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Ecology of the early life-history stages of shad *Alosa fallax fallax* (Lacépède, 1803) in the River Mira, with a note on *Alosa* spp. larvae in the River Guadiana

(Tese para a obtenção do grau de Doutor no ramo de Ecologia,
especialidade de Ecologia das Populações)

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Resumo

O Sável e a Savelha *Alosa* spp. são espécies migradoras anádromas, classificadas pelo ICN como "Em Perigo" ou "Vulnerável" (de acordo com critérios do IUCN), que ainda desovam em vários rios Portugueses. No âmbito dum projecto nacional de investigação, apresenta-se nesta dissertação o trabalho desenvolvido, entre Fevereiro de 1998 e Julho de 2000, com o intuito de estudar a biologia e ecologia das fases larvares da Savelha, *Alosa fallax fallax*, no Rio Mira (com uma nota final sobre *Alosa* spp. no Rio Guadiana).

A abundância de larvas de Savelha à superfície foi superior durante o dia. Pelo contrário, não se observaram ritmos circadianos no caso dos embriões (*i.e.* larvas com saco vitelino). As fases larvares de Savelha ocorreram, entre finais de Março e meados de Junho, nas estações localizadas a montante no Rio Mira. As características hidrológicas determinam a distribuição dos ovos e dos embriões. Pelo contrário, as abundâncias de larvas relacionam-se com várias variáveis ambientais (através de um modelo aditivo generalizado, GAM, não-linear).

Observaram-se pares de anéis, translúcidos e opacos, regularmente dispostos nos *sagittae* de *A. fallax fallax*. A elevada percentagem de *sagittae* utilizados, sugere a utilidade da análise micro-estrutural dos otólitos para estimar a idade e estudar o crescimento e a mortalidade larvar. As taxas de crescimento e de mortalidade das larvas variaram sazonalmente (0,36 a 0,60 mm d^{-1} e entre 16 e 30 % d^{-1}, respectivamente).

Num ano-tipo, as larvas encontram-se em melhores condições nutricionais (razão RNA/DNA) durante dois periodos de tempo relativamente curtos, em meados de Abril e em meados de Maio, quando as temperaturas são superiores a 22 ºC e as presas não ultrapassam 80 *nauplii* 100 m^{-3}. Pelo contrário, a razão RNA/DNA diminui significativamente sempre que a turbidez é superior a 2 mg DW m^{-3} e a abundância de presas potenciais >20 *nauplii* 100 m^{-3}.

O número reduzido de ocorrências de larvas de *Alosa* spp. no Rio Guadiana e na Ribeira de Odeleite, impediu a aplicação dos conhecimentos entretanto obtidos no Rio Mira e a comparação da biologia e da ecologia entre as populações.

Propõem-se, ainda, tópicos de investigação a desenvolver de modo a: 1) clarificar ou complementar algumas das conclusões; ou 2) abordar questões para as quais não foi possível obter e/ou analisar resultados.

Palavras-chave: Savelha, Ecologia larvar, RNA/DNA, Rio Mira.

Abstract

Allis and Twaite shad *Alosa* spp. are anadromous clupeid species, which still migrate into several rivers along Portugal to spawn but are regarded as Endangered or Vulnerable (using IUCN criteria). Within the framework of a countrywide research project, this dissertation reports the work carried out (from February 1998 until July 2000) to study the biology and ecology of the early life-history stages of Twaite shad, *Alosa fallax fallax*, in the River Mira (with a final note on *Alosa* spp. in the River Guadiana).

Twaite shad larvae were more abundant in the surface waters during the day whereas no diel abundance pattern was observed for embryos (*i.e.* yolk-sac larvae). Furthermore, eggs, embryos and larvae were only found in the upstream stations of River Mira from late-March until mid-June. As expected, no habitat selection occurs at either the egg or embryonic life stages. Conversely, shad larvae abundances were related to several environmental co-variates through a complex non-linear generalised additive model (GAM).

Paired translucent and opaque rings in a regularly-recurring sequence deposited around the *sagittae* of *A. fallax fallax* were readily visible. The high proportion of *sagittae* used suggests the usefulness of otolith microstructural analysis to estimate age and to study growth and morality of larvae. Growth and mortality rates varied seasonally (ranging from 0.36 to 0.60 mm d^{-1} and from 16 to 30 % d^{-1}, respectively).

In a typical year, larvae are in better nutritional condition (RNA/DNA ratio) during two relatively short time-periods, around mid-April and mid to late–May when water temperatures average 22 ºC and levels of prey are <80 *nauplii* 100 m^{-3}. Conversely, RNA/DNA are significantly reduced when water turbidity is higher than 2 mg DW m^{-3} and potential prey are readily available (>20 *nauplii* 100 m^{-3}).

The few occurrences of *Alosa* spp. embryos and larvae in the River Guadiana and the tributary Ribeira de Odeleite precluded sensible analysis.

Future topics of research to clarify some issues or directed at unanswered questions are proposed.

Keywords: Shad, Larval ecology, RNA/DNA, River Mira.

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Thank you the colleagues and staff of the Grupo de Investigação em Biologia Pesqueira e Hidroecologia and Faculdade de Ciências do Mar e Ambiente da Universidade do Algarve, that have contributed to research ideas, technical support, field and lab assistance, and cherished friendship. Teresa Pina, Jorge Palma, João Quintela and José António Bentes helped me extensively in the field, enduring hundreds of miles of road and long hours of labour. Professor Alexandra Chícharo was a tremendous help with sorting of ichthyoplankton and biochemical analysis of fish larvae. Professor Manuel Afonso Dias helped me with several statistical technicalities and doubts.

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I acknowledge all the support my friends gave even though not directly related to this dissertation (sorry for not mentioning you all individually!).

Last but not least, I am grateful to my family for their continuous support throughout my long education journey, and to my wife, Manuela Duarte, and son, Guilherme, for their love, patience and understanding, and for reminding me of the most important things in life.

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Chapter 1. General introduction

1.1. Shad who?

The Allis shad *Alosa alosa* (Linnaeus, 1758) and the Twaite shad *Alosa fallax* (Lacépède, 1803) (Figure 1.1) are both members of the numerous herring Family, Clupeidae. The shads (Subfamily Alosinae) form a relative large group of pelagic fishes found in seas all over the world except in the Antarctic. The Genus *Alosa* includes nearly half of the species of this Subfamily (Nelson, 1994). At present, no subspecies have been recognized in Allis shad whereas several subspecies have been proposed for *A. fallax* based mainly on geographical location and the number of gill rakers in the first gill arch (Aprahamian *et al.*, 2003; Baglinière *et al.*, 2003).

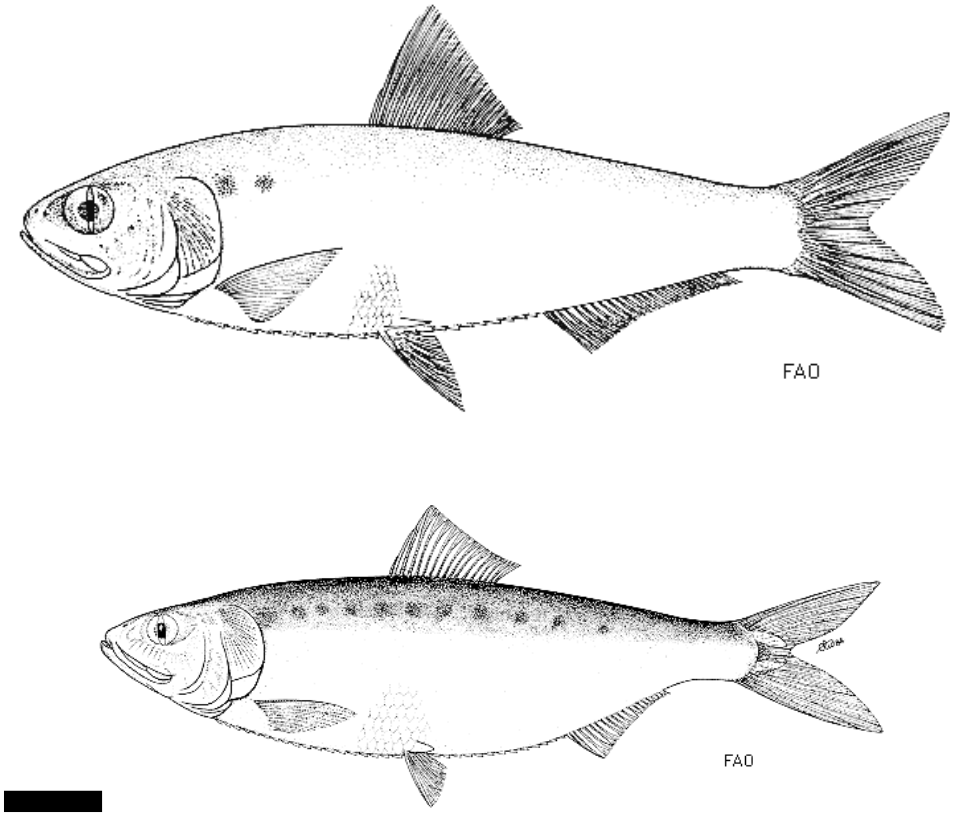

Figure 1.1 – Illustration of adult Allis shad (top) and Twaite shad (bottom) specimens (adapted from Whitehead, 1985). Bar = 5 cm.

Allis shad historically occurred along the Atlantic eastern coast, from Norway to Morocco, extending along the British Isles, the coasts of Germany, Belgium, France, and then southward to Spain and Portugal (see the references in Baglinière, 2000). Twaite shad has been reported from as far North as Iceland to Morocco in the South and as far East as Scandinavia and the Baltic Sea (Aprahamian *et al.*, 2003). Currently, the distribution of both species as regressed to limited areas, particularly in the case of Allis shad. This species only occurs in rivers along the Atlantic coasts of France and Portugal (Baglinière, 2000; Baglinière *et al.*, 2003), due mainly to anthropogenic intervention: dam or weir construction, deteriorating water quality and extraction of inert materials (Taverny *et al.*, 2000a) (Figure 1.2).

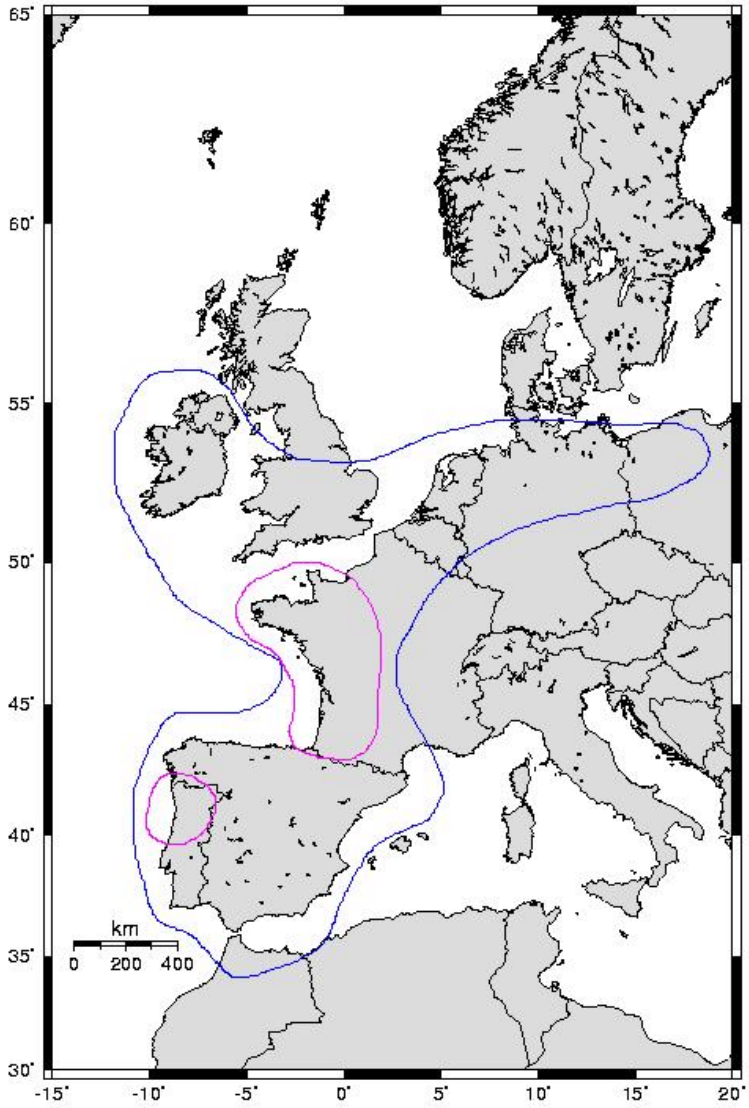

Figure 1.2 – Distribution of *Alosa* spp. species in Europe and North Africa (adapted from Aprahamian *et al.*, 2003; Baglinière *et al.*, 2003). At present, Twaite shad occurs from northern Germany to northern Morroco whereas the current distribution of Allis shad is limited to northern Portugal and Atlantic coast of France. Map obtained using OMC (at http://www.aquarius.geomar.de/omc_intro.html).

Most species of *Alosa* spp. are anadromous but some landlocked populations have been found in Greece, Italy, Ireland (Baglinière, 2000) and Portugal (Alexandrino, 1996; Collares-Pereira *et al.*, 1999; Eiras, 1981). Mature individuals (4 to 6 years-old) migrate from growing marine areas to spawning areas upriver from December to August (*A. alosa*) and between February and May (*A. fallax*), along a latitudinal gradient from South to North (Aprahamian *et al.*, 2003; Baglinière *et al.*, 2003).

The pattern of migratory dynamics of *A. alosa* corresponds to a dense anadromous migration of fish with one or two peaks of migration (Mennesson-Boisneau *et al.*, 2000b) whereas *A. fallax* enters the river in a series of waves (Aprahamian, 1981). Generally, anadromous Allis shad are considered semelparous since no more than 5-6% of the Atlantic stock spawn more than once (Mennesson-Boisneau *et al.*, 2000a). Conversely, nearly all populations of *A. fallax* have an iteparous life history (Aprahamian *et al.*, 2003). There is a higher proportion of males at the start of the migration (see Aprahamian *et al.*, 2003 for a review) but this unbalanced sex ratio tends to level out by the end of the spawning season (Mennesson-Boisneau *et al.*, 2000b).

Water temperature, which has a strong influence on hydrology and ecology both in estuaries and freshwater, is considered the most important environmental factor moderating migratory behaviour in both species. Spawning migrations generally take place when temperature ranges from 12 to 20 ºC (Aprahamian *et al.*, 2003; Baglinière *et al.*, 2003). Other environmental factors like tidal rhythm and water discharge are also deemed preponderant (Mennesson-Boisneau *et al.*, 2000b).

Allis shad spawns during the night at sites typically located in the middle or upstream reaches of the river. Spawning of *A. fallax* has been reported in tidal freshwater (Taverny, 1991; Thiel *et al.*, 1996) as well as in the nontidal freshwater areas of rivers (Caswell & Aprahamian, 2001) during the night. Some authors have narrowed down the maximum spawning activity to overnight between 2200 hours and 0300 hours (Cassou-Leins *et al.*, 2000). A commotion caused by tail splashing and rapid circular swimming near the surface (also known as "bull") is characteristic of spawning behaviour (Aprahamian, 1982; Baglinière *et al.*, 2003; Quignard & Douchement, 1991b). The typical spawning habitat of Allis shad is characterized by an area of coarse substrate limited upstream by a pool and downstream by shallow water with fast-moving currents (Cassou-Leins *et al.*, 2000), whereas the spawning habitat of Twaite shad in UK rivers comprises a fast-flowing shallow area of unconsolidated gravel-pebble or cobble substrate of

depths <1.0 m (Caswell & Aprahamian, 2001). In the River Elbe (Germany), *A. fallax* is reported as spawning in the upper reaches of the estuary in depths of up to 8.0 – 9.5 m (Thiel *et al.*, 1996). The relative fecundity of shads (number of ovules per kg weight) varies between 77000 and 576000 eggs kg^{-1} for *A. alosa* (Pina, 2000) and between *ca.* 90000 and 400000 eggs kg^{-1} for *A. fallax* (see other references in Cassou-Leins *et al.*, 2000). These estimates vary with habitat and estimation methodology (Taverny *et al.*, 2000c).

After spawning, the eggs of *Alosa* spp. (1 – 2 mm in diameter before hydration) drift in the current before sinking to the bottom where they become embedded in small crevices in the substrate. Eggs successfully develop at water temperatures above 17º C for Allis shad and between 15º and 25º C for Twaite shad with incubation taking 72 – 120 hours (Aprahamian, 1981; Taverny *et al.*, 2000c). Larvae of *A. alosa* are 7 – 12 mm TL at hatch but *A. fallax* larvae are smaller at hatch, 5 – 8 mm TL (Cassou-Leins & Cassou-Leins, 1981). They then move to open water, exhibiting a positive phototropism (Baglinière *et al.*, 2003; Véron *et al.*, 2003) and adopting a nektonic behaviour that persists until they are 36 days old (Gerkens & Thiel, 2001). Larvae of both species prefer low currents areas (*e.g.* side-channels or backwaters) which provide better nursery and feeding areas (Cassou-Leins & Cassou-Leins, 1981; Taverny *et al.*, 2000c). In the freshwater reaches of the Rivers Wye (Wales) and Sebou (Morocco), the diet of shad larvae is mostly comprised of larval aquatic insects and accessorily of crustacean zooplankton (Aprahamian, 1989; Cassou-Leins & Cassou-Leins, 1981). The early ontogeny of (reared) *A. fallax* has been described by Ehrenbaum (1894) and Mohr (1941) (cited in Quignard & Douchement, 1991a, b) and loosely classified according to their length and age into a few stages. Moreover, several recent studies addressed other aspects of the biology and ecology of early life-history stages of both species, particularly distribution and behaviour (e.g. Quignard & Douchement, 1991a; Quignard & Douchement, 1991b). Published information on age, growth, mortality or condition of European *Alosa* spp. larvae is anecdotal consisting of sizes-at-hatch and qualitative assessments (Taverny, 1991).

Age-0 and older fish migrate seaward during the Autumn in the surface layers of the water column (Thiel *et al.*, 1996). In the Elbe, the majority leave the estuary by the end of October, while in the Garonne it is not until the end of February that the majority have migrated seaward (Baglinière *et al.*, 2003). This occurs earlier in southern rivers. Juvenile migration is modulated by water temperature, river discharge and biological factors such as size and level of adaptability to marine conditions. The residence time in the estuary is not well known (Elie *et al.*, 2000).

1.2. Why shad larvae?

Shads, particularly the more valuable Allis shad, are harvested by commercial fisheries throughout their range in estuaries, middle sections of rivers or in their resident form in lakes. The fish are generally caught when they migrate towards the spawning grounds. In Portugal, the legal fishing period extends from 1 February until 14 June for both species and ≥35 cm TL for *A. alosa* and ≥20 cm TL for *A. fallax* are the minimum legal catch sizes[1]. Fishermen use traditional stationary or mobile fishing gears, *e.g.* stop net or drift trammel net (Baglinière *et al.*, 2003). Despite being an important resource, the economic value of the catches of Allis shad has been seriously reduced as a result of the decline or collapse of the stocks (Aprahamian *et al.*, 2003) (Figure 1.3). The best current estimates of the importance of *A. fallax* vary between 18 metric tons per year for the Gironde-Garonne-Dordogne sytem in France, and 0.5 – 1.5 metric tons for the River Lima (Portugal) or 0.2 – 0.6 metric tons for the River Sebou (Morocco). In several other rivers the catches are comparatively insignificant (*e.g.* the rivers Tagus and Guadiana in Portugal, or the River Severn in Ireland, after 1999) (Aprahamian *et al.*, 2003; Pina, 2000; Sousa *et al.*, 2003). Sousa *et al.* (2003) reported annual catches of 50 – 150 Allis shad and 3250 – 41000 Twaite shad in the River Guadiana, at several locations downriver of Pulo do Lobo (*e.g.* Azinhal, Guerreiros do Rio, Mértola), for the period 1999 to 2003. In the River Mira, no record of Allis shad exists for the past 40 years and Twaite shad is still harvested (although at unknown levels).

Moreover, shads constitute interesting material for the study of evolutionary biology, particularly of speciation. In fact, alosinids combine a considerable ecological and biological plasticity with several difficulties in taxonomic identification and genetics (genus, species). This complexity is pointed out by their ability of adaptation to lentic habitats, the hybridisation of *A. alosa* and *A. fallax* and the phylogenetical relationships within the Genus *Alosa* and among the Clupeidae (Mennesson-Boisneau *et al.*, 2000b).

Finally, *A. alosa* and *A. fallax* (as most members of the Genus *Alosa*) are also of ecological and patrimonial relevance. During the anadromous migration they can cover large distances, 800 km in the case of *A. alosa* (Baglinière, 2000; Hendricks *et al.*, 2002; Tomás *et al.*, 2005), from marine to freshwater habitats, and exhibit a pronounced homing behaviour similar to that of

[1] Direcção-Geral das Florestas (DGF) – Ministério da Agricultura, do Desenvolvimento Rural e das Pescas at http://www.dgrf.min-agricultura.pt/v4/dgf/ (revisited on 9 November 2005).

migratory salmonids (Baglinière, 2000). This stock-river relationship is important considering that environmental conditions during the embryo-larval period in the freshwater reaches of rivers play an important role in the future of populations (Assis, 1990). In contrast to salmonids shads are more sensible to the presence of obstacles (dams, weirs, etc.) in their migratory pathways because shads are incapable of jumping and cannot swim for long periods. This poses a threat to the survival of shad populations (Alexandrino, 1996), *e.g.* in <20 years *A. alosa* and *A. fallax* populations disappeared from the River Douro (Portugal) as a consequence of the construction of several dams in the estuary (cf. Larinier *et al.*, 2000). In recent years, adequate and more efficient fish passes have been developed and implemented (e.g. Quignard & Douchement, 1991a; Quignard & Douchement, 1991b). Notwithstanding, there has been very limited success (to say the least) in the attempts of artificial propagation, either to restore or augment population's abundance and distribution, of *Alosa* spp. in Europe (cf. Hendricks *et al.*, 2003) whereas in North America (especially in the USA) culture, stocking and translocation of *Alosa* spp. is a common and successful practice (Baglinière, 2000).

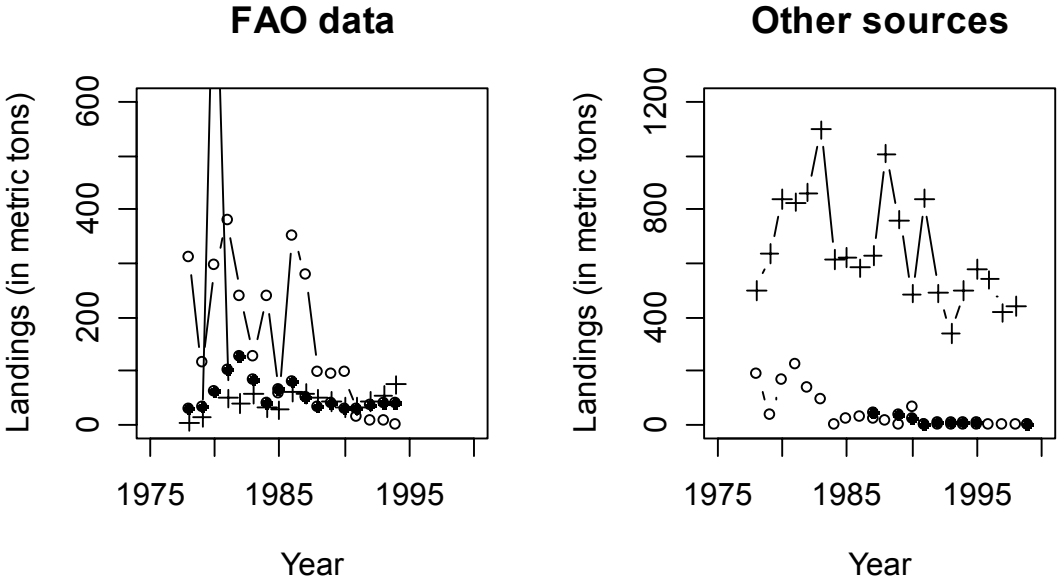

Figure 1.3 – Annual catches of Allis and Twaite shads reported to (left) the Fisheries Department of the FAO by national institutions of France (+), Portugal (●) and Morocco (○) and (right) compiled for key rivers from several other sources (Alexandrino & Boisneau, 2000; Baglinière, 2000; Legall, 2000; Sabatié *et al.*, 2000): Gironde-Garonne-Dordogne rivers system in France (+), River Lima in Portugal (●) and River Sebou in Morocco (○).

These attributes make *Alosa* spp. a privileged indicator-species of the biological (and physical) quality of the lower and middle reaches of rivers (Baglinière, 2000; Baglinière *et al.*, 2000). The

impact of human activities has led to a drastic restriction and fragmentation of the distribution area of shads, particularly of Allis shad (International Union for Conservation of Nature, 1994). In many countries, *A. alosa* and *A. fallax* are now either extinct or are regarded as rare or endangered according to IUCN criteria (Aprahamian *et al.*, 2003; Baglinière *et al.*, 2003). Besides other topics in need of further research (Crecco & Savoy, 1987b), the early life-history stages have been poorly studied in European shads as opposed to North American species (e.g. Crecco & Blake, 1983; Crecco & Savoy, 1984, 1987a; Crecco *et al.*, 1986; Limburg, 1996a, b; Savoy & Crecco, 1987, 1988), despite their importance in determining the recruitment variability, characteristic of the genus *Alosa*.

In this context, between 1997 and 2000 a national research project funded by the Fundação para Ciência e Tecnologia (ref. PRAXIS XXI 3/3.2/CA/1981/95) was carried out to characterize genetically and ecologically the Portuguese populations of Allis and Twaite shad (*Alosa* spp.) and lamprey (*Petromyzon marinus*) and establish the biological basis for its management and conservation. The field and laboratory work presented herein, which was directed at the valuable early life-history stages, took place within the scope of that project and was integrated with the results pertaining to other aspects of population biology (*e.g.* reproduction, adult growth) and genetics.

1.3. Objectives and organization of the thesis

The general objectives pursued during this thesis were two-fold: to estimate some important early life-history traits, *e.g.* abundance, age, growth and mortality rates, and nutritional condition of larvae; and to relate their variation to relevant environmental and biological covariates. More specific research objectives are outlined in each of the following chapters.

The organization of this thesis follows more or less closely the research strategy devised at the beginning of the research project. The dimension and accessibility of the River Mira and the abundance of Twaite shad *A. fallax fallax* early life-history stages therein favoured the fieldwork and thence constituted the core of most chapters that follow. First, the diel variation of major life-history traits, abundance, age (otolith microstructure analysis and time of increment completion) and nutritional condition (RNA/DNA ratios) was studied in River Mira during 1998 (Chapter 2). The conclusions were used subsequently to improve the sampling schedule in order

to: 1) study the seasonal abundance distribution; and 2) model its relationship to environmental and biological variables using generalised additive models (Chapter 3). Using the most appropriate data for River Mira (from year 2000), ages were determined and growth and mortality rates were estimated for 7 – 20 mm SL shad larvae. These rates were further related to putatively relevant environmental and biological covariates (Chapter 4). The usefulness of RNA/DNA ratios to assess the nutritional condition and to study the growth and mortality of Twaite shad larvae was investigated in Chapter 5. Moreover, Chapter 6 reports on the attempt to use the cumulative information of the previous chapters to assess shads' populations in the River Guadiana. The major conclusions of this work and some future topics of research needed to clarify some issues or directed at unanswered questions are presented in Chapter 7.

Chapter 2. Diel changes in abundance, condition and otolith growth of Twaite shad *Alosa fallax fallax* embryos and larvae in the River Mira

2.1. Introduction

Twaite shad *Alosa fallax fallax* (Lacépède, 1803) is an anadromous clupeid species, which still migrates into several rivers along Portugal to spawn (Eiras, 1977) but is regarded as "Vulnerable" by the Instituto de Conservação da Natureza (at http://www.icn.pt/) according to the IUCN criteria (Cabral *et al.*, 2006). Within the framework of a countrywide research project referred to earlier, different aspects of shad populations were studied in the River Mira, namely reproductive biology (Pina, 2000; Pina *et al.*, 2003). This paper is part of the initial work carried out to study the biology and ecology of the early life-history stages of Twaite shad in the River Mira.

In rivers, ichthyoplankton is generally collected in greatest abundance at night with both active and passive sampling gears (Gadomski & Barfoot, 1998). Conversely, early post-hatch larvae of Allis shad *Alosa alosa* move to open water where they exhibit a positive phototropism and a necktonic behaviour that persists until they are about 36 days old (Baglinière *et al.*, 2003). Aquarium experiments seemed to support the hypothesised positive phototropism of Allis shad (Cassou-Leins & Cassou-Leins, 1981). At present, there is no reason to believe that Twaite shad behaves otherwise. On the other hand, American shad *Alosa sapidissima* embryos in the middle-to-upper Delaware River were found to be more numerous after 1800 hours whereas postlarvae densities were similar over all hours sampled, from 0900 hours to 0100 hours (Ross *et al.*, 1993). Moreover, more shad larvae (*Dorossoma* spp.) were collected in daytime samples in embayments whereas their numbers were similar between diel samples in river habitats (Sammons & Bettoli, 2002). It seems that diel distributions of shads can vary depending on ecosystems, species and possibly years.

Few authors determined the age and/or studied growth of larval shads particularly American

shad (e.g. Crecco & Savoy, 1987a; Crecco *et al.*, 1983; Limburg, 1996a). For this species, Crecco & Savoy (1987) have validated the growth increment deposition as daily by monitoring known-age larvae.

In recent years, the RNA/DNA ratio has been used to assess the nutritional condition and growth of marine fish larvae (see Bergeron, 1997; Ferron & Leggett, 1994 for recent reviews) but no record of its use to study *Alosa* spp. condition was found to date. The RNA/DNA ratio is an ecophysiological index of condition, which reflects the potential for protein synthesis (Chícharo, 1997) and is therefore susceptible to changes in the environment. However the magnitude, timing and latency of variations in RNA/DNA ratios are poorly studied in natural larval fish populations. Evidence of diel periodicity of the nutritional condition of fish larvae have been reported for laboratory-reared red drum *Sciaenops ocellatus* (Rooker & Holt, 1996) and field-caught Atlantic sardine *Sardina pilchardus* (Chícharo *et al.*, 1998b). Conversely, Bailey *et al.* (1995) and Clemmesen (1996) found no significant diel effects on RNA content, for walleye pollock *Theragra chalcogramma* and herring *Clupea harengus* larvae, respectively.

Rhythmic behavioural processes are key aspects of survival. Obtaining accurate estimates of larval and young-of-year fish abundance, or any other life-history trait for that matter, is contingent on understanding their seasonal and diel variability. Hence, the aims of this study were to examine the daily variation in abundance, otolith increment deposition rate and RNA/DNA ratios of Twaite shad embryos and larvae and to relate those changes to some environmental and biological variables. Besides its biological and ecological relevance, this information is important in designing effective sampling programs that accurately estimate those life-history traits of field-caught Twaite shad embryos and larvae.

2.2. Material and Methods

Study area

The River Mira is a 145-km long, relatively narrow (<400 m near the mouth and narrower upriver) and shallow (<10 m deep near the mouth) watercourse located in SW Portugal (Figure 2.1). Tidal influence extends to approximately 40 km from the mouth, where mechanical effects and salinity changes have been observed. At the upper limit of tidal influence, water depth

varied between 0.5 and 4 m whereas river width did not exceed 30 m. The current velocity of water surface, averaged 0.23 – 1.20 m s^{-1} during ebb and 0.29 – 0.69 m s^{-1} during flood-tides (Author, unpublished results). Since 1968, the Santa Clara Dam (an embankment dam) has been used for local water supply and irrigation, and energy production. A detailed description of the lower River Mira can be found elsewhere (Andrade, 1986).

Sampling

In 27/28 April, 21/22 May and 18/19 June 1998, samples were collected every two hours during 24 hours at Odemira (station no. 4 of a larger study, Figure 2.1). At this site (*ca.* 34 km from the mouth), the River Mira is 20 – 45 m wide and less than 3.5 m deep with mixed sediment composition (very fine silt up to coarse sand, pebbles and cobbles) and abundant debris. During each sampling occasion, mesozooplankton was collected with a conical net (∅ 0.37 m, 1.6 m long, 0.5-mm mesh size) towed 1 m below the surface, at a constant speed of approximately 1 m s^{-1} for 10 minutes (an area of approximately 42000 m^2 was sampled). Plankton tows sampled equitatively the river margins and the main channel over a 42000 m^2-area of the river (Figure 2.2). A flowmeter (Hydrobios®) was attached inside the net to estimate the water volume filtered (median=62.8 m^3, range=14.9 to 113.1 m^3). Samples were sorted in a black tray, the fish larvae retrieved and immediately frozen in liquid nitrogen (-197º C). Zooplankters were stored in buffered 4% formaldehyde solutions. A further 26 – 39 L of water were sampled at 0.5-m depth and filtered through a 0.063-mm mesh size sieve to collect microplankters.

The surface temperature (º Celsius) and salinity (ppt) were measured using a hand-held thermometer and a refractometer, respectively. Additionally, the water level (in metres) at each sampling moment was estimated using the equations provided by the Instituto Hidrográfico da Marinha (I.H.M., 1997-1999), namely:

$$y = \frac{H+h}{2} + \frac{H-h}{2}\cos\left(\frac{\pi \cdot t}{T}\right) \tag{2.1}$$

$$y_1 = \frac{h+H_1}{2} + \frac{h-H_1}{2}\cos\left(\frac{\pi \cdot t_1}{T}\right) \tag{2.2}$$

where y is the water height (in metres) in any moment after high-tide, y_1 is the water height in any moment after a low-tide, H and H_1 are values of water height of two consecutive high-tides,

h is the water height at the intermediate low-tide, T and T_1 are the time intervals (in hours) between tides, and t and t_1 are the time intervals since the last tide. At the diel sampling site, times of high and low tide were obtained by adding 3 hours to the published time for the nearest port, at Sines. Equation (2.1) was used when $H - h > 0$ (ebb tide) and equation (2.2) was applied to data for flood tides ($H_1 - h < 0$).

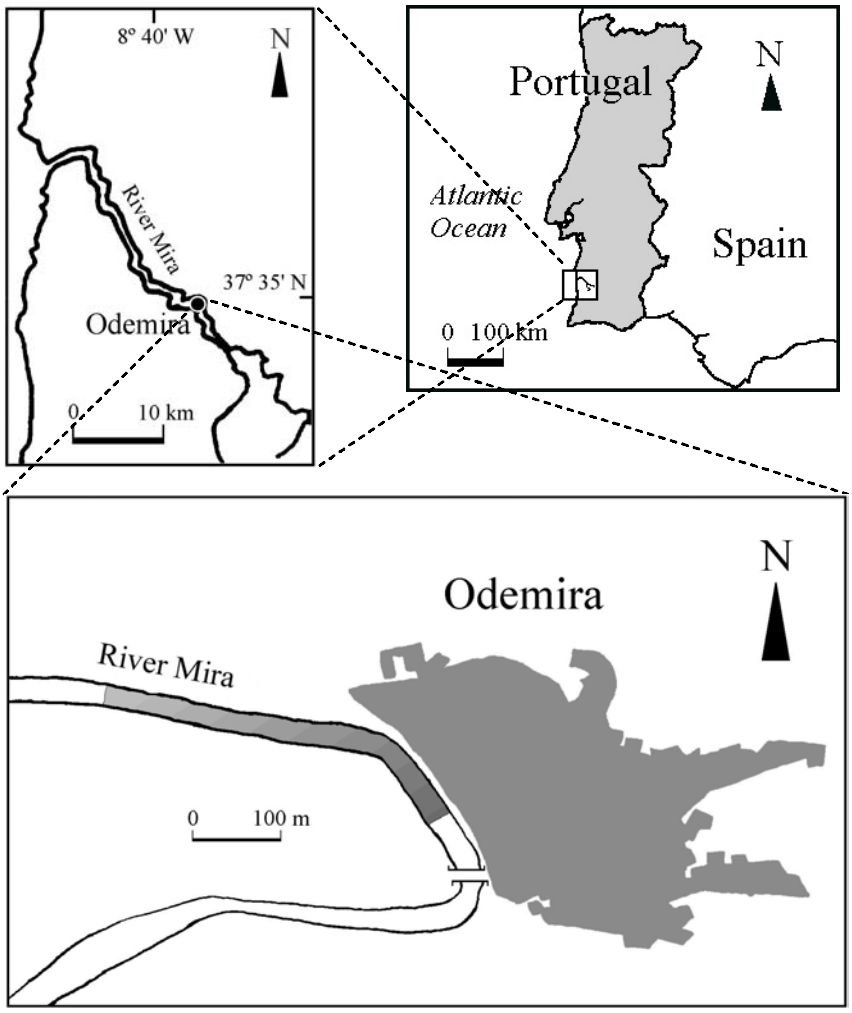

Figure 2.1– Location of sampling station no. 4 in the River Mira (shaded section of the river in the bottom plot) (approximate coordinates of Odemira are 37º 36' N, 8º 38' W).

Laboratory procedures

Twaite shad embryos and larvae were identified using published information (Johnson & Loesch, 1983; Quignard & Douchement, 1991b; Ramos, 1977). The remaining preserved mesozooplankters were identified using several sources (Newell & Newell, 1963; Smith, 1977; Todd & Laverack, 1991). According to Balon (1990), free embryos are fish from hatching until

the transition to exogenous feeding. Hereafter they will be referred to as embryos. Also, the observation of fish larvae under a dissection stereoscope allowed their classification into two groups, "gut with prey" and "empty gut", if prey items were visible or not in the gut, respectively. This information was incorporated into the data sets as the proportion of larvae with gut content $p_i = n_{gut}/(n_{gut} + n_{empty})$ (herein named *pwprey*). Whenever mesozooplankters were extremely abundant, successive sub-samples with a folsom-type splitter were analysed and at least 300 organisms counted (Omori & Ikeda, 1992). Plankters' density was expressed as number of individuals per 100 m^3 of water.

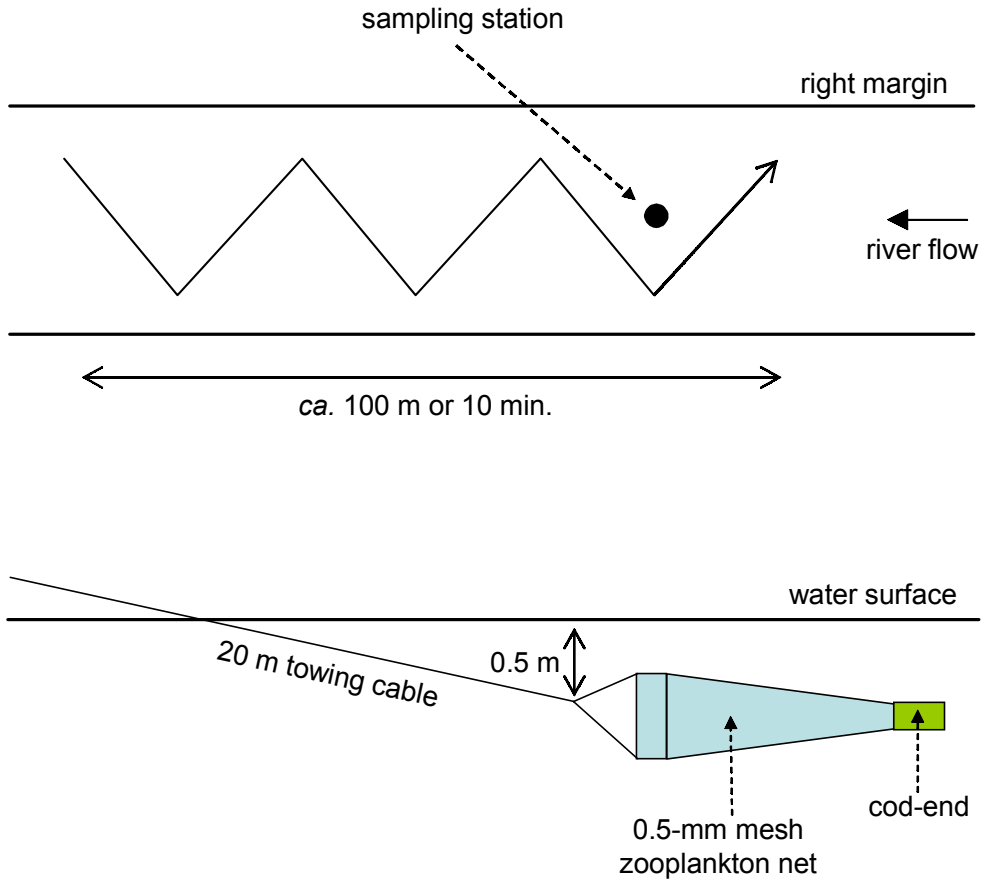

Figure 2.2 – Diagrams of sampling procedures (river section viewed from above, top, and lateral view of zooplankton tows, bottom).

To estimate microplankton biomass, aliquots of the homogenised samples were rinsed through a 1.2-µm milipore filter (∅ 25 mm, Macherey-Nagel® GF-3). Filters were dried in an electric oven at 60º C for 24 – 36 hours to obtain the dry weights (DW) and then incinerated at 450º C

for 4 hours to determine the ash-free dry weight (AFDW). Microplankton biomass was expressed as ash-free dry weight per cubic metre of water (mg AFDW m^{-3}). Dry weights allowed the estimation of water turbidity (as mg DW m^{-3}).

For the analysis of nutritional condition, individually frozen larvae (*n*=402) were thawed, identified and measured (to nearest 0.1 mm). Their condition was assessed using the RNA/DNA ratio. Nucleic acids in individual fish larvae were quantified according to a modified fluorimetric method and using ethidium bromide (EB) as the fluorophor. Fish larvae were extracted in 1% sarcosine in Tris-EDTA buffer (Trizma®, pH 8.0) to give a final concentration of 0.1%. After centrifugation, aliquots of the supernatant were: 1) combined with Tris-NaCl (Trizma®, pH 7.5) plus ethidium bromide (0.1 mg ml^{-1}) to give the total nucleic acids content; and 2) incubated for 30 min at 37 ºC with Tris-NaCl and 0.05 ml of ribonuclease A (Type-II A, 0.12 μg ml^{-1}) and stained with EB to give the DNA content. The fluorescence due to total RNA, mainly ribosomal, was calculated as the difference between the results from the previous two procedures. Fluorescence was determined by exciting at 365 nm and reading at 590 nm with a spectrofluorometer (Hitachi® Model 650-10). Concentrations were calculated by running standard curves of DNA-EB and RNA-EB every day with known concentrations of λ-DNA (Boehringer-Mannheim®, 0.25 μg μl^{-1}) and 16s-23s RNA (Boehringer-Mannheim®, 4 μg μl^{-1}), in the appropriate range of values. The limit of detection was 0.16 μg ml^{-1} for DNA and 0.46 μg ml^{-1} for RNA. Percent recovery of added λ-DNA (spikes) was 96.4%±5.9% and the recovery of 16s-23s RNA spikes was 97.6%±7.0%. Total amounts of nucleic acids were corrected base on these values. The coefficient of variation for estimates from eight homogenates was 1.5% for DNA and 3.5% for RNA (see Esteves *et al.*, 2000a for protocol details).

After the biochemical analyses, larval remains (mostly bony structures and connecting cartilage) were further examined for their otoliths (herein only the samples collected in 18/19 May were used). *Sagittae* can be removed from the larva's head using a combination of 45% NaOH rinse (<3 min) and fine-needle manipulation under a stereoscope. After washing (with 99% ethanol and water) and drying the otoliths they were mounted on a slide using Pro-Texx® mounting medium (see Secor *et al.*, 1991; Stevenson & Campana, 1992 for further details). Digitised images of the *sagittae* were obtained with a CCD camera connected to a microscope (at 400x) and analysed using image analysis software (ImagePro Plus®). The number of increments (herein an increment corresponded to paired translucent and opaque rings in a regularly-

recurring sequence deposited around the otolith) was counted and their width measured in three independent occasions without prior knowledge of length or sample information (see Stevenson & Campana, 1992 for a thorough review of methods).

Statistical analyses

Abundance: For the purpose of modelling the diel changes in the densities of shad embryos and larvae, the abundances of isopods, small insects, cladocerans and copepodites were pooled into a variable named *zoopl*. Another variable designated *pred* was the sum of the abundances of potential predators/competitors such as small medusae, mysids and amphipods. A simple index of diversity was calculated as the proportion of zooplankton *taxa* (microplankton excluded) present in any one sample (*index*).

