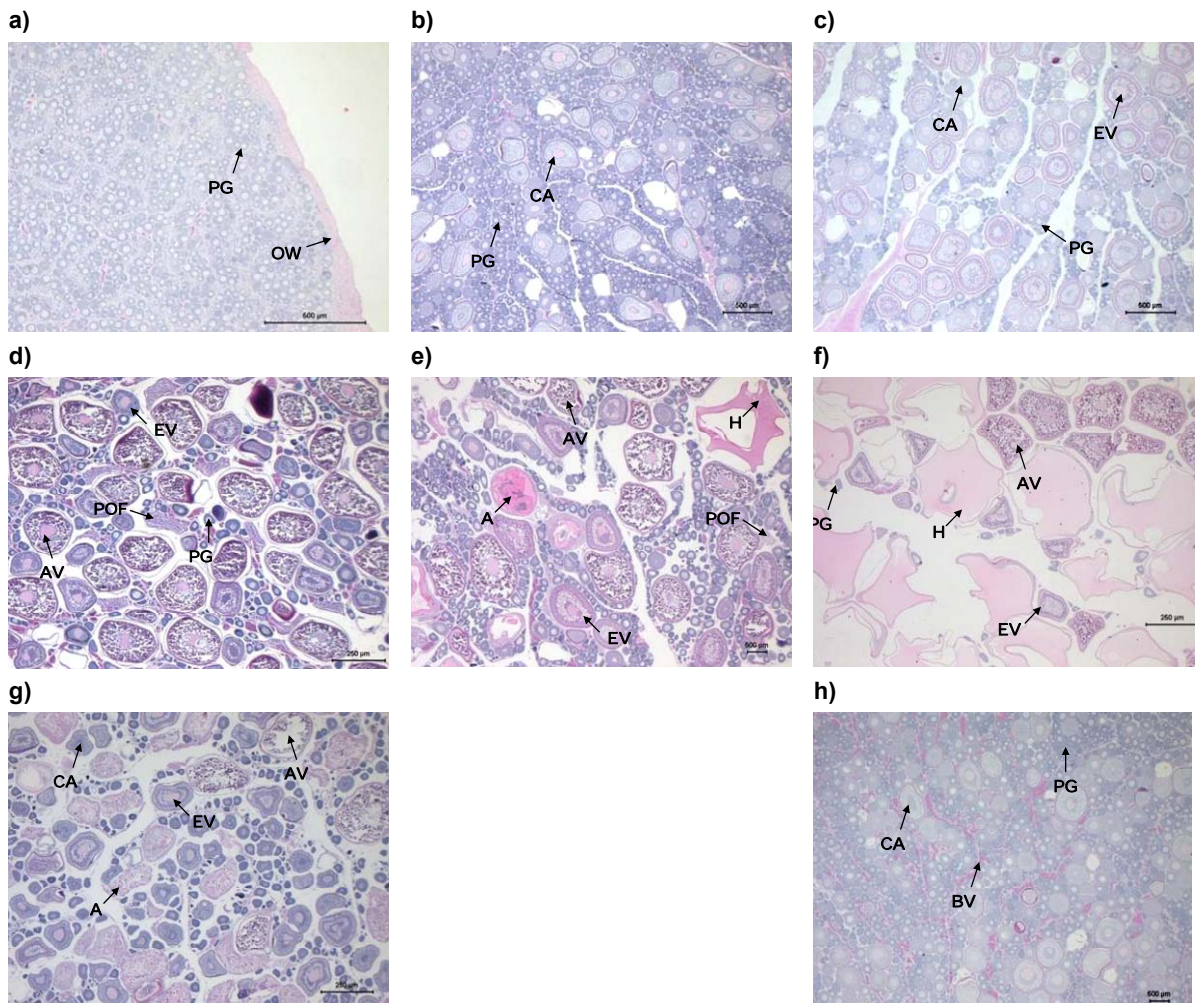
Figura 2: Estadios de desarrollo del ovario determinados histológicamente. a) ovario inmaduro (i; barra=500 μm); b) maduración temprana (m1, barra=500 μm); c) maduración (m1, barra=500 μm), d) ovario tras liberar un batch (m2, barra=250 μm), e) ovario al final de la puesta (m3, barra=500 μm), f) ovario hidratado/en ovulación (h, barra=250 μm), g) ovario maduro inactivo (im, barra=250 μm) and h) en recuperación (rc, barra=500 μm). PG=Ovocito een crecimeinto primario; OW=Pared del ovario; CA= ovocito en fase de alveolos corticales; EV=vitelogénesis temprana; AV=vitelogénesis avanzada; POF= Folículos post-ovulatorios; H= Ovocitos hidratados; A=Ovocitos atrésicos; BV=Vasos sanguíneos.