To identify diel patterns of larval density a statistical model that is resistant against outliers was used. Using median polish a simple additive model is fitted to the larval density data entered into a two-way table, rows representing hours and columns representing dates of sampling. The median polish for an additive model of the form

$$y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij} \tag{2.3}$$

with constant term μ (where y_{ij} is the larval density at hour i and date j, α_i is the effect of hour i, β_j is the effect of date j and ε_{ij} is the random error term) can be implemented in `R` by fitting the model (2.3) iteratively (Venables & Ripley, 1999). To test for non-additive structures in the residuals, ε_{ij}, these can be plotted against the comparison values $c = \alpha_i \beta_j / \mu$. If this diagnostic plot reveals a slope k, calculated by robust linear regression (Venables & Ripley, 1999), which is different from zero, the non-additive structure of the data can be removed by a power transformation of the response-variable with the power $p = 1 - k$. Herein, a ln(density+1) transformation was used instead of p = 0.66 and the model (2.3) was re-fitted adequately. The methods are fully described in Emerson & Hoaglin (1992).

Condition: An analysis of variance approach was used to study the diel changes in condition of shad larvae (the number of embryos collected precluded their analysis). The response variable (RNA/DNA ratios) was square root transformed to correct for non-normality (Shapiro-Wilk's W=0.996 and p=0.3031). Two outlier observations due to the unexpectedly low DNA content of larvae were removed. Factors *Hour* (0100 to 2300 hours), *Month* (April, May, June) and *Gut* (Prey, Empty) were considered fixed. Starting with the complete (full) model (*e.g.* Larvae =

Hour × Month × Gut)[2] several models were tested using sequential analysis of variance. Inclusion or deletion of terms obeyed the marginality principle and the effect of adding or removing individual terms from a model was assessed through model's statistics, *e.g.* residuals sum of squares. Models were further investigated using diagnostic plots (fitted values *versus* residuals and normal probability plots of residuals). *Post-hoc* comparison of significant factor levels was done using Tukey's HSD test (Venables & Ripley, 1999).

Otolith's increments: To study the diel rhythm of increment deposition in the *sagittae* of shad larvae a marginal increment ratio was used as the response variable. Variable *ratio* was calculated as the proportion of marginal increment width-to-previous increment width. Values close to zero indicate that the marginal increment is just being deposited whereas ratios of one are obtained when deposition is completed. Precision of increment counts was assessed using the coefficient of variation (Chang, 1982): $cv = s/\overline{x}$ (for each *sagittae*). Only information concerning larvae for which at least two-out-of-three readings coincided was used (n=62). The effect of factor *Hour* was studied with a simple one-way ANOVA (Venables & Ripley, 1999).
The degree of association between Twaite shad abundance or condition and relevant environmental variables was assessed using correlation analysis – Spearman's Rank Correlation coefficient (see e.g. Zar, 1996) as implemented in the `R` package `Hmisc` (see Alzola & Harrell, 2002). Non-parametric correlation analyses do not presume that variables are normally distributed or assume any particular type of relationship between variables. The significance level of r was corrected using the method of Bonferroni (Zar, 1996). Significant relationships were tentatively described using scatterplot smoothers.

All statistical procedures described above were implemented in `R` (R Development Core Team, 2003) and extracts of scripts are provided from the author upon request. For all statistical analyses, the level of significance was set at 0.05 and p-values greater than 0.10 were considered as indicative of non-significance.

[2] The "×" implies that the formula expands to Larvae = Hour + Month + Gut + Tide + Hour × Month + ... + Hour × Month × Gut (including 2nd and 3rd order interactions between terms).

2.3. Results

Environmental conditions varied somewhat during the diel sampling done at station no. 4 (Figure 2.3). Surface temperatures increased from April through May to June whereas salinity recordings were always inferior to 0.5 ppt. Temperature varied less than 2 ºC during each sampling cycle and peaked by late afternoon in every sampling. Tidal behaviour was different among sampling dates. In April, high tides occurred at dawn and dusk but in May and June water level was higher at about 0100 and 1300 hours. The right-hand plot in Figure 2.3 illustrates the hourly changes of turbidity that fluctuated between 1.4 and 2.7 mg DW m^{-3} except in two sampling occasions in June.

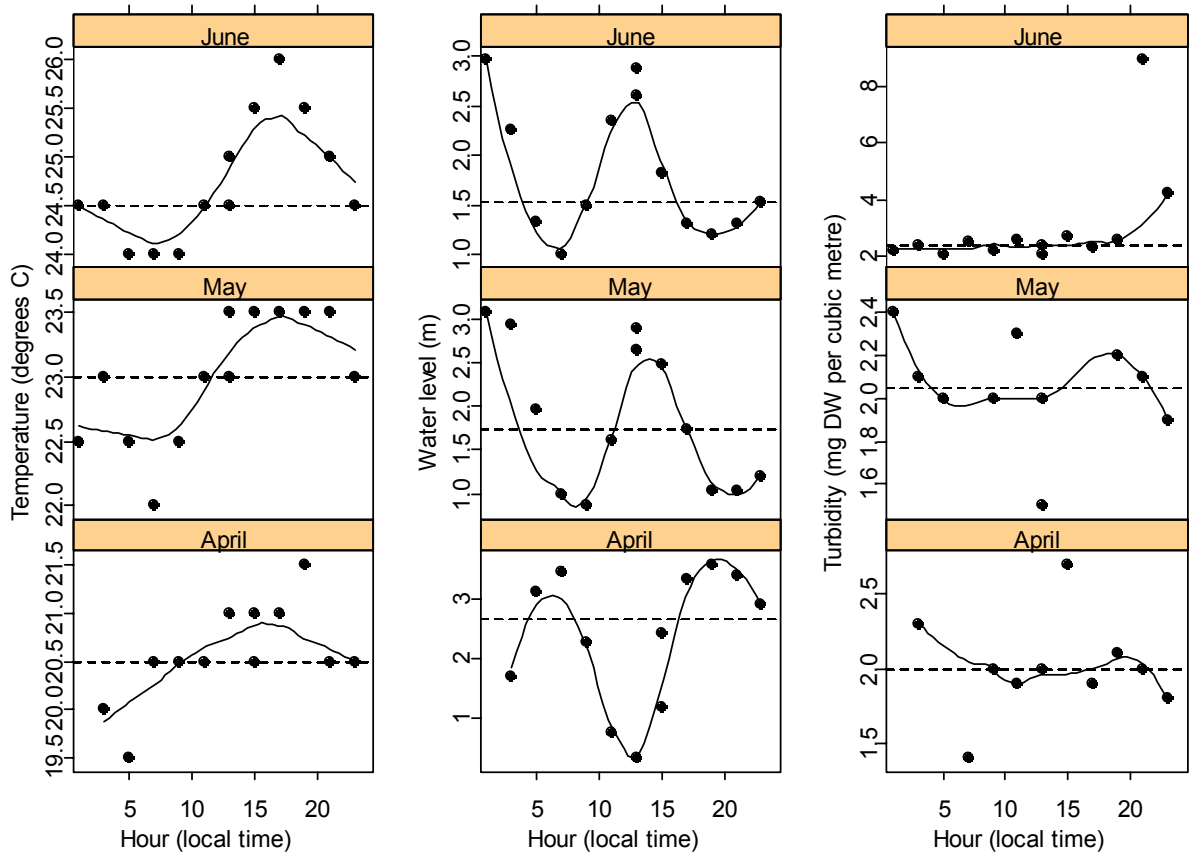

Figure 2.3 –Temperature, water level and turbidity changes over the 24-hour sampling periods accomplished in April, May and June (note the different *y*-scales). Horizontal dotted lines represent the monthly median. Spline models fitted are shown for illustrative purposes only. Approximate hours of sunrise and nightfall were 0611 to 0644 hours and 2025 to 2105 hours, respectively.

The hourly changes in microplankton biomass and decapods' *nauplii* density were similar and varied with sampling date (Figure 2.4). In April and May, values were lower at dawn and dusk

whereas in June biomass and density peaked during the night (respectively, >100 mg AFDW m^{-3} and >300 ind. 100 m^{-3}). A consistent diel pattern was observed for zooplankters and predators with greater densities during nigh time collections (Figure 2.4). A few exceptions to the patterns described above were noticed.

Fewer shad embryos were caught in June than in April or May, but no diel pattern could be depicted from data on their density (Figure 2.4). In fact, yolk-sac larvae were collected indiscriminately at any time of day, a situation that was reflected in the non-systematic results of the median polish analysis (Figure 2.5). On the contrary, larval Twaite shad were more common during daylight hours in comparison to samples collected during the night (Figure 2.4). Although larval abundance varied with sampling date they were found to be particularly (significantly) more abundant in the period 1300 – 1900 hours (Figure 2.5).

No diel pattern could be observed for the proportion of larvae with gut content. In May, a higher number of samples had $p_i>0$ when compared to other sampling dates. The proportion of all *taxa* present in a sample ("index of diversity") varied between 0.00 and 0.73 with greater values obtained for samples collected at dawn and dusk (Figure 2.6).

Table 2.1 summarises the correlation analyses of several variables prospectively relevant for the circadian distribution of shad embryos and larvae. Although none of the pairwise correlations including shad larvae densities was significant at the α-corrected level ($\alpha'=0.00076$) it appears that the more larvae are present, greater is the proportion of shad with prey items in their gut ($r=0.483$). Moreover, larvae seemed to feed mainly during daylight hours (*cf.* Figure 2.6). Furthermore, larval shad densities were inversely correlated with turbidity ($r=-0.46$) and predators' densities ($r=-0.33$).

On the other hand, important associations existed between zooplankton and abiotic predictors and among the various variables referring to zooplankton. In fact, temperature and water level were beneficial to the abundance of *nauplii* ($r=0.41$) and zooplankton ($r=0.33$) respectively, whereas turbidity, temperature and plankters' diversity were inversely correlated ($r=-0.39$ and $r=-0.40$). Also, microplankton biomass, decapods *nauplii*, zooplankters, predators' and zooplanktons' diversity (*index*) were positively correlated with each other ($0.38<r<0.60$).

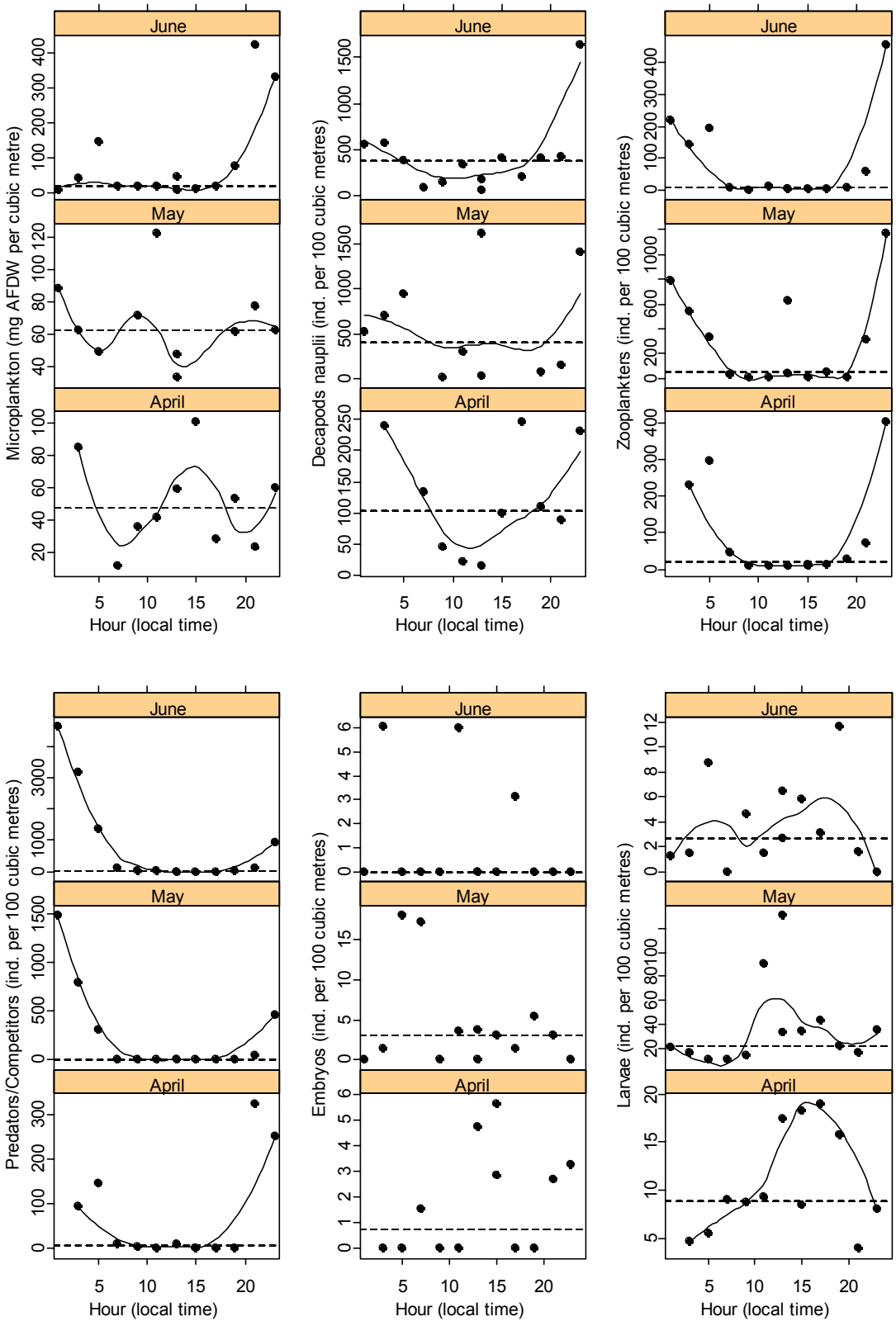

Figure 2.4 - Diel changes of microplankton biomass and decapods *nauplii*, zooplankters, predators and shad embryos and larvae abundance over the 24-hour sampling periods in April, May and June (note the different *y*-scales). Horizontal dotted lines represent the median. Spline models fitted are shown for illustrative purposes only. Approximate hours of sunrise and nightfall were 0610 to 0645 hours and 2025 to 2105 hours.

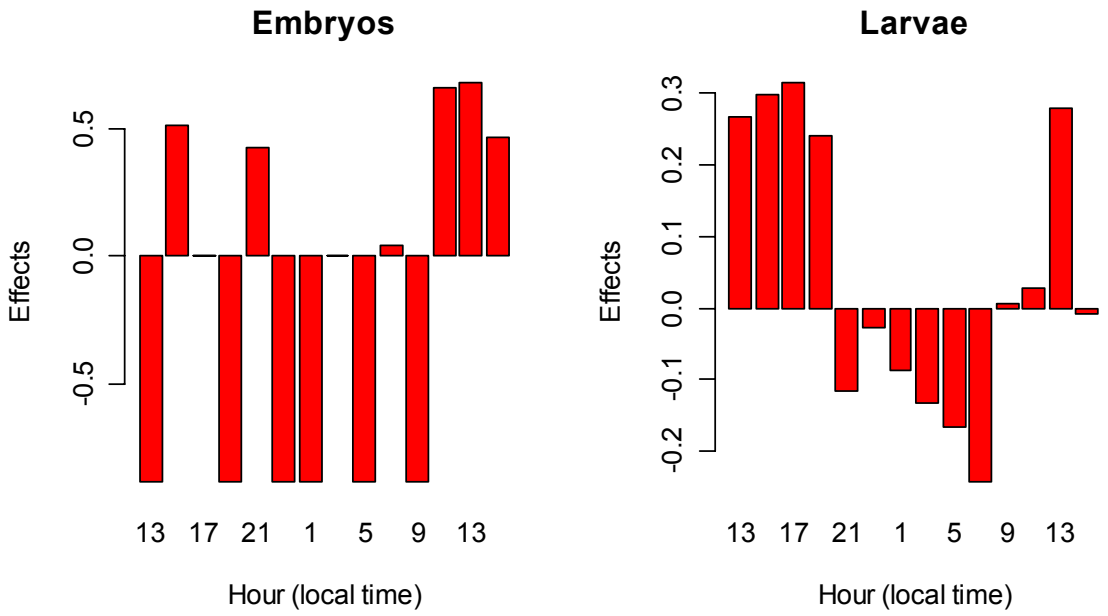

Figure 2.5 – Barplots of the effects of hour of sampling on embryos and larval density (results of the median polish analyses). These results are scaled to a median of zero. Positive values denote more specimens than expected from a random distribution. Conversely for negative values. Approximate hours of sunrise and nightfall were 0611 to 0644 hours and 2025 to 2105 hours.

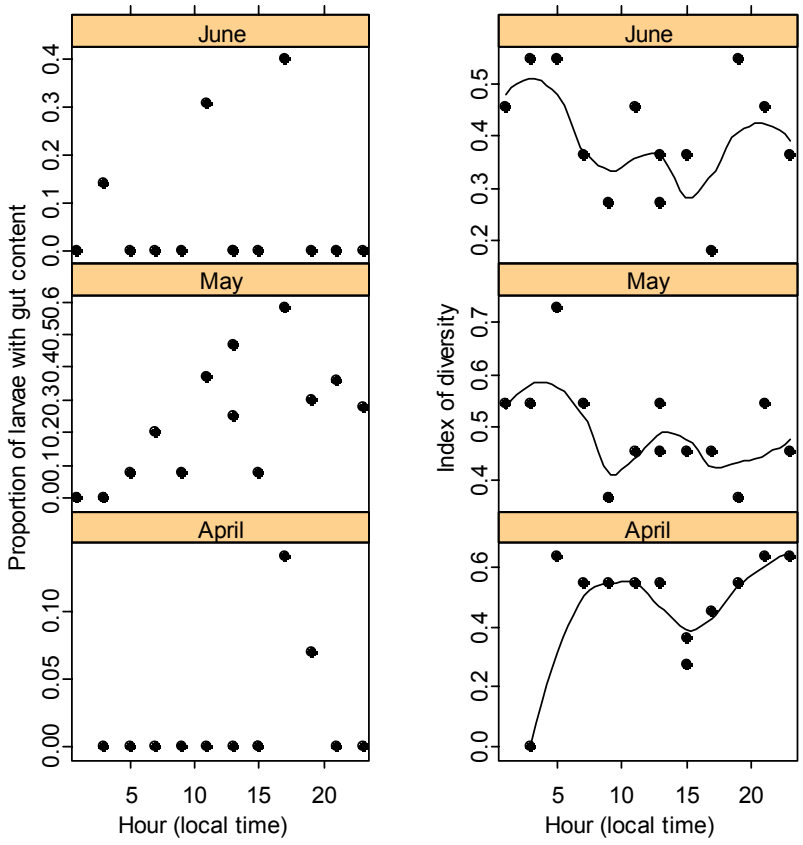

Figure 2.6 – Diel changes of the proportion of shad larvae with gut content (left) and index of diversity (right) over the 24-hour sampling periods in April, May and June. Spline models fitted are shown for illustrative purposes only. Approximate hours of sunrise and nightfall were 0611 to 0644 hours and 2025 to 2105 hours.

Table 2.1 – Pairwise Spearman's correlation coefficients *r* of presumed relevant variables to the circadian distribution patterns of Twaite shad. The superscripts a, b and c indicate significance at the α=0.05, α=0.01 and α'=0.00076 levels, respectively. Because of the number of pairwise correlations the corrected level of significance used was α' (see text for details).

Spearman *r*	Hour of sampling	Water height	Temperature	Turbidity	Embryos	Larvae	*Nauplii*	Micropankton	Zooplankton	Predators	Proportion
Water height	-0.05										
Temperature	0.14	-0.21									
Turbidity	-0.05	-0.14	0.48 [b]								
Embryos	0.03	-0.10	-0.10	-0.07							
Larvae	0.16	0.00	-0.31	-0.46 [b]	0.21						
Nauplii	-0.08	0.25	0.41 [a]	0.33	0.03	-0.09					
Microplankton	0.12	-0.22	-0.2	0.27	-0.01	0.27	0.21				
Zooplankton	-0.09	0.33 [a]	-0.31	-0.19	0.03	0.15	0.46 [b]	0.43 [a]			
Predators	-0.34 [a]	0.07	0.03	0.09	-0.07	-0.33 [a]	0.51 [b]	0.32	0.60 [c]		
Proportion	0.21	-0.14	0.18	-0.20	0.47 [b]	0.48 [b]	0.07	-0.03	0.06	-0.28	
Index	-0.12	0.30	-0.40 [a]	-0.39 [a]	0.18	0.19	0.08	0.17	0.54 [c]	0.38 [a]	-0.04

The nutritional condition of shad larvae varied throughout the day (Figure 2.7) being significantly lower than average during dawn and dusk hours (ANOVA, $F_{[11,355]}$=9.81 with p<0.0001 and Tukey HSD test p<0.05). Also, the amplitude (and diel behaviour) of RNA/DNA ratios was relatively different in May when compared to the other sampling occasions which accounted for the significant interaction *Hour* × *Month* term in the ANOVA model ($F_{[18,355]}$=4.19 with p<0.0001). Differences in RNA/DNA ratios between larvae with prey and emptied gut were found to be not significant ($F_{[1,355]}$=1.65 and p=0.1991).

Although individual RNA and DNA content increased markedly with the size of larvae as expected, a significant but very weak (r^2=0.020) relationship was found between RNA/DNA ratios and the length of shad larvae (Figure 2.8).

RNA/DNA ratios were weakly and not significantly correlated (p>0.05) with any of the variables tested (*nauplii* r=0.062, microplankton biomass r=0.135, zooplankters r=0.127, predators r=0.231, proportion of larvae with gut content r=-0.170 and diversity index r=-0.241).

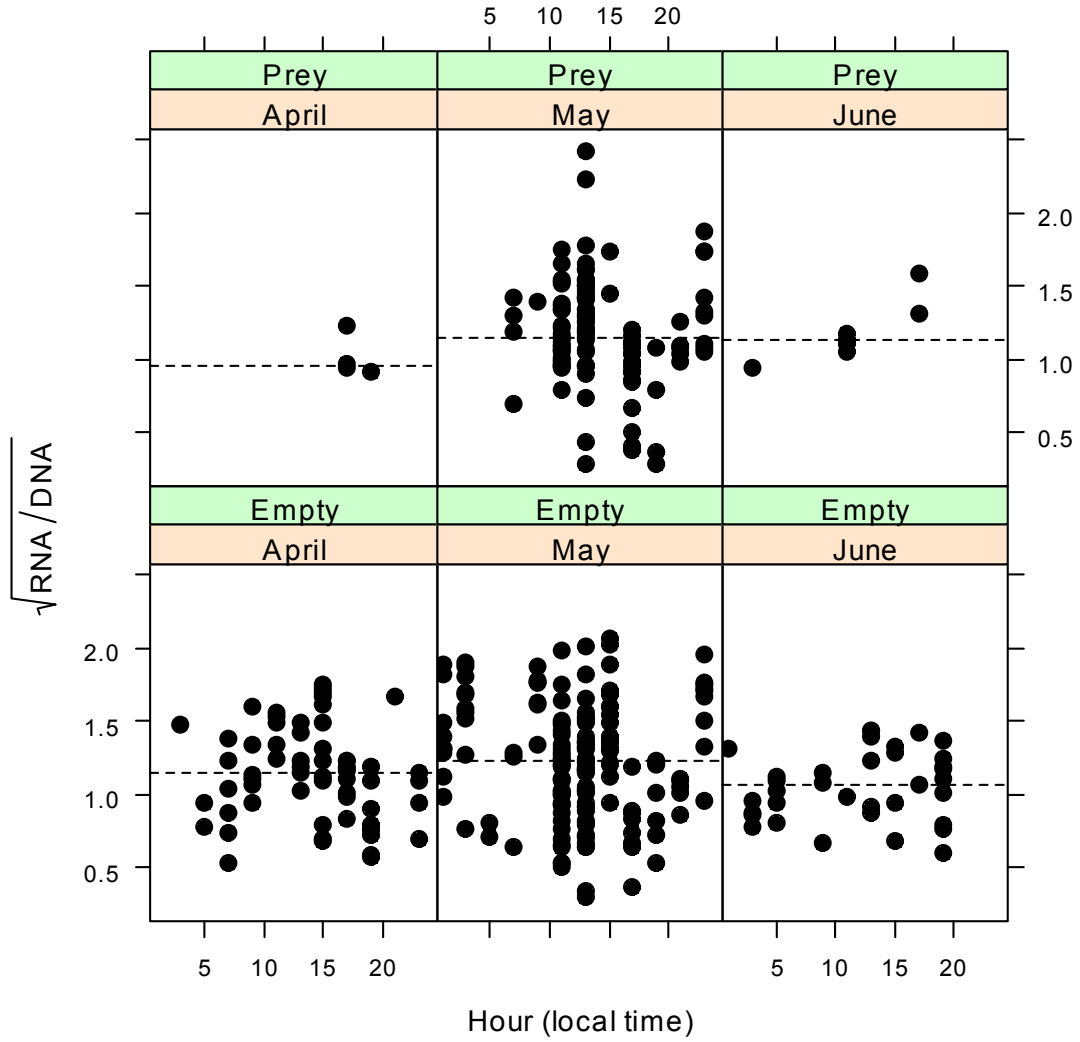

Figure 2.7 – Diel changes in square root-transformed RNA/DNA ratios of individual shad larvae with and without gut content over the 24-hour sampling periods in April, May and June. Horizontal dashed lines are monthly median values. Approximate hours of sunrise and nightfall were 0611 to 0644 hours and 2025 to 2105 hours.

The average precision of increment counts of the *n*=84 shad *sagittae* read was high (mean *cv*=7.3%). More than 73% of the *sagittae* analysed (*n*=62) were used herein because at least two-out-of-three counts coincided. The average increment width (5.00 $\pm$ 0.55 µm SD) and the marginal-to-previous increment ratios (which ranged from 0.26 to 1.90) were independent of the number of increments counted on shad larvae *sagittae* (Regressions *p*=0.1581 and *p*=0.2485 respectively) (Figure 2.9, previous page). No hourly differences (ANOVA, $F_{[8,53]}$=1.12 with *p*=0.3677) were found in the marginal-to-previous increment ratios, the measure of deposition rate in shad larvae *sagittae* used herein (Figure 2.10).

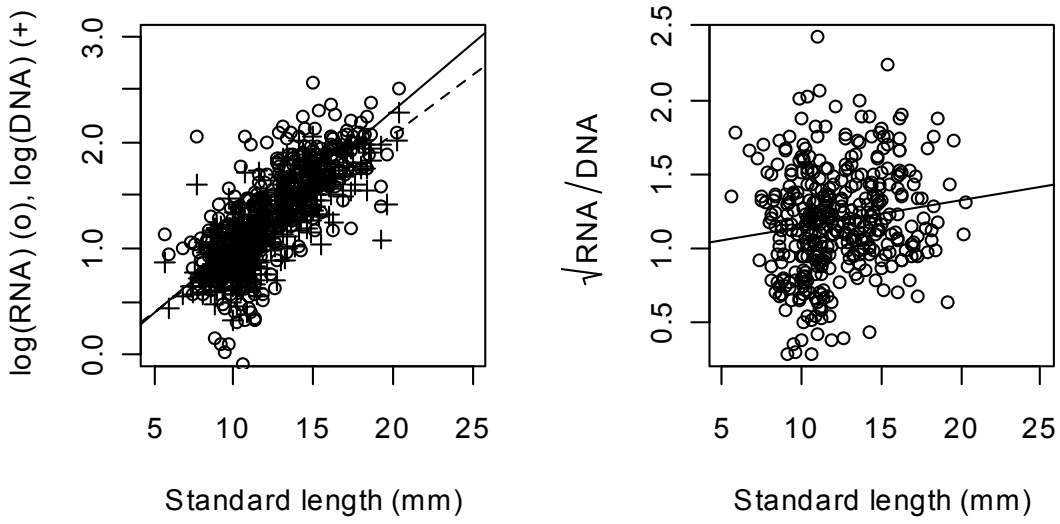

Figure 2.8 - Scatter plots of log(RNA), log(DNA) contents and square-root transformed RNA/DNA ratios versus the standard length of larvae. Regression lines are drawn following the equations: y = -0.262 + 0.074x (with r^2=0.551 and p<<0.001 for RNA; solid line), y = -0.158 + 0.052x (r^2=0.651 with p<<0.001 for DNA; dashed line) and y = 0.962 + 0.018x (r^2=0.020 and p=0.0052 for RNA/DNA).

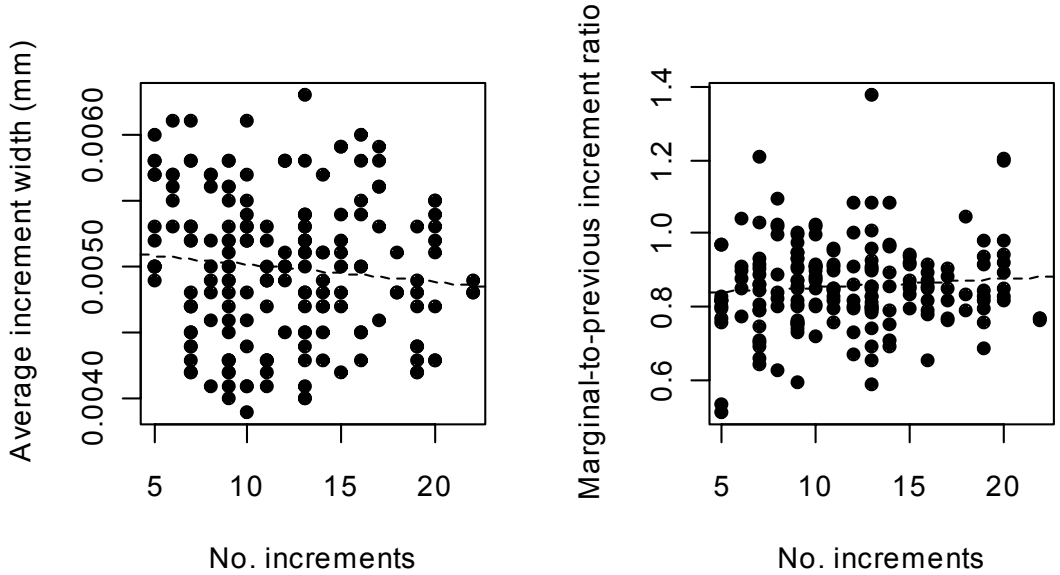

Figure 2.9 - Scatterplots of average increment width and marginal-to-previous increment ratio (Ratio) against the number of increments counted in *sagittae* (No. increments). Also plotted are dashed regression lines with non-significant slopes (left: y = 0.00514 - 0.00001x; right: y = 0.8279 + 0.0023x).

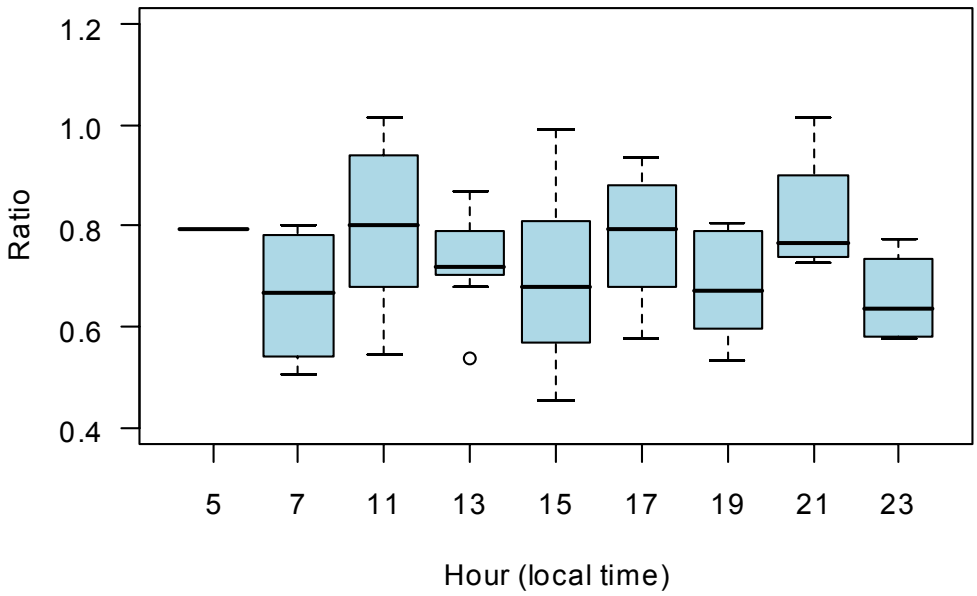

Figure 2.10 – Box-and-whisker plot of Ratio (the proportion of marginal increment width-to-previous increment width) per hour of sampling. Approximate hours of sunrise and nightfall were 0611 to 0644 hours and 2025 to 2105 hours.

2.4. Discussion

The greater availability of Twaite shad larvae at the surface during the day, unveiled by the results of the median polish analysis of River Mira abundance data, contrasts with the most common reports of greater abundance of riverine ichthyoplankton during the night. This later behaviour is usually attributed: (i) to the nightime downstream drift of embryos and larvae, although the reasons suggested for the night drift are largely speculative; (ii) to net avoidance during daylight, particularly for older, more active individuals; or (iii) to diel shifts in vertical distribution (cf. Gadomski & Barfoot, 1998). Positive phototropism has been observed in experimental conditions and in situ for shads (Baglinière *et al.*, 2003; Cassou-Leins & Cassou-Leins, 1981; Ross *et al.*, 1993) whereas data on other ichthyoplankton *taxa* seem to point otherwise (Gadomski and Barfoot 1998). A two-stage explanation for the results is proposed.

On one hand, the relatively higher rainfall (and thus river discharge) during April and particularly May 1998 (>50% than monthly average for the last 50 years) promoted the stratification of the water column (Andrade, 1986). This phenomenon, together with the tide patterns observed during sampling (*cf.* Figure 2.3), should have favoured the active vertical displacement of larvae towards the surface during the day (when flood tides predominated). This might be another example of tidally oriented (migration) movements by zoo- and

ichthyoplankton (Fuiman & Higgs, 1997; Kimmerer *et al.*, 1998). The vertical displacement of larvae towards the surface during flood tides benefits the retention of a higher number of shad larvae in the upper estuary before their physiological adaptation to conditions in the lower River Mira. Henderson (1987) also found that tides influence the vertical distribution of larval herring in the River Blackwater estuary: surface waters were avoided during both flood and ebb tides, but used during slackwater. This behaviour also contributed to a net upstream movement of larvae. The data seems to support both explanations depending on date of sampling. Moreover, young of *Alosa* spp. do not emigrate to the lower estuary before developing salinity tolerance (Zydlewski & McCormick, 1997) and until certain habitat conditions become unfavourable in the upper reaches (Aprahamian, 1988). This was not observed for embryos, which are incapable of active swimming, and is in accordance with previous findings concerning the embryos of *A. sapidissima* (Gadomski & Barfoot, 1998).

On the other hand, the greater incidence of larvae with visible gut contents during daytime hauls could justify, at least partially, the vertical displacements of larvae also as a function of potential prey, *e.g.* microplankton or crustacean *nauplii* (Blaxter & Hunter, 1982; Cassou-Leins & Cassou-Leins, 1981; Crecco & Blake, 1983). Actually, despite the non-significance of correlation analyses, some unexpected occurrences of prey were coincident with particularly higher peaks of shad larvae abundance (*cf.* Figure 2.4). In fact, clupeoid larvae are visual feeders, reacting to prey at a distance of 0.7-1.0 larval length (Blaxter & Hunter, 1982), and feeding is confined to daylight hours (Ré, 1994). Hence, Twaite shad larvae would migrate to superficial waters during the day in order to increase prey perception and feeding success. The results of the proportion of larvae with gut content seem to confirm this later hypothesis. Notwithstanding, the likely competition and/or the vulnerability to other *taxa*, *e.g.* medusae, copepods, juvenile or adult fishes (Bailey & Houde, 1989). This liability would not be largely increased considering the high turbidity observed throughout the sampling period (1.4 to 2.7 mg DW m^{-3} which corresponded to Secchi depths of less than 20 cm) and the expected lower predation pressure from planktivorous fishes in bright light.

The average RNA/DNA ratio of larvae collected at dawn and dusk were significantly lower when compared to larvae caught at night or during the day. This daily pattern is different from those reported for *Sciaenops ocellatus* (Rooker & Holt, 1996) and *Sardina pilchardus* (Chícharo *et al.*, 1998b) and could be related to the following: 1) under appropriate environmental conditions fish larvae minimise stage-specific mortality through increased growth (Houde,

1997a). However, at dawn and dusk the more appropriate light intensity would allow predators to forage and prey on energy-rich larvae and consequently reduce the mean RNA/DNA ratios at those periods of the day. Actually, within cohorts of larvae, predators consume preferably the larger, and eventually better-fit, individuals (Pepin *et al.*, 1992); or 2) the increase in RNA concentration, and thus of RNA/DNA ratios, during the night might take place in response to circadian periodicity in endocrine activity as suggested by Rooker & Holt (1996). The circadian periodicities are most likely under control of growth-regulating hormones, which may present cyclic variations driven by photoperiod; on the other hand, 3) some of the daytime rise in the RNA/DNA index might be the result of nucleic acids of the prey remaining in the gut of larvae after the period of higher feeding activity: up to 5% of the DNA and 12% of the RNA contents of individual shad larvae (Author, unpublished results). Nevertheless, the daily pattern of RNA/DNA ratio is still present in larvae without prey items visible in their gut (*cf.* Figure 2.7). Similarly, Clemmesen (1996) found no significant difference in RNA or DNA content of herring larvae with and without gut content.

A high proportion of the shad *sagittae* read (*ca.* 73%) was used in the analyses and estimates were highly reproducible (*cv*=7.3%) suggesting the usefulness of otolith microstructural analysis to study age and growth of shad larvae. Savoy and Crecco (1987) also obtained good precision for repeated age estimates of *A. sapidissima*. Despite the non-significant results of the marginal increment analysis it is reasonable to suspect that the deposition of a growth increment on shad larvae *sagittae* is completed at the end of the day (*ca.* 2100 – 2300 hours), when the marginal-to-previous increment ratio decreased somewhat. Unfortunately, no *sagittae* from larvae collected at 0100 – 0300 hours were readable and only a 24-hour period was studied to test the above suspicion. Geffen (1992) warns to the disadvantages of using marginal increment analysis to validate the timing of increment formation instead of other (more appropriate) methodologies. Nevertheless, current knowledge states that increments are deposited daily as a result of an endogenous circadian rhythm and microstructural growth patterns appear to be ubiquitous among all species (see e.g. Campana & Neilson, 1985). Moreover, Savoy & Crecco (1987) found close agreement between the number of increments and the true ages of known-age larvae. They concluded that increments are deposited daily on *sagittae* of *A. sapidissima* and thus reflect their age.

Fish larvae attempt to maintain high levels of condition and growth rates in order to reduce stage-specific duration and liability to starvation and predation (Houde, 1997a). The diel

variation in the RNA/DNA ratios of shad larvae reflects this life-strategy. Only at dawn and dusk the better-fit larvae might be removed by predation. Also, Twaite shad larvae seemed to migrate to superficial waters of the upper River Mira during daylight in order to profit from favourable tidal dynamics and in response to prey availability. The proposed behaviour ensured the retention of larvae in the beneficial waters of the upper estuary and might be an attempt to achieve optimal foraging strategy and habitat use. This was not observed for embryos, the distribution of which is apparently controlled largely by the hydrological characteristics of the river segment downstream of spawning area (Ross *et al.*, 1993). It was not possible to strictly confirm the daily deposition of growth increments on *sagittae* of larval shad, but the results suggest that increment's deposition be completed during the night. These findings require further confirmation under controlled conditions (experiments with constant light, temperature, prey densities, etc.), but support the choice of daytime sampling of shad early life-history stages in the upper River Mira.

Chapter 3. Modelling the seasonal distribution patterns of eggs, embryos and larvae of Twaite shad *Alosa fallax fallax* in a lowland tidal river (River Mira)

3.1. Introduction

Recruitment to commercial or adult stocks of fish is believed to depend primarily on survival during the annual re-colonisation of the environment by larval stages. Several hypothesis linking abiotic and biotic factors with larval survival have been presented (see e.g. Houde, 1987 for a review). However, it is not generally agreed whether density-independent (temperature, advection) or density-dependent mechanisms (starvation, predation) prevail in determining the survival of larvae. Perhaps the relative impact of those mechanisms is species- and/or ecosystem-dependent. The spatial and seasonal distribution and abundance of organisms in ecosystems is of crucial importance for understanding population ecology and ecosystems functioning (see e.g. Brosse & Lek, 2000 and references therein).

Several aspects of population biology and ecology of the anadromous clupeid, Twaite shad *Alosa fallax fallax* (Lacépède, 1803), have been reviewed recently (Aprahamian *et al.*, 2003; Maitland & Hatton-Hellis, 2003; Quignard & Douchement, 1991b). *A. fallax fallax* is presently classified as "Vulnerable" by the Instituto de Conservação da Natureza using IUCN criteria (Cabral *et al.*, 2006). The knowledge of Twaite shad biology and ecology, which still migrates into several rivers along Portugal to spawn (Eiras, 1977), has been addressed by several authors (Alexandrino, 1996; Assis, 1990; Assis *et al.*, 1992; Collares-Pereira *et al.*, 2000; Costa *et al.*, 1987; Pina, 2000; Sabatié *et al.*, 1996). In the River Mira, aspects of reproductive biology have been studied in recent years (Pina, 2000; Pina *et al.*, 2003) and just a few larvae of *Alosa* spp. were caught by Esteves (1999), in the upper River Mira estuary.

Quantitative assessment of the relative importance of abiotic and biotic factors on fish larvae

distribution is lacking for most Portuguese estuaries, *e.g.* Esteves *et al.* (2000b) used multiple linear regressions to relate several environmental variables with fish larvae abundance in the estuary of River Mira. Some statistical methods such as generalised additive models (GAM) provide an interesting approach comparable to regression analysis, but are particularly efficient for predicting non-linear data and explaining complex relationships between variables (Hastie & Tibshirani, 1990). These features are commonly used to describe the ecology of the early life-history stages of fish and therefore might be useful to study the relationship between environment and aspects of larval fish ecology (distribution abundance, etc.).

In this study, the aims were, first, to describe the putative seasonal and inter-annual patterns of distribution and abundance of Twaite shad *A. fallax fallax* early life-history stages in River Mira, a lowland tidal river. Secondly, to examine how changes in several environmental variables affect the within-year trends in the abundance and distribution of eggs, embryos and larvae of Twaite shad using GAM.

3.2. Material and Methods

Study area

The Mira River is a 145-km long, relatively narrow (<400 m near the mouth), shallow (<10 m deep near the mouth) and flat (mean slope of 17%) watercourse located in SW Portugal. Its drainage basin area is *ca.* 1576 km^2 at a mean altitude of 156 m. Tidal influence extends to approximately 40 km from the mouth (at Odemira), where mechanical effects and salinity changes have been observed. About 70 km upriver, the Santa Clara Dam was built in the late 1960's for irrigation and municipal water supply. With a reservoir area of 1986 ha (at full supply level) it can storage about 485×10^6 m^3 of water from a sub-catchment area of 552 km^2 (about 30% of total catchment). Equipped with a spillway (vertical well) capable of discharging up to 208 m^3 s^{-1} of water, the dam is sporadically used to regulate the river flow although mimicking the natural pattern. For further information visit the web site of the Serviço Nacional de Informação sobre Recursos Hídricos do Instituto Nacional da Água (SNIRH at http://snirh.inag.pt/snirh/). Table 3.1 summarises the information available in the literature and from personal observations describing the river sections sampled in this study.

Sampling

Between February 1998 and July 2000, samples were collected every two weeks in six stations located in the River Mira estuary (Figure 3.1). Sampling took place during daylight hours from 0900 hours to about 1900 hours (see Chapter 2). The stations covered two major regions of the estuary (sensu Almeida, 1996), the euhaline zone near the river mouth (stations no. 1 – 3) and the upstream transition area from the oligohaline to limnetic section of the river, close to the presumed spawning locations (stations no. 4 – 6). Occasionally, samples could not be collected due to equipment failure or severe weather, but at least six sampling dates each spawning season were accomplished. From 1998 to 2000 the sampling effort increased towards the upstream stations in an attempt to enlarge sample size near the suspected spawning areas.

Table 3.1 – Features of sampled stations in the River Mira (SW Portugal). River km (distance from river mouth) and Approximate area (inundated section of river) were obtained from digitised images (TIFF format) of 1:25000 topographic charts using ImagePro Plus® 3.0 (Media Cybernetics). Stations' designations were 1 – Vila Nova de Milfontes; 2 – Monte da Asneira; 3 – Alto do Zambujeiro; 4 – Cais de Odemira; 5 – Cemitério de Odemira; 6 – Cais 1º de Maio. Characteristics used for description of each station were compiled from Andrade (1986) (a) as well as from field observations (b).

Station no.	River km	Approximate area sampled (m^2)	Description
1 (a)	1	276708	250 – 450 m width and ≤5 m depth channel with sandy sediment and submerged vegetation (particularly *Zoostera* spp.) near the river margins; surface current velocity <0.7 m s^{-1}.
2 (a)	5	143346	125 – 200 m width and 8 – 10 m depth channel with river-bed consisting of sand and submerged vegetation (mainly *Zoostera* spp.) near the river margins; water surface current velocity <0.6 m s^{-1}.
3 (a)	9	194800	90 to 145 m wide and <3 m deep channel; mixed sediment composition (very fine silt up to sand and pebbles).
4 (b)	34	42311	20 – 45 m wide and less than 3.5 m deep; mixed sediment composition (very fine silt up to coarse sand, pebbles and cobbles) with abundant debris.
5 (b)	35	27720	Width of 25 – 35 m and depth of ranging from <0.5 to 3 m; sediment composed of very fine silt, sand, pebbles and cobble, with submerged vegetation and debris; range of superficial flow velocity 0.5 – 1.5 m s^{-1}.
6 (b)	37	17395	20 to 35 m wide and <0.5 to approximately 2 m deep, assorted sediment composition (very fine silt, coarse sand, pebbles, cobbles and even boulders) with submerged vegetation; river surface flow *ca.* 0.95 m s^{-1}.

At each station and sampling occasion, mesozooplankton was collected with a conical net (1.6 m long, ∅ 0.37 m, 0.5-mm mesh size) towed 1 m below the surface, at a constant speed of approximately 1 m s^{-1} for 10 minutes. Plankton tows sampled evenly the river margins and the main channel (see Chapter 2) over the area encompassed by each station (Table 3.1). A

flowmeter (Hydrobios®) was attached inside the net to estimate the water volume filtered (median=62.8 m^3, range=14.96 – 113.07 m^3). Zooplankters were stored in buffered 4% formaldehyde solutions. A further 26 – 39 L of water were sampled at 0.5-m depth and filtered through a 63-μm mesh size sieve to collect microplankters. Two consecutive samples of meso- and microplankton were collected in several randomly chosen occasions to assess the variability of density estimates of various plankton *taxa* (43 samples). Time elapsed between consecutive trawls averaged 16 min. The coefficient of variation, $CV = \overline{x}/s \times 100\%$ (where $\overline{x}$ is the average density of two consecutive tows and s is the corresponding standard deviation), was used to evaluate the precision of density estimates. In this study, the median CV of relevant *taxa* ranged from 15.4% (for shad), through 26.6% (for microplankton biomass) and about 40% (for crustaceans) to 60.8% (for insects).

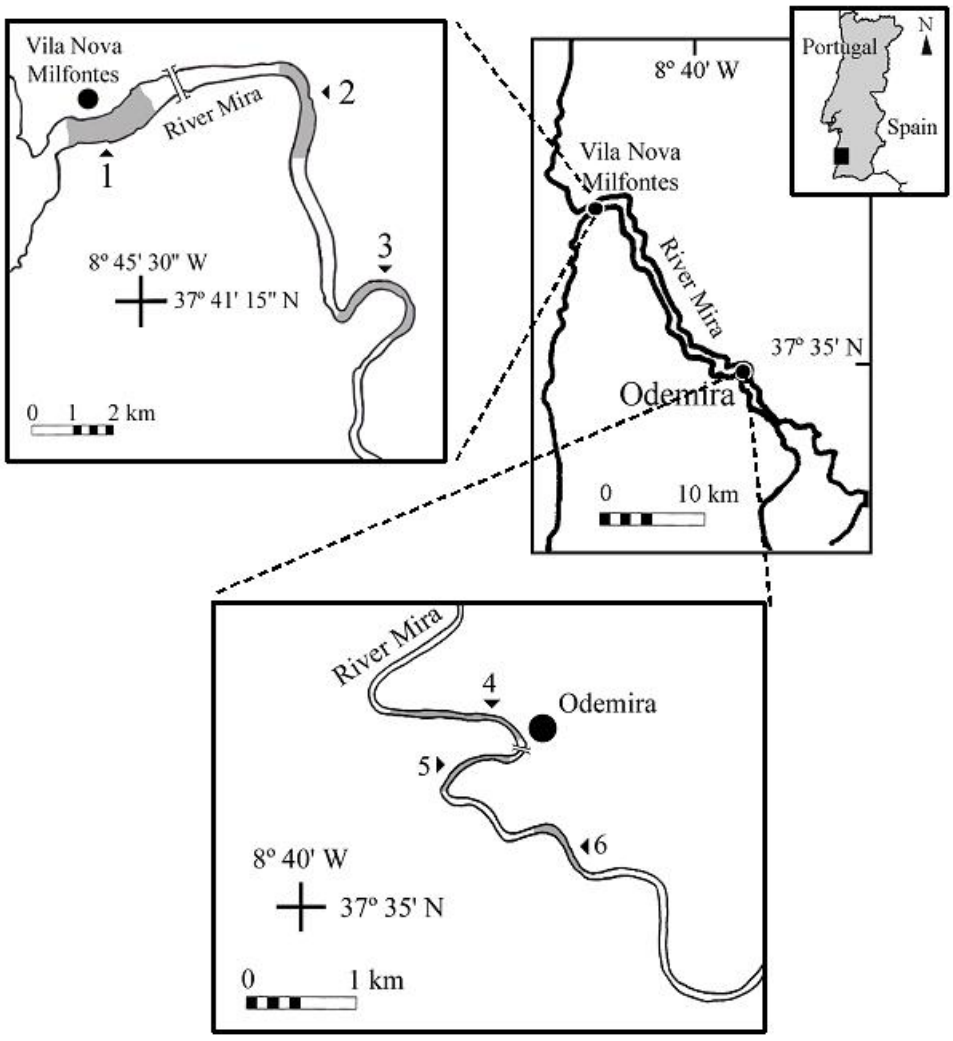

Figure 3.1 – Location of sampling sites (stations no. 1 to 6) in the River Mira (SW Portugal) near the mouth (Vila Nova Milfontes) and upriver at the limit of tidal intrusion (Odemira).

During plankton sampling, Secchii disk depth was determined, the state of tide was noted and the surface temperature (ºC) and salinity (ppt) were measured using a hand-held thermometer and a refractometer, respectively. Daily, monthly and annual rainfall data (mm) for the period 1 October 1950 – 30 September 2001 was obtained for a meteorological site (ref. 27G/01) near station no. 4 from the SNIRH (at http://snirh.inag.pt/snirh/). The SNIRH classifies hydrological years (*e.g.* 1 October 1997 to 30 September 1998, henceforth referred to as 1997/1998) comparing annual precipitation in one "year" to the average annual rainfall for the period of "hydrological years" 1940/1941 to 1997/1998. According to this classification, 1997/1998 was considered "humid" (110 – 125% of average annual rainfall), 1998/1999 was classified as "dry" (50 – 75%) and 1999/2000 was categorised as "average/dry" (75 – 90%). Daily cumulative rainfall values reflect the within-year changes as well as the inter-annual variation observed and therefore were used here to illustrate the classification suggested above (Figure 3.2). In 1997/1998 and 1999/2000 two heavy rainfall occurrences were registered during autumn (days 0 to 100) and late spring (around day 200) whereas during 1998/1999 no such events occurred.

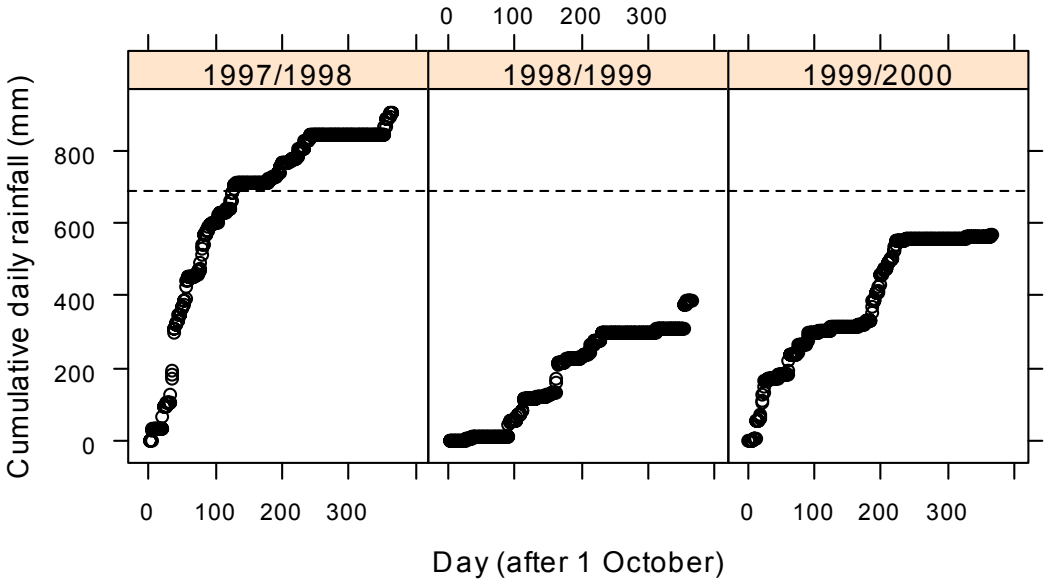

Figure 3.2 – Daily changes of cumulative rainfall (in mm) for the period February to July 1998 – 2000 scaled to day after 1 October. Average annual cumulative rainfall (in mm) for the period 1940/41 to 1997/98 was 686.7 mm and is represented by the horizontal dotted line (Source: SNIRH at http://snirh.inag.pt/).

Laboratory procedures

Fish early-life history stages and the remaining preserved mesozooplankters were identified using published information (Johnson & Loesch, 1983; Newell & Newell, 1963; Quignard &

Douchement, 1991b; Russell, 1976; Saville, 1964; Smith, 1977; Todd & Laverack, 1991). Apart from *Alosa* spp., fishes were identified to family level in order to minimise taxonomical errors and include the least represented species (Scheidegger & Bain, 1995). *Alosa fallax fallax* eggs are spherical, non-adhesive and relatively large (1.7 to 4.5 mm) with a wide periviteline space (see e.g. Aprahamian *et al.*, 2003) (Figure 3.3). Following Balon (1990), free embryos are fish from hatching until the transition to exogenous feeding (hereafter referred to as "embryos"). It is difficult, however, to determine if exogenous feeding has occurred in field caught specimens. Therefore, individuals with yolk sac were designated as shad embryos (Figure 3.3) and those with depleted yolk sac but still some evidence of a fin fold as shad larvae (Figure 3.4). During larvae processing the presence/absence of visible items in the gut was noted (Figure 3.4).

Figure 3.3 – Image of Twaite shad egg (top) and embryo (bottom) obtained with a CCD camera connected to a stereoscope.

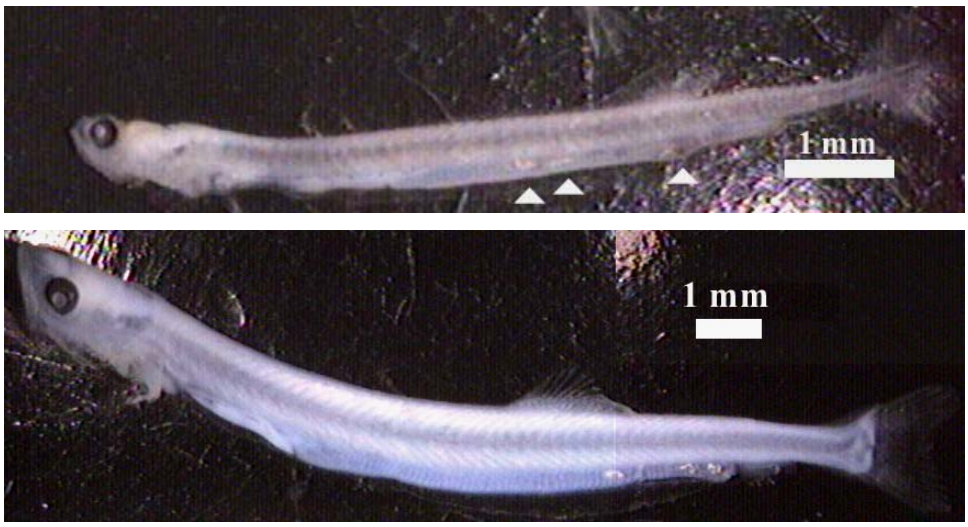

Figure 3.4 – Images of Twaite shad larvae with visible gut content (triangles point to food items on top) and without gut content (bottom).

Whenever mesozooplankters were extremely abundant, successive sub-samples with a folsom-type splitter were analysed and at least 300 organisms counted (Omori & Ikeda, 1992). Plankters' density was expressed as number of individuals per 100 m^3 of water.

To estimate microplankton biomass, aliquots of the homogenised samples were rinsed through a 1.2-µm milipore filter (∅ 25 mm, Macherey-Nagel® GF-3). Filters were dried in an electric oven at 60º C for 24 – 36 hours to obtain the dry weights and then incinerated at 450º C for 4 hours to determine the ash-free dry weight (AFDW). The results of microplankton biomass were expressed as ash-free dry weight per cubic metre of water (mg AFDW m^{-3}).

Statistical analyses

For the purpose of modelling the seasonal changes in shad embryos and larval densities several new predictor variables were created. After filtration of microzooplankton samples, the dry weights of filters were used as an estimate of water turbidity. This variable was named *turb* and expressed as mg DW per m^3. Precipitation in the upper River Mira area varies erratically (Figure 3.5). For this reason, moving averages of rainfall for the period 1 October 1997 – 30 September 2000 were used to tentatively describe the average rainfall conditions three, five and seven days prior to the sampling dates, *i.e.* variable *rainfall*. Unfortunately, this variable included 56% to 70% of zeros and hence was discarded. Instead standard errors of the moving averages (with a period of 7 days prior to sampling date; *e.g.* for day 139 refers to standard error of the moving average obtained from day 133 to 139) were used as an estimate of rainfall variability (*se.rain*). These two variables were significantly correlated (Spearman's rank correlation r=0.908, p<0.001), and the higher the estimate of *se.rain*, the greater the rainfall instability in the days that preceded sampling (Figure 3.6).

Densities of isopods, small insects, cladocerans and copepodites were pooled into a variable named, *zoo*. A second variable to describe these zooplankton *taxa, zoopl*, was used. It was derived from the product of the abovementioned *taxa* densities to (algebraically) reflect possible interactions. Another variable designated *pred* was the sum of the abundances of small medusae, mysids and amphipods. As a simple index of zooplankton diversity the proportion of *taxa* present in any one sample out of all the *taxa* collectable was named *index*. Only *taxa* that represented more than 1% of total abundance were used in further analyses.

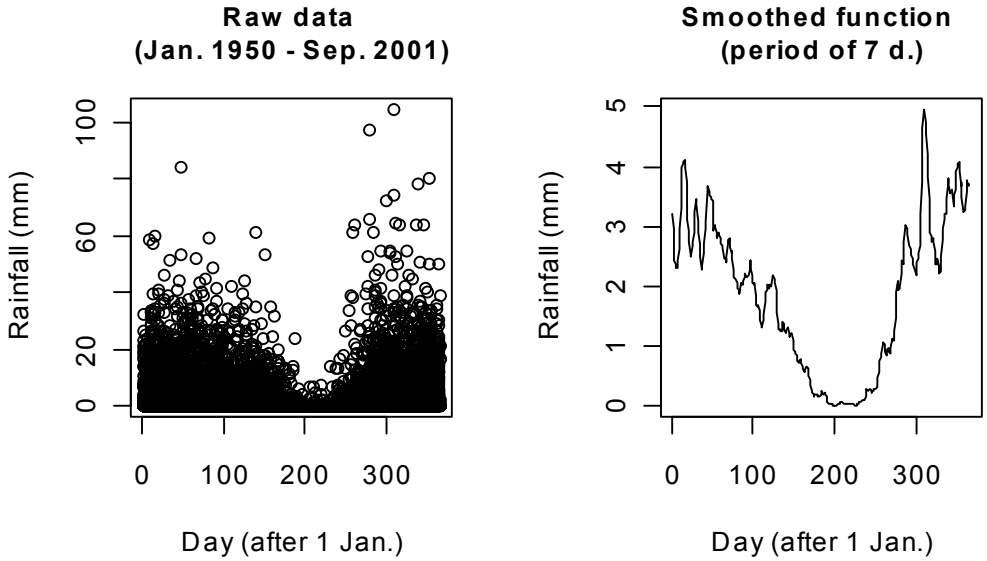

Figure 3.5 – Daily rainfall for a meteorological site (ref. 27G/01) near station no. 4 in the upper River Mira (source: SNIRH at http://snirh.inag.pt/). Note the different *y*-axis scales.

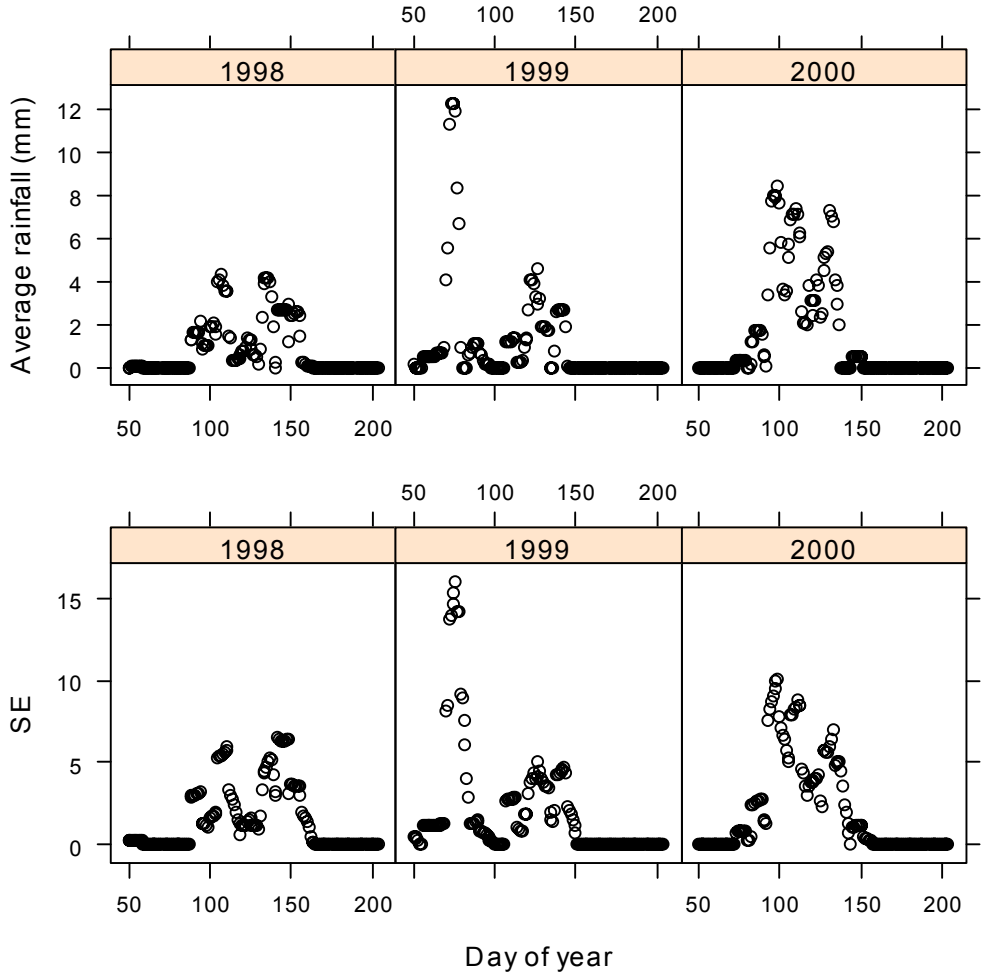

Figure 3.6 – Moving averages (7-day period) and standard errors (SE) of daily rainfall (mm) for the period February – July (days 50 to 205 after 1 January) at a meteorological site near station no. 4 in River Mira (see text for details).

Several predictor variables were transformed prior to analyses, *e.g.* $\log(x+1)$ or $\sqrt{x+0.5}$. Variables used for statistical analyses and modelling are presented in Table 3.2. All data were imported into `R` (R Development Core Team, 2003) as ASCII files and configured as data objects. The sampling and analytical procedures described above originated a 181 records by 25 variables data matrix (samples with no eggs, embryos or larval occurrences only comprised about 31% of the records).

Table 3.2 – List of variables used in this study of Twaite shad (units), their designations in our analysis and type of variables. *Trf.* – transformation used: none (blank space); logarithm (log); square-root (sqrt); [a] – pooled abundance of isopods, small insects, cladocerans and copepodites; [b] – includes small medusae, mysids and amphipods.

Variable	Designation	Type [transformation]
Density of larvae (ind. 100 m^{-3})	`larvae`	Continuous [log]
Presence/absence of larvae	`presence`	Binary (1/0)
Density of embryos (ind. 100 m^{-3})	`embryos`	Continuous [see text]
Density of eggs (ind. 100 m^{-3})	`Eggs`	Continuous [see text]
Year	`Year`	Discrete (1998 to 2000)
Month	`month`	Categorical (Feb. to July)
Day of year (from 1 January)	`Day`	Discrete (50 to 203 d)
Station no.	`Stat`	Categorical (1 to 6)
Tide	`Tide`	Categorical (Ebb, Flood)
Temperature (surface, º C)	`Temp`	Continuous
Salinity (ppt)	`sal`	Continuous
Turbidity (mg DW m^{-3})	`turb`	Continuous
Prior n-day average rainfall (mm)	`rainfall`	Continuous
SE of rainfall (period of 7 days, mm)	`se.rain`	Continuous
Microplankton biomass (mg AFDW m^{-3})	`micro`	Continuous
Insects (ind. 100 m^{-3})	`ins`	Continuous [log]
Copepodites (ind. 100 m^{-3})	`copep`	Continuous [log]
Isopods (ind. 100 m^{-3})	`iso`	Continuous [log]
Decapods nauplii (ind. 100 m^{-3})	`naupli`	Continuous [log]
Amphipods (ind. 100 m^{-3})	`anf`	Continuous [log]
Mysids (ind. 100 m^{-3})	`mys`	Continuous [log]
Zooplankters [a] (ind. 100 m^{-3})	`zoo`	Continuous [log]
Zooplankton interaction	`zoopl`	Continuous [log]
Potential predators [b] (ind. 100 m^{-3})	`pred`	Continuous [log]
Diversity	`index`	Proportion (0 to 1)

An analysis of variance (ANOVA) approach was used first to understand the general patterns of seasonal distribution of early life-history stages of Twaite shad. Factors *Year* (1998, 1999 and 2000), *Month* (February to July), *Station* (no. 4 to 6) and *Tide* (Ebb and Flood) were considered fixed. Stations no. 1 to 3 were excluded from further statistical analyses because no eggs, embryos or larvae of Twaite shad were collected at those sites during this study. For shad eggs,

ANOVA could not be performed due to the severely unbalanced design. Several combinations of factors were not observed and therefore their effects on the density of eggs could not be estimated. Instead visual exploration of possible distribution patterns was done. Due to non-constant variance of the response-variable (densities of embryos or larvae) in the design (Figure 3.7, top plots), further insight on the choice of transformation was required. The Box-Cox profile likelihood function (3.1) for the largest linear model is commonly suggested (Venables & Ripley, 1999) as a guide for choosing the value of λ (see below), which will then remain fixed. The profile likelihood function is

$$\hat{L}(\lambda) = const - \frac{n}{2}\log RSS(z^{\lambda}) \tag{3.1}$$

where $z^{\lambda} = y^{\lambda} / \dot{y}^{\lambda-1}$, $\dot{y}$ is the geometric mean of the observations and $RSS(z^{\lambda})$ is the residual sum of squares for the regression of z^{λ}. The transformed response-variable y^{λ} is obtained through analysis of variance of the model

$$y^{\lambda} = \begin{cases} (y^{\lambda} - 1)/\lambda & \lambda \neq 0 \\ \log y & \lambda = 0 \end{cases}$$

where $y = \text{density} + 1$. Calculated values of λ were in the range –1.5 to –2 for embryos and –0.4 to –0.2 for larvae (Figure 3.7, lower plots). Consequently, the inverse abundances of embryos were squared, $1/(y+1)^2$ and the inverse of larval abundances was cube root transformed, $\sqrt[3]{1/(y+1)}$.

Starting with the complete (full) model (*e.g.* Larvae = Year × Month × Station × Tide)[3] several models were tested using sequential analysis of variance. Inclusion or deletion of terms obeyed the marginality principle and the effect of adding or removing individual terms from a model was assessed through model's statistics, *e.g.* residuals sum of squares (Venables & Ripley, 1999). Models were further investigated using diagnostic plots (fitted values *versus* residuals and normal probability plots of residuals).

To model the relationship between several abiotic and biotic predictors (see Table 3.2) and seasonal density distribution of shad eggs, embryos and larvae living in the upper River Mira, Generalised Additive Models (GAM) were used (Hastie & Tibshirani, 1990). Data was screened

[3] The "×" implies that the formula expands to Larvae = Year + Month + Station + Tide + Year x Month + ... + Year × Month × Station + ... + Year × Month × Station × Tide (including 2nd, 3rd and 4th order interactions of terms).

to reveal characteristics of data sets and scatterplots were made for each pair of variables to visually assess any relationships. The very few occurrences of shad eggs (just ten samples during the year 2000 included eggs) precluded this analysis for that life stage.

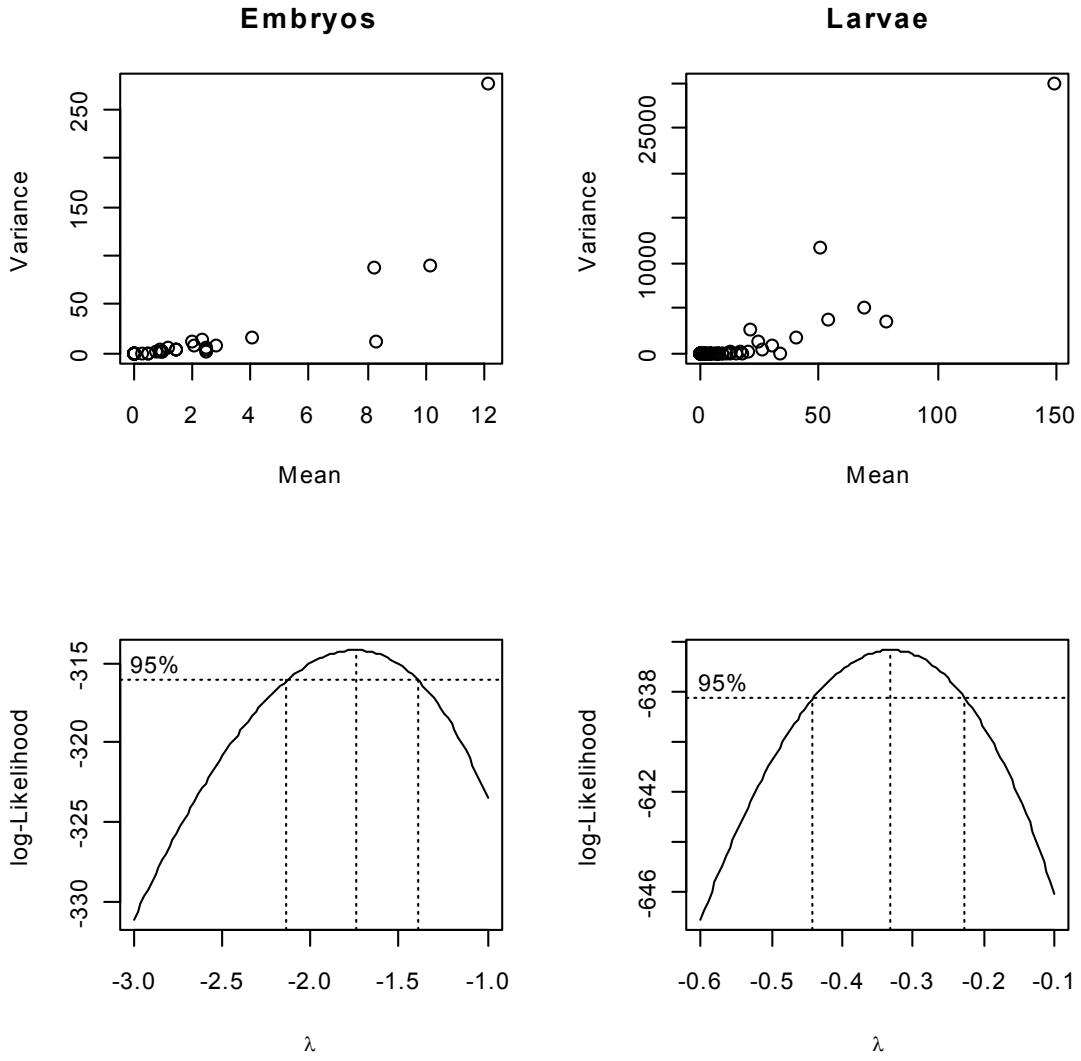

Figure 3.7 – Variance-to-mean plots of abundance data for Twaite shad (top) and plots of the Box-Cox profile likelihood function (bottom) with superimposed 95% confidence intervals for λ (see text for details).

GAM extend the range of applications of Generalised Linear Models by allowing non-parametric smoothers in addition to parametric forms combined with a range of link functions (linear, log, logit, etc), which describe the error structure of the model, *i.e.* the variation of $E\left(Y\middle|X_j\right)$. The relationship between a dependent variable Y and a set of predictor variables X_1, X_2, …, X_p can be modelled as

$$Y = \alpha + \sum_{j=1}^{p} f_j(X_j) + \varepsilon \qquad (3.2)$$

where f_j are rather general (unspecified) functions that can be estimated in a flexible manner, using a simple algorithm. This algorithm is basically a scatterplot smoother and the estimated function $\hat{f}_j(x_j)$ can then reveal possible non-linearity in the effect of x_j. A modern alternative is to use *spline* functions, which impose smoothness directly on $f(x)$. A function $f(x)$ that minimises

$$\sum w_i[y_i - f(x_i)]^2 + \lambda \int (f''(x))^2 dx \tag{3.3}$$

is sought. Notice that $\int f''(x)^2$ measures the "wiggliness" of the function *f*. λ is a non-negative smoothing parameter that must be chosen by the data analyst. It governs the trade-off between the goodness-of-fit to the data and "wiggliness" of the function. Large values of λ force the *f* to be smoother. For any value of λ, the solution is a cubic *spline*, *i.e.* a piecewise cubic polynomial function. It is convenient to express the smoothness of f_j using the "degrees of freedom" or "effective number of parameters" *df*. The number of *df* can be chosen arbitrarily or calculated automatically by cross-validation. The *df* can be interpreted as penalties for the complexity of the non-parametric curve fitted.

A *backfitting algorithm* fits the smooth functions f_j in (3.2) one a time by taking the residuals

$$Y - \sum_{k \neq j} f_k(X_k) \tag{3.4}$$

and smoothing them against X_j using *smoothing splines*. This process is repeated until it converges (formally this is a Gauss-Seidel algorithm). The algorithm separates the parametric from the non-parametric parts of the fit and any linear terms in the model are fitted by least squares. Predictors were modelled using smoothing *splines* with *df* chosen by cross-validation (Venables & Ripley, 1999). Herein, raw data on larval density was used as the response variable and a Poisson distribution together with a log link function was used to describe the error structure because it is often appropriate for count data (in this case, fish density was estimated as larval numbers) (Swartzman *et al.*, 1992). Moreover, GAM modelled predictors using smooth functions with about 3 *df* (range of 2.8 to 3.1). Inclusion or deletion of predictor variables obeyed the marginality principle and the effect of adding or removing individual terms from a model was first assessed visually using the model's effects plot. Furthermore, models were compared and chosen using sequential analysis of deviance (with χ^2–test statistic), Akaike's Information Criterion (AIC, see below) and interpretation of diagnostic plots (fitted *versus* residuals and normal probability plots of residuals). The AIC can be computed using

$$AIC = -2\log_e(\ell(\hat{\theta} \mid data)) + 2K \tag{3.5}$$

where $\log_e(\ell(\hat{\theta} \mid data))$ is the value of the maximised log-likelihood over the unknown parameters (θ) and K is the number of parameters of the fitted model (Anderson *et al.*, 2000; Burnham & Anderson, 1998). The AIC relates Kullback-Leibler information (a dominant paradigm in information and coding theory) and maximum likelihood (the predominant paradigm in statistics) (Anderson *et al.*, 2000; Burnham & Anderson, 1998). The model where AIC is minimised is selected as the best for the empirical data at hand. Models were ranked using ΔAIC, the difference between a model's AIC and minimum AIC, and the likelihood of models i=1, 2, ..., R assessed by the Akaike's weights w_i:

$$w_i = \frac{\exp(-\frac{1}{2}\Delta_i)}{\sum_{i=1}^{R}\exp(-\frac{1}{2}\Delta_i)} \tag{3.6}$$

The w_i can be interpreted as approximate probabilities that model i is, in fact, the most adequate in the set of models considered. To measure the goodness-of-fit of each model, a pseudo-coefficient of residual determination, *PCf*, was estimated (Bellido *et al.*, 2001; Swartzman *et al.*, 1992):

$$PCf = 1 - \frac{RD}{ND} \tag{3.7}$$

where *RD* is the residual deviance, *i.e.* the deviance of the full model, similar to the residual sum of squares in a linear model, and *ND* the null deviance, *i.e.* the deviance of the model with only the intercept term. Finally, Spearman's rank correlation coefficients between observed and predicted values of shad density were used to assess the predictive potential of the models.

All statistical procedures described above were implemented in `R` (R Development Core Team, 2003), namely GAM fitting through `R` package `mgcv` (Wood, 2001), and are provided as text files upon request from the author. For all statistical analyses, the level of significance was set at 0.05 and *p*-values greater than 0.10 were considered as indicative of non-significance.

3.3. Results

No shad eggs, embryos or larvae were collected near the river mouth during this study. At the estuarine stations (no. 1 to 3) surface temperature and salinity increased gradually reaching about 24 ºC and >30 ppt by mid-July whereas turbidity fluctuated between 1 and 2.5 mg DW m^{-3}

during the sampling period (Figure 3.8). All *A. fallax fallax* specimens mentioned herein were sampled upstream of station no. 4 (inclusive). At these stations, temperature increased consistently from about 16 ºC in late-Winter to more than 26 ºC in early-Summer. The patterns of salinity and turbidity changes were somewhat different between years. In 1998, salinity averaged 1 ppt whereas in 1999 and 2000 conflicting dynamics were depicted: salinity increased with day of sampling during 1999 (up to 4 ppt in the summer) and decreased during the year 2000 spawning season (from 5 ppt to about 0.5 ppt). In 1998 and 1999, turbidity averaged 2.2 and 1.3 mg DW m^{-3} respectively while in 2000 the quantity of suspended materials in the water exhibited a distinct pattern and peaked during April (about 6 mg DW m^{-3}). The rainfall regimes during the sampling periods in 1998 and 2000 were quite similar, raining persistently from late-March until late-May (from around day 80 to day 150). Conversely, in 1999 a clear bimodal rainfall pattern was registered with higher values around days 70 and 125, *i.e.* early-March and early-May (*cf.* Figure 3.6).

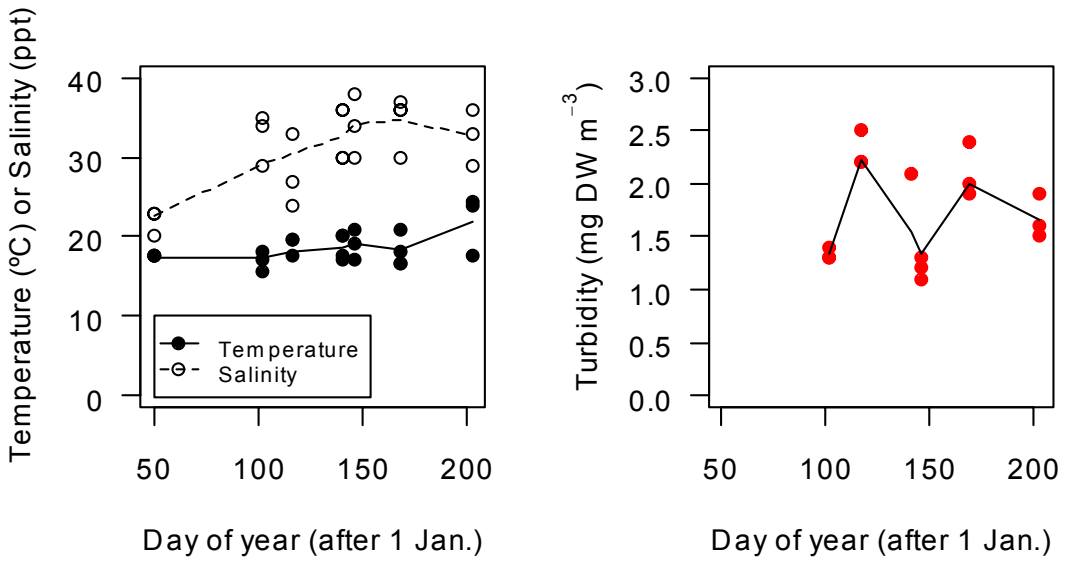

Figure 3.8 – Changes in surface temperature, salinity and turbidity with day of year (after 1st January) measured during larval sampling at the estuarine stations (downriver stations no.1 to 3). Smoothed lines are shown for illustrative purposes only. Days of year 100 and 150 corresponded to 9 April and 29 May, respectively.

At the riverine stations (no. 4 to 6), where Twaite shad occurred, no more than 69 eggs 100 m^{-3}, 36 embryos 100 m^{-3} or 380 larvae 100 m^{-3} were collected. Shad eggs were caught only during the spawning season of the year 2000 and there were only ten registered occurrences ranging in density from 1.3 to 68.3 eggs 100 m^{-3}. During that year a greater number of shad embryos and larvae were caught in comparison to 1998 or 1999. Yearly mean densities of embryos did not

exceed 3 ind. 100 m^{-3} (1998: 0.81; 1999: 0.32; and 2000: 2.61 ind. 100 m^{-3}) while the average density of larvae varied between 4.94 ind. 100 m^{-3} in 1999 and 26.47 ind. 100 m^{-3} in 2000 (in 1998: 11.17 ind. 100 m^{-3}).

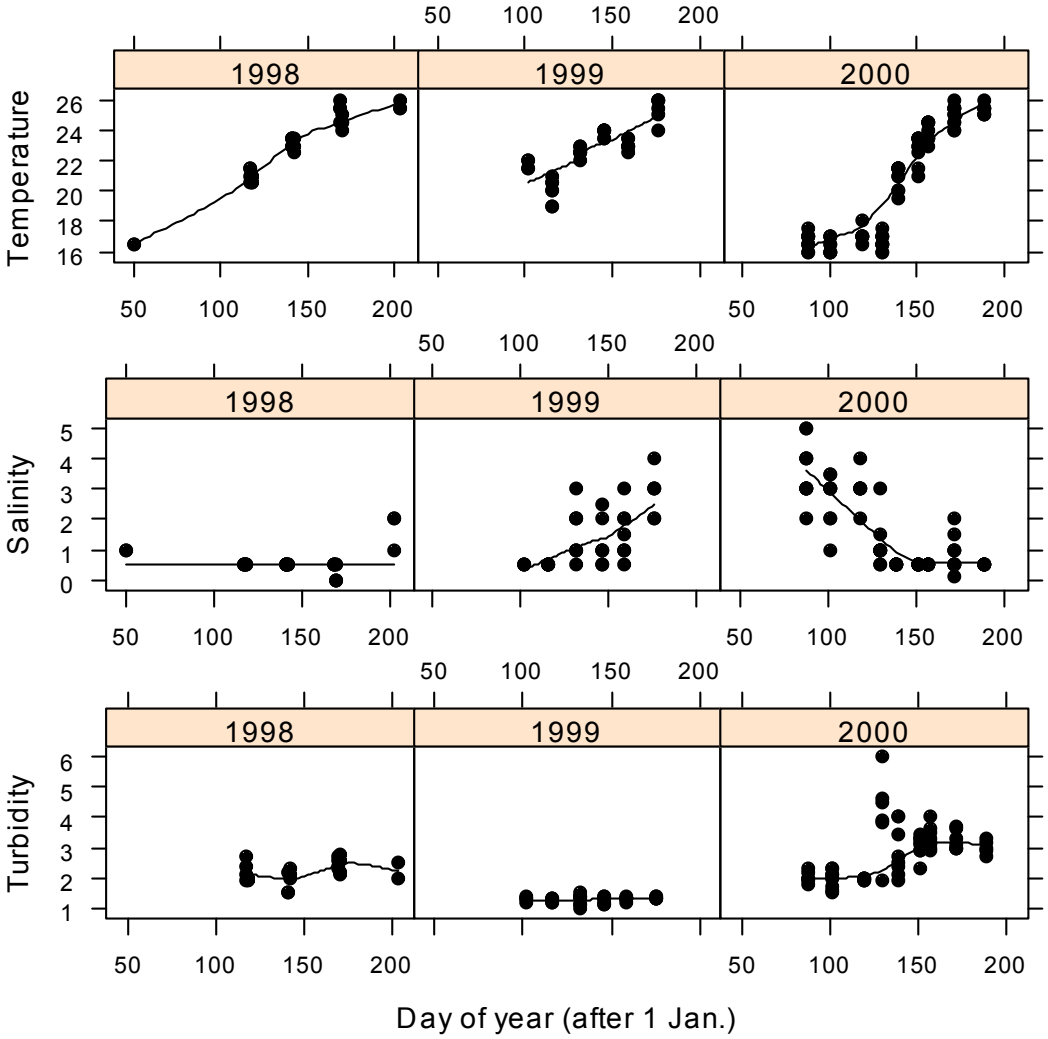

Figure 3.9 – Changes in surface temperature (º C), salinity (ppt) and turbidity (mg DW m^{-3}) with day of year (after 1st January) at the riverine stations (no. 4 to 6) sampled in River Mira. Spline models fitted are shown for illustrative purposes only. Days of year 100 and 150 corresponded to 9 April and 29 May, respectively.

The few occurrences of shad eggs precluded the analysis of variance but relatively simple ANOVA models with few terms were found to significantly describe the seasonal changes of shad embryos and larvae abundance, transformed according to Box-Cox likelihood function, in the upper River Mira ("Best Model", Table 3.3). These models can be written as:

$$(Embryos+1)^{-2} = Year + Month + Station + Tide + Year \times Station$$

and

$$(Larvae+1)^{-\frac{1}{3}} = Year + Month + Station + Tide + Year \times Month + Year \times Station + Month \times Tide\ .$$

The significance of the interaction terms in both equations indicates that the behaviour of the response-variable (*i.e.* embryos or larvae density) varied within levels of factor *Year* or *Month*. Consequently data were analysed separately by year (Table 3.4). The significance of factor *Month* in several of these later (yearly) models suggests a noticeable temporal pattern of embryos and larval densities. In 1999, also *Station* and *Tide* (interacting with factor *Month*) were found to have an effect on larval abundance.

Table 3.3 – Sequential ANOVA table for the models of seasonal changes in the density of Twaite shad embryos and larvae. Legend: *RSS* – Residual sum of squares; *RSS df* – RSS degrees of freedom. The superscripts after model designation group non-significantly different models (see p-values of models). Model designations are self-explanatory except for: "High-order" – similar to the full-model but without the 4th order interaction Year x Month x Station x Tide; "Special" – model with main effects and 2nd order interaction involving factor *Year*; and "Best model" – model selected using an iterative procedure based upon the analysis of deviance and AIC (see text for details).

Model	Significant terms (total number of terms)	RSS	RSS df	p-value
Embryos				
Full model [a]	3 (15)	13.46	98	
High-order [a]	3 (14)	13.85	136	0.4229
"Special" [a]	4 (7)	16.34	141	0.9826
"Best" model [a]	4 (5)	17.27	143	0.4988
Additive [b]	3 (4)	18.25	146	0.0751
Intercept [c]	(1)	27.43	156	<0.001
Larvae				
Full model [a]	5 (15)	4.01	98	
High-order [a]	5 (14)	4.16	101	0.2887
"Best" model [a]	5 (7)	5.10	110	0.8823
Additive [b]	1 (4)	8.89	146	<0.0001
Intercept [c]	(1)	12.06	156	<0.0001

Table 3.4 – Summary statistics of the ANOVAs by year performed to study the seasonal variation densities of early life-history stages of Twaite shad. Legend: *No. terms* – number of terms estimated; *RSS* (*df*) – Residual sum of squares (and respective degrees of freedom).