Del total de hembras muestreadas, 79 fueron seleccionadas para analizar bioquímicamente sus tejidos. Se cuantificó el contenido lipídico, proteico, de glucógeno, de materia inorgánica y de agua en gónada, hígado y músculo a partir de muestras previamente congeladas a -22ºC. La extracción lipídica se realizó conforme al método desarrolldo por Bligh and Dyer (1959). Para la cuantificación lipídica se utilizó el método gravimétrico de Herbes y Hallen (1983). Se tomaron 100 mg de tejido congelado para analizar el contenido de proteínas usando Seroalbúmina Bovina (BSA) con una concentración de 0.33 mg/ml como Standard (Lowry *et al*., 1951). El contenido de glucógeno fue determinado de acuerdo con el método desarrollado por Strickland and Parsons (1968). Para la cuantificación del contenido de humedad de los tejidos las muestras fueron secadas 24 horas a 100ºC y para la estimación del contenido de materia inorgánica (cenizas) se incineraron a 500ºC durante 12 horas. El contenido energético de cada muestra se calculó en base a las siguientes relaciones:

Lípidos → 39.5 kJ/g de peso seco
Proteínas → 23.6 kJ/g de peso seco
Glucógeno → 17.1 kJ/g de peso seco

Puesto que en peces de fecundidad indeterminada no es posible estimar la fecundidad potencial, puesto que incorporan nuevos ovocitos al pool de ovocitos en desarrollo durante la estación reproductiva, se creó un nuevo término NDO (Nº de ovocitos en desarrollo) que es el nº de ovocitos en proceso de maduración más o menos avanzada que se encuentran en las gónadas de una hembra en un momento determinado de la estación de puesta. También se estimó la fecundidad parcial que es el nº de ovocitos que van a ser liberados en cada batch y se estima a partir del número de ovocitos hidratados presentes en la gónada. Para estimar el NDO se emplearon dos métodos, el método gravimétrico combinado con análisis de imagen y el método estereológico. En el caso de la fecundidad parcial, además de estos dos métodos se aplicó también el conteo manual.

En el primer método se toma una muestra de ovario de peso conocido y tras la eliminación del tejido conectivo mediante lavado con agua a presión y filtrado, los ovocitos son contados y medidos por un analizador de imagen digital, el nº de ovocitos contados en la muestra es extrapolado al peso total de la gónada. El método esterológico conlleva una serie de cálculos matemáticos más o menos complejos que relacionan parámetros tridimensionales que definen una estructura con medidas bidimensionales obtenidas de una sección de dicha estructura, es decir, es la interpretación tridimensional de la sección bidimensional de una estructura (Weibel, 1979). La fórmula desarrollada por Weibel and Gómez (1962) para partículas de perfil constante es:

$$N_V = \frac{1}{\beta} \cdot \frac{N_A^{3/2}}{V_V^{1/2}}$$

Donde N_A es el nº de partículas por unidad de área en cada sección que depende del nº de partículas por unidad de volumen de la estructura (N_V), V_V es el volumen ocupado por las partículas en el volumen total de la estructura y β es un coeficiente que viene dado por la relación no dimensional entre el volumen de la partícula y el área media de la sección.

Finalmente, el tercer método aplicado en la estimación de la fecundidad parcial fue el conteo manual. Para ello, se toma una muestra de peso conocido de ovocitos de un ovario con signos de ovulación inmediata, se le aplican unas gotas de glicerina de forma que los ovocitos que van a ser ovulados, y que se encuentran hidratados, se vuelven transparentes, permitiendo su conteo a través de la lupa. El nº de ovocitos hidratados contenido en la muestra es extrapolado al peso total de la gónada.

Debido a la enorme asincronía poblacional del ciclo de puesta de la merluza no es posible conocer la duración de la estación reproductiva individual por lo que, en comparación con otros gádidos, se asume una puesta individual de 2 meses. La producción de huevos relativa de la población (nº de huevos producidos por unidad de peso de hembras maduras activas, EP) puede ser calculada como el producto del nº medio de puestas (batches) por mes (NB), el nº relativo de huevos producidos en cada batch (fecundidad parcial relativa, BFrel) y el porcentaje de hembras activas de la población (AF) en cada mes:

$EP = NB \cdot BF\ rel \cdot AF$

El nº medio de batches por mes es calculado según la siguiente fórmula:

$NB = S^{-1} \cdot nº\ days\ of\ each\ month$

Donde S es la fracción de hembras en puesta estimada como se explicó anteriormente.

En los peces existe un mecanismo de regulación de la fecundidad conocido como atresia que consiste en la reabsorción de las reservas energéticas depositadas en los ovocitos en desarrollo que finalmente no van a ser puestos. Las características del mecanismo de atresia pueden variar entre especies. En algunas especies la atresia se produce al final del ciclo reproductivo para recuperar la energía que se encuentra en los ovocitos remanentes que ya no van a ser liberados o cuando la condición de la hembra no le permite continuar con la puesta, de manera que en la naturaleza pueden observarse atresias masivas que suponen un aborto de la estación reproductiva de ese año. También pueden reabsorberse pequeñas proporciones de ovocitos en desarrollo a lo largo de la estación reproductiva como sistema de regulación inmediata de la fecundidad. La atresia en merluza se estimó según el método estereológico explicado anteriormente.