Year	No. model terms	Significant terms	*p*-value	Model RSS (df)
Embryos				
1998	5	none		3.86 (19)
1999	7	none		2.36 (22)
2000	7	Month	<0.0001	7.23 (57)
Larvae				
1998	5	Month	<0.0001	0.16 (19)
1999	7	Month Station Month x Tide	<0.001 0.0085 0.0282	0.48 (22)
2000	7	Month	<0.0001	3.37 (57)

Twaite shad eggs and embryos were found in samples from day 88 (late-March) through day 151 (late-May) but peaked two-weeks apart: around day 120 (late-April) for eggs and day 130-140 (mid-May) for embryos (Figure 3.10). No embryos were collected at station no. 5 and 6 in 1998 and no. 4 in 1999. On the other hand, larvae were already present in zooplankton samples at day 90 (late-March) but no larvae were collected after day 171 (mid-June). Higher densities were observed between days 130 and 150 (mid to late-May) (Figure 3.10). The pattern of larvae occurrences seemed slightly different amongst years.

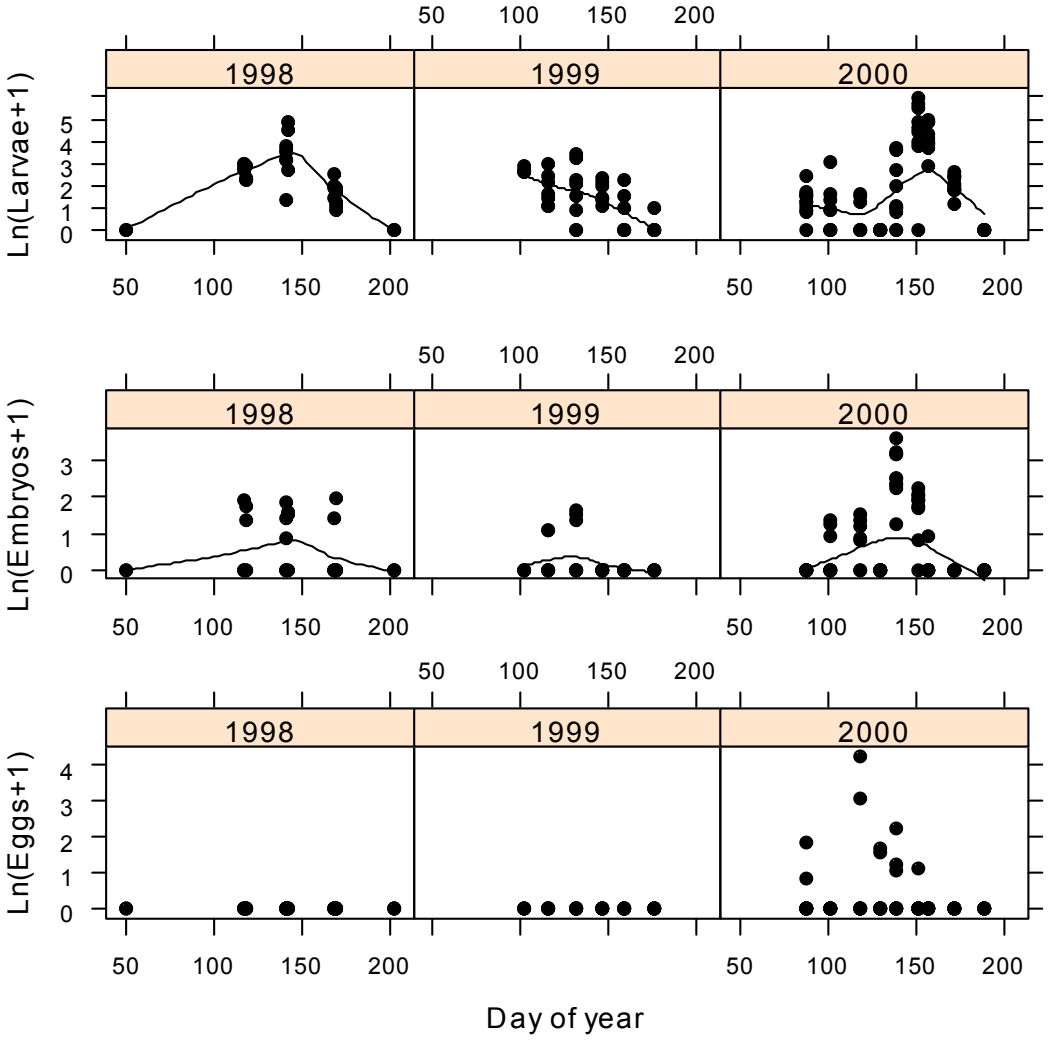

Figure 3.10 - Changes in density of Twaite shad eggs, embryos and larvae with day of year (after 1 January) plotted by year (1998 to 2000). Spline models fitted are shown for illustrative purposes only. Days of year 100 and 150 corresponded to 9 April and 29 May, respectively.

Highest numbers of Twaite shad eggs were collected when water temperature, salinity, turbidity and rainfall variability were 17 ºC, 3 ppt, 2 mg DW m^{-3} and 3.7 mm respectively. A much smaller but discernible increase in eggs' numbers was observed for water surface temperature of

21 ºC, salinity of 0.5 ppt, turbidity of 4 mg DW m^{-3} and 1.5 mm SE(rainfall) (Figure 3.11).

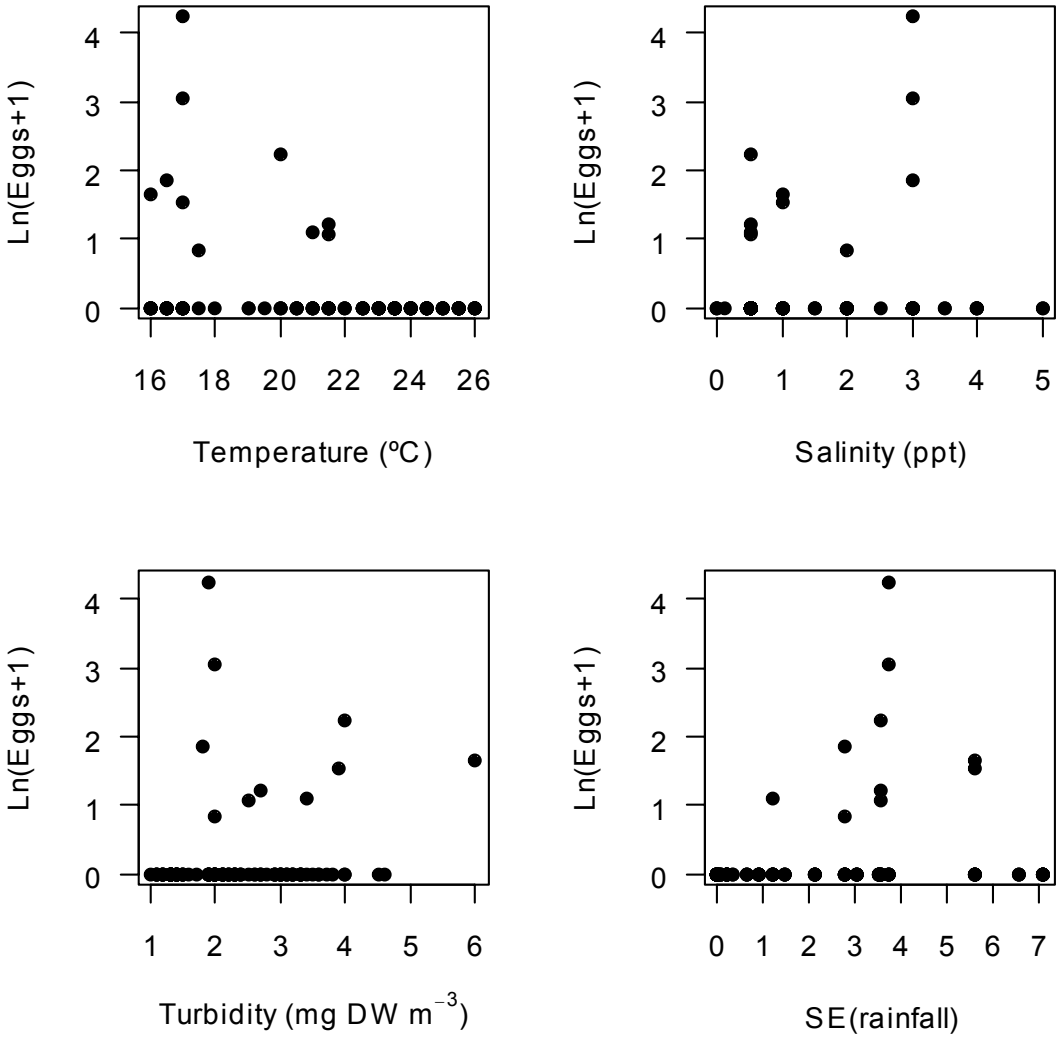

Figure 3.11 – Scatterplots of log-transformed shad eggs' abundance *versus* surface temperature, salinity, water turbidity, and rainfall variability [*i.e.* SE(rainfall)].

An additive model (GAM), which included abiotic predictors (temperature, salinity and rainfall) besides factors *Year* and *Tide*, was used initially to model the densities of shad embryos. It accounted for inter-annual and between-tides differences (seaz.29.gam). After several attempts to include/exclude environmental covariates or include other predictors of biological relevance (*e.g.* turbidity, diversity, zooplankters' and predators' abundances), model seaz.30.gam was chosen as the "best" model (lowest AIC and w_i=0.396). Two other GAM, seaz.41.gam and seaz.42.gam, were also found suitable for our data with $0.279 < w_i < 0.299$ (Table 3.5). During

the spawning season of the year 2000 more shad embryos were collected than in previous years. The effect of *Tide* was not significant. All other predictor variables had a non-linear effect on embryos' abundance (Figure 3.12). Density of embryos was higher for water surface temperature in the range 19 – 21 ºC and intermediate values of rainfall. More embryos were collected when salinity was less than 1 ppt whereas turbidity values higher than 4 mg DW m^{-3} contributed to a density decline of shad embryos in samples. A substantial proportion of the variance in the data was explained by each of the models tested (Pseudo-r^2>0.7) but problems with non-homogeneity of variances and distribution of residuals remained. Predicted densities by the "best" model were significantly correlated with observed values (Spearman's r=0.631, p<0.001) however discrepancies are readily visible and the model predicts the occurrence of embryos when none were actually collected (Figure 3.13).

Table 3.5 – Summary statistics of the generalised additive models (GAM) fitted to seasonal data on shad embryos' density in the upper River Mira. Also included are the results of the analysis of deviance performed on these models. Legend: *k* – no. of terms (apart from "intercept"); RD – residual deviance; df – RD degrees of freedom; p-value – significance of the analyses of deviance with χ^2 test; AIC – Akaike's Information Criterion (the higher the AIC of a model the less plausible it is for the data at hand); ΔAIC – difference between models' AIC and minimum AIC; w_i – Akaike's weights are approximate probabilities of model adequacy; and PCf – pseudo-coefficient of determination (see text for details).

Model	k	RD	df	p-value	AIC	ΔAIC	w_i	PCf
seaz.29.gam	4	266.37	140.40		325.55	43.76	0.0000	0.720
seaz.30.gam	5	210.27	108.54	0.0051	281.79	0.00	0.3957	0.742
seaz.41.gam	6	197.96	104.66	0.0139	282.49	0.70	0.2789	0.757
seaz.42.gam	7	185.64	100.75	0.0140	282.35	0.56	0.2991	0.772
seaz.43.gam	8	176.91	96.82	0.0655	287.21	5.42	0.0263	0.783

Several Generalised Additive Models (GAM) were fitted to the density data of shad larvae. The set of candidate models is presented in Table 3.6. All of the GAM fitted the data well (Pseudo-r^2>0.85). Apart from the starting and most complete model (seaz.10.gam), one model was singled-out from the remaining (seaz.19.gam). This GAM with 12 terms (2 factors and 10 continuous variables) had the lower value of AIC, explained a large proportion of the variation in the data (Pseudo-r^2=0.9634) and was just slightly different from the starting model (p-value=0.0343). Observed and GAM-predicted values of larval density were highly correlated (r=0.836, p<0.0001) (Figure 3.14). Other models attempted to reduce the number of covariates and thence simplify ecological interpretation, *e.g.* substituting the densities of particular zooplankton *taxa* by their pooled abundance or diversity, without valuable results.

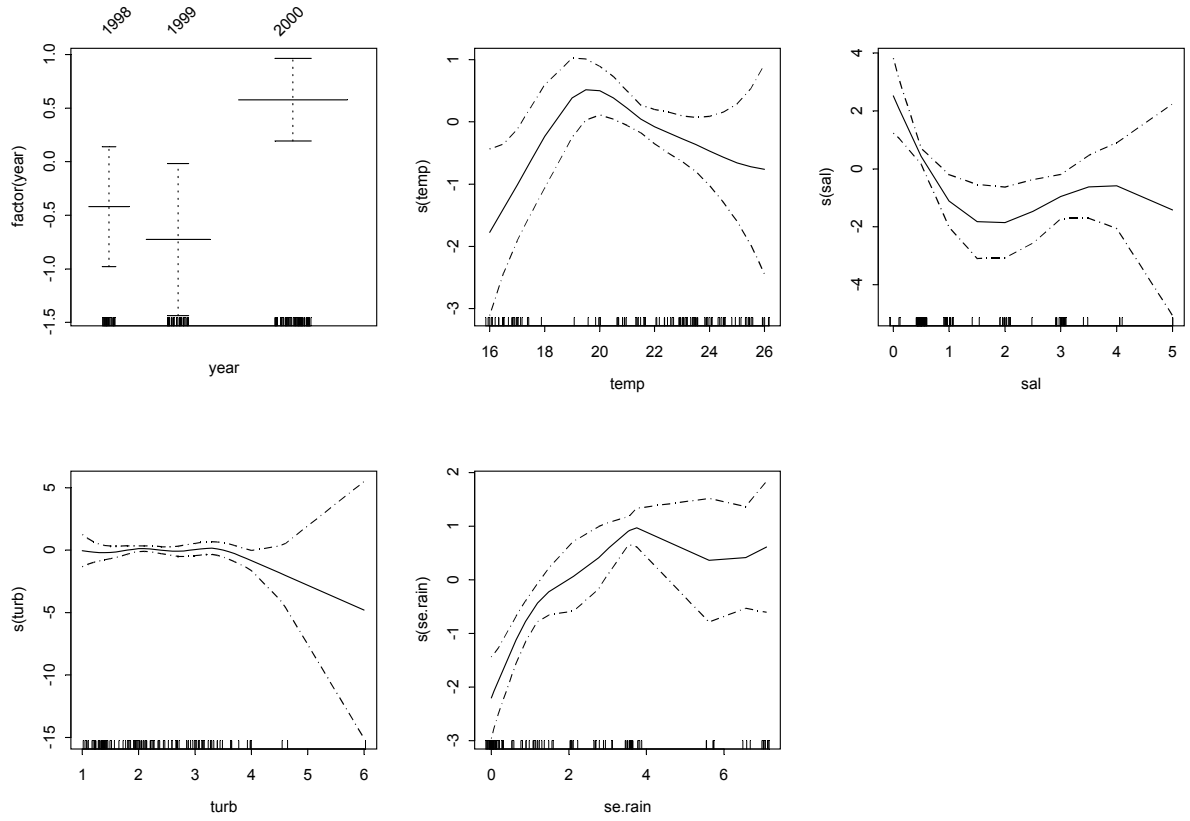

Figure 3.12 – Results of fitting model seaz.30.gam to the seasonal data on shad embryos' abundance. The effect of year (as a factor) on shad embryos' density is represented as a whisker plot. Horizontal lines indicate mean response with 95% confidence intervals. Width of bars is proportional to number of observations. Effects on the density of shad embryos are also represented as smoothing functions of temperature (temp), salinity (sal), turbidity (turb) and SE of rainfall (se.rain). These plots represent the effect of a particular covariate while maintaining the other predictors at a constant level. Fitted lines in each panel are adjusted for the effects of all other variables. Effects are standardised because the estimated density at a given value of a variable is dependent upon levels of all other variable. The dashed lines are approximate 95% pointwise confidence intervals. The tick marks near the *x*-axis show the location of the observations on that variable.

Table 3.6 – Summary statistics of the generalised additive models (GAM) fitted to seasonal data on larval shad density in the upper River Mira. Also included are the results of the analysis of deviance performed on these models. Legend as in Table 3.5.

Model	k	RD	df	p-value	AIC	ΔAIC	w_i	PCf
seaz.10.gam	14	204.65	39.03		749.53	149.27	0.0000	0.9642
seaz.11.gam	13	198.77	42.99	0.2043	642.72	42.46	0.0000	0.9652
seaz.19.gam	12	209.26	47.05	0.0343	600.26	0.00	0.9999	0.9634
seaz.12.gam	10	399.54	55.07	<0.0001	920.81	320.55	0.0000	0.9301
seaz.15.gam	9	433.83	58.91	<0.0001	906.45	306.19	0.0000	0.9241
seaz.16.gam	7	1059.17	103.41	<0.0001	1542.34	942.08	0.0000	0.8590

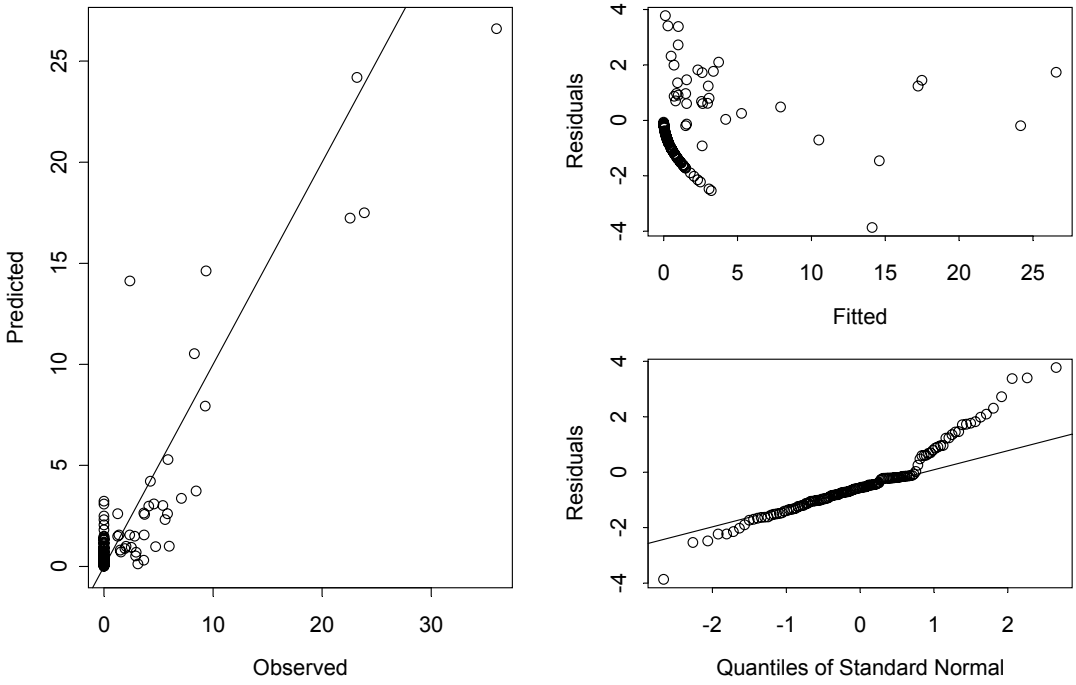

Figure 3.13 – Plots of observed versus predicted densities of shad embryos (ind. 100 m^{-3}) (left), residuals versus fitted (top-right) and normal probability plot (bottom-right) for the upper River Mira obtained from the fitted additive models (see text for details). The continuous line on the left plot as slope of 1.

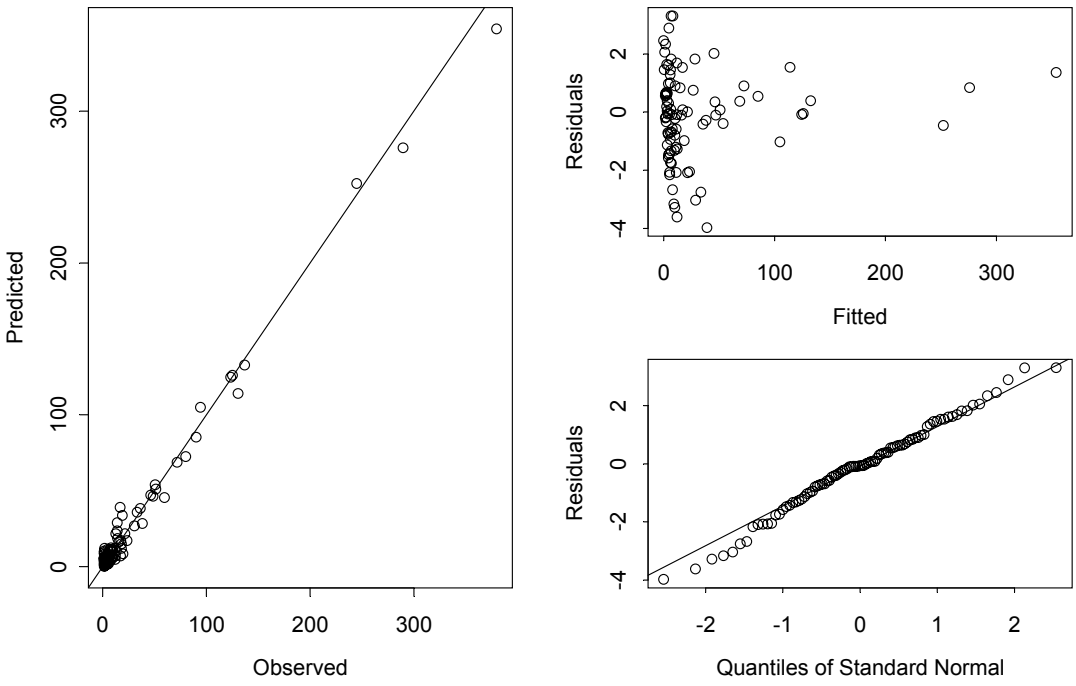

Figure 3.14 - Plots of observed versus predicted densities of shad larvae (ind. 100 m^{-3}) (left), residuals versus fitted densities (top-right), and normal probability plot of residuals (bottom-right) for the upper River Mira obtained from the fitted additive models (see text for details). The continuous line on the left panel as slope of 1.

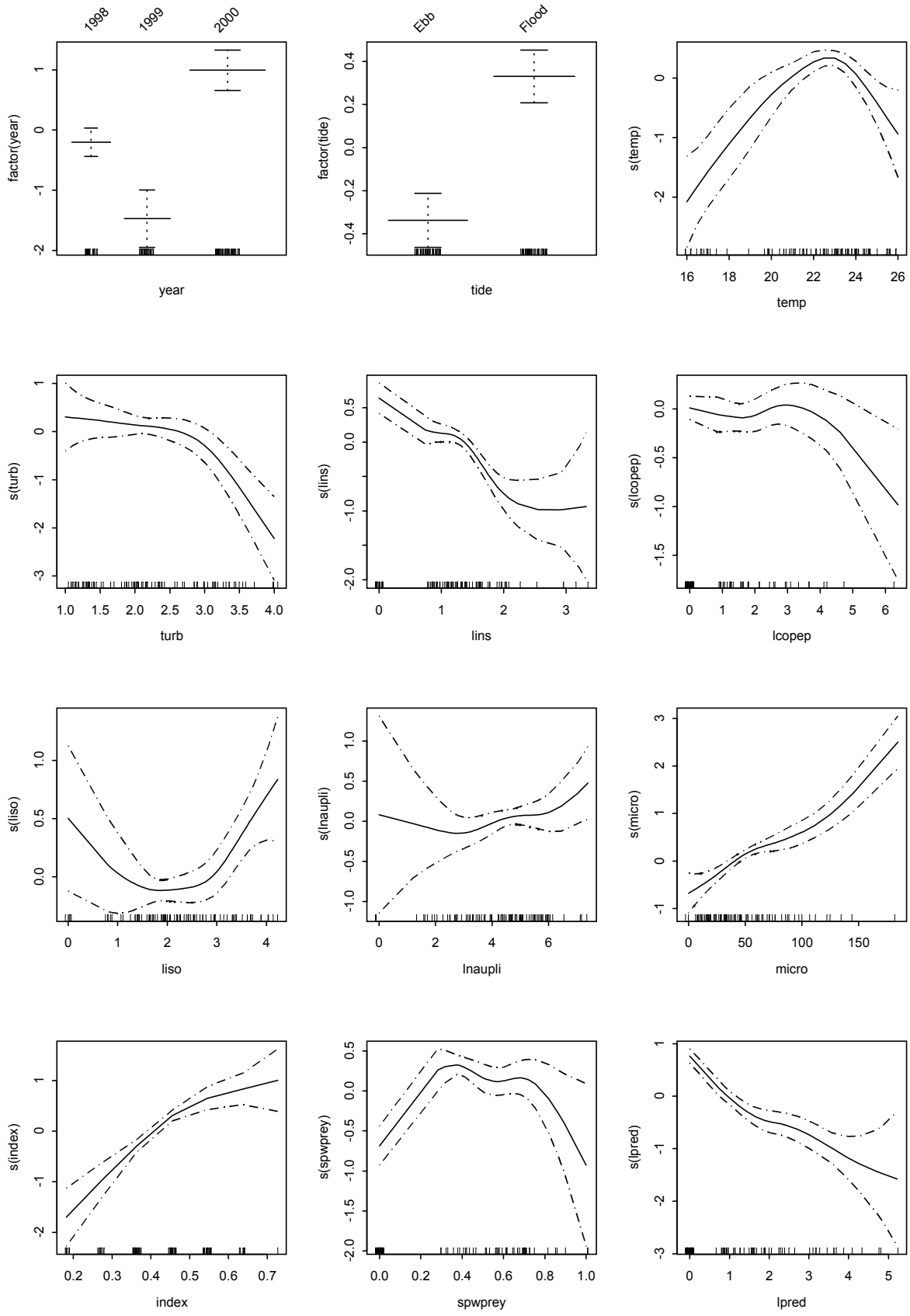

Figure 3.15 – Results of fitting model seaz.19.gam to the seasonal data on shad larvae abundance. For abbreviations' meaning and plots' interpretation details see Figure 3.12.

In the GAM found to best describe the seasonal changes in larval density apart from year and tide all other predictors were included as non-linear smoothed functions (Figure 3.15, facing page). More larvae were collected during the year 2000 than in 1998 or 1999 as well as during flood tides compared to ebb tides. The effect of temperature and turbidity on larval density was highest when values were in the range 21 – 24 ºC and lower than 2.5 mg DW m^{-3} respectively. The number of shad larvae seemed to clearly benefit from increasing microplankton biomass and decapods *nauplii* density, and decreasing predators' abundance respectively for values higher than 50 mg AFDW m^{-3}, 8.4 *nauplii* 100 m^{-3} and 5.5 predators 100 m^{-3} (these estimates are the result of back-transforming the logarithms actually used in the analyses). The "response" of shad to the abundance of small insects, copepodites and isopods strongly differed but the higher the diversity of zooplankton *taxa* the more abundant were shad larvae (Figure 3.15). Whenever the proportion of larvae with gut content was within the range 20 – 80%, shads were more abundant (Figure 3.15).

At this time, a note of caution concerning the correlation between predictors is in order. Just 26 out of 120 pairwise correlations between all the covariates were significant (at $\alpha'=0.00042$) but very few cases had $r > 0.5$ namely *temperature–se.rain* (r=-0.67), *temperature-nauplii* (0.52), *turbidity-microplankton* (0.62), *isopods–zooplankton* (0.64), *copepodites–index* (0.50), *copepodites–zooplankton* (0.53), *amphipods–predators* (0.57), *mysids–predators* (0.61) and *index–zooplankton* (0.55) (*cf.* Table 3.2 for meanings).

3.4. Discussion

The results support previous findings concerning the ecology of early life-history stages of Twaite shad *A. fallax fallax* and analogous species (see the reviews by Aprahamian *et al.*, 2003; Maitland & Hatton-Hellis, 2003; Quignard & Douchement, 1991b). During this study, eggs, embryos and larvae of Twaite shad were only found in the upstream stations (no. 4 – 6) of River Mira from late-March until mid-June (particularly higher densities were observed during a 5-week period from late-April through to the last week of May). During this time, surface temperature ranged from 16 to 26 ºC and salinity never exceeded 5 ppt. The first record of shad in zooplankton samples roughly coincided with the onset of springtime rain. Inter-annual and within-year deviations to this general pattern are discussed below.

In this study, stations no. 4 to 6 were located close to the upper boundary of estuarine influence, near the probable estuarine turbidity maximum (ETM), about 30 km from the river mouth. The ETM is part of a particle-trapping mechanism that lengthens the residence time of river-borne organic material in an estuary, increasing that materials' availability to estuarine bacteria and the estuarine food web (Crump & Baross, 1996), *e.g.* larval fish (Chícharo *et al.*, 2001a). River width and depth at these locations rarely exceeds 30 m and 3 – 4 m, respectively. At each station a mixture of river substrates varying from plain mud or silt (near the river margins) to coarse-gravel or even stones (in the main channel), with many submerged vegetation and debris, was observed. Apparently, these were riverine areas of riffle to fast flowing currents but river flows of less than 1.5 m s^{-1} were found to be typical at those sites. These features were also reported as characteristic of the spawning grounds of *A. fallax fallax* in other European rivers (Aprahamian *et al.*, 2003; Maitland & Hatton-Hellis, 2003; Quignard & Douchement, 1991b; Sousa *et al.*, 2003). On the other hand, *A. alosa* can spawn successfully in untypical areas (Boisneau *et al.*, 1990) and this behaviour is also possible in *A. fallax*, thus explaining the differences from published descriptions.

The period of Twaite shad eggs, embryos and larvae occurrence described for River Mira is in agreement not only with concurrent data on spawning behaviour of adults in River Mira (Pina, 2000) but also with published information regarding other shad populations in Portugal (Alexandrino, 1996; Eiras, 1977; Pina, 2000; Sousa *et al.*, 2003) and elsewhere (Aprahamian *et al.*, 2003; Leggett & Whitney, 1972; Maitland & Hatton-Hellis, 2003; Maniukas, 1989; Moller & Dieckwisch, 1991; Quignard & Douchement, 1991b). The upstream migration of mature shad appears to be triggered by temperature. Adult shads start their movements towards spawning grounds when water temperatures are above 10 – 12º C (Alexandrino, 1996; Aprahamian, 1981, 1988; Boisneau *et al.*, 1990; Leggett & Whitney, 1972). Moreover, Ross *et al.* (1993) found that water temperature was the only physical habitat variable, which significantly affected spawning activity of American shad *A. sapidissima* in the Delaware River (USA). The span of water surface temperatures at which the early life-history stages of shad were collected in this study is within the temperature range reported for shad spawning (and subsequent occurrence of eggs and larvae) in other rivers in Europe (see the reviews by Aprahamian *et al.*, 2003; Maitland & Hatton-Hellis, 2003; Quignard & Douchement, 1991b). Spawning runs of shads are also influenced by estuarine tides and river flows. Twaite shad runs in the rivers Severn and Wye (UK) use the flood tides and seemed facilitated during neap tides (Aprahamian, 1981, 1988). Allis shad, *A. alosa*, seems to move up estuaries on spring tides and if flows are relatively low.

Currents exceeding 2 m s^{-1} and depths less than 10 cm make swimming upstream difficult (Cassou-Leins & Cassou-Leins, 1981). Moser & Ross (1994) found that American shad migrate upstream more frequently during ebbing tides. According to Sabatié *et al.* (1996) the start of the spawning run of *A. alosa* in the Oued Sebou River (Morocco) is mainly influenced by river discharge. Richkus (1974) also found that the upstream migration of Alewife (*Alosa pseudoharengus*) (number of fishes counted at a fish passage) was correlated with the river outflow two days previously. That author proposed that higher outflows should increase the concentration of stream water at the river mouth and therefore increase the probability of adult fishes locating their home stream. The usual hydrological conditions of the River Mira by mid-March would be appropriate to the start of upriver migration and subsequent spawning of adult shad. In fact, the markedly seasonal distribution of rainfall in River Mira (Loureiro *et al.*, 1984), with >90% of precipitation concentrated in the period November-March, is a proxy of increased river discharge by late winter (considering the lag period between rainfall and discharge together with the artificial discharge due to the Santa Clara Dam, a few km upstream of Odemira).

It is likely that the tides and river flow also influence the distribution patterns of early life-history stages of shad. Although no statistically significant relationship was observed between water level and diel abundance of larvae (*cf.* Chapter 2), herein more larvae were collected at the surface during flood tides (see Figure 3.15). The vertical displacement of shad larvae towards the surface during flood tides would ensure the retention of larvae in the favourable upper reaches of the river during this developmental stage. There are numerous examples available in the literature of this mechanism of tidally oriented migration by zooplankton and fishes (Fuiman & Higgs, 1997; Kimmerer *et al.*, 1998). This mechanism would also enable larvae to avoid the more saline bottom layer of water during flood tides and enhance feeding success (see below). On the other hand, heavy rainfall events, which are relatively common in the River Mira area during spring, should contribute significantly to higher runoff (Quintela, 1984) and can wash larvae out of favourable nursery areas. We believe that this was the case for samples collected on day 130 of the year 2000.

Shad larvae are believed to stay in the upper stretches of rivers until they reach the juvenile stage before migrating downstream and reach the lower regions of the estuaries by mid-summer or early-Autumn (Aprahamian *et al.*, 2003; Cassou-Leins & Cassou-Leins, 1981; Quignard & Douchement, 1991b; Thiel *et al.*, 1996). In this study, by early-July no Twaite shad eggs, embryos or larvae were collected neither in the riverine stations (no. 4 to 6) nor in the estuarine

stations (no. 1 to 3). It was outside the scope of this study the collection of juveniles thus the confirmation of young-of-year arrival at the lower estuary was not done. Nevertheless, a few juveniles (17 – 23 mm in standard length) were collected with the plankton net in stations no. 4 and 5 in 20 June and 7 July 2000. This migration is temperature-dependent (Limburg, 1996a; O'Leary & Kynard, 1986) whereas Zydlewski & McCormick (1997) demonstrated that *A. sapidissima* only develop salinity tolerance at the onset of metamorphosis, between 26 and 45 post-hatch, after which the downstream movement is physiologically possible.

The average number of shad eggs, embryos and larvae differed between years of sampling while within-year analyses confirmed the major effect of time of year (factor *Month*) on the seasonal pattern of embryos and larvae described earlier (ANOVA results, *cf.* Table 3.3 and Table 3.4). Embryos and larvae were several-times more abundant during the sampling season of year 2000 than in 1999 or 1998. The differences in the rainfall regimes of those years (*cf.* Figure 3.6) and associated habitat conditioning might be a plausible explanation (see above). Only for shad larvae and year 1999 significant differences between stations and tides were found. As expected, no habitat selection occurs at either the egg or embryonic life stage. The distribution of these life stages is apparently controlled largely by the hydrological characteristics of the river segment downstream of spawning area (Ross *et al.*, 1993). Conversely, larvae most probably react to habitat conditions in an attempt to increase survival. A fraction of the inter-annual differences might be attributed to the non-existence of collections before day 100 in 1999 and the absence of larvae (or any other zooplankton *taxa* for that matter) on day 130 in 2000 (after an abnormal rainfall event) might explain the fact. Presumably, the general patterns of larvae abundance observed are linked to hydrology (see above) and biology (see previous chapter and text below).

The main associations, found between the density of Twaite shad early life-history stages and habitat features in River Mira, were approximated by non-linear functions to a very satisfactory extent. The likely non-linear nature of the relationships between potential explanatory variables and patterns of shad abundance suggested that generalised additive models (GAM) would provide a more informative tool than traditional regression techniques for data exploration. Despite the lack of formal procedures of inference, the use of GAM as gained widespread popularity as a means of devising prediction rules for fast and repeated evaluation, as screening method for variables and for summarising multivariate data sets (Bellido *et al.*, 2001). Moreover, the precise form of the relationships and mechanisms underlying the relationships between ecological variables are generally unclear (Bellido *et al.*, 2001) making GAM a useful

tool in population ecology.

The GAM found to be most relevant for the data at hand included several explanatory variables and most probably reflect the intrinsic complexity of distribution patterns of Twaite shad embryos and larvae. In this study, surface temperature, salinity, turbidity and rainfall were important abiotic factors in determining shad eggs, embryos and larvae abundance in the upper River Mira (Figure 3.12 and Figure 3.15). Several authors have demonstrated relationships between temperature, turbidity and river flow and the distribution or abundance of various anadromous clupeids (Bain *et al.*, 1988; Ross *et al.*, 1993; Sabatié *et al.*, 1996; Talbot, 1954; Thiel *et al.*, 1995). Moreover, temperature and river flow/turbidity have been linked to larval fish mortality (Crecco & Savoy, 1984, 1987a), to larvae ecology (Turner *et al.*, 1994), to the reproductive cycle of adults and to the onset of spawning (Aprahamian, 1988; Moser & Ross, 1994; Richkus, 1974), and to cycles of primary and secondary production of plankton (Odum, 1988). As mentioned above, higher rainfall is one of the major factors contributing to increase river discharge (Quintela, 1984) and stream flow speeds, which in turn result in lower water temperatures and transparency. Shad larvae are expected to grow less (Crecco & Savoy, 1985) and feed less frequently due to high turbidity and reduced zooplankton patchiness. Conversely, Sirois & Dodson (2000) demonstrated that lower energetic costs are incurred by smelt, *Osmerus mordax*, larvae that exploit similar feeding conditions at higher turbidities. In fact, larval smelt in the ETM fed during the coincidence of daylight hours and flooding tide. Herein, the proportion of larvae with gut content decreased slightly with increasing water turbidity and was greater for zooplankters' abundance, in the range 100 – 10000 ind. 100 m^{-3} (Figure 3.16).

Other covariates were included in the GAM obtained for Twaite shad larvae. Of those variables microplankton biomass, decapods' *nauplii* and predators' (medusae, mysids and amphipods) density and zooplankton diversity clearly affected the abundance of larval shad. The relationship of *A. fallax fallax* larvae with other zooplankton *taxa* (*e.g.* small insects, isopods and copepodites) was much less straightforward (*cf.* Figure 3.15). Microplankton, which consisted almost entirely of crustacean *nauplii*, most probably copepod *nauplii* (Esteves *et al.*, 2000b) and nauplii of *Palaemon longirostris* (Paula, 1993), and small insects and isopods are described in the literature as potential prey for shad larvae (Blaxter & Hunter, 1982; Cassou-Leins & Cassou-Leins, 1981; Crecco & Blake, 1983). The co-occurrence of both larvae and prey could result from the active search by fish larvae for patches of prey (Hunter, 1984). In fact, when swimming within patches of food, larvae tend to reduce their activity thus remaining within the aggregation

– area-restricted foraging (Ross *et al.*, 1996). Also, fish larvae only perceive food items at a relatively short distance, less than one larval length for clupeids, and search less than a fraction of a litre per hour (Blaxter & Hunter, 1982). Hence, the presence of shad larvae in patches of potential prey might increase the encounter probability and, therefore, enhance feeding success (Laurence, 1982; Letcher & Rice, 1997). Food deprivation has been shown to significantly affect growth and survival of American shad (Johnson & Dropkin, 1995). Probably the distribution of shad larvae observed in this study is partly related to spatial and/or seasonal displacements as a function of potential prey. DeVries & Stein (1992) and Stein *et al.* (1995) also observed coincident distributions of larval *Dorossoma cepedianum* and zooplankton. Moreover, Ross & Backman (1992) found that American shad larvae foraged and fed independently of one another, and neither schooling nor forms of behaviour leading to co-ordinated swimming were observed in laboratory experiments. This should contribute to the complex shape of the inter-specific relationships and to lessen the empirical evidences of relationship between shad larvae and their prey. Furthermore, using a game theoretic approach Hugie & Dill (1994) found that fish larvae (acting as predators) tend to be distributed as function of productivity instead of prey abundance. This could account for the relevance of microplankton biomass in the models presented herein.

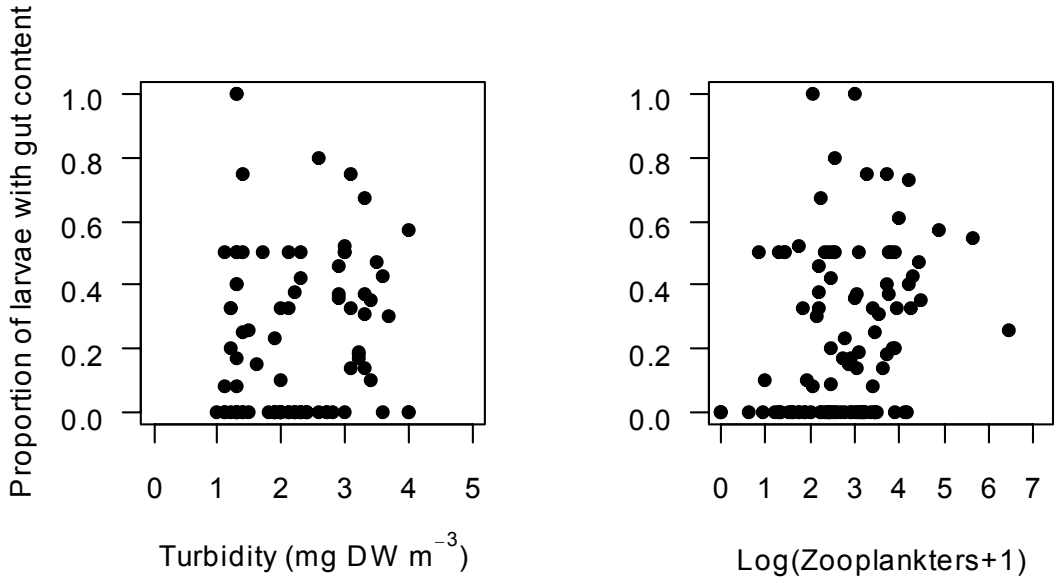

Figure 3.16 – Scatterplots of the proportion of larvae with gut content against turbidity (left) and log(abundance) of zooplankters (right).

The variable *pred* included *taxa* referred to in the literature as potential predators of fish eggs and larvae (see Bailey & Houde, 1989 for a review) which also can act as competitors for the same prey, *e.g.* mysids were found to be the most important predators on rotifers, cladocerans

and copepods of all sizes in the St. Lawrence River (Canada) (Winkler *et al.*, 2003). The negative effect of that variable upon shad larvae abundance found herein (*cf.* Figure 3.15), could reflect: 1) the predatory potential of those *taxa*, which consume shad larvae reducing their numbers; and/or 2) the similar patterns of feeding, *i.e.* competing *taxa* avoid sharing the same *ecological niche* – character displacement (Odum, 1988). In fact, adult prawns *P. longirostris* and carnivorous copepods (*e.g. Oithona* spp.) were found to migrate upstream to the freshwater Mira during late-Spring and Summer months (Cartaxana, 1994; Mattos, 1995). Moller (1984) and Suthers & Frank (1990) stressed the predatory potential of medusae and ctenophores based upon negative correlations between *taxa*. Contrasting results of correlation analyses have been rationalised as a trophic advantage for those predators and potentially increase their predatory efficiency on fish larvae (Esteves *et al.*, 2000b; Moller, 1984; Suthers & Frank, 1990). Frank & Leggett (1985) criticised these evidences and claimed that the inverse relationships could reflect the adaptive responses of larvae or the evolutionary impact of predation on the reproductive behaviour of adults (but these aspects were not investigated herein). Besides the groups considered in this study, several other invertebrate and vertebrate *taxa* are potential predators of fish larvae but were not investigated, *e.g.* amphibians, planktivorous fishes (*Micropterus salmoides*) or birds. Their impact on shad larvae remains unknown. For example, the number of fishes that feed on shad larvae and the average number of shad consumed per predator increased with stocking density, indicating short-term aggregation of predators at the release sites of American shad larvae (Johnson & Ringler, 1998).

The smoothed functions obtained for some variables, e.g. temperature, salinity, turbidity, or zooplankton number in GAM (*cf.* Figure 3.12 and Figure 3.15) are similar to the relationships calculated using Habitat Suitability Index (HSI) models for river herring (*A. pseudoharengus* and *A. aestivalis*) (Pardue, 1983) and for *A. sapidissima* (Ross *et al.*, 1993). The shape of the functions found herein agrees with the presumed relationships between shad abundance and environmental variables. This emphasises the potential use of GAM as a tool for population management and conservation.

Non-linear modelling methods, such as GAM, were able to define Twaite shad eggs, embryos and larvae microhabitat precisely and to provide insight into early life-history stages distribution in the upper River Mira. The results showed that the microhabitat used is influenced by a complex combination of several environmental variables mainly in a non-linear way. The additive models are less restrictive in what concerns the statistical distribution of the data

(Gaussian, Binomial, Poisson, etc). However, it is necessary to choose the error and link distribution applied (Hastie & Tibshirani, 1990). At present, methods of statistical inference and formal goodness-of-fit tests are not available for GAM (Bellido *et al.*, 2001) thus interpretation of results is more complicated and relies mainly upon visual examination of effects and residuals. Another constraint is the large proportion of zeros in the response variable (shad abundance), which sometimes seems to show the density as a discrete process rather than a continuous process. The samples predicted as being (densely) populated by the GAM, but where no fish were captured (*cf.* Figure 3.13), could represent areas or occasions were the environment is suitable for shad embryos or larvae but not inhabited by fish. This supports the hypothesis of Brosse & Lek (2000) that sometimes there may not be enough fish to fill all suitable habitats despite the existence of favourable habitat conditions. Moreover, additive models do not consider the possible interactions between co-variates and therefore can be judged to over-simplify. However, the attempts to fit models, which included "more complex" variables (*e.g. zoopl*), proved to be inefficient. Finally, few predictor variables included in the GAM obtained herein were correlated. The largest correlations were between temperature and rainfall (r=-0.67) or *nauplii* (r=0.52), microplankton biomass and turbidity (r=0.62) and between zooplankters density and diversity ($0.50 < r < 0.64$). River flow and temperature are invariably confounded and their effects may generally be difficult to separate (see above). Crustaceans, being ectotherms, are strongly influenced by water temperature. Therefore, reproduction is expected to grow with increasing water temperature (up to the thermal limit) and the abundance of nauplii and copepodites should peak during the Summer. In fact, Mattos (1995) found that the abundance of copepods in the River Mira (particularly *Acartia* sp.) peaked during March-April. The biomass of microplankton and turbidity were associated because both estimates are obtained at different stages of the same procedure. The confounding between density and diversity of zooplankters was probably due to the index of diversity used. The occurrence of "rare" *taxa* is more probable in samples with a greater abundance of zooplankters. For the index of diversity used in this study, every *taxon* was considered with an equal weight despite being conspicuous or "rare". Thus, the *index* was found to be higher in congested samples. All these remarks should be taken into consideration whenever using the GAM provided herein.

In addition to increasing the sample size, the difficulties mentioned above could be overcome using a two-stage model: modelling first the presence/absence as a binomial distribution and then modelling density solely when and where embryos or larvae occurred (e.g. Mueter & Norcross, 2002).

Chapter 4. Effects of biotic and abiotic factors on annual growth and mortality of Twaite shad larvae in the River Mira

4.1. Introduction

The use of daily growth rings on the otoliths of many fish species not only provided a method for age determining (and timing of life history events), model growth and estimate survival-at-age in larval and juvenile fish but also provided a valuable tool with which to analyse within-season changes in growth and survival of larval fish (Jones, 1992). Small changes in the rates of growth and survival during early life history of fish are believed to have pronounced effects on later recruitment success (Houde, 1987). In fact, the central theme of the critical period and match-mismatch hypotheses (Cushing, 1975; Hjort, 1914) is that food availability and/or larval predation are the proximate determinants of larval survival and the later year-class strength, whereas hydrographical events are the ultimate causes that drive the system (Houde, 1996).

The anadromous Twaite shad, *A. fallax fallax* (Lacépède, 1803), is classified as "Vulnerable" by the Instituto de Conservação da Natureza using IUCN criteria (Cabral *et al.*, 2006) and still migrates upriver to spawn in several rivers along Portugal to spawn (Eiras, 1977), namely the River Mira. Published information on age and growth and/or mortality of *A. fallax fallax* larvae is anecdotal, consisting mainly of sizes-at-hatch and qualitative assessments (Aprahamian *et al.*, 2003; Baglinière & Elie, 2000; Quignard & Douchement, 1991b), none of which concerns populations from Iberian peninsula's rivers. The work on the early life-history stages of a closely-related alosinid, American shad *Alosa sapidissima*, in the Connecticut River (USA) during the 1980s (Crecco & Savoy, 1984, 1985, 1987a, b; Crecco *et al.*, 1983; Crecco *et al.*, 1986) and in the Hudson River (USA) in the 1990s (Limburg, 1996a) constitutes the bulk of the investigations on this subject outside Europe.

Therefore, this study which is part of a larger research project was designed to: 1) determine the ages and model growth and mortality; 2) study the seasonal variation in those life-history traits;

and 3) to examine how short-term changes in environmental factors affect within-year changes in growth and relative survival of larvae from the 2000-year class of Twaite shad in the River Mira (SW Portugal).

4.2. Material and Methods

Study area

The Mira River is a 145-km long, relatively narrow (<400 m near the mouth), shallow (<10 m deep near the mouth) and flat (mean slope of 17%) watercourse located in SW Portugal. Its drainage basin area is *ca.* 1576 km^2 at a mean altitude of 156 m. Tidal influence extends to approximately 30 km from the mouth, where mechanical effects and salinity changes have been observed. About 70 km upriver, the Santa Clara Dam was built in the late 1960's mainly for irrigation and municipal water supply. Equipped with a spillway (vertical well) capable of discharging 208 m^3s^{-1} of water, the dam is sporadically used to regulate the river flow although mimicking the natural pattern. Further information is provided in the web site of the Serviço Nacional de Informação sobre Recursos Hídricos do Instituto Nacional da Água (SNIRH at http://snirh.inag.pt/snirh/).

Sampling

Plankton samples were collected every other week from 28 March until 7 July 2000 at stations no. 4 – 6 in the upper River Mira (*cf.* Figure 3.1, Chapter 3). From each sample at least 10% of the shad larvae caught was subsampled and analysed herein. Zooplankters were stored in buffered 4% formaldehyde solutions and later were identified using published information (Johnson & Loesch, 1983; Newell & Newell, 1963; Quignard & Douchement, 1991b; Russell, 1976; Saville, 1964; Smith, 1977; Todd & Laverack, 1991). Plankters' density was expressed as number of individuals per 100 m^3 of water.

A further 26 – 39 L of water were sampled at 0.5-m depth and filtered through a 63-µm mesh size sieve to collect microplankters. To estimate microplankton biomass, aliquots of the homogenised samples were rinsed through a 1.2-µm milipore filter (∅ 25 mm, Macherey-Nagel® GF-3). Filters were dried in an electric oven for 24 – 36 hours at 60º C to obtain the dry weights and then incinerated at 450º C for 4 hours to determine the ash-free dry weight

(AFDW). The results of microplankton biomass were expressed as ash-free dry weight per cubic metre of water (mg AFDW m^{-3}).

Otolith analysis

Otolith microstructure was analysed to determine age and estimate growth and mortality rates. In the present study, *sagittae* from *n*=469 (post-yolk-sac) larvae were examined from a total of 570 otoliths processed (*ca.* 82%). Each larva was measured to the nearest 0.01 mm standard length (SL) using the ImagePro Plus® image analysis system (MediaCybernetics). According to Theilacker (1980) lengths were corrected for shrinkage during collection and preservation. *Sagittae* from the 7.21 to 20.40 mm SL larvae were removed using a combination of 45% NaOH rinse (<3 min) and fine-needle manipulation under a stereoscope (Figure 4.1). After washing (with 99% ethanol and water) and drying the otoliths, these were mounted on a slide using Pro-Texx® mounting medium (Baxter Diagnostics Inc.) (see Secor *et al.*, 1991; Stevenson & Campana, 1992 for further details).

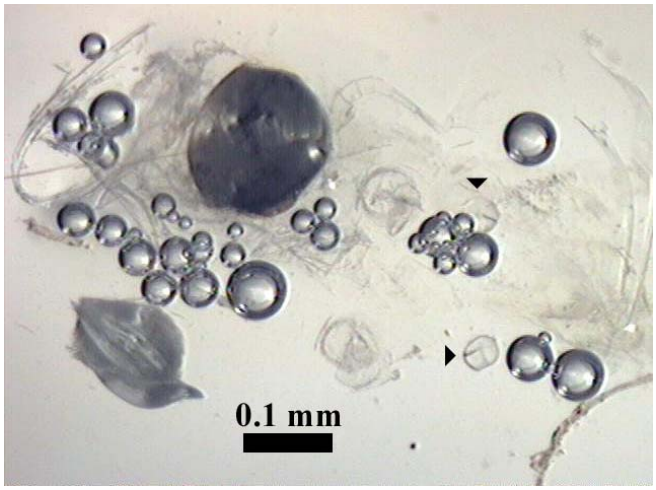

Figure 4.1 – Head of Twaite shad larva (16.1 mm SL) after 3 minutes rinse with 45% NaOH. Triangles point to individualised *sagittae*.

Digitised images of the *sagittae* were obtained with a CCD camera connected to a microscope (at 400x magnification) and analysed using the same image analysis software. The number of increments was counted on three separate occasions without prior knowledge of length or sample information (see Stevenson & Campana, 1992 for a thorough review of methods). Herein, an increment corresponded to paired translucent and opaque rings in a regularly-recurring sequence deposited around the otolith. The final age was regarded as the mode of the three estimates. Significant agreement between the number of sagittal rings and the true daily

age of *A. sapidissima* has been found (Crecco & Savoy, 1985; Savoy & Crecco, 1987), indicating that the chronological ages from the otoliths were good estimates of the true ages. Precision of each age estimate was assessed using the coefficient of variation $cv = s/\overline{x}$, where s and $\overline{x}$ are the standard deviation and mean of replicate readings (Chang, 1982).

Hatching dates were then back-calculated from the date of capture and grouped into 7-day classes. Increment's widths were measured (to the nearest 0.1 μm) in a sub-sample of thirty larvae, randomly chosen from each sampling date.

Growth rates

Growth in length of larvae (mm d^{-1}) was estimated from the slopes of the linear regressions of mean back-calculated SL on ages from daily otolith-increment analysis:

$$L_t = \alpha + gt \tag{4.1}$$

where L_t is the back-calculated SL (mm) at age t (days), g is the instantaneous growth rate (mm d^{-1}) and α is the estimated SL (mm) at hatch (y-intercepts). Only larvae 4 to 15 d-old were used. Individual standard lengths-at-age i were back-calculated considering the body-proportional hypothesis (BPH) approach of Francis (1990) and using the following algorithm for each larvae aged:

$$L_i = (c + d \cdot S_i) \cdot (c + d \cdot S_c)^{-1} \cdot L_c \tag{4.2}$$

where c and d are the intercept and slope of the regression of standard length of larvae on *sagittae* radius, L_i (L_c) and S_i (S_c) are the lengths of larvae and size of *sagittae* (radius) at age i and capture (c) respectively. Average back-calculation error (sensu Francis, 1990) was lower than 1.2 mm and no evidence of Lea's-phenomenon was apparent (Figure 4.2).

Coefficients in growth-model regressions were compared between sampling dates using analysis of covariance (ANCOVA). The ANCOVA tested for differences in slopes (growth rates) and/or y-intercepts (size-at-hatch) in the growth equations. When significant, ANCOVA results (estimates of g) were studied further using Tukey's simultaneous confidence intervals (Zar, 1996).

Mortality rates

A main age-length key was developed to convert larval length distributions to age distributions

from which mortality rates then were estimated. To derive the main key, the frequencies of age groups within 0.5-mm length classes were obtained from the sample of otolith-aged larvae. Seven age-length keys were constructed from the length-frequency data for each sampling date. This maintained the seasonal-specific integrity of size-at-age data, allowing estimation of mortality rates.

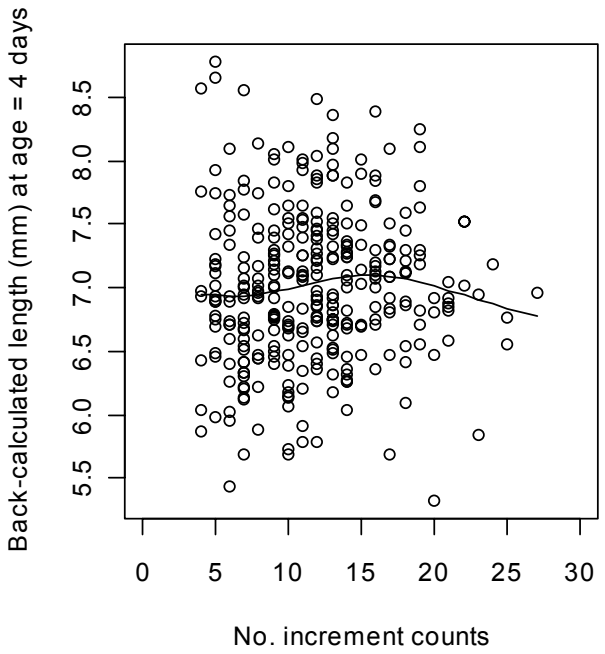

Figure 4.2 – Relationship between individual back-calculated length-at-age equal to 4 days and the number of increments (age at capture) counted on sagittae of Twaite shad larvae from the River Mira (March-June 2000).

Instantaneous daily mortality rates of larvae were estimated from an exponential model of decline in abundance of unadjusted numbers with respect to age (time):

$$N_t = N_0 \cdot e^{-Z \cdot t} \tag{4.3}$$

where N_t is the abundance (no. 100 m^{-3}) at age t (d), N_0 is the estimated initial abundance at time t=0 (y-intercept of regression; no. 100 m^{-3}), Z is the instantaneous mortality coefficient (d^{-1}) and t is age (d). The data were fitted to the log-linear form of the model after ln-transformation of the abundance data: $\ln(N_t) = \ln(N_0) - Z \cdot t$. Daily mortality percentages M were expressed as follows:

$$M = (1 - e^{-Z}) \cdot 100\% \tag{4.4}$$

Date-specific mortality coefficients were estimated and compared by analysis of covariance

(ANCOVA) as described earlier for growth rates. Herein, larval mortality rates were estimated for two separate groups (yolk-sac larvae were excluded from analyses). First, *M* was estimated for larvae SL<13 mm (*i.e.* 5 to 14-day-old) that were ubiquitous in all sampling dates. Then, *M* rates were also obtained for larvae 13 mm≤SL<17.5 mm (15 to 20-days-old), present only in the later sampling occasions (after day 150). No estimates were obtained for larvae ≥17.5 mm (*i.e.* >20-day-old) because of the low numbers collected. When significant, ANCOVA results (estimates of *Z*) were studied further using Tukey's simultaneous confidence intervals (Zar, 1996).

Correlations and predictions

Nine independent variables (Table 4.1) besides growth and mortality rates (for 5 to 14 days-old larvae) were considered. Plankter's abundance estimates were log-transformed, log(x+1), to correct for non-normality. A simple index of plankters diversity was calculated as the proportion of *taxa* present in each sample out of all the *taxa* collectable. Only *taxa* that represented more than 1% of total abundance were used in further analyses.

Table 4.1 – List of variables used in this study, units and summary statistics (mean ± standard deviation (SD) and range). *Zooplankters* included isopods, insects, cladocerans and copepodites, *predators* included small medusae, mysids and amphipods, and *plankters* included *nauplii*, zooplankters and predators.

Covariate, units	Mean ± SD	Range
Temperature, ºC	20.58 ± 3.69	16 – 26
Rainfall, mm	2.65 ± 2.43	0.0 – 7.1
Microplankters, ind. 100 m^{-3}	54.33 ± 36.87	7.51 – 184.8
Crustacean nauplii, ind. 100 m^{-3}	63.41 ± 92.21	0.0 – 396.7
Zooplankters, ind. 100 m^{-3}	23.95 ± 36.79	0.0 – 284.3
Predators, ind. 100 m^{-3}	63.96 ± 453.48	0.0 – 4239.9
Plankters “diversity”	0.407 ± 0.078	0.273 – 0.545
Shad larvae, ind. 100 m^{-3}	26.97 ± 63.19	0.0 – 379.9
Proportion of larvae with gut content	0.22 ± 0.24	0.00 – 0.75

Prior to regression analysis, Spearman correlation analyses were run to determine which independent variables were related (*i.e.* r>0.70) and thus might be collinear and unsuitable for inclusion in the predictive regression models. Regression analyses (using least squares procedures) of appropriate models were used to determine if Twaite shad larval growth and

mortality rates could be related to biological or environmental factors. Whenever significant regression models were not plausible, simple descriptive statistics and/or parametric tests were used. All analyses were conducted using R (R Development Core Team, 2003) at the 0.05 and 0.10 significance levels and *p*-values greater than 0.10 were considered as indicative of non-significance.

4.3. Results

Seasonal variation in water temperature and rainfall was pronounced (Figure 4.3). Mean water temperature increased sharply after mid-May (day of year 130), from about 17º C to >25º C in early-July (day 172) whereas rainfall variability was higher during April (days 100 – 140) and decreased to zero after day 150 (late May).

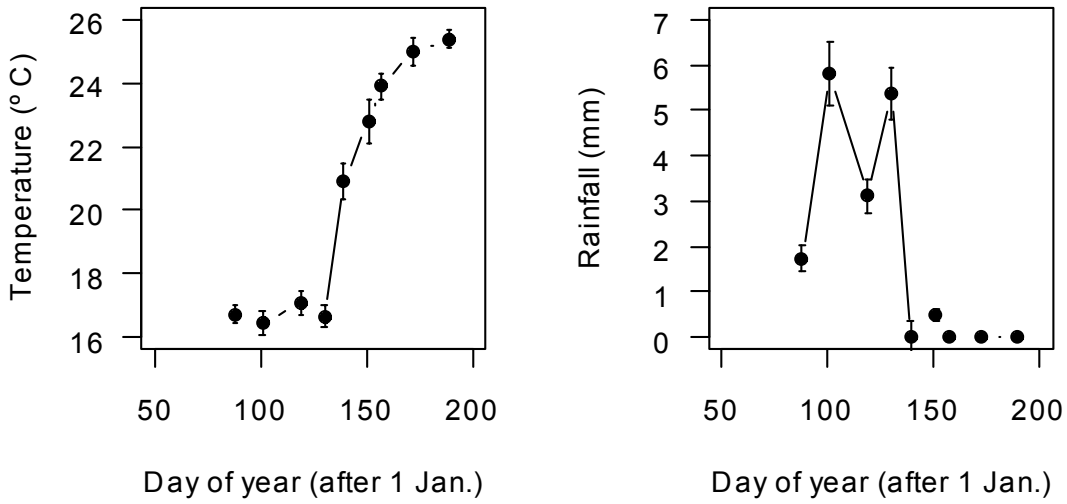

Figure 4.3 – Mean temperature (± 95% confidence intervals), left, and precipitation (previous 7-day moving average ± 0.1×standard error), right, recorded for the River Mira, late March-early-July 2000 (days of year =100 and 200 corresponded to 9 April and 18 July, respectively).

Microplankton biomass changed from less than 50 mg AFDW m^{-3} before day 130 to about 75 – 100 mg AFDW m^{-3} afterwards (Figure 4.4). Densities of crustacean *nauplii* and other zooplankters showed a similar seasonal pattern. A first abundance peak was observed at day 100 (*ca*. 10 – 30 ind. 100 m^{-3} in early-April), followed by slight decrease and a pronounced peak during early-June (>300 ind. 100 m^{-3}, around day 160) after which densities decreased notably (Figure 4.4). The numbers of potential predators of shad larvae increased steadily from 1 – 3 ind. 100 m^{-3} in late-March to around 30 ind. 100 m^{-3} at the end of the sampling period, early-July (Figure 4.4).

Larval shad were immediately detected during the first sampling occasion (March 28) and peak densities were observed in late-May and early-June (averages of 145.3 and 72.6 larvae 100 m^{-3}, respectively). By early-July, only two larvae were caught (Figure 4.5). No larvae were collected in May 9 (day 130) after a week of high rainfall. Gut content analysis showed that mean proportions of larvae with gut content were consistently low (<13%) from March 28 through May 30 and sharply increased after early June to >32% (Figure 4.5).

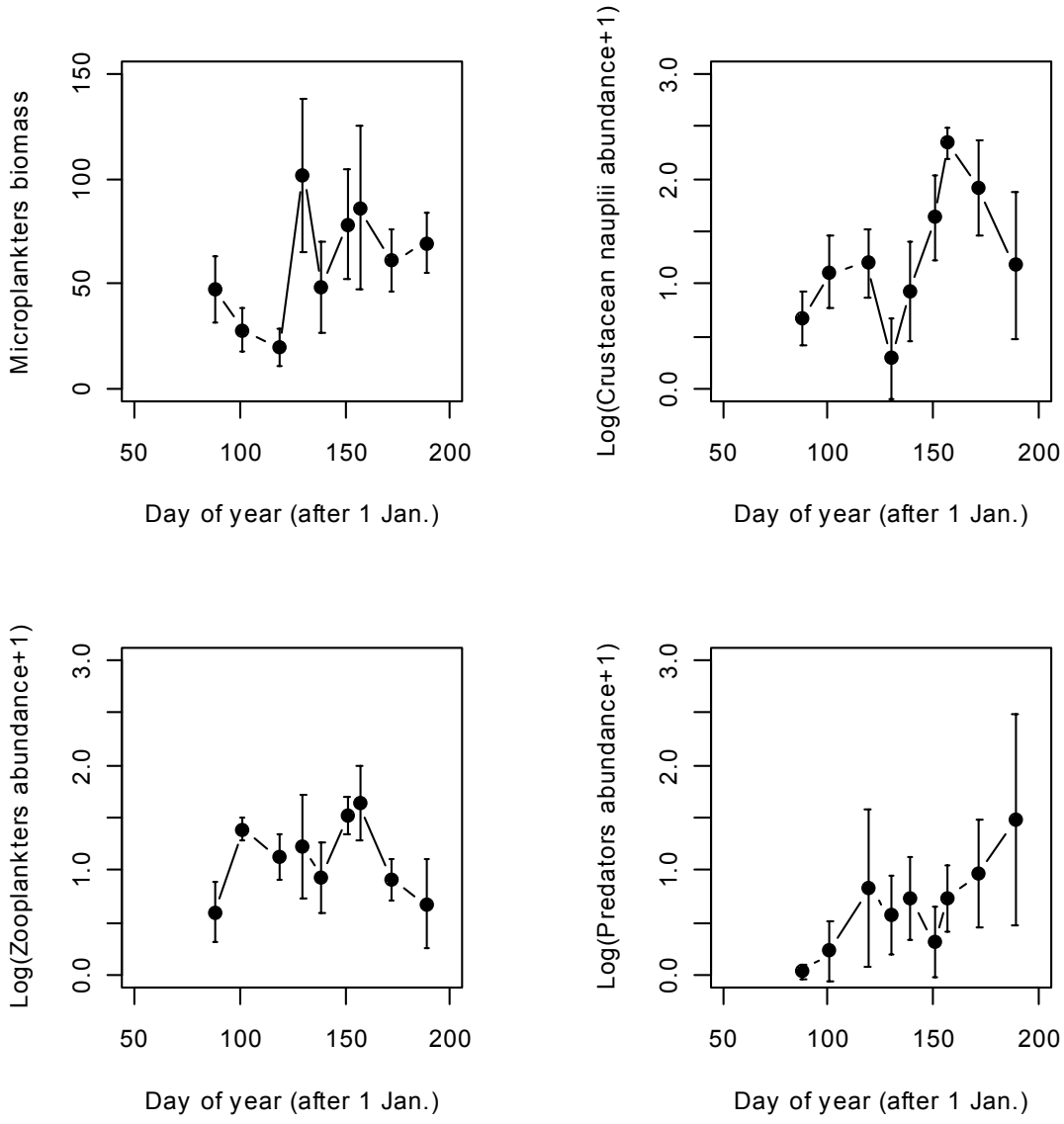

Figure 4.4 – Mean microplankters biomass (AFDW m^{-3}) and log-transformed zooplankters abundance (ind. 100 m^{-3}) (± 95% confidence intervals) in the River Mira, late March-early-July 2000 (days of year =100 and 200 corresponded to 9 April and 18 July, respectively).

The size of aged shad larvae ranged from 7.21 mm to 20.40 mm (mean ± SD: 12.21 ± 2.37 mm). Their *sagittae* were round and disc-shaped (Figure 4.6). As larvae grew the otoliths acquire a slightly oval and concave in shape. Notwithstanding, a significant linear relationship (r^2=0.77,

n=326, p<10^{-4}) was calculated between larval length and otolith size (sagittal radius) (Figure 4.7 left). No relationship was observed between the residuals of this regression and age-at-capture (Figure 4.7 right).

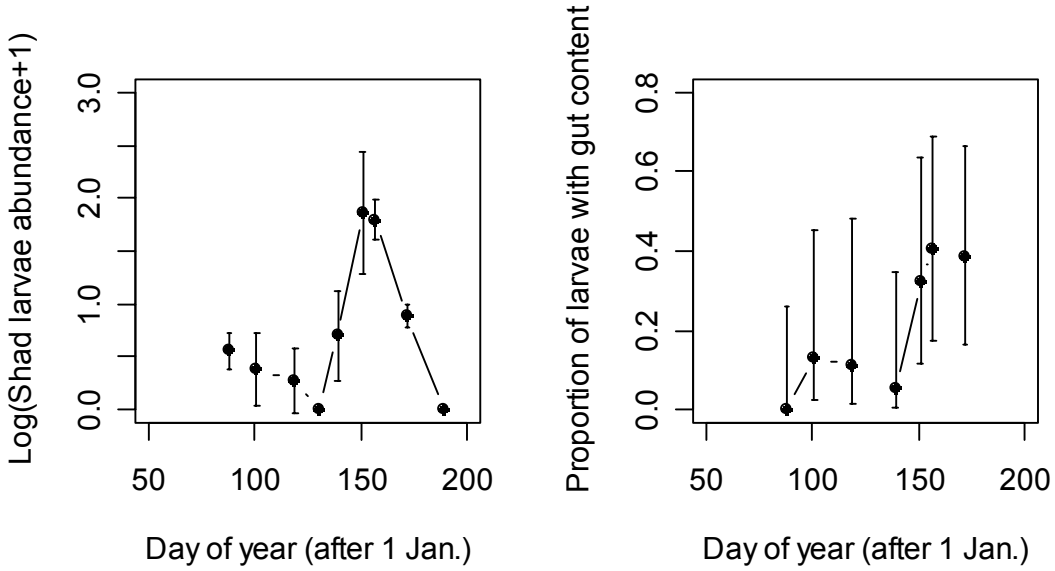

Figure 4.5 – Log-transformed abundance of shad larvae (ind. 100 m^{-3}), *left*, and average feeding incidence, measured as the proportion of shad larvae with gut content, *right*, (± 95% confidence intervals) in the River Mira, late March-early-July 2000 (days of year =100 and 200 corresponded to 9 April and 18 July, respectively).

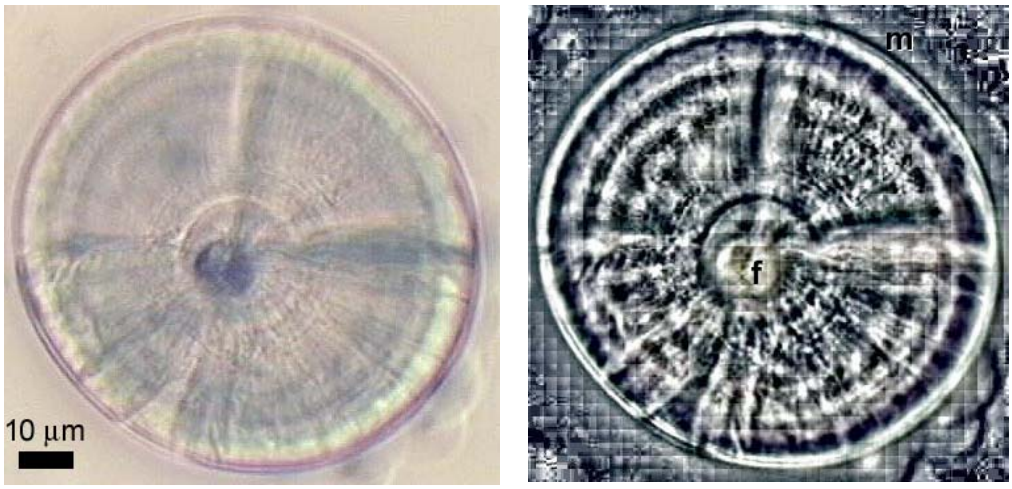

Figure 4.6 – Digitised images of *sagitta* from a 9.1 mm SL Twaite shad estimated to be 7 days-old. Image on the left was obtained with a CCD camera mounted on a microscope at 400x magnification. The image was processed using ImagePro Plus® (right) and paired light-and-dark rings were counted from the focus (f) to the margin (m).

Shad larvae were aged 4 d to 27 d-old (mean ± SD: 11.8 ± 4.7 d). The average precision of repeated increment counts was high (mean cv=5.7%) with a mean error ≤ 2 increments in 99% of the cases. Almost 70% of the *sagittae* analysed were used (n=327) because at least two-out-of-three counts coincided. The mean width of the first increment deposited on *sagittae* of Twaite

shad larvae was substantially higher than overall average (6.8 μm *versus* 5.1 μm ± 1.05 μm). Afterwards, the pattern of average increments' widths fluctuated around 5 μm up to the 20th increment and was quite variable for older ages (Figure 4.8). The distribution of hatching dates, backcalculated from estimated ages-at-capture and partitioned among 7-d cohorts, shown that spawning events and the following larval emergence were sparse before late-April (day 120). A strong peak in the hatchdate distribution was found during May (Figure 4.9).

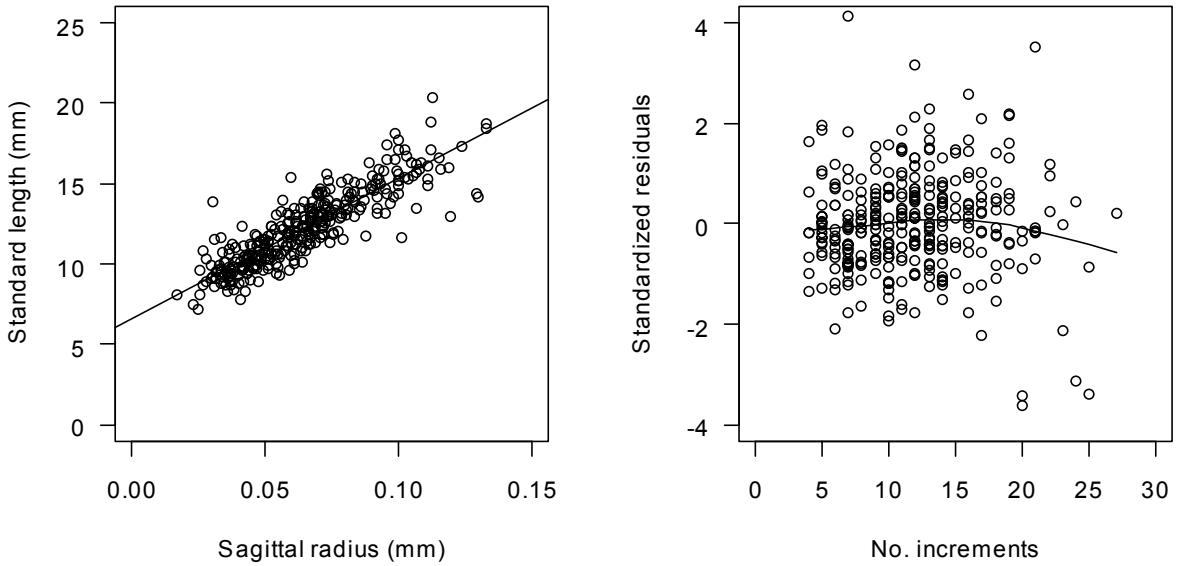

Figure 4.7 – (left) Linear relationship between sagittal radius and larval length of individual Twaite shad larvae (y=6.56+87.56x, n=327, r^2=0.7671, p<10^{-4}) of Twaite shad caught in River Mira during the sampling season of 2000 (late-March to early-July). (right) Scatterplot of residuals from the previous regression against number of increments counted on *sagittae*. The smooth (spline) curve is shown for illustrative purposes only.

Overall growth-in-length of shad larvae was significantly described by the linear equation $\hat{L}_t = 7.18 + 0.43t$ (r^2=0.7293, n=326, p<10^{-4}), which yielded a daily (instantaneous) growth rate of 0.43 mm d^{-1}. Significant differences were found in the instantaneous growth rates (regressions' slopes) between sampling dates and the estimated sizes-at-hatch (intercepts) changed during the spawning season (ANCOVA using mean back-calculated lengths-at-age, p<10^{-4}) (Table 4.2). Date-specific instantaneous growth rates were higher earlier in the spawning season (maximum of g=0.603 mm d^{-1} ± 0.105 SE in early-April, day of year=101) and decreased towards the end of June (minimum of g=0.388 mm d^{-1} ± 0.029 SE in late-June, day of year=172). Shad embryos hatched at approximately the same estimated size throughout the spawning period (4.75 mm – 5.69 mm), but smaller sizes were found earlier in the season (Figure 4.10).

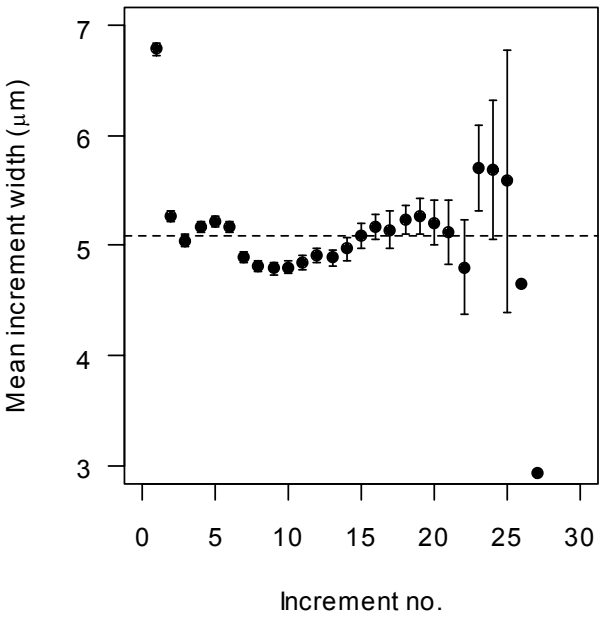

Figure 4.8 – Average otolith increment widths (± SE) per age-group of Twaite shad caught in River Mira during the sampling season of 2000 (late-March to early-July). Horizontal dashed line corresponds to the overall mean (5.1 μm).

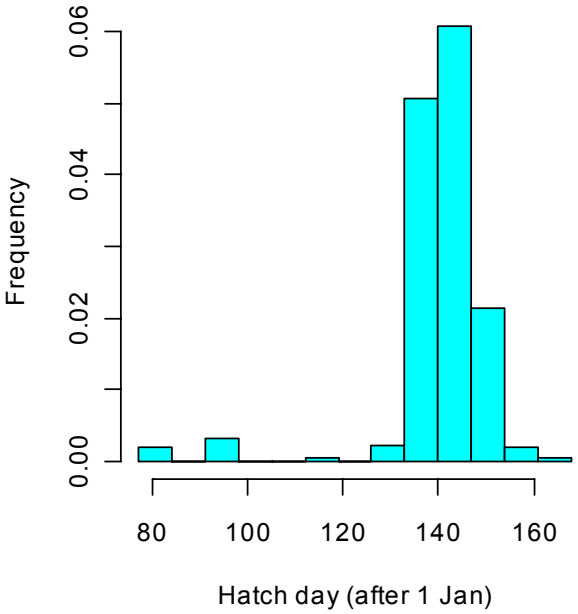

Figure 4.9 – Hatchdate distribution (per 7-days bins) of Twaite shad larvae backcalculated from specimens collected in the River Mira (days of year = 80 and 140 corresponded to 20 March and 19 May, respectively).

Larvae experienced high mortality rates (Table 4.3). When the data analysis included only larvae <15-days old (the oldest age-length key derived age-group represented in all sampling dates), estimated Z ranged from -0.36 d^{-1} (M=30.1 % d^{-1}) in late-March (day of year 88) to *ca.* -0.04 d^{-1} (M≈4 % d^{-1}) in late-May/early-June (the positive Z estimate for day 172 was set to zero). For the

oldest larvae and later collections, estimates of Z were in the range -0.30 to -0.48 d^{-1} (that is 26 % d^{-1}<M<38 % d^{-1}) (Figure 4.11).

Table 4.2 – Date-specific estimates of size-at-hatch (α, mm) and instantaneous growth rates (g, mm d^{-1}) for Twaite shad larvae from the River Mira (March-June, 2000). Mean back calculated lengths-at-age 4 to 15 d were used. Legend: No. – number of larvae aged; SE – standard error; Superscripts (a, b, c) group similar estimates of α or g (ANCOVA, Tukey HSD); p is the p-value for H_0: α=0 or H_0: g=0.

Day of year (Date)	No.	α (± SE)	p	g (± SE)	p	Age (d) and length (mm) intervals
88 (28 March)	6	4.754 (0.1871) [a]	<10^{-4}	0.532 (0.0304) [a]	<10^{-4}	5 – 8 d 8.4 – 10.5 mm
101 (10 April)	7	5.608 (0.1542) [b]	<10^{-4}	0.603 (0.0229) [b]	<10^{-4}	5 – 9 d 10.9 – 13.3 mm
119 (28 April)	1	5.097 (0.2409) [a]	<10^{-4}	0.489 (0.0429) [c]	<10^{-4}	7 d 9.6 mm
130 (9 May)	---	---		---		--- ---
139 (18 May)	5	5.687 (0.2409) [b]	<10^{-4}	0.447 (0.0429) [a]	<10^{-4}	4 – 7 d 8.1 – 10.8 mm
151 (30 May)	170	5.316 (0.0811) [b]	<10^{-4}	0.438 (0.0080) [c]	<10^{-4}	4 – 21 d 8.3 – 15.8 mm
157 (5 June)	127	5.125 (0.0811) [b]	<10^{-4}	0.452 (0.0080) [c]	<10^{-4}	4 – 27 d 7.2 – 18.8 mm
172 (20 June)	11	5.233 (0.0811) [b]	<10^{-4}	0.356 (0.0080) [c]	<10^{-4}	11 – 25 d 11.5 – 14.3 mm
189 (7 July)	---	---		---		---

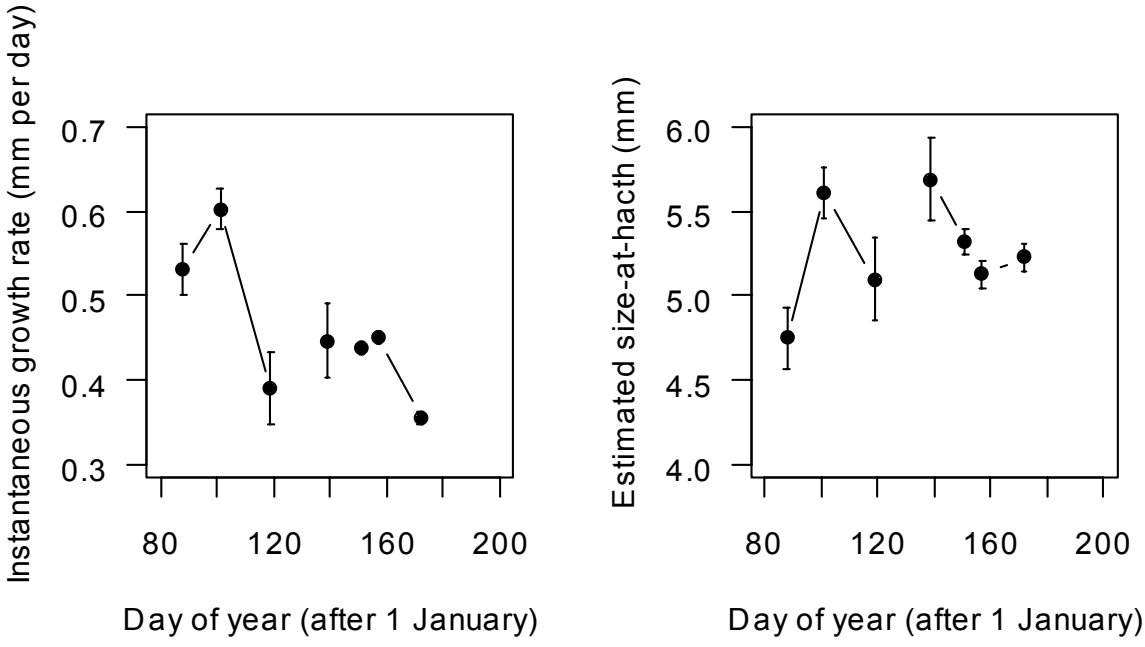

Figure 4.10 – Date-specific estimates of instantaneous growth rates (g±SE) and size-at-hacth (α±SE) obtained from regressions of mean back calculated lengths-at-age on age of Twaite shad larvae from the River Mira (March-June, 2000). Days of year 130 and 189 not represented because of insufficient number of larvae.

Table 4.3– Date-specific estimates of the initial abundance $\log_{10}(N_0)$ and the mortality coefficient Z (d^{-1}) and corresponding instantaneous mortality rates M (% d^{-1}) of Twaite shad larvae collected in the River Mira (March-June 2000). Legend: ‡ - Age-class range of 9 to 15 d instead of 5 to 15 d; M marked with # was set to 0.0%; * denotes estimates for older and later collected larvae; Superscripts a, b, c group similar estimates of Z (using Tukey's HSD); and p is the p-value for H_0: Z=0.

Day of year (Date)	$\log_{10}(N_0)$ (± SE)	Z (± SE)	p	M	$\log_{10}(N_0)$ (± SE)	Z* (± SE)	p	M
88 (28 March)	3.935 [a] (0.342)	-0.357 [a] (0.034)	$<10^{-4}$	30.0%				
101 (10 April) ‡	2.554 [a] (0.869)	-0.173 [b] (0.075)	0.0248	15.9%				
119 (28 April)	1.460 [b] (0.342)	-0.273 [a] (0.034)	$<10^{-4}$	23.9%				
130 (9 May)	--- ---	--- ---						
139 (18 May)	3.901 [a] (0.342)	-0.278 [a] (0.034)	$<10^{-4}$	24.3%				
151 (30 May)	4.590 [a] (0.342)	-0.044 [b] (0.034)	0.2038	4.3%	10.793 (2.204)	-0.479 [c] (0.110)	0.0005	38.1%
157 (5 June)	3.992 [a] (0.342)	-0.037 [b] (0.034)	0.2931	3.6%	8.839 (2.204)	-0.303 [c] (0.110)	0.0146	26.1%
172 (20 June)	0.544 [b] (0.342)	+0.066 [b] (0.034)	0.0616	[#] 0.0%	6.861 (2.204)	-0.322 [c] (0.110)	0.0103	27.5%
189 (7 July)	---	---				---		

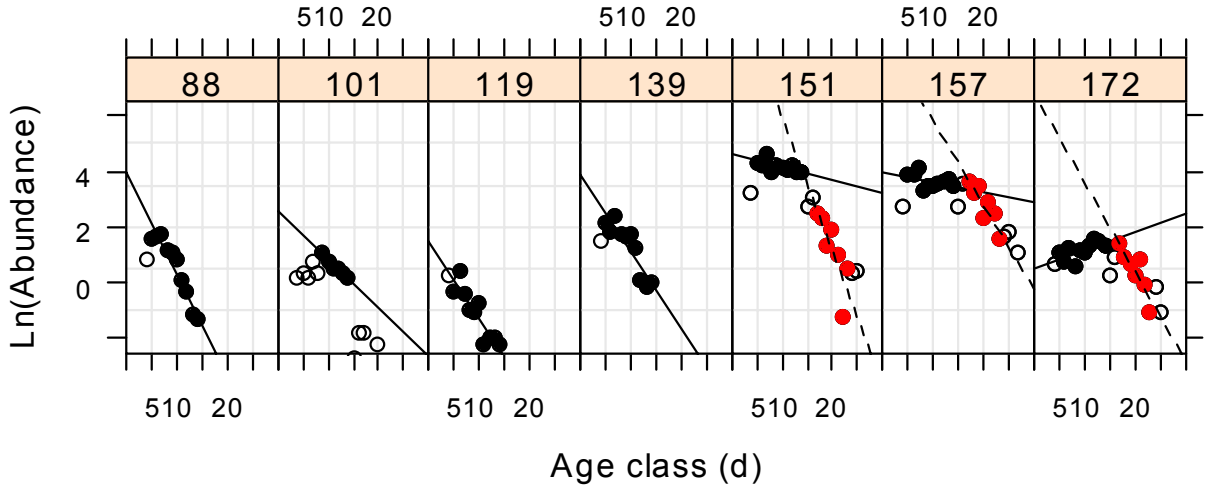

Figure 4.11 - Age-specific mortality curves for Twaite shad larvae in the River Mira during the year 2000. The abundance estimates for each age-class were derived from age-length keys. Number above each plot is day of sampling (from 1 January). Two size-groups are represented: larvae SL<13 mm (•, continuous line) and 13 mm≤SL<17.5 mm (•, dashed line). Days of year 101 and 151 corresponded to 10 April and 30 May, respectively.