Más adelante, dada la fuerte asincronía no sólo poblacional sino también individual del fenómeno reproductivo de la merluza y la dificultad que de esto deriva para establecer una clasificación de los estados de madurez robusta, se aplicaron una serie de técnicas estadísticas para tratar de delimitar los diferentes estadios de desarrollo de la gónada con más exactitud. Por un lado, se analizó la variabilidad de la composición bioquímica de los tejidos a lo largo de la estación reproductiva empleando el análisis factorial para crear nuevas variables que nos permitieran identificar grupos de madurez entre las hembras. Por otro lado, se analizó estadísticamente en profundidad la distribución de tallas de los ovocitos presentes en un ovario y se combinaron los diferentes descriptivos de la distribución (Moda, Media, Máx, Mín, Cuartiles, etc.) con la intención de encontrar algún patrón que permitiera diferenciar entre los distintos grupos de madurez.

Finalmente, para medir el potencial reproductivo de la merluza en aguas de Galicia se analizó la relación del NDO y la fecundidad parcial con los atributos maternales, la condición de las hembras y sus reservas energéticas mediante regresiones múltiples.

RESULTADOS Y DISCUSIÓN

CICLO REPRODUCTIVO

Los resultados de este estudio indican que la merluza del stock sur muestra un desarrollo asincrónico del ovario, fecundidad indeterminada y que es un ponedor parcial (batch spawner), confirmando lo ya encontrado por otros autores en diferentes poblaciones de la misma especie (Murua *et al*., 1996; Murua and Motos, 2006). En las gónadas de merluza se encuentran ovocitos cercanos a la ovulación, así como signos de puesta reciente (folículos post-ovulatorios) durante todo el año. La prevalencia de estos estadios indica que la merluza en Galicia presenta una estación reproductiva prolongada a nivel poblacional que puede extenderse a lo largo de todo el año. Sin embargo, la duración de la puesta individual sigue siendo desconocida y no ha sido posible estimarla con exactitud en este trabajo, en parte debido a la larga duración de la estación reproductiva de la población (asincronía poblacional). Este fenómeno ha sido también observado en otras poblaciones de la misma especie, por ejemplo en el Golfo de Vizcaya (Lucio *et al*., 1998) y en el Mar Adriático (Ungaro *et al*., 2001). Garvey *et al*. (2002) sugiere que una estación reproductiva prolongada a nivel poblacional en condiciones ambientales variables puede incrementar las expectativas de supervivencia de la descendencia e incrementar su nivel de reclutamiento a la población de adultos ya que aumenta las posibilidades de que algunas de las larvas coincidan con condiciones ambientales favorables y cantidad de alimento suficiente. En el caso de la costa gallega, la supervivencia de huevos y larvas de la merluza dependen enormemente de los episodios de upwelling que ocurren esporádicamente durante un periodo del año más o menos largo (Wooster *et al*., 1976; Fraga, 1981 and Castro *et al*., 1994).

De acuerdo con las ojivas macroscópicas (*de visu*), las hembras de merluza maduran mayoritariamente entre los 40 cm (21.9% de maduras) y los 50 cm (84.6% de maduras), aunque se encontraron hembras maduras de menor tamaño (39 y 37.1 cm en 2003 y 2004 respec.). Por el contrario, las hembras inmaduras más grandes registradas presentaban una talla de 60.5 cm en 2003 y 54 cm en 2004, aunque realmente podría ser que estas hembras se encontraran en el estado de recuperación que macroscópicamente puede ser confundido con una gónada inmadura, aunque no es común. Probablemente es esta posible confusión la que dio lugar a las diferencias significativas existentes entre las ojivas macroscópicas y las microscópicas. En 2003 la talla de maduración estimada macroscópicamente fue significativamente más grande que la estimada microscópicamente. Sin embargo, ambas curvas eran básicamente paralelas, indicando la existencia de un error sistemático en la asignación del estado de madurez en todas las tallas analizadas, probablemente debido a diferencias en la metodología de muestreo (época del año en que se muestrea, tipo de arte empleado, etc.). Por el contrario, la principal diferencia hallada en 2004 fue el cambio de la pendiente de la curva macroscópica, que no era paralela a la estimada microscópicamente,

ni a ninguna de las dos curvas del año anterior, lo que sugiere un error en la clasificación de los estadios de madurez probablemente provocado por la confusión entre hembras inmaduras y en recupueración. En cuanto a la talla de primera maduración (L_{50}) se observaron diferencias significativas entre años independientemente del método empleado para su cálculo. En 2003 la L_{50} microscópicamente estimada fue de 44.45 cm mientras que en 2004 fue 42.97 cm. Una disminución similar fue observada en la L_{50} macroscópicamente calculada decreciendo de 47.05 cm en 2003 a 45.73 cm en 2004).

La fracción de hembras en puesta (S) en los años 1999, 2000, 2003 y 2004 fue mayor entre Enero y Marzo con un pequeño pico secundario que se observó especialmente en Junio-Julio en algunos años. Más de un pico de puesta ha sido observado también por otros autores en las poblaciones del Mediterráneo, por ejemplo, Biagi *et al*. (1995) detectaron un pico de actividad reproductiva en Febrero-Marzo y otra en Septiembre en el Mar Tirreno, mientras que Orsi-Relini *et al*. (2002) sugiere la existencia de tres picos de puesta en la merluza de la costa tunecina, uno en verano, otro en invierno y otro en primavera. La existencia de dos o más picos de puesta podría ser explicada por la presencia de diferentes cohortes (clases de edad) más o menos abundantes que llegan al área de puesta en distintos momentos del año, aunque tendrían que hacerse más estudios para corroborar esta hipótesis. Algunos autores sugieren que podría deberse a diferencias en la duración de la puesta de hembras grandes y pequeñas (Alarcón *et al*., 2004) lo cual parece una explicación más plausible.