Larval (instantaneous) mortality rates differed significantly among sampling dates (ANCOVA, $F_{[13,52]}$=178.6, $p<10^{-4}$). Mortality rates of the most common age-classes (4 – 15 days-old larvae) were significantly higher (Tukey simultaneous confidence intervals, $p<0.05$) earlier in the

spawning season (day 88) and during a 2-3 weeks period from late-April to mid-May. No date-specific differences were found for older larvae. Estimated initial abundances were significantly lower in mid-April (day of year 119, N_0=28.8 ind 100 m^{-3}) and late-June (day of year 172, N_0=3.5 ind 100 m^{-3}) than in other sampling occasions (N_0 ranged 354.8 to $38.9 \cdot 10^3$ ind 100 m^{-3}). The older larvae (17 – 23 days-old) collected later in the spawning season (late-May to mid-June) belonged to cohorts which emerged in appreciable numbers ($7.9 \cdot 10^6$ ind 100 m^{-3} $<N_0<63.1 \cdot 10^9$ ind 100 m^{-3}).

Few correlations between biological traits and environmental variables were considered significant at the $\alpha = 0.05$ level (). Several coefficients were high enough to suggest possible correlations particularly among environmental variables (*e.g.* temperature, rainfall and plankton). Only one significant correlation involving growth rates of larval shad was found. Shad larvae growth was inversely correlated with potential predators' abundance (r=-0.86, p=0.0137). Instantaneous mortality rates (M) were negatively correlated with several co-variates: temperature (r=-0.75, p=0.0522); abundances of crustacean *nauplii* (r=-0.93, p=0.0025); zooplankters' diversity (r=-0.89, p=0.0068); and the feeding incidence (r=-0.96, p=0.0005). The proportion of shad larvae with gut content (*i.e.* feeding incidence) was directly related with water temperature (r=0.71, p=0.0713), microplankton biomass (r=0.68, p=0.0938), abundance of crustacean *nauplii*, and with the diversity of plankters (all these with r=0.96, p=0.0005) (Table 4.4).

Table 4.4 – Spearman correlation coefficients for date-specific data collected in the River Mira (March – June 2000). Legend: *g* – instantaneous growth rates; *M* – instantaneous mortality rates; *Nauplii* are crustacean *nauplii*; Predators include small *medusae*, mysids and amphipods; Zooplankters include isopods, insects, cladocerans and copepodites; Index – index of diversity; Larvae – Twaite shad larvae; Feeding – feeding incidence; superscripts a = p<0.10, b = p<0.05 e c = p<0.01.

	G	*M*	Temp.	Rain.	Micro.	Nauplii	Zoopl.	Pred.	Index	Larvae
M	0.32									
Temperature	-0.64	-0.75 [b]								
Rainfall [(a)]	0.39	0.64	-0.95 [c]							
Microplankters	-0.07	-0.61	0.33	-0.34						
Nauplii	-0.36	-0.93 [c]	0.67 [a]	-0.61 [a]	0.12					
Zooplankters	0.25	-0.46	-0.17	0.28	0.37	0.40				
Predators	-0.86 [b]	-0.61	0.77 [b]	-0.59 [a]	0.13	0.50	-0.18			
Index [(b)]	-0.07	-0.89 [c]	0.47	-0.44	0.73 [b]	0.68 [b]	0.65 [a]	0.30		
Shad larvae	-0.11	-0.57	0.31	-0.37	0.16	0.62 [a]	0.40	-0.18	0.44	
Feeding	-0.18	-0.96 [c]	0.71 [a]	-0.61	0.68 [a]	0.96 [c]	0.64	0.54	0.96 [c]	0.61

(a) Standard error of past 7-days moving average; (b) Proportion of *taxa* present in any given sample.

No satisfactory regression model could be fit for larval growth. Larval mortality rates (*M*) were higher at water temperatures below 20º C and intermediate levels of rainfall instability (Figure 4.12). Moreover, estimated values of *M* decreased linearly with increasing log(abundance) of crustacean *nauplii*, plankters diversity (Figure 4.13) and proportion of shad larvae with gut content (Figure 4.14).

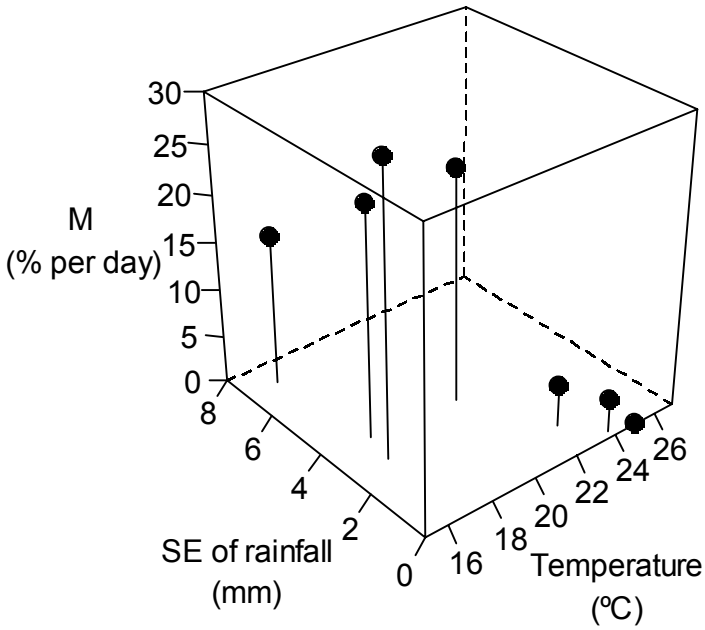

Figure 4.12 – 3D representation of date-specific mortality rates variation with rainfall (standard-error of previous 7-days moving average) and temperature for Twaite shad larvae (5 to 14 days-old) collected in the River Mira during the year 2000 (late-March to early-July).

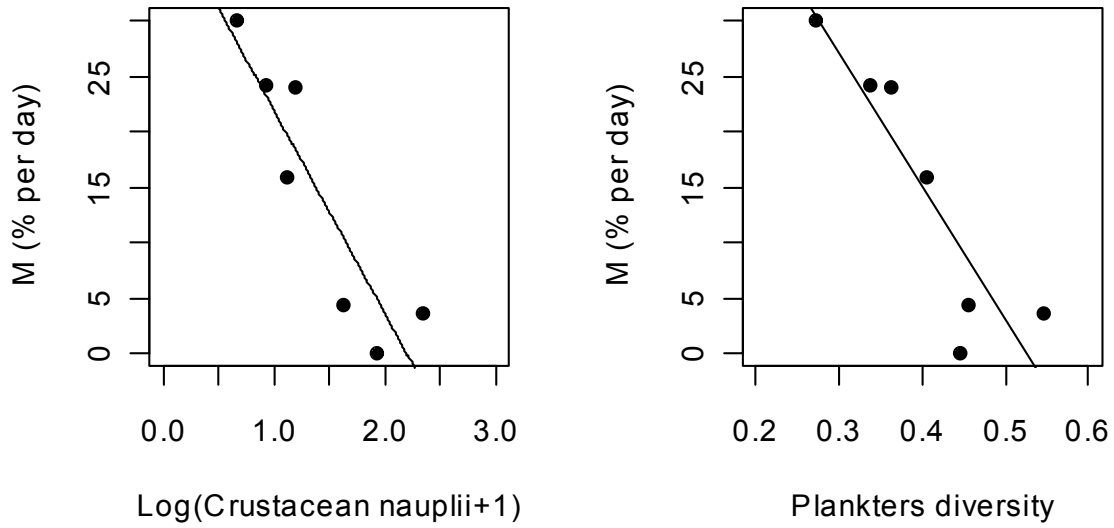

Figure 4.13 - Relationships between mortality rates (*M*, % d^{-1}) of Twaite shad larvae (5 to 14 days-old) and crustacean *nauplii* density (left) and zooplankters diversity (right) (larvae were collected in the River Mira from late-March to early-July 2000). Equations are: M=40.28-18.36n with r^2=0.82 and p=0.0049 for crustacean *nauplii* n and M=63.20-120.52x with r^2=0.80 and p=0.0016 for plankters diversity x.

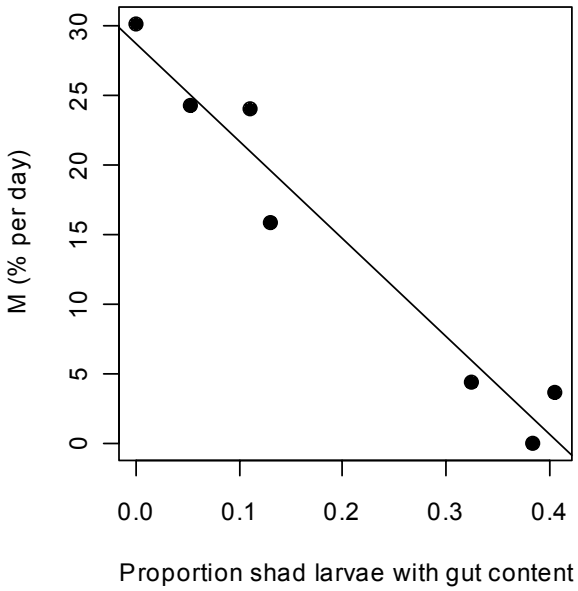

Figure 4.14 - Relationships between mortality rates (*M*, % d^{-1}) of Twaite shad larvae (5 to 14 days-old) and the proportion of larvae with visible gut content (larvae were collected in the River Mira from late-March to early-July 2000). Equation: M=28.70-70,33x with r^2=0.95 and p=0.0002.

4.4. Discussion

When the digitised images of *sagittae* of Twaite shad *A. fallax fallax* larvae were analysed, increments were almost readily visible as composed of paired translucent and opaque rings in a regularly recurring sequence deposited around the otolith. A reduced percentage (<20%) of the 570 *sagittae* processed was not used due to preparation difficulties (*e.g.* over-bleached or lost during handling or mounting). Of the remaining otoliths, *ca*. 70% were used to estimate the age of Twaite shad larvae since at least two out of three replicate readings coincided. Although no formal validation of ages (see Geffen, 1992) was attempted here (*cf.* Chapter 2), the number of increments counted on the *sagittae* of *A. fallax fallax* is expected to reflect the age of larvae considering that significant agreement between the number of sagittal rings and the true daily age has been found for another alosinid, the American shad, *A. sapidissima* (Crecco & Savoy, 1985; Savoy & Crecco, 1987).

There was a moderate (r^2=0.77) but highly significant ($p<10^{-4}$) linear relationship between the growth of the otolith and the larvae, which partially supports the validity of the ageing results and the back-calculation method used here for Twaite shad larvae. Crecco & Savoy (1985) and

Savoy & Crecco (1987) obtained similar results for American shad larvae (r=0.77 and r^2=0.89, respectively).

Herein, the widths of increments deposited on *sagittae* of Twaite shad (1.8 to 12.2 μm), well above the practical resolution limit of light microscopy, 0.5 – 1.0 μm (Campana, 1992; Neilson, 1992), were within the range observed by Limburg (1996a) for American shad collected in the Hudson river estuary (USA), 2 to 7 μm for larvae 9 to 27 mm SL, but were substantially narrower than the increments measured by Savoy & Crecco (1987) on the *sagittae* of American shad captured in the Connecticut River (USA) (13.5 to 24.6 microns for larvae 4 to 24 days-old). Furthermore, average increment widths measured in this study exhibited a rather convoluted pattern with increasing age, being higher than average for the first, *ca*. 5th and 15-20th increments. These correspond roughly to milestones in shad ontogeny namely hatch [in fact, the otoliths are visible immediately after hatch (Taverny *et al.*, 2000b)], yolk-sac resorption and end of larval period respectively (Quignard & Douchement, 1991b; Taverny *et al.*, 2000b) and may reflect the improved ontogenic conditions for larval (and otolith) growth at these transition periods (end of embryonic development, completion of yolk-sac resorption and increased swimming and feeding capabilities, respectively).

Larval hatching extended from 20 March to 16 June 2000, with most Twaite shad larvae emerging after early-May when water temperatures exceeded 20 ºC. These results agree with previous studies on *A. fallax fallax* spawning and hatching behaviour in the River Mira (Pina, 2000) and elsewhere in Europe (Acolas *et al.*, 2004; Adlandsvik *et al.*, 1999; Aprahamian, 1988; Boisneau *et al.*, 1990; Maitland & Lyle, 2005; Mennesson-Boisneau *et al.*, 1986; Thiel *et al.*, 1996). In fact, adult Twaite shad have a protracted spawning season extending from February to July, and are multiple spawners (Aprahamian *et al.*, 2003; Quignard & Douchement, 1991b). Frequency counts may not reflect true hatching intensity, as no age-specific mortality corrections were made (Savoy & Crecco, 1987). However, hatchdate distributions are valuable when compared to egg or recently hatched larval densities and might suggest processes responsible for differential cohort survivorship (Powell *et al.*, 2004). The distribution of hatch dates calculated for Twaite shad in the River Mira along with collections of newly-hatched larvae (Figure 4.15) indicate that shad spawn between March and June and that the majority of spawning occurs above 20 ºC and was concurrent with the seasonal variation in the abundance of potential prey. Intriguingly, a large portion of the embryos collected between days 100 and

130 did not survive to be aged on subsequent sampling dates (see below) thence the discrepancy between the histogram and the line in Figure 4.15.

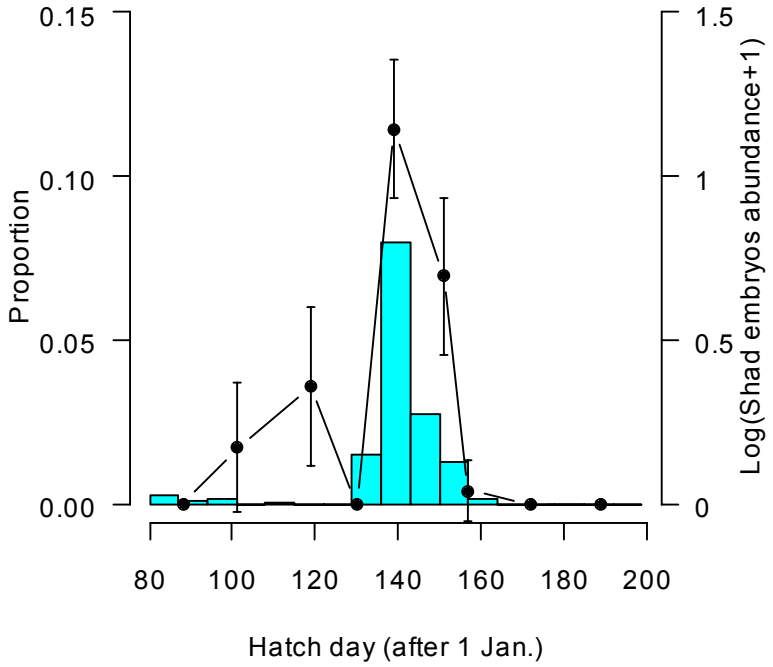

Figure 4.15 – Histogram (proportion of total number of larvae) of backcalculated hatch dates divided into 7-d bins with superimposed line plot of log(abundance) estimates of Twaite shad embryos – newly-hatched larvae with visible yolk-sac (with 95% confidence intervals).

The synchrony between peak larval emergence and occurrence of 20-24 ºC water temperatures reported here and elsewhere may be an adaptive feature (Leggett & Whitney, 1972) because it ensures that egg development coincides with rising water temperatures, shown to be beneficial to larval survival. The eggs can successfully develop within a temperature range of 15 to 25 ºC and incubation lasts for 96 h at a temperature of 19 ºC in the River Seine (France) (e.g. Quignard & Douchement, 1991b). Also, previous studies demonstrated that egg incubation time for American shad declines from 15 days at 14ºC to 3 days at 22ºC (Crecco & Savoy, 1987a). According to Limburg (1996a), egg development time (EDT) of American shad is a monotonic decreasing function of temperature (T) and can be expressed as: $\ln(EDT) = 8.9 - 2.484 \cdot \ln(T)$ (r^2=0.987, $F_{(1,4)}$=223, p<0.0001). Spawning of Twaite shad begins when the water temperature has warmed above 12-16 ºC in northern Europe and 18-22 ºC elsewhere in Europe or Northern Africa (Aprahamian *et al.*, 2003; Boisneau *et al.*, 1990; Quignard & Douchement, 1991b). Moreover, the onset of American shad spawning begins in the Connecticut River (USA) at temperatures of 13-18ºC (Leggett & Whitney, 1972). On the other hand, hatching of Twaite shad larvae seemed to roughly precede the peaks in abundance of potential prey (Figure 4.4

versus Figure 4.5). Cohorts that hatch before *maxima* should encounter increasing prey density during subsequent weeks, whereas cohorts hatching in synchrony with prey *maxima* will encounter progressively reduced prey availability as the cohort ages (Betsill & Avyle, 1997). Hence, the higher growth rates of early-spawned larvae (see below) might reflect, at least partially, the more favourable environmental and biotic conditions.

Overall growth-in-length of Twaite shad larvae from the River Mira was significantly described by a linear equation, which yielded a daily (instantaneous) growth rate of 0.430 mm d^{-1}. Nevertheless, date-specific growth rates of shad larvae indicated that growth varied seasonally (range: 0.356 – 0.603 mm d^{-1}). These estimates are within the range of mean length increments of American shad larvae reported by Crecco & Savoy (1985) (0.38 to 0.52 mm d^{-1}) and the values reviewed by Blaxter & Hunter (1982) for several clupeoids (0.3 – 1.0 mm d^{-1}). Instantaneous growth rates of Twaite shad larvae in the River Mira are higher than those found by Ré & Gonçalves (1993) for anchovy *Engraulis encrasicolus* also spawning in the River Mira (0.27 to 0.45 mm d^{-1}) and the rates obtained by Secor & Houde (1995) for anadromous striped bass *Morone saxatilis* from the Patuchent river (Chesapeake Bay), 0.15 mm d^{-1} to 0.22 mm d^{-1}. Estimated length-at-age using the models provided here agreed well with sizes at age referred to by Quignard & Douchement (1991b), 5-8 mm at hatch, 8-9 mm at 6 days-old and 13-15 mm for 15-20 days-old larvae. Herein, the difference between fast- and slow-growing cohorts was about 0.250 mm d^{-1}. Over a 20-d period this could mean a 5-mm difference in larval length. Alternatively, this level of variation in growth rate would mean a 23-d difference in the duration of the larval age, assuming that metamorphosis to juvenile stage, the "transitional stage" (sensu Quignard & Douchement, 1991b), occurs at ≥20 mm. On the other hand, estimated size-at-hatch corresponded well to published data from field and rearing studies of Twaite shad (Aprahamian *et al.*, 2003; Quignard & Douchement, 1991b). In the River Mira and elsewhere, larvae of Twaite shad seem to hatch at relatively smaller sizes than other alosinids, *e.g. ca.* 7 mm for Allis shad (Ramos, 1977) or 6.5 – 10 mm TL for American shad (Crecco & Savoy, 1985).

The failure to detect any significant influence of temperature and/or prey density on date-specific growth rates was surprising. Laboratory, pond (mesocosm) and model-simulation studies have demonstrated repeatedly that growth and survival of fish larvae increase as temperature and/or prey density increases, *e.g.* American shad (Crecco & Savoy, 1985; Johnson & Dropkin, 1995; Leach & Houde, 1999; Letcher *et al.*, 1997), Threadfin shad *Dorosoma petenense* (Betsill & Avyle, 1997) or Striped bass (Betsill & Avyle, 1997; Rilling & Houde,

1999; Secor & Houde, 1995). It is possible that the failure to demonstrate a relationship between growth rates and temperature and/or prey densities was an artefact that resulted from backcalculating growth rates from mostly older larval survivors which endured the unusual rainfall events of early-Spring (Figure 4.3). Growth rates of these larvae conceivably may have been higher than growth rates of larvae that died (Pepin, 1989) and may have obscured impacts of lower temperature and lower prey densities on growth (Rutherford & Houde, 1995). Remarkably, date-specific growth rates of Twaite shad were inversely correlated with the abundance of potential predators (*e.g.* small medusae, mysids and amphipods). However, this was probably a fortuitous result because no other meaningful correlation (*e.g.* between predators and shad larvae abundances or predators and prey abundances) was statistically significant.

Cohorts of Twaite shad larvae in the River Mira do not experience uniform mortality during the spawning season. Date-specific estimates indicated that mortality rates were lowest for 5 to 14 days-old Twaite shads hatched during late-May and June (<5% d^{-1}), when densities of post-larvae and their potential prey reached maximum levels. Conversely, mortality rates for early-season samples of those larvae were in the range 16 to 30 % d^{-1}. This pattern of seasonal variation in mortality rates agrees with the results of Crecco & Savoy (1987a) for American shad in the Connecticut River (USA). Using a different methodology to estimate mortalities (the proportion of larvae surviving to the juvenile stage – which yielded comparatively lower estimates), these authors found that cohort-specific mortality rates were higher for cohorts early-spawned (*ca.* 6 to 7 % d^{-1}) and declined to about 2 – 3% d^{-1} later in the spawning season. On the other hand, older (17 – 23 days-old) and later collected (days 151 – 172) larvae of Twaite shad in the River Mira experienced relatively higher mortality rates, *ca.* 26 to 38% d^{-1}. It was expected that these larvae survived better than first-feeding and younger larvae because of their likely increased swimming and feeding skills (Blaxter & Hunter, 1982). In fact, Crecco *et al.* (1983) found that instantaneous mortality rates of larval American shad in the Connecticut river dropped from *ca*. 25% d^{-1} for 9 – 10 days-old larvae to about 18% d^{-1} (18 – 20 days-old larvae) and 6% d^{-1} for >26 days-old larvae. The results obtained in this study might be related to a detrimental combination of falling abundance of potential prey and growing density of potential predators (or competitors for same food resources) coincident with those larval stages.

The exponential models provided acceptable estimates of date-specific mortality rates of Twaite shad. These rates could be influenced by the range of ages analysed for each cohort (Secor & Houde, 1995). Herein, this was the case for only one sampling date (day of year=101) when the

smaller and presumably younger larvae were not completely sampled. Notwithstanding, estimates of initial abundance (N_0) per sampling date were in agreement with the abundance of recently hatched (yolk-sac) larvae (Table 4.3 *versus* Figure 4.15). On the other hand, if gear avoidance increases with the size of shad larvae the estimates of mortality rates for the larger individuals should be biased. Even though the same gear was used to collect all sizes of larvae, mortality rates were not estimated for larval lengths ≥17.5 mm (≥24-d old) to overcome this potential problem.

Temporal variability in Twaite shad larvae mortality rates was related both to temperature and rainfall as well as zooplankton density and diversity and shad larvae feeding incidence. For temperatures above 22ºC, M dropped below 5% d^{-1}. Significant but non-linear relationships between cohort-specific mortality rates and water temperature have also been found for other alosinids, *e.g.* Threadfin shad *Dorosoma petenense* (Betsill & Avyle, 1997) and American shad (Crecco & Savoy, 1987a), suggesting that mortality rates fall rapidly at water temperatures above 21 – 22 ºC. Interestingly, the steep rise in surface water temperatures measured in the upper River Mira (from *ca*. 17 ºC to more than 20 ºC) in a short period of time (between days 120 and 140) coincided with an episodic increase in the mortality estimates (Figure 4.3 *versus* Figure 4.11). Leach & Houde (1999) demonstrated that environmental pulses of temperature and pH, *e.g.* 5 ºC rise in temperature or depressions of pH from 7.0 to 6.0 that may occur in association with rainfall events, had strong detrimental responses in survival probability of reared American shad larvae. According to Crecco & Savoy (1985), the magnitude of larval American shad mortality (and juvenile production) among discrete cohorts depends largely on the coupling of larval emergence and river flow conditions one week later. They found that high larval mortality (9 to 11% d^{-1}) and low feeding rates of first-feeding larvae during late-May resulted from storms and high river runoff that lowered water temperatures and transparency. As a result, first-feeding larvae from early cohorts sustained higher mortality because they grew more slowly at low (15 – 18ºC) water temperatures (Crecco & Savoy, 1985) and/or fed less frequently due to high turbidity and reduced zooplankton patchiness (Lasker, 1975, 1978). Moreover, unexpected high rainfall events and the resulting high river flows registered in the River Mira during early-May may have advected larvae and their zooplankton prey some distance from "safe" sites, where conditions are favourable, to nearby sections of the river of lower food density, high predator abundance (Crecco & Savoy, 1987a) or even downriver towards the euryhaline estuary. Zydlewski & McCormick (1997) found that there was no

survival in seawater of reared American shad larvae before 36 days post-hatch and that most mortalities occurred within 2 h of exposure to a saline (35 ppt) milieu.

In this study, the mortality rates of 5 to 14 days-old shad larvae were also inversely and significantly correlated ($r \leq -0.90$, $p < 0.01$) with the abundance and diversity of potential prey (*e.g.* crustacean nauplii) and the proportion of larvae with visible gut content (*i.e.* feeding incidence). The coupling of mortality variability and zooplankters densities suggests that differential larval mortality is due partly to temporal changes in food availability in both quantity and quality. Short periods of food deprivation among first-feeding larvae can result in death from malnutrition (Blaxter & Hunter, 1982; Hunter, 1981) because the foraging ability of young herring-like larvae is greatly limited by their small mouth and poor swimming ability (Hunter, 1981). Furthermore, the significant inverse correlation obtained herein between the mortality rates and feeding incidence of shads and the direct relationship between the proportion of larvae with gut contents and the abundance of potential prey again stresses the importance of food availability upon survival of the larvae. Lack of food would also enhance larval mortality indirectly by reducing larval growth and stamina, thus rendering the larvae vulnerable to predation for prolonged periods (Hunter, 1982). But despite the potential linkage between predation-induced mortality and year-class strength, larval predation is a complex process whose interaction with biotic and abiotic factor is difficult to measure in field surveys (Crecco *et al.*, 1983).

The results of this study indicate that temporal changes in river flow and temperature as well as variation in the abundance and quality of potential prey, coincident with early larval development, are important regulators of mortality of Twaite shad in the River Mira. Climatic factors such as heavy rainfall events may sweep Twaite shad larvae and their zooplankton prey from “safe sites” to unfavourable reaches of the river. On the other hand, elevated river flows coincident with peak larval emergence may also increase the water turbidity that has been shown to favour feeding success of fish larvae through enhanced prey contrast on the small perceptive scale used by the larvae (Boehlert & Morgan, 1985; Utne-Palm, 2001). Larval residence in turbid environments may contribute to reduce predation from larger, visual planktivores substantially affected by turbidity, while searching ability in the small larval perceptive field is not decreased (Boehlert & Morgan, 1985). No evidence of this suggestion could be produced herein. Conversely, low transparency can interfere with photosynthesis and river phytoplankton production (Mallin *et al.*, 1999; Marker & Collett, 1997), causing the selective elimination of

major larval prey such as crustacean *nauplii* and immature insects (Aprahamian *et al.*, 2003; Crecco & Blake, 1983; Taverny *et al.*, 2000b), leading to higher incidences of malnutrition among first feeding larvae (Crecco & Savoy, 1987b) and eventually reducing larval growth. The subsequent use of nucleic acid-based indices, *e.g.* RNA/DNA ratios – an ecophysiological index of condition, which reflects the potential for protein synthesis – allows to further study the nutritional condition of larvae and its relationship with several abiotic and biotic factors and their relative importance in determining larval mortality and recruitment.

Chapter 5. RNA/DNA of Twaite shad *Alosa fallax fallax* (Lacépède, 1803) larvae from a lowland tidal river (River Mira) and its relationships to ontogeny and the environment

5.1. Introduction

The variability in recruitment is determined by survival during the early life history stages of fish. This is particularly evident in the Genus *Alosa* (Crecco & Savoy, 1987a; Lorda & Crecco, 1987). Several hypotheses linking abiotic and biotic factors with larval mortality have been presented (Cushing, 1975; Hjort, 1914; Lasker, 1975). However, it is not generally agreed whether density-independent (temperature, advection) or density-dependent mechanisms (starvation, predation, competition for food) prevail in determining the survival of larvae. Although hydrological factors and predation seem to regulate larval mortality (Bailey & Houde, 1989), starvation is also responsible, directly or through increasing vulnerability to predation and liability to detrimental abiotic factors (Houde, 1997b).

The nutritional condition of larval and juvenile fish is a means of assessing the significance of hydrological features for fish populations during the critical planktonic stage of early life history (Suthers, 1996). Since *Alosa* spp. are anadromous species, exhibiting a pronounced homing behaviour (e.g. Hendricks *et al.*, 2002; Tomás *et al.*, 2005), environmental conditions during the embryo-larval period in the freshwater reaches of rivers play an important role in the future of populations (Baglinière, 2000). Habitat quality of nursery areas depends on the interplay of both natural environmental and anthropogenic factors. High quality nursery habitats are presumed to be those where the growth, the condition and thence, the survival of the early life-history stages are enhanced (Gilliers *et al.*, 2004).

Measures of growth, mortality and condition of young fish have been used to assess the effects of environmental conditions on individuals (e.g. Bailey *et al.*, 1995; Chícharo, 1998; Chícharo *et*

al., 1998a; Clemmesen, 1994; Crecco & Savoy, 1985; Esteves *et al.*, 2000b; García *et al.*, 1998; Hovenkamp & Witte, 1991; McGurk *et al.*, 1992; Secor & Houde, 1995). Many plausible condition indices have been proposed (for reviews see Bergeron, 1997; Ferron & Leggett, 1994). The amount of DNA in a cell is constant; the amount of RNA indicates how actively the cell is synthesising proteins, and thus perhaps how healthy it is (Buckley *et al.*, 1984; Clemmesen, 1994). Short-term changes in feeding activity and development rates are reflected in RNA/DNA level within a few days (Bergeron, 1997; Clemmesen, 1996; Ferron & Leggett, 1994). There is evidence from laboratory and field studies that a decrease in average nucleic acid ratios is associated with reduced survival (Canino, 1994; Canino *et al.*, 1991; Clemmesen *et al.*, 1997) and growth rates (Hovenkamp, 1990).

The anadromous Twaite shad, *A. fallax fallax*, is classified as "Vulnerable" by the Instituto de Conservação da Natureza using IUCN criteria (Cabral *et al.*, 2006) and still migrates upriver in several rivers along Portugal to spawn (Eiras, 1977), namely the River Mira. At present there is no published information on nutritional condition of reared or field-caught *A. fallax fallax* larvae except for the RNA/DNA ratios of a few *Alosa spp.*larvae (n=4) collected in the River Guadiana (SW Portugal) that averaged 4.07 (1.01 to 8.56) (Esteves, 1999).

The main objectives of this work were two-fold: to determine the RNA/DNA ratios of individual *A. fallax fallax* larvae and to combine that information with ontogenic variables (length, age or gut content) and other more classical indices of population “health” (growth and mortality rates); and to examine the influence of environmental (temperature, rainfall and turbidity) and biological (abundances of potential prey, predators, shad larvae and feeding incidence) covariates on the seasonal changes of RNA/DNA ratios.

5.2. Material and Methods

Study area

The Mira River is a 145-km long, relatively narrow (<400 m near the mouth) and shallow (<10 m deep near the mouth) watercourse located in SW Portugal. Tidal influence extends to approximately 30 km from the mouth, where mechanical effects and salinity changes have been observed. At the upper limit of tidal influence, water depth varied between <0.5 and 3.5 m

whereas river width did not exceed 45 m. The current velocity of surface water averaged 0.23 – 1.20 m s^{-1} during ebb, and 0.29 – 0.69 m s^{-1} during flood-tides (Author, unpublished results). River sediment is composed of very fine silt, sand, pebbles and cobble with submerged vegetation and debris (see Chapter 3). Further information is provided in the web site of the Serviço Nacional de Informação sobre Recursos Hídricos do Instituto Nacional da Água (SNIRH at http://snirh.inag.pt/snirh/).

Sampling and laboratory procedures

Between February 1998 and July 2000, samples were collected every two weeks in three stations (no. 4 to 6) located in the upper River Mira (*cf.* Figure 3.1, Chapter 3). Sampling took place during daylight hours from 0900 hours to about 1900 hours. The stations covered the oligohaline to limnetic reaches of the river that were accessible by boat and close to the presumed spawning locations. Occasionally, samples could not be collected due to equipment failure or severe weather conditions, but at least six sampling dates on each spawning season were accomplished.

At each station and sampling occasion, mesozooplankton was collected with a conical net (1.6 m long, ∅ 0.37 m, 0.5-mm mesh size) towed 1 m below the surface, at a constant speed of approximately 1 m s^{-1} for 10 minutes. Plankton tows sampled evenly the river margins and the main channel along the area covered by each station. A flowmeter (Hydrobios®) was attached inside the net to estimate the water volume filtered (median=62.8 m^3, range=15.0 – 113.1 m^3). Samples were sorted in a black tray, the fish larvae retrieved and immediately frozen in liquid nitrogen (at -197 ºC) for later biochemical analysis. The remaining zooplankters were stored in buffered 4% formaldehyde solutions and later were identified using published information (Johnson & Loesch, 1983; Newell & Newell, 1963; Quignard & Douchement, 1991b; Russell, 1976; Saville, 1964; Smith, 1977; Todd & Laverack, 1991). Plankters' density was expressed as number of individuals per 100 m^3 of water. During plankton sampling, Secchii disk depth was monitored, the state of tide was noted and the surface temperature (º C) and salinity (ppt) were measured using a hand-held thermometer and a refractometer, respectively. A further 26 – 39 L of water were sampled at 0.5-m depth and filtered through a 63-µm mesh size sieve to collect microplankters.

To estimate microplankton biomass, aliquots of the homogenised samples were rinsed through a 1.2-µm milipore filter (Macherey-Nagel® GF-3, ∅ 25 mm). Filters were dried in an electric

oven at 60º C for 24 – 36 hours to obtain the dry weights (DW) and then incinerated at 450º C for 4 hours to determine the ash-free dry weight (AFDW). The results of water turbidity were expressed as mg DW m^{-3} and microplankton biomass was expressed as mg AFDW m^{-3}.

For the analysis of nutritional condition, individually frozen larvae (*n*=1699) were thawed, identified and measured (to nearest 0.1 mm). Their condition was assessed using the RNA/DNA ratio. Nucleic acids in individual fish larvae were quantified according to a modified fluorimetric method and using ethidium bromide (EB) as the fluorophor. Fish larvae were extracted in 1% sarcosine in Tris-EDTA buffer (Trizma®, pH 8.0) to give a final concentration of 0.1%. After centrifugation, aliquots of the supernatant were: 1) combined with Tris-NaCl (Trizma®, pH 7.5) plus ethidium bromide (0.1 mg ml^{-1}) to give the total nucleic acids content; and 2) incubated for 30 min at 37 ºC with Tris-NaCl and 0.05 ml of ribonuclease A (Type-II A, 0.12 μg ml^{-1}) and stained with EB to give the DNA content. The fluorescence due to total RNA, mainly ribosomal, was calculated as the difference between the results from the previous two procedures. Fluorescence was determined by exciting at 365 nm and reading at 590 nm with a spectrofluorometer (Hitachi® Model 650-10). Concentrations were calculated by running standard curves of DNA-EB and RNA-EB every day with known concentrations of λ-DNA (Boehringer-Mannheim®, 0.25 μg $μl^{-1}$) and 162-23s RNA (Boehringer-Mannheim®, 4 μg $μl^{-1}$), in the appropriate range of values. The limit of detection was 0.16 μg ml^{-1} for DNA and 0.46 μg ml^{-1} for RNA. Percent recovery of added λ-DNA (spikes) was 96.4%$\pm$5.9% and the recovery of 16s-23s RNA spikes was 97.6%$\pm$7.0%. Total amounts of nucleic acids were corrected base on these values. The coefficient of variation for estimates from eight homogenates was 1.5% for DNA and 3.5% for RNA (see Esteves *et al.*, 2000a for further protocol details).

Yolk-sac larvae (*n*=156) 4.92 – 10.79 mm SL were analysed but not considered further because the condition of fed and starved larvae in the first days after hatching is not significantly different due to the process of internal yolk absorption and learning to capture food (Clemmesen, 1996).

After biochemical analysis, larval remains (mostly bony structures and connecting cartilage) were further examined for their otoliths (herein only the samples collected during 2000 were used). *Sagittae* were removed using a combination of 45% NaOH rinse (<3 min) and fine-needle manipulation under a stereoscope. After washing (with 99% ethanol and water) and drying the

otoliths these were mounted on a slide using Pro-Texx® mounting medium (see Secor *et al.*, 1991; Stevenson & Campana, 1992 for further details). Digitised images of the *sagittae* were obtained with a microscope (at 400x magnification) and analysed using image analysis software (ImagePro Plus®). The number of increments (herein an increment corresponded to paired translucent and opaque rings in a regularly-recurring sequence deposited around the otolith) was counted and their width measured (to 0.1 μm) in three independent occasions without prior knowledge of length or sample information (see Stevenson & Campana, 1992 for a thorough review of methods).

Statistical analyses

The relationships between larval nutritional condition (RNA/DNA ratios) and otolith–related traits, *e.g.* number of increments counted (age), recent otolith growth (determined by measuring the width of the three last complete increments of the otoliths to 0.1 μm), and average increment width and otolith radius (measured to 0.1 μm) were studied through Spearman correlation analysis using data for the individual n=151 larvae caught during the year 2000. The use of the widths of otolith increments was justified because a strong linear relationship existed between the size of the fish and size of the otolith: $y=6.06+97.77x$, $r^2=0.84$ with $p<10^{-4}$ (Campana & Neilson, 1985). Furthermore, Spearman correlation coefficients were used to study if nucleic acids-based results were related to estimates of "classical" (instantaneous) growth and mortality rates previously determined by analysing otolith micro-structural features (see Chapter 4).

A 3-way ANOVA ratio was used to test the (general) effect of factors *Year* (1998 – 2000), *Station* (no. 4 – 6) and *Gut* contents (Empty or Prey visible) on RNA/DNA ratios. For this ANOVA, the ratios were cube root-transformed prior to analysis to correct for normality. The Box-Cox profile function (Venables & Ripley, 1999) was used to select the appropriate transformation. Furthermore, in order to study the within-year variation in nutritional condition of shad larvae, values of RNA/DNA ratios were first scaled using $(x-\bar{x})/s$, where $\bar{x}$ and s are the station-wise yearly average and standard deviation. This approach allowed the pooling of ratios across stations and years of collection considering that there was a significant station × year interaction effect upon RNA/DNA ratios (see above). Then, average (scaled) values where plotted against day of collection to expose possible seasonal trends.

Finally, the following predictor variables (Table 5.1) were used to model the relationship

between environmental conditions and (average) nutritional condition of shad larvae: water surface temperature (ºC); water turbidity (expressed as mg DW per 100 m^3 and estimated from the dry weights of filters used for microzooplankton samples); standard errors of the moving averages of rainfall (with a period of 7 days prior to sampling date) were used as an estimate of rainfall variability in mm (the higher the estimate, the greater the rainfall instability in the days that preceded sampling); densities of isopods, small insects, cladocerans and copepodites were pooled into a variable named *zoo* (ind. 100 m^{-3}); another variable designated *pRed* was the sum of the abundances of potential predators/competitors such as small medusae, mysids and amphipods grouped into three abundance classes, 0, 1 – 10 and >10 plankters 100 m^{-3}; the abundance of shad larvae (ind. 100 m^{-3}); the proportion of larvae with visible gut content; and the individual nutritional condition (RNA/DNA ratios). Several predictor variables were transformed prior to analyses, e.g. $\log(x+1)$ for abundances or $\sin^{-1}\left(\sqrt{x}\right)\cdot 180/\pi$ for proportions[4].

To study the effects of abiotic (temperature, rainfall and turbidity), biotic (abundance of prey and predators) and biological co-variates (abundance of shad larvae and feeding incidence) on the nutritional condition of Twaite shad larvae, multiple linear regression and generalized additive models were used to determine the best fitting models. Models were built using each separate category of co-variates and all categories in combination. The (adjusted) coefficient of determination (adjusted-R^2) was used to measure the portion of variation (of RNA/DNA ratios) explained by the models and the correlation coefficient (R) between predicted and observed ratios was used herein as a measure of model performance (or predictive accuracy).

Stepwise linear regression (Crawley, 2002; Venables & Ripley, 1999) was performed to build models using potential predictor variables (based on Spearman rank correlations and visual inspection). Addition and removal of variables were conducted manually based on *p*-values, biological interpretation and examination of plots. Residual plots to examine final models for homoscedasticity and normality have been used.

[4] The actual proportions (the x in the above formula) were obtained as follows: if $y/n=0$ then $p=1/(4n)$ or if $y=n$ then $p=(n-¼)/n$ (otherwise $p=y/n$) where y is the number of outcomes of interest, n is the sample size and p is the proportion (Robin High, University of Oregon, http://darkwing.uoregon.edu/~ronih/arcsin.txt, on 18 Aug 2005).

Table 5.1– List of variables used in this study of Twaite shad (units) and respective designations for analysis, descriptive statistics and transformations used.

Variable	Designation	Median (Range)	Type (Transformation)
Abiotic			
Temperature (surface, º C)	`temp`	23.0 (16.0 – 26.0)	Continuous (None)
SE of rainfall (7-day period, mm)	`rain.se`	1.9 (0 – 7.1)	Continuous (None)
Turbidity (mg DW m^{-3})	`turb`	2.2 (1.0 – 4.0)	Continuous (None)
Biotic			
Microplankton biomass (mg AFDW m^{-3})	`Micro`	42.3 (0 – 184.4)	Continuous (Log)
Decapods nauplii (ind. 100 m^{-3})	`Naupli`	78.6 (0 – 1617.0)	Continuous (Log)
Zooplankters (ind. 100 m^{-3})	`Zoo`	16.6 (0 – 622.7)	Continuous (Log)
Potential predators [a] (ind. 100 m^{-3})	`pRed`	1.4 (0 – 128.5)	Continuous (Group [b])
Biological			
Density of larvae (ind. 100 m^{-3})	`Larvae`	10.1 (1.3 – 379.9)	Continuous (Log)
Proportion of larvae with gut content	`pWprey`	0.167 (0.006 – 0.800)	Continuous (Arcsine)
Response			
RNA/DNA ratio	`RNADNA`	1.50 (0.55 – 7.19) [c]	Continuous (Log)

Legend: [a] – also includes *taxa* that competes with shad for the same niche, b – data grouped into three abundance classes (0, 1 – 10 and >10 plankters 100m^{-3}), c – two extreme values (due to a underestimated estimate of larval DNA content) were not considered herein.

Generalised additive models or GAM (Hastie & Tibshirani, 1990; Wood & Augustin, 2002) extend the range of applications of generalised linear models by allowing non-parametric smoothers in addition to parametric forms combined with a range of link functions (linear, log, logit, etc) which describe the error structure of the model, *i.e.* the variation of $E(Y \mid X_j)$. The relationship between a dependent variable Y and a set of predictor variables $X_1, X_2, \ldots, X_p$ can be modelled as

$$Y = \alpha + \sum_{j=1}^{p} f_j(X_j) + \varepsilon \tag{5.1}$$

where f_j are rather general (unspecified) functions that can be estimated in a flexible manner, using a simple algorithm. This algorithm is basically a scatter plot smoother and the estimated function $\hat{f}_j(x_j)$ can then reveal possible non-linearities in the effect of x_j. A modern alternative is to use *spline* functions, which impose smoothness directly on $f(x)$. The model is fitted by

iteratively smoothing partial residuals in a process known as backfitting (see Chapter 3 for further details), Herein, RNA/DNA ratios were log-transformed and a gaussian probability distribution together with a identity link function was used to describe the error structure. GAM were fitted through the `R` package `mgcv` (Wood, 2001).