En este trabajo se eligió una duración individual de la puesta para la merluza del stock sur de dos meses como promedio de los resultados de otros estudios basándose en tres razones: i) no se conoce exactamente la duración de la estación de puesta individual de la merluza, aunque algunos autores han asumido que es tres meses (Sarano, 1984; Murua *et al*., 1996); ii) el merlán, otro Gádido, muestra una estación reproductiva de tres meses en cautividad que podría ser más corta en especímenes salvajes como sugiere Kjesbu (1989) y iii) la duración de la puesta del bacalao ha sido empíricamente registrada entorno a un mes. Así, una duración de puesta de dos meses es una aproximación en base a los estudios en otros Gádidos. En cualquier caso, son necesarios más estudios para estimar con precisión la duración de la puesta individual de la merluza, aunque no es una tarea fácil en esta especie. Hasta ahora, se han hecho algunos intentos para mantener especímenes en cautividad sin éxito en la reproducción porque las especies de este género son muy sensibles a la manipulación (Bjelland and Skiftesvik, 2006). Por otro lado, algunos experimentos de captura – recaptura se han llevado a cabo pero no se ha obtenido información de buena calidad debido a la fragilidad de estas especies (Lucio *et al*., 2000; de Pontual *et al*., 2003). Es necesario mejorar la metodología en el cultivo de merluza para alcanzar un mejor conocimiento de su comportamiento reproductivo. Además, es también necesario investigar los efectos de los factores ambientales, edad, talla y condición en el

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momento y duración de la puesta como cambios en la actividad reproductora que pueden afectar al stock de reproductores y su potencial.

En la costa de Galicia los valores más altos de S para la merluza están en torno a 0.2 durante el pico de puesta, aunque en 1999, al contrario que en otros años, el pico de verano de S fue mayor que en Febrero. Basándose en estos resultados, la frecuencia de puesta poblacional (PBF) fue estimada entre 5 y 7 días durante el pico de puesta aumentando a 10 días o más al disminuir la actividad reproductiva en los siguientes meses. Estos valores son similares a los observados por otros autores en el Golfo de Vizcaya (Murua *et al*., 1996; Murua and Motos, 2006) y en Merluza Argentina (Macchi *et al*., 2004).

COMPOSICIÓN BIOQUÍMICA Y RESERVAS ENERGÉTICAS

En el presente estudio el contenido lipídico de la merluza se estimó entre un 0.5 y un 1.5% del peso húmedo para el músculo, entre 8.5 y 56% para el hígado, y entre un 2 y un 13% para la gónada. Se observaron algunas diferencias con respecto a otros stocks de merluza que podrían deberse a diferencias ambientales, por ejemplo, se ha demostrado que la temperatura afecta al contenido de lípidos y la composición de ácidos grasos en peces (Greene and Selinovchick, 1987).

Las proteínas son el componente principal del tejido muscular y no sólo son la principal fuente de energía del metabolismo activo de los peces, sino que también el crecimiento de la gónada se produce a expensas de las proteínas corporales (Love, 1980; Tyler and Colow, 1985; Black and Love, 1986). El contenido de proteínas de la merluza de la costa gallega se estimó entre el 3 y el 25% para la gónada, entre 1.8 y 15% para el hígado y entre el 7.5 y el 20% para el músculo. Los valores del músculo son ligeramente inferiores a los reportados para merluza en aguas del sur de España (Soriguer *et al*., 1997), pero mayores que los estimados en aguas de Escocia (Pérez-Villareal and Howgate, 1987).

Tradicionalmente, el glucógeno ha sido considerado un componente bioquímico insignificante en peces a pesar de su indisputable importancia en otras especies marinas como los moluscos que usan el glucógeno como fuente principal de energía en ausencia de oxígeno (Wilbur and Hochachka, 1983; de Zwaan and Mathieu, 1992), consecuentemente no hay muchos datos disponibles en peces. Sin embargo, Love en 1970 ya describió el importante papel del glucógeno no sólo en la reproducción sino también en los procesos metabólicos en general y principalmente en situaciones de estrés. El glucógeno del hígado y del músculo es la primera reserva energética usada por los peces, antes incluso que los lípidos, lo que sugiere que podría ser un sustrato energético inmediato fácilmente movilizable (Chellappa *et al*., 1989).

En este estudio el glucógeno varió en los tres tejidos analizados aunque ni en gónada ni en músculo las diferencias fueros tan marcadas como en hígado donde su contenido varió entre el 2 y el 10%.

El contenido de agua normalmente siguió el patrón opuesto al de los componentes orgánicos porque la proporción de agua en los tejidos crece cuando su energía va siendo consumida, por lo que se le considera un buen índice de la condición del individuo (Lambert and Dutil, 2000; Dutil *et al*., 2003b). En este estudio, el contenido de agua de la gónada estuvo entre 61 y 85%, en el hígado entre el 10 y el 60% y en el músculo entre 79 y 85%, lo que concuerda con lo estimado para el músculo en aguas de Escocia (Pérez-Villareal and Howgate, 1987).

Toda la variabilidad de la composición bioquímica puede ser resumida en la variación de la energía contenida en los tejidos que en ste estudio varió entre 24 y 21.5 kJ/g en gónada, entre 27 y 38 kJ/g en hígado y entre 17 y 24 kJ/g en músculo, valores similares a los observados en bacalao (Lambert and Dutil, 1997a). Los valores más altos de energía en la gónada de merluza fueros registrados en las hembras en ovulación porque justo antes de liberar los huevos éstos contienen la máxima cantidad de vitelo. Sin embargo, la densidad energética (kJ/g) de los tejidos no se relacionó significativamente ni con los atributos maternos ni con la condición de la hembra, excepto en el músculo cuya energía se relacionó negativamente con la talla y el peso de la hembra, aunque el poder predictivo de esta regresión fue muy bajo. Otros estudios revelan resultados similares para distintas especies del Mediterráneo (Somarakis *et al*., 2004; Lloret *et al*., 2005). Estos resultados sugieren que la composición relativa de la merluza es independiente del tamaño.

Los cambios en la composición bioquímica y en la condición no sólo se deben al gasto de energía durante la reproducción sino que también pueden ser una consecuencia de la falta de alimento o de un balance negativo entre la ingesta y el gasto de energía (Black and Love, 1986; Lambert *et al*., 2003; Weber *et al*., 2003; Eckmann, 2004), aún más, los factores ambientales pueden también afectar a la condición y provocar diferencias temporales como se ha visto en el bacalao (Krohn *et al*., 1997; Lambert and Dutil; 1997b; Lloret and Rätz, 2000), el lenguado (Buckley *et al*., 1990), en *Mysis mixta* (Richoux *et al*. 2004), en especies Mediterráneas (Lloret *et al*., 2005) o en especies de agua dulce de zonas templadas como la trucha (Berg *et al*., 2000). Estos cambios en la condición pueden provocar variaciones en el éxito reproductivo de la especie.

La falta de relación entre la energía del hígado y del músculo y el ciclo reproductivo pueden ser consecuencia de que estos tejidos actúan como reservas por cortos periodos de tiempo contribuyendo a la maduración de la gónada, aunque no son críticos para la misma. En otras palabras, el hecho de que la energía permanezca constante a lo largo de la estación reproductiva apoya la idea de que la merluza no deja de alimentarse durante la

reproducción por lo que la producción de huevos no depende completamente de las reservas energéticas. En general, los peces inmaduros dedican su energía a supervivencia y crecimiento, pero una vez que maduran, parte de esa energía es usada para la producción de gametos y el cortejo. Por tanto, debe existir un equilibrio en el balance de distribución de energía que afecta a la dinámica reproductiva y de crecimiento (Iles, 1984; Saborido-Rey and Kjesbu, in press).

FECUNDIDAD Y PRODUCCIÓN DE HUEVOS

En este estudio se observó que la fecundidad parcial (BF) estaba comprendida entre 18,710 y 577,302 huevos hidratados (media=182,537 huevos hidratados, con una BF relativa entre 140 y 170huevos/g de hembra). Estos valores son ligeramente superiores a los observados en el Golfo de Vizcaya variando la magnitud de las diferencias en función del año considerado (Murua *et al.*, 1996; 1998). En otras especies se han observado diferencias de la BF entre años causadas por diferentes condiciones nutricionales de la hembra, ambientales o una combinación de ambas (Kjesbu *et al.*, 1998; Kraus *et al.*, 2000; Wang and Houde, 1994; Milton *et al.*, 1994; Somarakis *et al.*, 2005). En este estudio la BF no parece tener relación con la condición de la hembra, por lo que los cambios interanuales podrían estar relacionados con la disponibillidad de alimento, las condiciones ambientales, factores de estrés, diferencias entre cohortes o en la estructura del stock (Wootton, 1990)

En aguas de Galicia el número de ovocitos en desarrollo (NDO) fluctúa entre 311 y 3,366,352 (media=748,907ovocitos). Valores similares fueron encontrados previamente tanto en Galicia como en el Cantábrico (Pérez and Pereiro, 1985); en el Golfo de Vizcaya (Sarano, 1984) y en el mar Tirreno (Biagi, 1995). En aguas griegas el NDO es bastante inferior al observado en Galicia (Tsimendis, 1985), aunque en su cálculo sólo se contaron los ovocitos mayores de 500 micras, mientras que en este estudio se han tenido en cuenta todos los ovcitos mayores de 150 micras que según estudios histológicos realizados en la presente tesis, es el umbral menor de tamaño de los ovocitos en la primera fase de maduración del ovocito. Las diferencias en el NDO entre areas pueden deberse también a diferencias en el momento del muestreo (más o menos cerca del pico de puesta). Los resultados indican que el NDO se relaciona significativamente con la condición de la hembra en merluza de la costa gallega.