All statistical procedures described above were carried out using `R` (R Development Core Team, 2003) and are available from the author upon request. For all statistical analyses, the level of significance was set at 0.05 and *p*-values greater than 0.10 were considered as indicative of non-significance.

5.3. Results

A total of 1699 Twaite shad larvae were analysed for RNA and DNA contents, of which 316 were colleted in 1998, 117 in 1999 and 1266 in 2000. Larval size varied between 5.7 and 20.4 mm SL (5.7 – 19.3 mm SL in 1998, 6.3 – 15.4 mm SL in 1999 and 6.1 – 20.4 mm in 2000). DNA and RNA contents increased exponentially with larval size (Figure 5.1). Moreover the two nucleic acids showed a strong linear relationship with each other (r^2=0.75, $F_{[1,1697]}$=5153 with $p<10^{-4}$). The RNA/DNA ratio correlated positively with larval size (Regression: y=-0.40+0.06·x, $F_{[1,1697]}$=124,8 with $p<10^{-4}$), although the coefficient of determination was rather low (r^2=0.07). Hence, no size-effect correction of RNA/DNA ratios was made in further analysis[5]. Individual RNA/DNA ratios ranged from *minima* of <0.1 for 9.05 to 16.64 mm larvae (n=7) to *maxima* of >10 for 10.28 and 14.10 mm larvae (n=3). The mean RNA/DNA ratio of the sampled larvae was 1.64 $\pm$ 1.12 (SD).

Using data for the year 2000 (n=151), RNA/DNA ratios were significantly correlated with otolith radius and age (respectively, r=0.53 and r=0.49; both with $p<10^{-4}$) but not with the sum of the last three complete increments (herein the index of recent of otolith growth) or the average increment width (respectively, r=0.09 with p=0.2508 and r=0.02 with p=0.8089). The sum of the last three complete increments was only significantly correlated with the average increment width (r=0.33 with $p<10^{-4}$) (Figure 5.2).

[5] The analyses described here for the RNA/DNA ratios were also performed using the residuals of the RNA-to-SL regression and the residuals of the RNA-to-DNA regression as alternative indices of nutritional condition with similar conclusions, thus they were not included herein for succinctness.

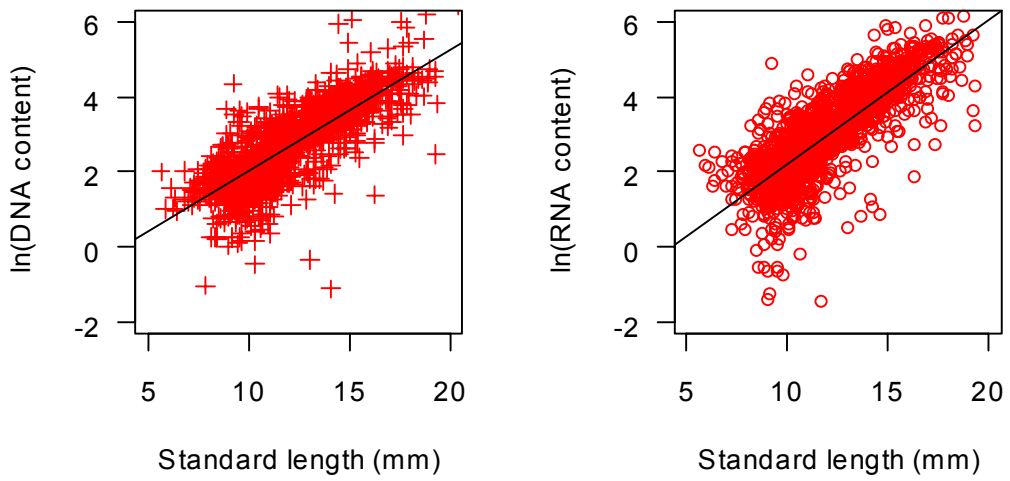

Figure 5.1 – Relationship between nucleic acids contents and larval standard length. Equations are: ln DNA = -1.22 + 0.32 SL (r^2=0.67, $F_{[1,1697]}$=3449 with p<10^{-4}); and ln RNA = -1.63 + 0.39 SL (r^2=0.66, $F_{[1,1697]}$=3224 with p<10^{-4}).

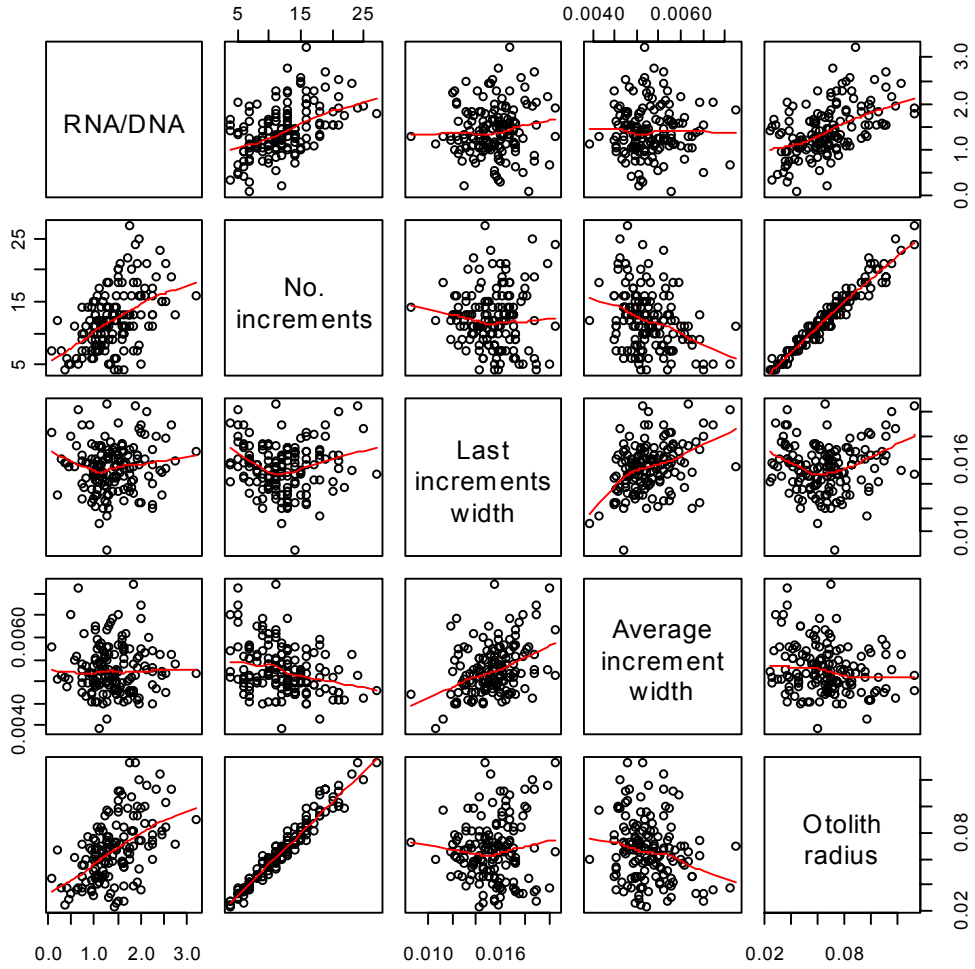

Figure 5.2 – Scatter plot matrix of RNA/DNA ratios and otolith growth (number of increments, last three increments width, average increment width and otolith radius) of Twaite shad larvae. Each dot (○) represents an individual larva. Continuous lines are spline functions shown for illustrative purposes only.

When comparing the RNA/DNA ratios and the growth and mortality rates derived from otolith microstructure analysis (Figure 5.3), mean ratios were inversely correlated with instantaneous growth and mortality rates (r=-0.71 and r=-0.25, respectively) although not significantly (p=0.0713 and p=0.5887; n=7). If data pertaining to day 139 was not considered, correlation coefficients rose up to r=-0.81 and r=-0.71, respectively (with p=0.0416 and p=0.1108; n=6).

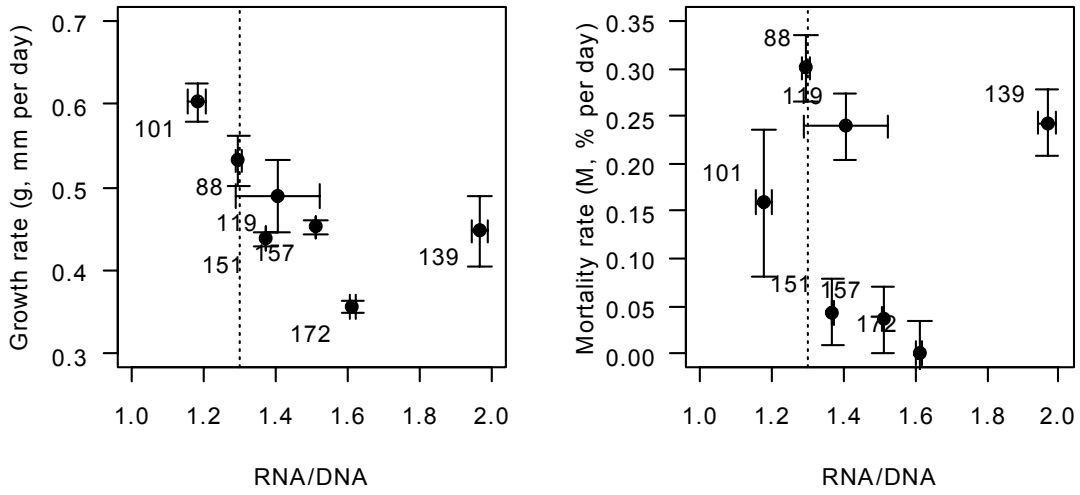

Figure 5.3 – Plots of average RNA/DNA ratios versus growth rates (left plot) and instantaneous growth rates (right plot). Numbers are day of year (after 1 Jan.) and error bars are ± 1 standard error. Vertical dotted lines on the plots indicate the "critical" RNA/DNA ratio of 1.3.

Differences in larval condition among years, stations of collection and gut content were studied using a 3-way ANOVA. A relatively complex behaviour was found for the RNA/DNA ratios since the significant interactions *Year* × *Station* and *Year* × *Gut* were evident (Table 5.2). Mean RNA/DNA ratios were higher for larvae caught during 1999 (3.71±1.68 SD) than those collected in 1998 and 2000 (1.49±0.86 and 1.46±0.64, respectively). Furthermore, average RNA/DNA ratios were highest for larvae collected on station no. 5 in 1998 and 2000 (1.86 and 1.53, respectively) whereas during 1999 the ratios declined steadily from station no. 4 to station no. 6 (from 5.44 to 3.44). On the other hand, larvae with prey visible in the gut collected during 2000 had higher RNADNA ratios than those with empty guts (1.67 *vs*. 1.34, respectively) while the effect of gut content on larval nucleic acid content was negligible in 1998 and 1999 (Figure 5.4). During 1998 and 2000 a large proportion of larvae had RNA/DNA ratios <1.3 (almost 45% each year) whereas during 1999 only a small portion (*ca.* 5%) were considered as "starving". The percentages of starving Twaite shad larvae were higher for 8 to 12 mm length classes and decreased considerably with increasing size during 1999 and 2000 but not for larvae collected in

1998 (Figure 5.5).

Table 5.2 – 3-way ANOVA to test for the effect of year (1998 to 2000), station (no. 4 to 6) and gut content (empty or with visible prey items) on cube root RNA/DNA. Only the significant factors (or terms) (i.e. $p<0.05$) are shown here.

Source of variation	df	SS	MS	*F*-value	*p*-value
Year	2	18.46	9.23	277.70	$<10^{-4}$
Station	2	0.22	0.11	3.36	0.0349
Gut	1	1.93	1.93	58.15	$<10^{-4}$
Year x Station	3	0.70	0.23	7.02	0.0001
Year x Gut	2	0.50	0.25	7.44	0.0006

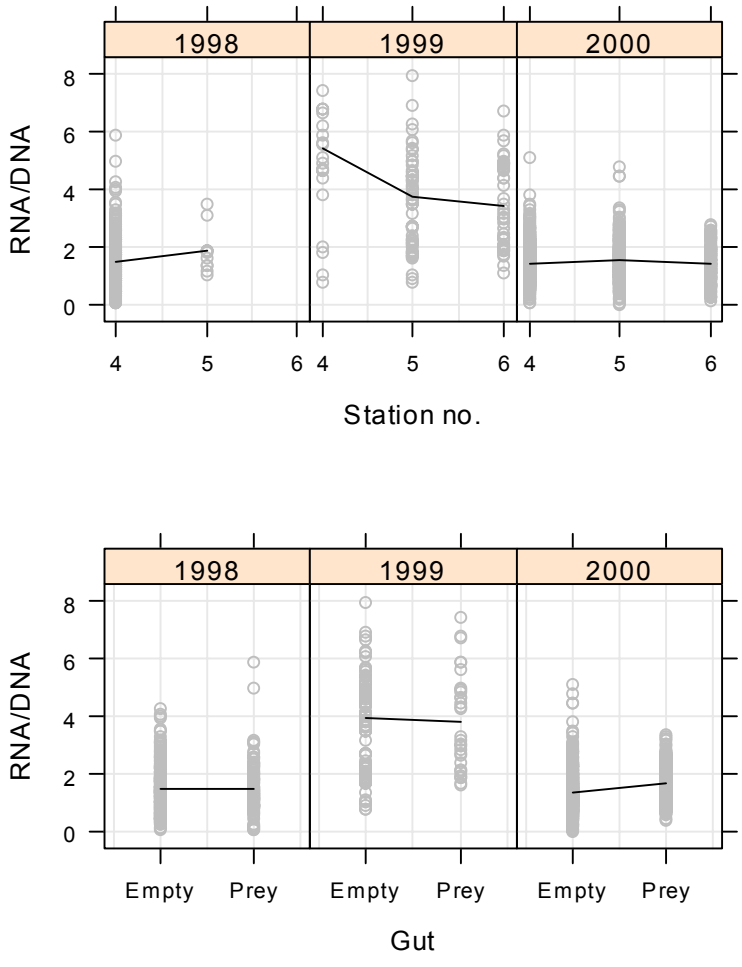

Figure 5.4 – Scatter plots of changes in RNA/DNA ratios of Twaite shad larvae between years of collection (1998 – 2000), stations (no. 4 – 6) (top panel) and gut content (bottom panel). Continuous lines join average RNA/DNA per station or gut content.

The within-year variation of the scaled nutritional condition showed that Twaite shad larvae were in better condition in late March-early April (days 80 to 100) and late May-early June

(days 140 to 150) relatively to other periods during the spawning season (Figure 5.6). The seasonal changes of environmental co-variates monitored in this study are illustrated in Figure 5.7 to Figure 5.9 (sampling dates were pooled for illustrative purposes).

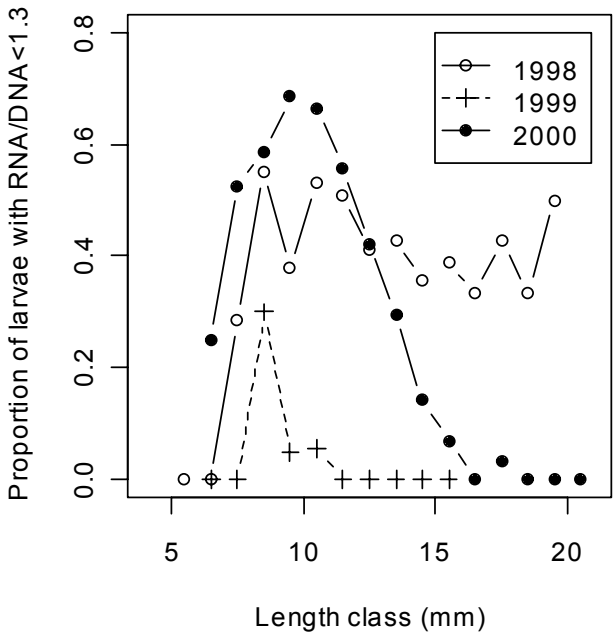

Figure 5.5 – Proportion of starving Twaite shad larvae (*i.e.* RNA/DNA<1.3) per length class and year.

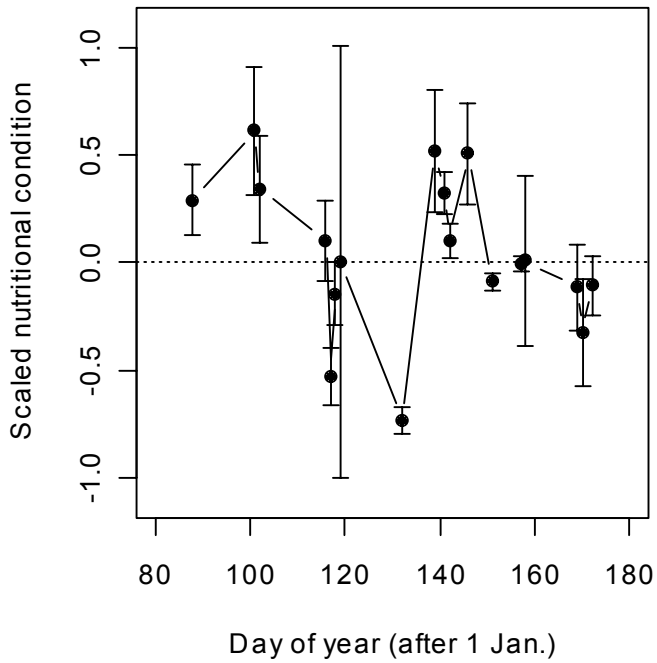

Figure 5.6 – Variation of average scaled RNA/DNA ratios of Twaite shad larvae per day of year (see text for details). Larvae in better nutritional condition should have (scaled) values of RNA/DNA ratios above zero whereas the opposite is expected for larvae in poorer condition. Whiskers represent $\pm$ 1 standard error of the mean. Note: the values for days 175 and 189 were for only one larva and therefore were not included in this figure. Days of year 100 and 160 correspond to 9 April and 8 June, respectively.

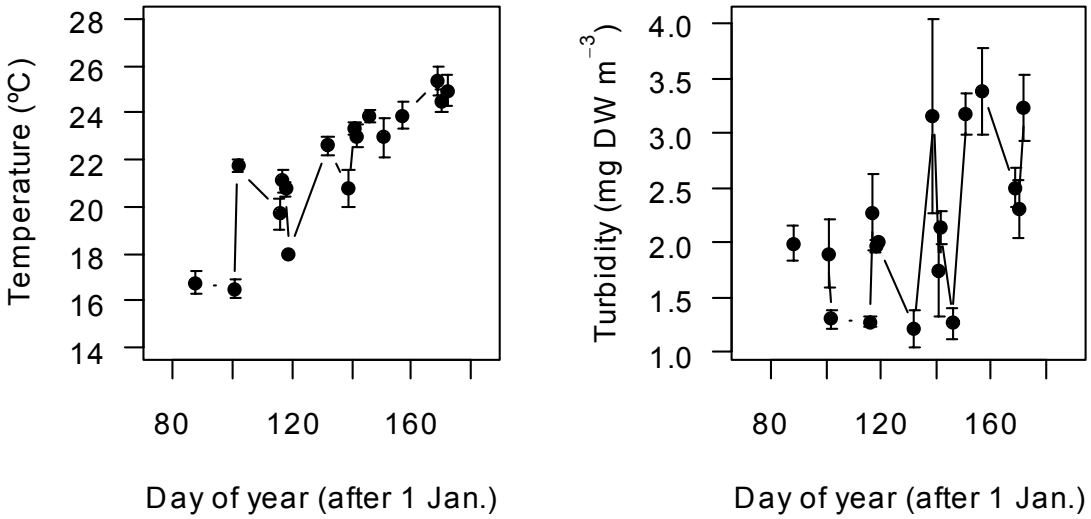

Figure 5.7 – Seasonal changes in average water temperature (left) and turbidity (right). Whiskers are ± 1 standard deviation of the mean. Days of year 100 and 160 correspond to 9 April and 8 June, respectively.

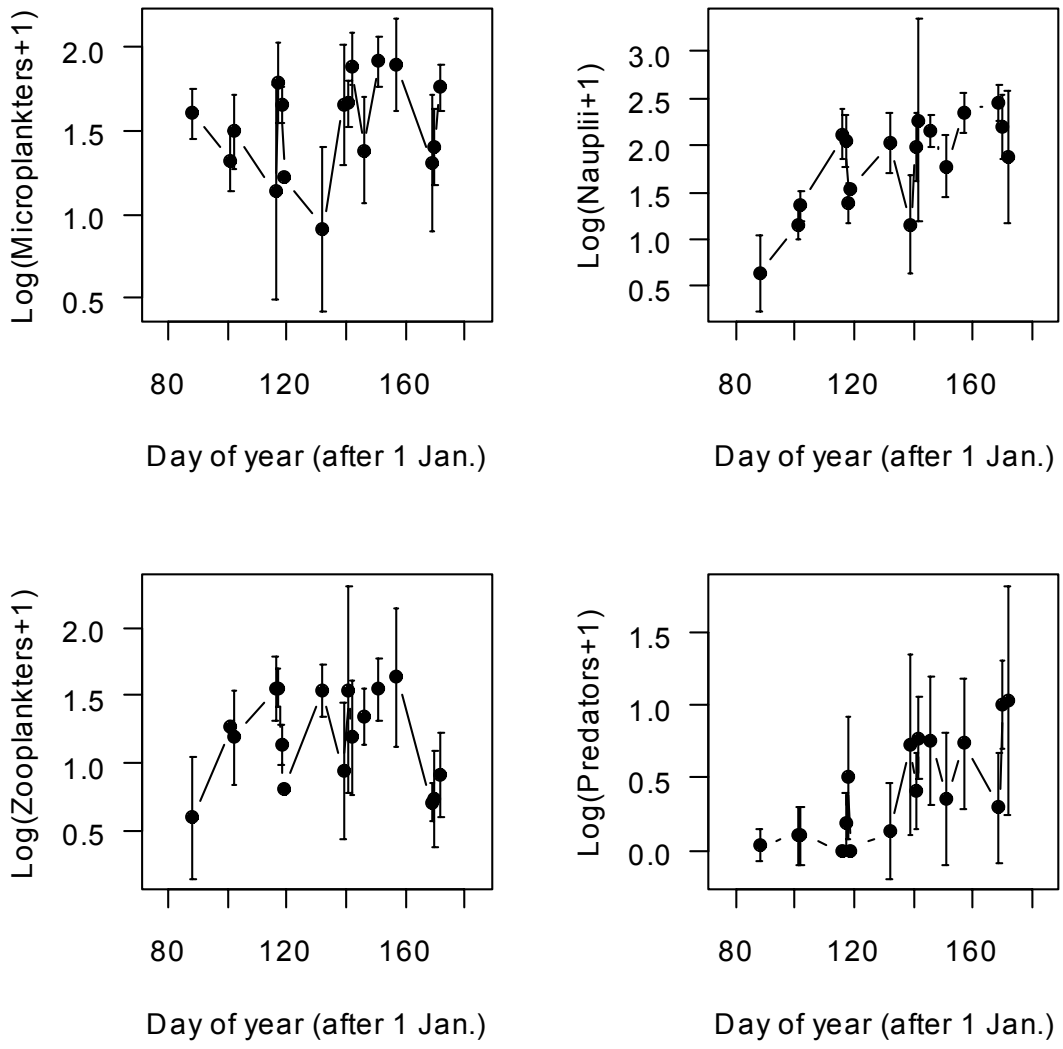

Figure 5.8 – Seasonal variation of log(plankters abundance). Whiskers are ± 1 standard deviation.

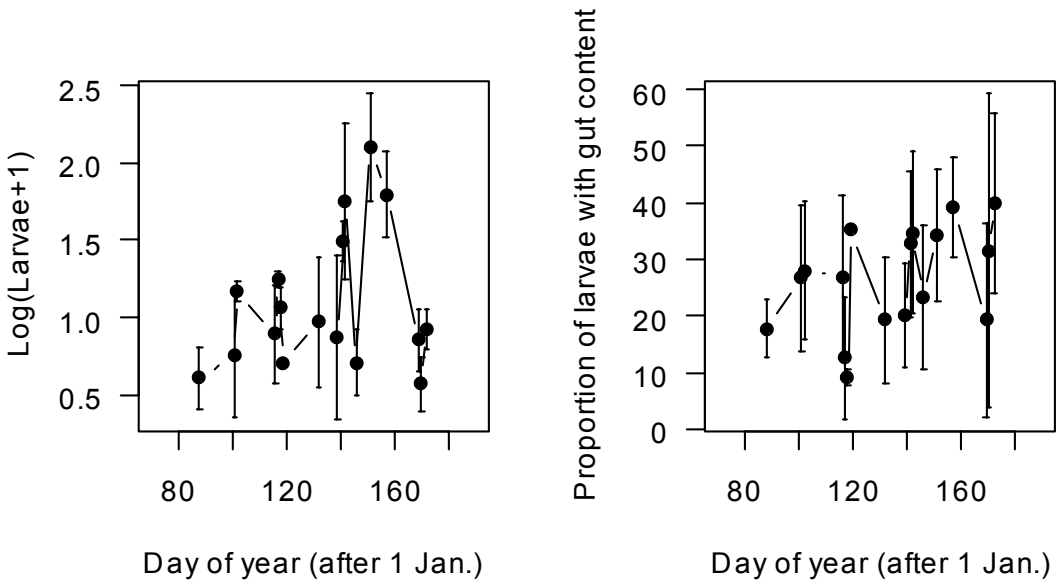

Figure 5.9 – Seasonal changes in log(shad larvae abundance) (left) and respective proportion of larvae with gut content (right). Whiskers are ± 1 standard deviation of the mean. Days of year 100 and 160 correspond to 9 April and 8 June, respectively.

A highly significantly although complex linear regression model (see equation below; $F_{[6,77]}$=27.46 with $p<10^{-4}$) was obtained using a stepwise procedure to relate RNA/DNA ratios and environmental covariates. However, the high dimensionality prevents its adequate illustration. The model below explained a large proportion of larval condition variability (r^2=0.66) but possibly some non-linear features of data could not be seized by this approach (Figure 5.10):

$$\log(RNA/DNA) = -0.441 + 0.093 \cdot Temp - 0.677 \cdot Turb + 0.014 \cdot Turb^2 + 0.476 \cdot \log(Nauplii + 1)$$
$$-0.036 \cdot Temp \times \log(Nauplii + 1) + 0.052 \cdot Turb \times \log(Nauplii + 1)$$

where *Temp* is surface temperature (ºC), *Turb* is turbidiy (mg DW m^{-3}) and *Nauplii* are decapods *nauplii* (ind. 100 m^{-3}). A positive coefficient in front of a variable means that (all other variables being constant) the variable positively influences the log RNA/DNA of shad larvae: as the value of the variable increases, the condition of larvae is predicted to increase as well. Conversely, a negative coefficient means a negative influence: as the variable increases, the condition of shad larvae is predicted to decline (all other variables being constant). Quadratic terms and interactions between explanatory variables may confuse somewhat the simple interpretation outlined above (Figure 5.11).

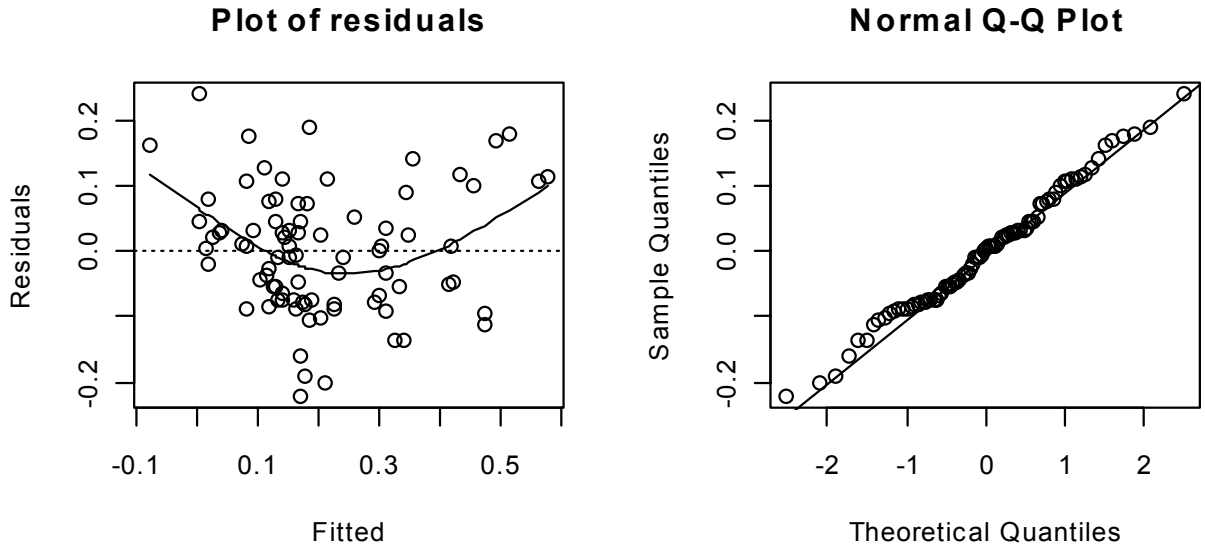

Figure 5.10 – Diagnostic plots for the multiple regression model relating the nutritional condition (RNA/DNA ratio) of Twaite shad larvae and environmental covariates (see text for further details).

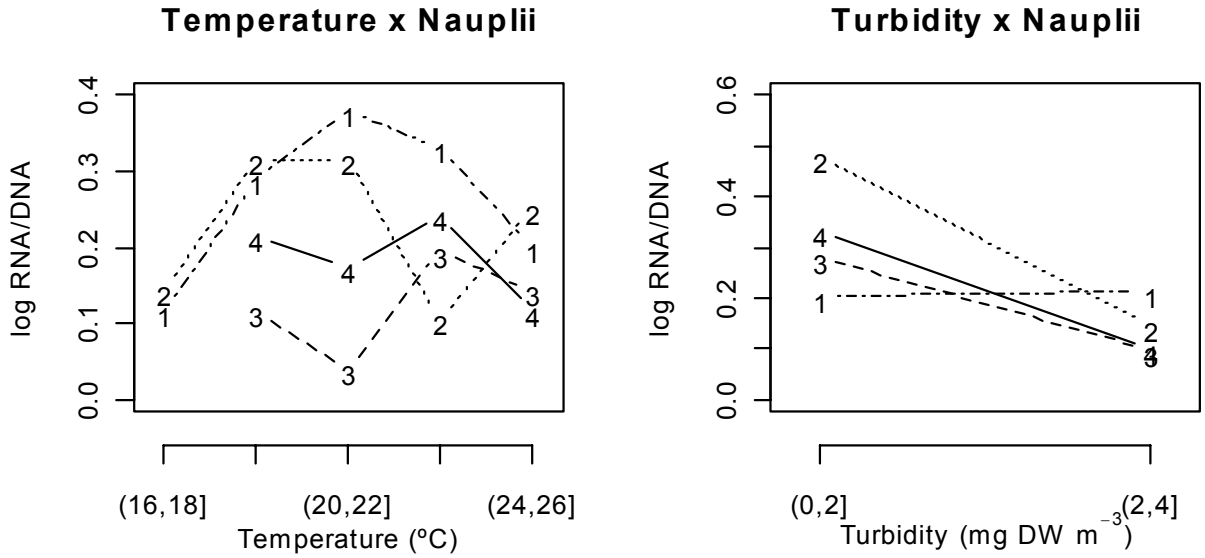

Figure 5.11 – Plots of significant interactions between covariates (Temperature x *Nauplii* and Turbidity x *Nauplii*) in the regression model, found to influence the log RNA/DNA ratio of Twaite shad larvae (The numbers 1 to 4 in the lower panels refer to log(*Nauplii*+1) abundance classes: 1 – (0, 1.3], 2 – (1.3, 1.9], 3 – (1.9, 2.3], and 4 – (2.3, 3.2]).

On the other hand, in the generalized additive model found to best describe the relationship (r^2=0.60) between the changes in larval condition and environmental predictors, turbidity, temperature and log(decapods *nauplii* abundance) were included as non-linear smoothed

functions (Figure 5.12):

$$RNA/DNA = 0.21 + \beta_1(Temp) + f_2(Turb) + f_3(\log(Nauplii + 1)) + f_{13}(Temp \times Nauplii)$$

where β_1 is a (linear) regression coeficient and f_j are (non-linear) piecewise cubic polynomial functions, *Temp* is surface temperature (ºC), *Turb* is turbidiy (mg DW m^{-3}) and *Nauplii* are decapods *nauplii* (ind. 100 m^{-3}). Moreover, a significant interaction effect between temperature and *nauplii* abundance was found to influence the nutritional condition of shad larvae. Condition was negatively affected by increasing turbidity; values <2 mg DW m^{-3} largely contributed to improve the nutritional condition of shad larvae (Figure 5.12, left panel). The combination of temperatures of *ca.* 22 ºC and abundances of decapods *nauplii* between 50 and 100 ind 100 m^{-3} enhanced larval nutritional condition (Figure 5.12, right panel).

Figure 5.13 illustrates the relationships between the observed and predicted values of nutritional condition of shad larvae using multiple linear regression (MLR) and generalised additive models (GAM). The predictive accuracy was lower for MLR (r=0.67) when compared to GAM (r=0.82). Both models performed better for log RNA/DNA ratios between 0 and 0.6 (*i.e.* values 1≤RNA/DNA≤4).

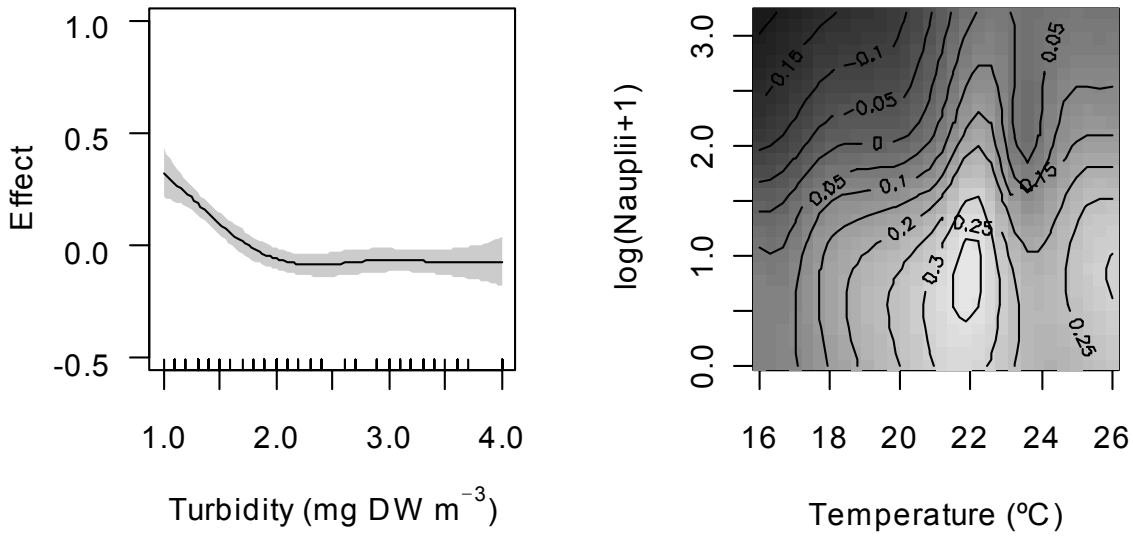

Figure 5.12 - Results of fitting GAM to sample-wise data of larval shad condition and environmental predictors. The effects on the nutritional condition of Twaite shad larvae are represented as smoothing functions of turbidity (left) and temperature × log *nauplii* abundance (right). These plots represent the effect of a particular covariate while maintaining the other predictors at a constant level. Effects are standardised because the estimated density at a given value of a variable is dependent upon levels of all other variable. The shaded area in the left plot corresponds to the approximate 95% pointwise confidence intervals. The tick marks near the *x*-axis show the location of the observations on that variable.

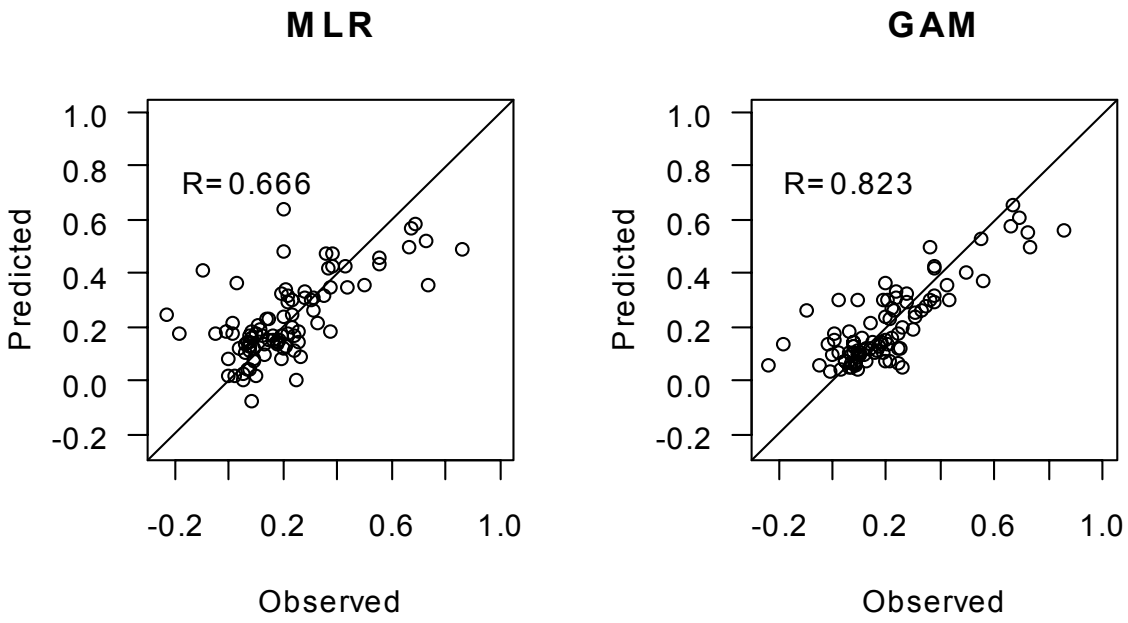

Figure 5.13 – Plots of predicted *versus* observed of log-transformed values of log RNA/DNA (index of larval nutritional condition) for the two predictive models obtained: multiple linear regression (MLR) and generalised additive model (GAM) (see text for further details).

5.4. Discussion

Nucleic acids contents in individual Twaite shad *A. fallax* larvae were determined using a recommended (Bergeron, 1997) fluorimetric methodology that was already applied to other estuarine fish larvae (Chícharo *et al.*, 2001b; Esteves *et al.*, 2000b; McGurk *et al.*, 1992; Rooker *et al.*, 1997). The mean RNA/DNA ratio of the larvae sampled was 1.64 ($\pm$ 1.12 SD, n=1699), which was close to the lower limit of values obtained for a few *Alosa spp.*larvae (n=4) in a previous study, 1.01 to 8.56 (Esteves, 1999). Furthermore, RNA and DNA concentrations for *A. fallax fallax* larvae (5.7 to 20.4 mm SL) were within the range of values published for other clupeiforms of comparable size (e.g. Chícharo *et al.*, 1998b; Clemmesen, 1996; Esteves *et al.*, 2000b; García *et al.*, 1998; Mathers *et al.*, 1994; McGurk *et al.*, 1992; Suneetha *et al.*, 1999). Yolk-sac larvae were analysed (0.01 to 21.84 μg DNA larva^{-1}, 1.13 to 12.08 μg RNA larva^{-1} and 0.25≤RNA/DNA≤5.47) but not considered further because the processes of internal yolk absorption and learning to capture food mask the influence of feeding upon the nutritional condition of larvae (Clemmesen, 1996).

Nucleic acids contents were significantly related to larval size (SL). Despite the high variability in RNA/DNA ratios within size classes, which can be attributed to periods of cell proliferation and growth during the development of organs, they were directly (although weakly) related to

size. Other authors have observed increases in RNA/DNA ratios with fish larvae size (Buckley *et al.*, 1984; Chícharo *et al.*, 1998b; Clemmesen, 1994; García *et al.*, 1998; Ramirez *et al.*, 2001; Rooker & Holt, 1996). Clemmesen (1996) theorized a fluctuating (due to a diel periodicity effect) increase in RNA/DNA ratios with length of fish larvae. In contrast, Suneetha *et al.* (1999) found no significant length dependency of the RNA/DNA ratio of herring larvae in experimental cages. In fact, once exogenous feeding has been established, standard length, dry weight, protein, RNA and DNA content are all highly correlated (Folkvord *et al.*, 1996; Richard *et al.*, 1991). On the other hand, Pepin *et al.* (1999) suggested that an increase in RNA/DNA ratio in fish larvae is an indication of improved survival since individuals in better condition (*i.e.* those with higher RNA/DNA ratios) are more susceptible to survive to later stages (selective mortality) (Ramirez *et al.*, 2001).

The (positive) relationship between RNA/DNA and age is less documented in field-caught larvae but common in laboratory studies. In actively feeding larvae, nucleic acid contents increases with larval age and size (Buckley, 1982; Buckley, 1984; Buckley *et al.*, 1999). Herein, it was expected since age and size of larvae were significantly related (y=7.19+0.43x, r^2=0.73, $F_{[1,324]}$=867.5 with $p<10^{-4}$) and RNA/DNA ratios were correlated with SL. Using a multiple linear regression (r^2=0.76), Buckley *et al.* (1984) described, how the age of larvae (together with rotifer densities) affected (positively) the RNA/DNA ratios of laboratory-reared sand-lance (*Ammodytes* sp.). Clemmesen (1994) found that RNA/DNA ratios of fed *Clupea harengus* larvae showed a tendency to increase with age of larvae. Folkvord *et al.* (1996) obtained polynomial regression equations to describe the relationship between RNA/DNA ratios and age of *C. harengus* reared in mesocosms. An increase in the ratio with age of larvae might be a consequence of cells growing without increasing the DNA content (hypertrophy) which is specific to locomotory muscle DNA (Clemmesen, 1994).

RNA/DNA of Twaite shad larvae was not related to recent otolith growth (sum of the increments of the last three complete increments) but was significantly correlated with the long-term otolith growth (average increment width). Several authors have found positive (although weak or moderate) relationships between nutritional condition (RNA/DNA ratios or RNA/Dry weight) and recent otolith growth of fish larvae (Clemmesen & Doan, 1996; Hovenkamp & Witte, 1991; Ramirez *et al.*, 2001; Suthers, 1996). However, other studies have questioned the use of the RNA/DNA ratio as a reliable index larval growth (Bergeron & Boulhic, 1994; Mathers *et al.*, 1994; Suthers *et al.*, 1996). Furthermore, the uncoupling between otolith and

somatic growth rates has already been reported for young pond-reared striped bass *Morone saxatilis* (Secor & Dean, 1989) and the experimental results of Baumann *et al.* (2005) with post-larval sprat *Sprattus sprattus* suggest that perturbation in environmental conditions (namely food levels) can temporarily decouple otolith from somatic growth. Moreover, Fey (2005) disputes the use of marginal increment analysis to compare the growth rate (or condition) of larval and juvenile herring exposed to drastic temperature fluctuations and changes in feeding conditions. Thus, the non-significant relationship between RNA/DNA ratios and recent otolith growth while a correlation existed between RNA/DNA and average increment width of Twaite shad larvae might not be unexpected.

During 2000, RNA/DNA ratios were inversely related to instantaneous growth rates obtained from otolith microstructure analysis. RNA/DNA are supposed to reflect the nutritional condition and growth potential of fish larvae (Clemmesen, 1996 and references therein). Strong correlations between RNA/DNA ratios and growth have been observed in a variety of species (Buckley, 1984; Folkvord *et al.*, 1996; Rooker & Holt, 1996). Notwithstanding, the results obtained herein are consistent with the findings of Clemmesen & Doan (1996) for starved cod *Gadus morhua* larvae reared from 1 to 12 d after hatching. In fact, a large proportion of the shad larvae collected during 2000 had RNA/DNA ratios<1.3 and could be considered as "starving" (see below). On the other hand, RNA/DNA ratios and mortality rates (estimated from otolith microstructure analysis) were inversely related. Larvae in poorer nutritional condition are more liable to die directly by inanition or indirectly through increased susceptibility to predation (Bailey & Houde, 1989). The correlations between RNA/DNA ratios and growth and mortality rates were much improved if the data for day 139 (May 18) was removed. In the days that preceded that sampling date, heavy rainfall events and the resulting higher river flows may have contributed to increased mortality (see Chapter 4) of otherwise average-growing larvae in good nutritional condition. In fact, the previous sampling date May 9 (day of year 130) produced no shad larvae.