Al revisar la literatura, en la mayoría de los casos, los autores no diferencian entre NDO y fecundidad potencial lo cual es completamente erróneo. Algunas especies son determinadas *sensu stricto* y es posible conocer exactamente cuantos huevos va a poner muchos meses antes de que comience la estación reproductiva mediante conteo de los ovocitos en desarrollo y corrección de las pérdidas por atresia; en estas especies, como en el bacalao, el NDO y la fecundidad potencial son iguales. En otras especies, como la

merluza, es imposible conseguir esta información porque el reclutamiento de ovocitos continúa después de comenzar la puesta, por tanto, sólo se puede calcular el NDO que es menor que la fecundidad potencial. Sin embargo, en algunas especies con desarrollo asincrónico de los ovocitos pero fecundidad determinada, el numero exacto de huevos que van a ser liberados puede ser calculado justo antes del comienzo de estación reproductiva, pero no previamente. Una revisión de las estrategias reproductivas de especies del Atlántico se encuentra en Murua and Saborido-Rey (2003). Realmente el término de determinado e indeterminado son conceptos que se refieren más a la capacidad de los investigadores para saber exactamente el número de huevos que van a ser puestos por una sóla hembra que a la estrategia reproductiva real de los peces. Las hembras siempre pueden modular su propia producción de huevos en función de una serie de factores a través de diferentes mecanismos, como la atresia, o reclutando más o menos ovocitos. En el caso de que la fecundidad potencial no pueda ser estimada, se recomienda emplear el concepto de NDO.

La Producción Anual de Huevos (AEP) basada en las estimaciones de producción diarias de huevos varía en función del periodo de puesta considerado. Los mayores valores de AEP, asumiendo una puesta individual de dos meses se producen en invierno y principios de la primavera (enero-marzo) y los más bajos a final de verano y otoño (septiembre-noviembre). En función de esto, el AEP varía entre 1947 y 4366huevos/g de hembra al principio del año, entre 366 y 2806huevos/g de hembra en junio-julio y finalmente, entre 549 y 793huevos/g de hembra durante el último trimestre del año. Estos valores son más altos que los registrados en el Golfo de Vizcaya entre enero y marzo (Murua *et al*. 2006), sin embargo, son similares a los estimados por estos autores en el periodo abril-octubre.

El impacto que el número de hembras inactivas o en condición de atresia podría tener en la producción diaria de huevos relativa de la población no ha sido considerado en este estudio. Sin embargo, es conocido por la literatura que la proporción de hembras inactivas o con atresia masiva puede suponer una fracción importante del stock de reproductores (Rideout *et al*., 2000; Rideout *et al*., 2005; Engelhard and Heino, 2006; Jørgensen *et al*., 2006; Murua and Motos, 2006; Rideout and Rose, 2006; Rideout *et al*., 2006).

NIVELES DE ATRESIA

Los resultados de este estudio muestran que la proporción de hembras de merluza con atresia masiva no fue muy alta generalmente (en torno a 10%). La atresia masiva en la merluza de la costa gallega se observa principalmente durante el último trimestre del año y puede asociarse con el cese de la actividad reproductora (post puesta). Este fenómeno a sido observado por otros autores (Sarano, 1984; Murua and Motos, 2006). Fedorov (1971)

observó una atresia masiva relativamente frecuente en las hembras maduras de fletán de Groenlandia como consecuencia de las condiciones ambientales, al igual que Oven (2004) que observó que los niveles de atresia de los peces del Mar Negro eran mayores durante periodos de fuertes perturbaciones antrópicas. En otras especies la intensidad de atresia parece relacionarse más con la condición de la hembra (Kjesbu, 1991; Kurita *et al*., 2003), que en ocasiones debe realizar grandes esfuerzos reproductivos, como migraciones a las áreas de puesta, lo que le supone un gasto energético excesivo que podría traducirse en un salto o aborto de la puesta (Skip spawning; Privalikhin, 2003), reabsorbiendo los ovocitos mediante atresia (Fedorov, 1971; Silverstein *et al*., 1998; Bromley *et al*., 2000; Óskarsson *et al*., 2002; Morgan and Lilly, 2004).

Millner *et al*. (1991) explican este fenómeno de salto de la puesta en especies de vida larga como el lenguado, sugiriendo que el objetivo de estas especies es conseguir suficiente comida para crecer y mejorar su condición en lugar de incrementar su potencial reproductivo. El incremento del crecimiento indirectamente conlleva una mayor producción de huevos. Esta estrategia maximiza el esfuerzo reproductivo, siendo más importante para especies de vida larga reproducirse regularmente a lo largo de su vida, en diversas ocasiones que invertir grandes cantidades de energía en la reproducción a expensas del crecimiento o la condición. Jørgensen *et al.* (2006) se aproximan al fenómeno de salto de la puesta desde dos puntos de vista: ecológico (el salto de la puesta es debido a la plasticidad fenotípica de las estrategias de vida de los peces como respuesta a factores fisiológicos y ecológicos) y evolutivo (cómo pueden los cambios modificar la estrategia vital de los peces y qué consecuencias tiene ésto en el "plan" reproductivo de la población).

En especies de fecundidad indeterminada, como la merluza europea, el nº de ovocitos a desarrollar se acomoda a la cantidad de energía disponible en el medio, por lo que las hembras producirán más o menos huevos en función de su capacidad para incorporar energía progresivamente al proceso reproductivo.

ESTADIOS DE DESARROLLO DEL OVARIO

Se ha mencionado varias veces que la alta asincronía de la actividad reproductiva observada en merluzas no permite estudiar su reproducción usando los protocolos tradicionales. La mayoría de estadios de desarrollo gonadal son fácilmente identificables, pero es prácticamente imposible diferenciar entre las hembras maduras en pre-puesta (no han liberado aun su primer batch) y las hembras en puesta (ya han liberado uno o más batches).

La clasificación macroscópica de los estadios de desarrollo del ovario es un método rápido y común, pero es limitado porque proporciona escasa información, lo que puede

inducir a errores (West, 1990; Kjesbu, 1994; Domínguez *et al*., 2005). La utilidad de la clasificación macroscópica depende de la necesidad de información del investigador y de la estrategia reproductiva de la especie, pero en cualquier caso, se recomienda calibrar los resultados con análisis microscópicos. En el presente trabajo se trató de resolver este handicap usando dos aproximaciones: la variación de la condición/composición bioquímica a lo largo de la puesta y el análisis de la distribución de tallas de los ovocitos. El primero ofrece más información sobre la dinámica energética de la reproducción en peces, pero debido a la gran variabilidad entre hembras pertenecientes al mismo estadio de madurez microscópico, los resultados son bastante inexactos.

El análisis de la distribución de tallas de los ovocitos combinando moda y media, parece ser un método aceptable, es relativamente rápido ya que la información se obtiene automáticamente y de forma rutinaria cuando se estima la fecundidad con análisis de imagen, podría ser aplicada a otras especies de fecundidad indeterminada y no se requiere una experiencia previa especial en reproducción de peces. En cualquier caso, son necesarios más estudios enfocados a las hembras con folículos post-ovulatorios ya que en la presente tesis no se han incluido en los análisis de fecundidad.

POTENCIAL REPRODUCTIVO DE LA MERLUZA

El potencial reproductivo debe ser determinado en cada stock de forma regular debido a diferentes razones. Primero, porque depende de factores poblacionales como el pool genético, talla/edad de maduación o estructura talla-edad de la población que se sabe cambia con el tiempo. Segundo, porque el potencial reproductivo de un stock depende también de las características individuales como la composición bioquímica, condición, incidencia de atresia o fecundidad que también fluctúan. No todas las hembras contribuyen en igual medida a la producción de descendencia, las hembras más grandes producen huevos y larvas más grandes y más competitivos que tienen más probabilidades de sobrevivir (O´Brien, 2003). El potencial reproductivo del stock no siempre está exclusivamente relacionado con la condición de la hembra. A veces, la variabilidad en la producción de huevos y larvas o incluso cambios en las características genotípicas que determinan dicha producción, son debidos a largos periodos de condiciones ambientales desfavorables o a altas tasas de mortalidad selectiva, por ejemplo, por pesca (Moneda and Wright, 2004; Morgan and Brattey, 2005). Sin embargo, no todas las especies ni poblaciones se ven afectadas en igual medida por la presión del medio externo, algunos stocks muestran una sorprendente resiliencia a los cambios del ecosistema (Helser and Brodziak, 1998; Junquera *et al*., 1999). Debido a estas particularidades, es necesario un estudio individualizado del potencial reproductivo, no sólo a nivel específico, sino también poblacional.

El nº de ovocitos en desarrollo (NDO) está influenciado por la condición bioquímica de la hembra, incrementando al aumentar la energía de la misma. Las hembras con mayor energía son capaces de movilizar mayor nº de ovocitos en un periodo más corto, aumentando la probabilidad de producir mejores batches en mejores condiciones. Por otro lado, tanto el NDO como la fecundidad parcial (BF) incrementan con la talla y peso de la hembra. Sin embargo, el proceso de hidratación tiene requerimientos energéticos bajos, especialmente comparado con la vitelogénesis. Esto sugiere que, probablemente, el NDO movilizado en cada periodo depende de la las reservas energéticas a corto plazo, pero el número de ovocitos liberados en cada batch (BF) depende más del tamaño de la hembra. En otras palabras, en algunas especies como el bacalao, las hembras almacenan energía durante la estación de alimentación que suele tener lugar bastante antes de la estación reproductiva, periodo durante el cual prácticamente no se alimenta, dependiendo el desarrollo de los ovocitos de las reservas acumuladas. Por el contrario, la merluza continúa alimentándose durante la estación reproductiva, acumulando reservas energéticas a corto plazo que determinarán el NDO que se movilizará periódicamente. Asumiendo esta hipótesis, las hembras más grandes liberarán valores similares de NDO más rápido que las pequeñas, lo que les permite reclutar más ovocitos al NDO, lo que a su vez resulta en una mayor producción total de huevos, aunque siempre modulado por la captura de energía externa (alimento). También es posible que la tasa de desarrollo de los ovocitos dependa del la talla y peso de la madre o de las reservas energéticas a corto plazo, pero son necesarios más estudios para esclarecer la relación existente entre los atributos y la condición de la hembra, la dinámica de desarrollo de los ovocitos y la producción de huevos de esta especie.

En general, este trabajo corrobora la importancia de la composición bioquímica como reserva energética a corto plazo en la producción de huevos de la merluza europea en aguas de Galicia, que es diferente a la observada en especies de aguas más frías. Sin embargo, como el desarrollo de los ovocitos en la merluza sigue un patrón asincrónico, la clasificación exacta de los estadios de maduración de la gónada es un gran handicap a superar. Más estudios para relacionar la intensidad de atresia con la condición de las hembras, cubriendo periodos de tiempo y áreas de muestreo más amplias deberían ser llevados a cabo. También es necesario estudiar cómo afectan los factores externos y la presión pesquera al potencial reproductivo de la merluza europea, así como más análisis de la estructura poblacional, de la dinámica anual de los factores de condición y una exploración de las relaciones funcionales entre las características maternales y la producción de huevos, ya que permitiría evaluar con mayor exactitud las tasas de supervivencia de los descendientes y los niveles de reclutamiento de los mismos a la población adulta.

CONCLUSIONES

1. La merluza europea es un ponedor parcial con fecundidad indeterminada. Esta especie presenta una modalidad reproductiva altamente asincrónica tanto a nivel poblacional (patrón de puesta) como individual (desarrollo ovocítico).

2. Presenta una estación reproductiva prolongada que abarca todo el año, pero básicamente desde Enero a Julio con un pico principal de puesta entre Enero y Marzo y, eventualmente, un pico secundario en Junio-Julio dependiendo del año.

3. La fecundidad potencial no puede ser estimada en especies indeterminadas por lo que la única estimación posible es la del nº de ovocitos en desarrollo (NDO).

4. El NDO fluctúa entre 78,873 y 3,366,352 ovocitos por hembra con un valor medio 721,821 ovocitos. La fecundidad parcial (BF) varía entre 470 y 835,230 huevos hidratados con un valor medio de 124,663 ovocitos. Para una hembra promedio de 60 cm el NDO es igual a 881,000 ovocitos y la BF es igual a 243,000 huevos por batch.

5. La fracción de hembras en puesta en el periodo 1999-2000 y 2003-2004 estuvo entre 4% al comienzo y final de la estación de puesta y 25% durante el pico de actividad reproductora. La frecuencia de puesta poblacional (PBF) se estimó entre 3 y 10 días.

6. La producción diaria y anual de huevos (DEP y AEP respec.) varía a lo largo de la estación de puesta. Para todo el año, la DEP está comprendida entre 4.2 y 91 huevos/g de hembra y la AEP entre 366 y 4,366 huevos/g de hembra, lo que resulta en una producción real de huevos para una hembra prototipo de 1kg de 549,000 a 4,366,000 huevos.

7. La talla de primera maduración sexual de las hembras fue de 44.45 cm en 2003 y 42.97 cm en 2004.

8. No se observaron patrones temporales de variación en las reservas energéticas. Probablemente porque la producción de huevos en la merluza depende de las condiciones ambientales y la disponibilidad de alimento

durante la época de puesta, aunque la asincronía poblacional también podría estar enmascarando los patrones de variación de las reservas energéticas.

9. El desarrollo de la gónada no depende de las reservas acumuladas en hígado y músculo, aunque estos órganos pueden actuar como compartimentos intermediarios durante la traslocación de energía, especialmente el hígado.

10. La talla y el peso de la hembra influye tanto al NDO como a la BF, mientras que las reservas energéticas a largo plazo y los factores de condición tienen menor influencia. El NDO aparentemente depende más de las reservas a corto plazo, es decir, la asimilación de energía durante el periodo de reclutamiento de ovocitos.

11. Hipotéticamente, las hembras más grandes tienen una mayor fecundidad, movilizando más ovocitos a lo largo de la estación reproductiva, aunque esta movilización es modulada por su capacidad para capturar energía externa.

12. La pérdida de ovocitos por atresia abarca desde 1.59 a 84.92%, aunque la intensidad media de atresia es 6% y no se relaciona con ninguno de los atributos materno. La incidencia de atresia observada es menor durante el período reproductivo, aumentando durante la etapa de post-puesta.

13. La composición bioquímica podría ser un buen índice del estadio de desarrollo del ovario, aunque es necesario establecer criterios más precisos para diferenciarlos.

14. El análisis de imagen es una ténica rápida, automática y precisa para estimar el NDO, la BF y la distribución de tallas de los ovocitos y podría ser aplicada directamente sobre cortes histológicos en un futuro próximo.

15. Es importante incluir información sobre la composición del stock y tener en cuenta las características de talla y condición de los reproductores en la evaluación y gestión de los stocks de merluza europea.

16. Son necesarios más análisis de la composición bioquímica de la merluza y su relación con la atresia y la fecundidad para predecir con mayor exactitud la supervivencia de la descendencia y los niveles de reclutamiento, así como estudios de la influencia de los factores ambientales y la estructura de la población en ellos.