Larval Twaite shad RNA content and RNA/DNA ratios varied over time and space. The higher average nutritional condition of Twaite shad larvae collected during 1999 corresponded to particular habitat conditions namely: mean temperature of 22.1 ºC; reduced mean annual rainfall (corresponding only to 50-75% of the 1941-1997 average; SNIRH at http://snirh.inag.pt/) and water turbidity (1.26 mg AFDW m^{-3} against 2.17 and 2.87 mg AFDW m^{-3} in 1998 and 2000); *ca.* 90 crustacean *nauplii per* 9 larvae 100 m^{-3} and lower predation pressure (only 38% of the

samples had ≥1 predators 100 m^{-3} *versus* 74% in 1998 and 57% in 2000). In addition, according to the results of the 3-way ANOVA there was an important effect of gut content on nutritional condition of shad larvae collected during 2000. Wagner *et al.* (1998) obtained nucleic acids contents of 7.5 μg RNA per copepod and 1.5 μg DNA per copepod (0.65 to 2.14 mm prosome length, *Calanus finmarchichus*). Thus, the RNA contents of items visible in the gut of Twaite shad larvae could have been significant for about 25% of the larvae while the DNA content of food items could be important for only 1% of the larvae. Conversely, Clemmesen (1996) found no significant difference between RNA and DNA content of 8-d old herring larvae with or without visible food in the gut from laboratory-rearing experiments as well as from field sampling in the Orkney/Shetland area and from the English Channel (UK). The influence of exogenous nucleic acids was in the range of the method's variability and could be neglected. The limit of detection of the method used herein was 0.16 μg ml^{-1} for DNA and 0.46 μg ml^{-1} for RNA, and the variability of estimated nucleic acids content was rather low (*cv*=1.5% for DNA and 3.5% for RNA) (Esteves *et al.*, 2000a). The contribution of prey items to the whole-larvae nucleic acids content (particularly of RNA) should be considered whenever smaller larvae are found to be in poor condition.

There are no experimental data on critical values of RNA/DNA of *Alosa spp.*with which our estimates could be compared. Critical values close to 1.0 have been established for first-feeding *Sardina pilchardus* larvae starved ≤6 d, 1.3 (Chícharo, 1997), and for 10 mm herring larvae starved for 6-9 d, 1.2 (Clemmesen, 1994; Robinson & Ware, 1988). Using the threshold value of 1.3 to preliminarily distinguish starved larvae, a fairly high proportion of the Twaite shad larvae caught in the River Mira was starving during 1998 and 2000 (about 45%) but not in 1999 (*ca.* 5%). Because RNA/DNA ratios of field caught larvae are generally higher than those observed in reared larvae (Buckley, 1984; Clemmesen, 1989; Robinson & Ware, 1988), it was expected that few larvae with an RNA/DNA ratio under 1.3 in the field, since these are more liable to die. Notwithstanding, a closer inspection of the results (*cf.* Figure 5.5) showed that during 1998 proportions were independent of larval length, while in 2000 the high overall proportion of starving larvae, *i.e.* those in poorer condition, was mainly due to individuals 7 to 13 mm SL. These individuals constituted *ca*. 67% of all larvae caught. In fact, low feeding incidences (<39% of the larvae with visible gut content) were found in the great majority (>80%) of the samples with smaller larvae (average SL <13mm). Moreover, Barron & Adelman (1984) examined the influence of sublethally concentrations of toxicants on fathead minnows

(*Pimephales promelas*) and found RNA, DNA, protein and RNA/DNA ratios to be sensitive to toxicant stress. The major sources of pollution in River Mira are sewage discharges (of which about 53% are treated) from small urban areas, since there are no registered industries within the drainage area, and land is used mostly (*ca*. 82%) for agriculture and forestry (INAG at http://snirh.inag.pt/snirh). Available data (for a few collections during years 2002 through 2004, check the INAG web site at http://snirh.inag.pt/snirh) concern concentrations of selected heavy metals determined at a sampling station *ca*. 12 km downstream of stations sampled in this study: Al (7310 mg kg^{-1}); Fe (4.2-6.3 µg L^{-1}); Mn (177-1715 mg kg^{-1}); Zn (12.5-2287 mg kg^{-1}); Cu (0.6-1.0 µg L^{-1}); Ni (1.1-2.1 µg L^{-1}); Pb (0.4-0.6 µg L^{-1}); Cd (0.09-0.15 µg L^{-1}); and Hg (0.010-0.040 µg L^{-1}).

In a typical year, Twaite shad larvae are in better nutritional condition during two relatively short time-periods, around days 100 (mid-April) and 145 (mid to late–May). These periods correspond roughly to peak occurrences of the early life-history stages of the species in River Mira (*cf.* Chapter 3). Moreover, higher growth rates were calculated for shad larvae collected during April and lower mortality rates were obtained for larvae caught during late-May and early-June in River Mira (*cf.* Chapter 4). Many biological and physical factors can interact to affect the growth and mortality of young fish during their first year (Fogarty *et al.*, 1991). Starvation and predation, acting independently or together with the modifying impact of abiotic factors (physical processes) have often been suggested as the main sources of mortality (e.g. Cushing, 1975; Hjort, 1914; Lasker, 1975). The extent to which these potential effects are realized is, however, the subject of debate (Pepin *et al.*, 1992). Two different regression techniques were used to model the possible relationships between RNA/DNA ratios and environmental co-variates: multiple linear regression and generalized additive models. Similar variables (main effects and interactions of variables) were included in the final models relating (mean) nutritional condition of Twaite shad larvae and environmental variables: water temperature and turbidity, and prey abundance. According to the multiple regression model nutritional condition of Twaite shad larvae is enhanced at water temperatures near 22 ºC (particularly for moderate levels of prey availability, <80 crustacean *nauplii* 100 m^{-3}) and is significantly reduced when water turbidity is higher than 2 mg DW m^{-3} and potential prey are readily available (>20 crustacean *nauplii* 100 m^{-3}) (*cf.* Figure 5.11). The simpler conclusions of the generalized additive model obtained herein (see Figure 5.12) are in accordance with previous findings. It has been shown that the RNA/DNA ratio is related to water temperature (Buckley,

1984; García *et al.*, 1998; Suneetha *et al.*, 1999). Temperature governs the rate of chemical reactions, pacing metabolic requirements, digestion and the rate of growth (Fry, 1971). Buckley (1982) concluded that temperature was the dominant growth factor when larvae have adequate food supply. However, when the temperature range is narrow, food availability becomes the predominant factor for growth and condition (Buckley, 1979, 1984). Herein, RNA/DNA ratios of Twaite shad larvae also correlated well with crustacean *nauplii* abundance. Actually, RNA/DNA ratios in larval fish should correlate positively with prey densities (Ferron & Leggett, 1994). Better feeding conditions in terms of prey density and food type usually determine enhanced growth rates and nutritional condition (Battini *et al.*, 1995; Buckley *et al.*, 1984; Canino, 1994; Martin *et al.*, 1985). Several authors (Bailey *et al.*, 1995; Chícharo *et al.*, 1998a, b; Esteves *et al.*, 2000b; García *et al.*, 1998) concluded that RNA/DNA ratios of fish larvae were related to prey biomass or abundance. Kashuba & Matthews (1984) observed that midgut and digestive tract's organs of shad, *Dorosoma* spp., showed evidence of poor condition during the period of low zooplankton abundance. In addition, it was expected that, together with *nauplii*, microplankton biomass correlated with Twaite shad larvae nutritional condition (see Chapter 3). Considering that zooplankton is known to be patchily distributed on the spatial scale searched by the feeding larval fish (Leggett & DeBlois, 1994), it is possible that the sampling procedure used to collect microplankters (see Material and Methods) produced biased estimates of effective concentrations available to Twaite shad larvae (if these larvae have the ability to exploit micro-scaled food patches) (Chícharo, 1998). The feeding incidence of shad larvae (measured herein as the proportion of larvae with gut content) was not included in the predictive models aforementioned and further confirmed this hypothesis. Moreover, (micro)zooplankton abundance estimates may underestimate prey availability because zooplankton production may be neglected in the estimates since it is consumed as quickly as it is produced (Hunter, 1981). Both statistical methods used herein highlighted the detrimental effect of turbidity upon larval Twaite shad nutritional condition. In fact, low transparency can interfere with photosynthesis and river phytoplankton production (Mallin *et al.*, 1999; Marker & Collett, 1997), causing the selective elimination of major larval prey such as crustacean *nauplii* and immature insects (Aprahamian *et al.*, 2003; Crecco & Blake, 1983; Taverny *et al.*, 2000b), leading to higher incidences of malnutrition among first feeding larvae (Crecco & Savoy, 1987b) and eventually reducing larval growth. On the other hand, competition and/or predation pressure should presumably have an impact upon the nutritional condition of Twaite shad larvae (see above). Notwithstanding, neither the (raw or grouped) abundance data of small medusae, mysids and amphipods (potential predators or competitors) or the densities of shad larvae were included in

the models referred to above. Few studies dealt simultaneously with field-caught fish larvae and their potential predators (Bailey & Houde, 1989). Esteves *et al.* (2000b) and Chícharo (1998) obtained models relating the nutritional condition of sardine larvae and predators abundance (medusae and chaetognats, respectively). Purcell & Grover (1990) concluded that predation was a major cause of mortality of herring (*Clupea harengus pallasi*) larvae in Kulleet Bay and that food limitation was not important. Leggett & DeBlois (1994) suggested that the influence of food availability may be indirect and may operate principally through its regulation of the timing and intensity of mortality due to predation, the ultimate cause of mortality. The action of predation soon removes larvae in poorer condition and thereby contributes to an increase in the average condition of the larvae not consumed. No evidence of this was produced herein. Moreover, Savoy & Crecco (1988) found that only 18% of the total pre-recruitment mortality of American shad eggs and early larvae was due to density-dependent mechanisms (most likely intra-specific competition for food or space and predation) but stressed the difficulty to quantify density-dependent losses of eggs and larvae.

Relating larval condition to environmental variables is complex due to small-scale temporal and spatial patchiness in the distribution of fish larvae and prey (and/or predators). Herein, the regression models included interaction terms, which tried to account for at least part of that complexity. The condition of shad larvae seemed conditioned by temperature, turbidity and food availability in the plankton. These findings are in accordance with the results of modelling the seasonal distribution (see Chapter 3), growth and mortality (see Chapter 4) of early life-history stages of shad. However, a few difficulties persist. The fraction of starving larvae (*i.e*. those more likely to die of inanition or through an increased exposure to predation pressure) was estimated based upon threshold values derived for other clupeid species. It would be advisable to determine the starvation threshold in terms of RNA/DNA ratio under controlled laboratory conditions or *in situ* (see e.g. Chícharo, 1997). On the other hand, the RNA/DNA ratio is believed to indicate overall condition and survival probability. Nevertheless, condition might have little to do with survival if predators take good- and poor-condition larvae indiscriminately (*i.e.* remove larvae with values in the lower- and upper-tail of the probability distribution). The contrast of the average response (larval nutritional condition measured by the RNA/DNA ratio) under set or observed conditions using either MLR or GAM (or other statistical technique estimating average values) provides little information about the distribution of individuals about this mean and misses the point (Pepin *et al.*, 1999). Therefore, the ecological effect of interest concerns the possible change in scatter (say the difference between the 10^{th} and 90^{th} percentile)

with age, or any other covariate, and not the mean ('the average larva is dead') (Evans, 2000). The methods described in Pepin *et al.* (1999) and Evans (2000) could be used to further study the relationship(s) between shad larvae condition and environmental factors.

Chapter 6. A note on Allis and Twaite shad *Alosa spp.* larvae in the rivers Guadiana and Odeleite

6.1. Introduction

Extensive development of the River Guadiana estuary basin over the last century has resulted in significant alteration of river flow regimes and in anthropogenic nutrient enrichment. The construction of the Alqueva dam (inaugurated in 2002) and pollution, *e.g.* BOD_5 in excess of 20000 t O_2 y^{-1} and nitrogen plus phosphorous load of *ca*. 12000 t y^{-1} (Plano Nacional da Água at http://www.inag.pt/ on 10/3/2006) will pose problems for maintenance of water quantity and quality in the River Guadiana estuary (Chícharo *et al.*, 2001b). Thus, all the contributions to an ecological evaluation of this important Iberian estuary and an assessment of anticipated impacts of environmental change are important.

Allis shad *Alosa alosa* (Linnaeus, 1758) and Twaite shad *A. fallax fallax* are anadromous clupeid species, which still migrate into several rivers along Portugal to spawn (Eiras, 1977) but are regarded as "Endangered" and "Vulnerable", respectively, by the Instituto de Conservação da Natureza according to IUCN criteria (Cabral *et al.*, 2006). In Portugal, the various sub-populations of spawning individuals and their respective areas of functional habitat are regressing, particularly that of *A. alosa* in the River Guadiana (Plano Sectorial da Rede Natura 2000 at http://www.icn.pt/ on 28/02/2006). Within the framework of a countrywide research project referred to earlier (*cf.* Chapter 1), different aspects of shad populations were studied in the rivers Mira and Guadiana, namely reproductive biology (Pina, 2000; Pina *et al.*, 2003) and larval biology and ecology (Esteves *et al.*, 2000b and previous chapters). Moreover, Sousa *et al.* (2003) monitored the abundance of spawning adult shads, studied the behaviour during spawning and characterized some locations of spawning and eggs occurrence in the River Guadiana downriver of Pulo do Lobo.

The two main goals of this study were: 1) to describe the diel and seasonal variation of abundances and nutritional condition of larval shads, *Alosa* spp.; and 2) to relate those changes

to environmental conditions namely temperature, turbidity and abundances of potential prey and predators.

6.2. Material and Methods

Study area

The River Guadiana extends for 810 km from its origin (Ruidera lagoon in Spain) to the river mouth located in SE Portugal (Figure 6.1). Its catchment basin is the fourth in the Iberian Peninsula (*ca.* 67000 km^2) of which about 12000 km^2 are in Portugal (González, 1995).

Near the mouth, the River Guadiana can be 550 m wide and 7 – 10 m deep but maximum depths of 15 to 17 m have been recorded (Rodrigues da Costa, 1980). Tidal influence extends to approximately 70 km from the mouth (just upriver from Mértola), where mechanical effects have been observed but salinity is rather low (*ca.* 0.1 ppt). At the upper limit of tidal influence, water depth is generally ≤5 m and river width does not exceed 30 – 40 m. In the estuary, the current velocity of water at the surface averages 1.75 – 2.0 m s^{-1} during ebb and a decreases to about 1 m s^{-1} during slack tides. Mean monthly river flow volumes vary markedly on a seasonal and yearly basis. However, the Alqueva dam and associated infrastructures are expected to artificially regulate river flow when the whole project is completed (planned for the year 2012). Water inflow near Mértola can reach 200 – 600 m^3 s^{-1} in Winter but decrease to 0.1 – 20 m^3 s^{-1} in Summer. The year-to-year variability of inflow is also high, totalling as little as 20×10^7 m^3 in a dry year (1980), and as much as 140×10^7 m^3 in a wet year (1962). The mean yearly rainfall in the River Guadiana estuary catchment area ranges from 400 to 600 mm, but the Summer months are usually very dry and hot. The average water temperature in the cooler months (December–January) is less than 18 °C, and in the warm months greater than 22° C. It can be classified as a mesotidal (average amplitude of 2 m) partially stratified estuary, with a highly variable freshwater inflow. During periods of low inflows, water salinity becomes vertically homogenous within the estuary and, depending on position in the tidal cycle, mesohaline conditions are produced whereas during high inflows the estuary is stratified.

Recent and detailed descriptions of the (lower) River Guadiana can be found elsewhere (Plano Nacional da Água at http://www.inag.pt/ on 10/3/2006).

Sampling

Between February 1998 and July 2000, samples were collected every two weeks in six stations located in the River Guadiana estuary and Ribeira de Odeleite, a small tributary (Figure 6.1). Sampling took place during daylight hours from 0900 to about 1900 hours except on three occasions in 1998 (27/28 April, 21/22 May and 18/19 June) when samples were collected every two hours during 24 hours at Foz de Odeleite (station no. 20). The stations were located in three major regions of the River Guadiana estuary (see Chícharo *et al.*, 2001b), upper (station no. 30, about 65 km upriver), middle (station no. 20, *ca.* 20 km upriver and near the ETM) and lower (station no. 11, close to the river mouth), and the navigable portion of the Ribeira de Odeleite (stations no. 21 to 23). Occasionally, samples could not be collected due to equipment failure or severe weather, but at least eight sampling dates during the spawning seasons of 1998 and 2000 were accomplished (during 1999 the sampling was very limited).

During each sampling occasion, mesozooplankton was collected with a conical net (∅ 0.37 m, 1.6 m long, 0.5-mm mesh size) towed 1 m below the surface, at a constant speed of approximately 1 m s^{-1} for 10 minutes. Plankton tows sampled equitatively the river margins and the main channel. A flowmeter (Hydrobios®) was attached inside the net to estimate the water volume filtered (median=67.4 m^3, range: 23.9 to 250.1 m^3). Samples were sorted in a black tray, the fish larvae retrieved and immediately frozen in liquid nitrogen (-197º C). Zooplankters were stored in buffered 4% formaldehyde solutions. A further 26 – 39 L of water were sampled at 0.5-m depth and filtered through a 63-μm mesh size sieve to collect microplankters.

The surface temperature (º C) and salinity (ppt) were measured using a hand-held thermometer and a refractometer, respectively. Additionally, the water level (in metres) during diel sampling periods was estimated through the equations used earlier (*cf.* Chapter 2).

Laboratory procedures

Shad embryos and larvae were identified using the information available in the literature (Johnson & Loesch, 1983; Quignard & Douchement, 1991b; Ramos, 1977). At these early life-history stages the two species, Allis shad *A. alosa* and Ttwaite shad *A.fallax*, are especially difficult to distinguish. No attempt was made here to separate the two species because of greater (laboratory) processing difficulties. The remaining preserved mesozooplankters were identified using generic literature (Newell & Newell, 1963; Russell, 1976; Smith, 1977; Todd & Laverack,

1991). Whenever mesozooplankters were extremely abundant, successive sub-samples with a folsom-type splitter were analysed and at least 300 organisms counted (Omori & Ikeda, 1992). Plankters' density was expressed as number of individuals per 100 m^3 of water.

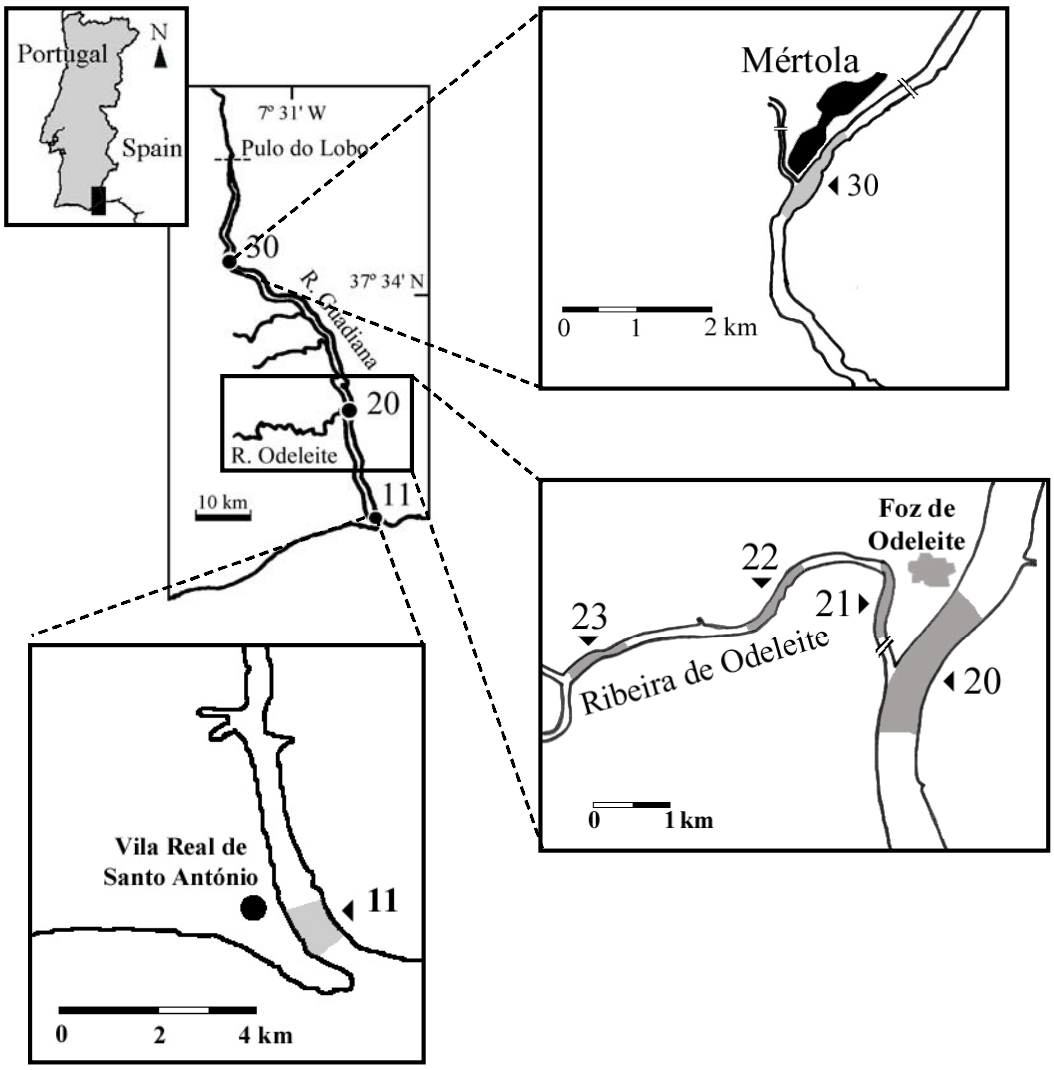

Figure 6.1 – Location of sampling stations in River Guadiana and Ribeira de Odeleite (SE Portugal) (approximate coordinates are: Vila Real de Santo António, 37º 12' N 7º 25' W; Foz de Odeleite, 37º 21' N 7º 27' W; Mértola, 37º 38' N 7º 40' W).

To estimate microplankton biomass, aliquots of the homogenised samples were rinsed through a 1.2-µm milipore filter (∅ 25 mm, Macherey-Nagel® GF-3). Filters were dried in an electric oven at 60º C for 24 – 36 hours to obtain the dry weights (DW) and then incinerated at 450º C for 4 hours to determine the ash-free dry weight (AFDW). Microplankton biomass was

expressed as ash-free dry weight per cubic metre of water (mg AFDW m^{-3}). Dry weights allowed the estimation of water turbidity (expressed as mg DW m^{-3}).

For the analysis of nutritional condition, individually frozen larvae (*n*=19) were thawed, identified and measured (to nearest 0.1 mm). Their condition was assessed using the RNA/DNA ratio. Nucleic acids in individual fish larvae were quantified according to a modified fluorimetric method and using ethidium bromide as the fluorophor (see Esteves *et al.*, 2000a for protocol details).

Data analysis

The few occurrences and low catches of larval shad precluded the use of complex statistical methods to analyse data. Descriptive statistics and simple linear regression were used to present data and study the relationship between variables, respectively.

6.3. Results and Discussion

Early life-history stages of shad were observed only in six of the 135 samples collected over the three-year period of sampling in the River Guadiana estuary. Larval shad occurred during late-May and June 1998, 1999 and 2000 in stations 30 (Mértola, 4.78 ind. 100 m^{-3}), 20 (Foz de Odeleite, 27.90 and 106.24 ind. 100 m^{-3}) and 21 or 22 (Ribeira de Odeleite, 2.1 or 2.5 ind. per 100 m^3), respectively. These (few) occurrences of shad larvae corresponded to water temperature ≥22 ºC and turbidity in the range 1.3 – 3.3 mg DW m^{-3}, intermediate levels of microplankters' biomass (11.7 – 87.9 mg AFDW m^{-3}) and copepods' densities (3.2 – 1028.1 ind. 100 m^{-3}) but were independent of the abundances of potential predators (mysids and decapods) (Figure 6.2).

More occurrences and greater numbers of shad larvae were expected in this study, because sampling sites were chosen to be near known spawning locations (*e.g.* Ribeira de Odeleite or Mértola) (Pina, 2000; Sousa *et al.*, 2003). Nevertheless, the few shad larvae were found when environmental parameters were close to conditions that have been reported to enhance larval abundances (see *e.g.* Chapter 3).

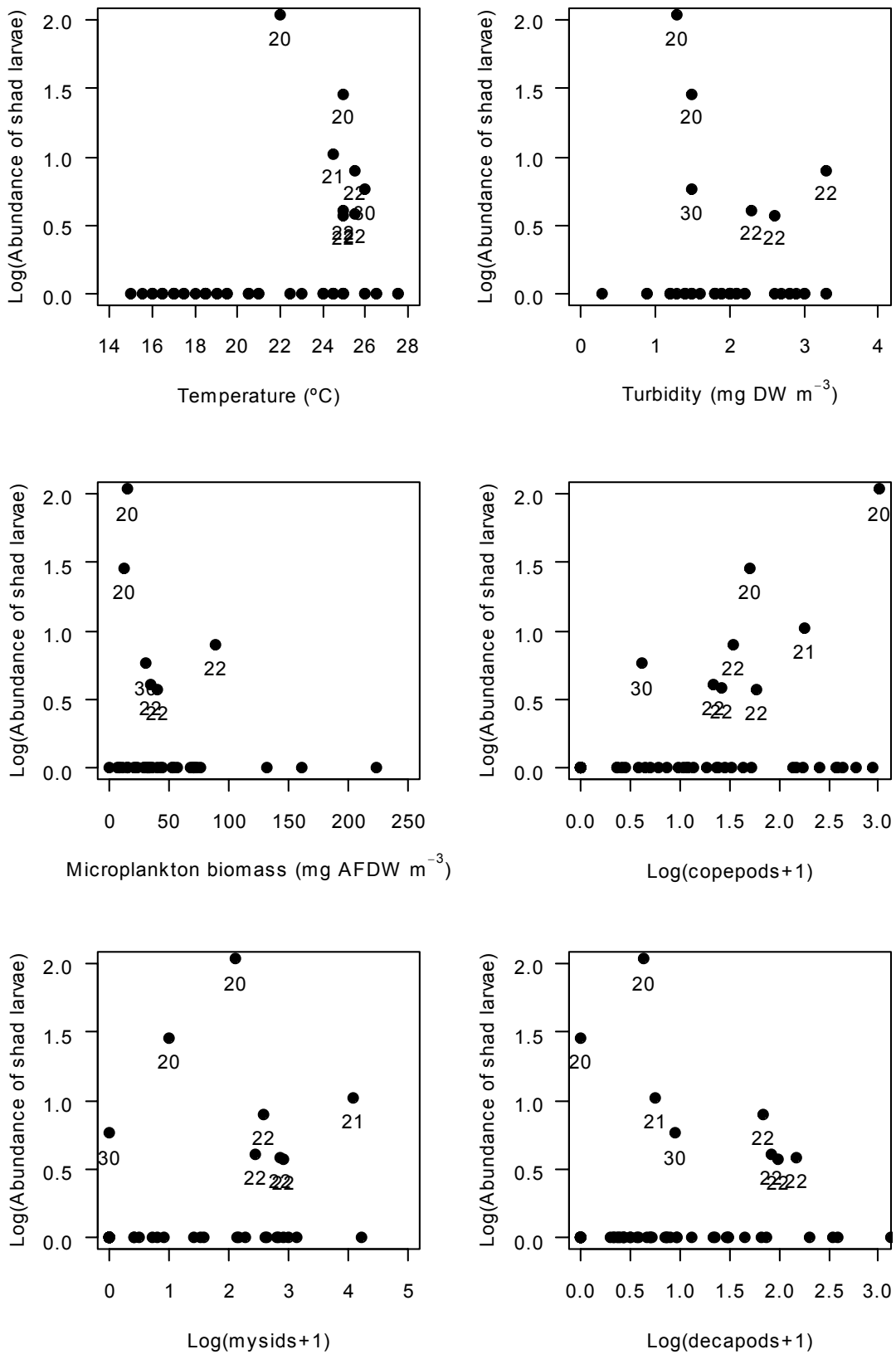

Figure 6.2 – Scatterplots of Twaite shad larval log-transformed abundances versus environmental covariates in the River Guadiana Estuary. Labels refer to station of collection (see Figure 6.1).

The average nutritional condition of (only) nineteen shad larvae (3.8 to 18.4 mm SL)[6], expressed in terms of RNA/DNA ratio, ranged from 1.19 (±0.33 SD, n=3, Station 30 on 22 June 1998) to 2.4 (Station 21 on 31 May 2000). Nucleic acids contents were related to larval size (Figure 6.3) and a significant (p=0.0037) relationship existed between size and condition if an outlier value was removed (Figure 6.4).

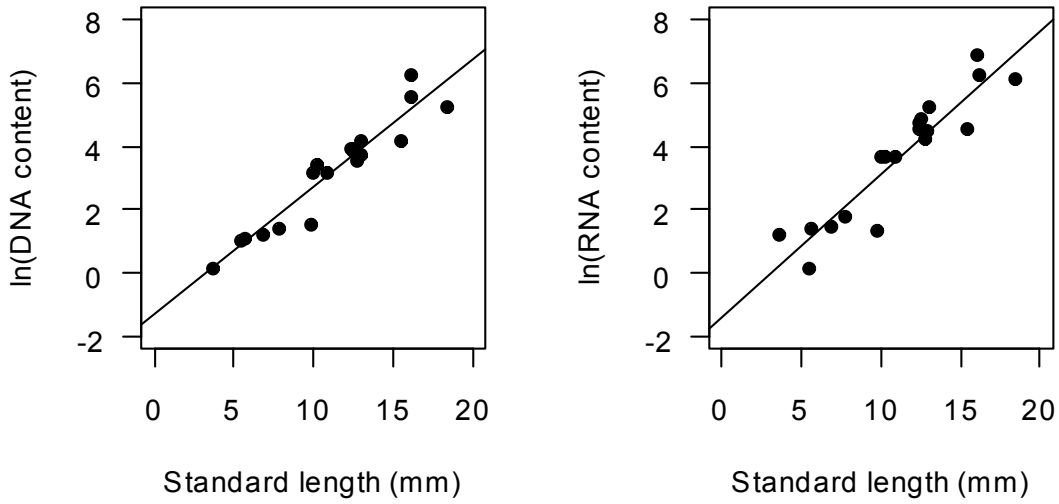

Figure 6.3 – Relationship between nucleic acids contents and larval length of Twaite shad. Equations are: ln DNA = -1.30 + 0.40 SL (r^2=0.90, $F_{[1,17]}$=158.1 with $p<10^{-4}$); and ln RNA = -1.36 + 0.45 SL (r^2=0.86, $F_{[1,17]}$=104.5 with $p<10^{-4}$).

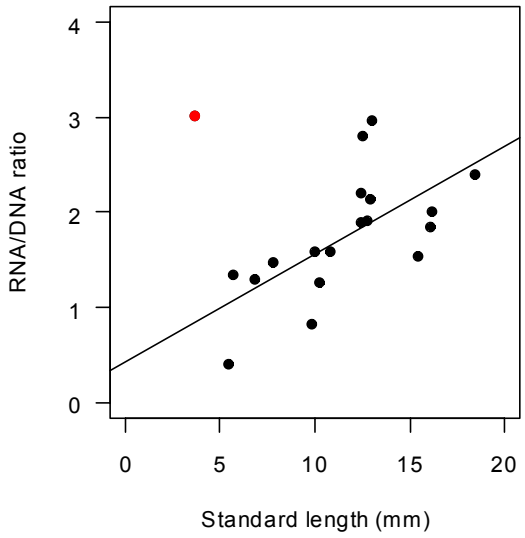

Figure 6.4 - Relationship between RNA/DNA ratio and standard length of Twaite shad larvae. Equation of the line is RNA/DNA = 0.43 + 0.11 SL (r^2=0.42, $F_{[1,16]}$=11.53 with p=0.0037). The point, at approximate coordinates (x=5,y=3), was considered to be an outlier (see text).

[6] The value 3.8 corresponds to an incomplete larva.

Shad larvae were in better condition earlier in the spawning season (day of year=152 *i.e.* 31 May) than in later collections (day of year≥158 *i.e.* 6 June) (Table 6.1). Higher RNA/DNA ratios corresponded to increased turbidity and microplankton biomass as well as higher abundance of both copepods and mysids. Nevertheless, these findings were derived from pooling data from different years and stations and their inclusion in this study serves informative purposes. Average nutritional condition of these larvae agrees with the values found for *A. fallax fallax* in the River Mira (*cf.* Chapter 5).

Table 6.1 – Average RNA/DNA ratio of shad larvae collected in the River Guadiana and Ribeira de Odeleite and correspondent environmental conditions. Legend: SD – standard deviation; Number – number of larvae considered; C – Copepods (ind. 100 m^{-3}); M – Mysids (ind. 100 m^{-3}); D – Decapods (ind. 100 m^{-3}).

Day of year (Date, Station)	Mean RNA/DNA (SD)	Number	Temperature (ºC)	Salinity (ppt)	Turbidity (mg DW m^{-3})	Microplankton biomass (mg AFDW m^{-3})	Log(C+1)	Log(M+1)	Log(D+1)
152 (31 May 2000, 21)	2.40 (0.00)	1	24.5	0.1	3.3	71.81	2.25	4.08	0.76
152 (31 May 2000, 22)	2.04 (0.56)	9	25.3	0.1	2.3	34.09	1.33	2.43	1.92
158 (6 June 2000, 22)	1.80 (0.24)	3	25.2	0.7	2.6	39.46	1.43	2.85	2.18
173 (22 June 1998, 30)	1.20 (0.33)	3	26.0	0.5	1.5	29.78	0.62	0.00	0.95
179 (28 June 1999, 20)	1.58 (1.32)	3	25.0	10.0	1.5	11.71	1.70	0.98	0.00

Catches of early life-history stages of *Alosa* spp. in the River Guadiana and tributaries might be increased if sampling frequency is increased and/or if other more appropriate sampling gears are used, *e.g.* dip net, fixed (channel) net, sampling (light) traps or electrofishing, particularly in the Ribeira de Odeleite and near Mértola.

Chapter 7. Conclusions

7.1. Main conclusions

This thesis tried to address topics of biological (estimates of larval abundance, age, growth, mortality and condition) and ecological (modelling the relationships between life-history traits and environmental variables) relevance to the early life-history stages of Twaite shad *Alosa fallax fallax* in the River Mira, a lowland tidal river that is relatively common in temperate regions. The methods were devised to favour the study of those topics at different time scales (diel, seasonal and inter-annual). Furthermore, the planned application of the results to the larval stages of *Alosa* spp. in the River Guadiana and the tributary Ribeira de Odeleite was not possible owing to scarce results. The main conclusions are summarized below and further topics of research are proposed later.

Diel and seasonal distribution of early life-history stages

Twaite shad larvae were more abundant in the surface waters of River Mira during the day whereas no diel abundance pattern was observed for embryos (yolk-sac larvae). Larvae seem to exhibit tidally oriented migratory movements in order to benefit the retention of a higher number of larvae in the upper estuary and as function of potential prey. Furthermore, eggs, embryos and larvae of Twaite shad were only found in the upstream stations (no. 4 – 6) of River Mira from late-March until mid-June (particularly higher densities were observed during a 5-week period from late-April through to the last week of May). During this time, surface temperature ranged from 16 to 26 ºC and salinity never exceeded 5 ppt. The first record of shad in zooplankton samples (28 March) followed the upstream migration of spawners and roughly coincided with the onset of springtime rainfall. Inter-annual and within-year deviations to this general pattern are discussed below.

The average number of Twaite shad eggs, embryos and larvae changed among years of sampling while within-year analyses strengthen the major effect of time of year (factor *Month*) on the seasonal pattern of embryos and larvae. The differences in the rainfall regimes of studied years

and associated habitat conditioning might be a plausible explanation. As expected, no habitat selection occurs at either the egg or embryonic life stages. Apparently the distribution of these stages is largely controlled by the hydrological characteristics of the river segment downstream of spawning area. Conversely, larvae most probably react to habitat conditions in an attempt to increase survival. Presumably, the general patterns of larvae abundance observed are linked to hydrology and biology. The generalised additive models (GAM) fitted to the data included several explanatory variables (temperature, rainfall, turbidity, prey and other zooplankters' abundance and diversity) that described the relationships by non-linear functions to a very satisfactory extent. These models reflect the intrinsic complexity of distribution patterns of Twaite shad embryos and larvae.

Growth and mortality of larvae

When the digitised images of otoliths from *A. fallax fallax* larvae were analysed, rings were almost readily visible as composed of paired translucent and opaque rings in a regularly-recurring sequence deposited around the otolith. A high proportion of the shad *sagittae* read (*ca.* 73%) was used in the analyses (since at least two out of three replicate readings coincided) and estimates were highly reproducible (*cv*=7.3%). A reduced proportion of the processed *sagittae* was not used due to preparation difficulties (*e.g.* over-bleached or lost during handling or mounting). These results suggest the usefulness of otolith microstructural analysis to study age and growth of Twaite shad larvae.

Using data from diel sampling, it is reasonable to suspect that the deposition of a growth increment on shad larvae *sagittae* is completed at the end of the day (*ca.* 2100 – 2300 hours), when the marginal-to-previous increment ratio decreased somewhat. Unfortunately, no *sagittae* from larvae collected at 0100 – 0300 hours were readable and only a 24-hour period was studied to test the above suspicion.

Larval hatching (deduced from hatch-date distributions derived from increment counts) extended from 20 March to 16 June 2000, with most Twaite shad larvae emerging after early-May when water temperatures exceeded 20 ºC. These results agree with previous studies on adult and larval *A. fallax fallax* spawning and hatching behaviour in the River Mira and other rivers in Europe. Intriguingly, a large portion of the embryos collected between days 100 and 130 did not survive

to be aged on subsequent sampling dates (*i.e.* differential cohort survivorship) (see below).

Overall growth-in-length of Twaite shad larvae from the River Mira was significantly described by a linear equation that yielded an instantaneous growth rate of 0.43 mm d^{-1}. Nevertheless, growth varied seasonally. In fact, date-specific growth rates of shad larvae ranged from 0.36 to 0.60 mm d^{-1}. The failure to detect any significant influence of temperature and/or prey density on date-specific growth rates was surprising. It is possible that this was an artefact that resulted from back calculating growth rates from mostly older larval survivors, which endured the unusual rainfall events of early Spring.

Cohorts of Twaite shad larvae in the River Mira do not experience uniform mortality during the spawning season. Mortality rates were lowest for 5 to 14 days-old Twaite shads produced during late-May and June (<5% d^{-1}), when densities of post-larvae and their potential prey reached maximum levels. Conversely, mortality rates for early-season samples of those larvae were in the range 16 to 30 % d^{-1}. This pattern of seasonal variation in mortality rates is in accord with the observations for American shad in the Connecticut River. On the other hand, older (17 – 23 days-old) and later collected (days of year 151 – 172) larvae of Twaite shad in the River Mira experienced relatively higher mortality rates, *ca.* 26 to 38% d^{-1}. These results might be related to a detrimental combination of declining abundances of potential prey and growing density of potential predators (or competitors for same food resources) coincident with those larval stages. Temporal changes in river flow and temperature, and variation in the abundance and quality of potential prey, coincident with early larval development, seem to be important regulators of mortality of Twaite shad larvae in the River Mira.

Nutritional condition of larvae and environmental covariates

Nucleic acids content in individual Twaite shad larvae were determined using a recommended fluorimetric methodology that has already been applied to other estuarine fish larvae. Nucleic acids (RNA and DNA) contents were significantly related to larval size (standard length, SL). Despite the high variability in RNA/DNA ratios within size classes, which can be attributed to periods of cell proliferation and growth during the development of organs, they were directly (although weakly) related to size.

The average RNA/DNA ratio of *A. fallax fallax* larvae collected at dawn and dusk were significantly lower when compared to larvae caught at night or during the day. Fish larvae attempt to maintain high levels of condition throughout the day but the more appropriate light intensity at those periods of the day would allow predators to forage and prey on energy-rich larvae and consequently reduce the mean RNA/DNA ratios.

The positive relationship between RNA/DNA and age is less documented in field-caught larvae but common in laboratory studies. An increase in the ratio with age of the larvae might be a consequence of cells growing without the DNA content increasing (hypertrophy), which is specific to locomotory muscle DNA. RNA/DNA of Twaite shad larvae was not related to recent otolith growth (sum of the increments of the last three complete increments) but was significantly correlated with the long-term otolith growth (average increment width). Some studies have questioned the use of the RNA/DNA ratio as a reliable index of larval growth (Bergeron & Boulhic, 1994; Mathers *et al.*, 1994; Suthers, 1996).

RNA/DNA ratios were inversely related to both (instantaneous) growth and mortality rates estimated through microstructure analysis of *sagittae*. Ratios are supposed to reflect the nutritional condition and growth potential of fish larvae. Higher rates of protein synthesis (growth) correspond to higher values of RNA/DNA. Notwithstanding, the result obtained herein is consistent with the findings for reared larvae starved for several days. In fact, a large proportion of the Twaite shad larvae collected had RNA/DNA ratios lower than 1.3 and could be considered as "starving" (see below). On the other hand, larvae in poorer nutritional condition (with lower RNA/DNA ratios) are more liable to die either directly by inanition or indirectly through increased susceptibility to predation.

Larval Twaite shad RNA content and RNA/DNA ratios varied over time and space. The higher average nutritional condition of Twaite shad larvae collected during 1999 corresponded to particular habitat conditions, namely: 1) average water temperature of 22 ºC; 2) reduced mean annual rainfall (corresponding only to 50-75% of the 1941-1997 average); 3) diminished water turbidity (1.3 mg DW m^{-3} against 2.2 and 2.9 mg DW m^{-3} in 1998 and 2000); prey availability of *ca.* 90 crustacean *nauplii* 100 m^{-3}; and lower predation pressure (during 1999 only 38% of the samples had ≥1 predators 100 m^{-3} *versus* 74% in 1998 and 57% in 2000). In addition, there was an important effect of gut content on nutritional condition of shad larvae collected during 2000.

The contribution of prey items to the whole-larvae nucleic acids content (particularly of RNA) should be considered whenever smaller larvae are found to be in poor condition.

Using the threshold value of 1.3 to preliminarily distinguish starved larvae, a fairly high proportion of the Twaite shad larvae caught in the River Mira had RNA/DNA ratios <1.3 during 1998 and 2000 (about 45%) but not in 1999 (*ca.* 5%). Considering that RNA/DNA ratios of field caught larvae are generally higher than those observed in reared larvae, it was expected that few starving larvae (*i.e.* with RNA/DNA ratio lower than 1.3) were collected in the field, since these are more liable to die. A closer analysis showed that the high overall proportion of starving larvae (collected during 2000) was mainly due to individuals 7 to 13 mm SL that constituted *ca*. 67% of all larvae caught. In fact, low feeding incidences (<39% of the larvae with visible gut content) were found in the great majority (>80%) of the samples with smaller larvae (average SL <13mm).

In a typical year, Twaite shad larvae are in better nutritional condition during two relatively short time-periods, around days 100 (mid-April) and 145 (mid to late–May). These periods corresponded roughly to peak occurrences of the early life-history stages of the species in the River Mira (see Chapter 3). Moreover, higher growth rates were found for shad larvae collected during April and lower mortality rates were obtained for larvae caught during late-May and early-June in the River Mira (see Chapter 4). According to the regression models obtained herein, the nutritional condition of Twaite shad larvae is enhanced at water temperatures of *ca.* 22 ºC, particularly for moderate levels of prey, <80 *nauplii* 100 m^{-3}, and is significantly reduced when water turbidity is higher than 2 mg DW m^{-3} and potential prey are readily available (>20 *nauplii* 100 m^{-3}).

Alosa spp. *in the River Guadiana and Ribeira de Odeleite*

Early life-history stages of shad *Alosa* spp. were observed only in six of the 135 samples collected over the three-year period of sampling in the River Guadiana estuary. Larval shad occurred during late-May and June 1998, 1999 and 2000 in Mértola, Foz de Odeleite and Ribeira de Odeleite, respectively. These (few) occurrences of shad larvae corresponded to water temperature ≥22 ºC and turbidity in the range 1.3 – 3.3 mg DW m^{-3} and intermediate levels of microplankters' biomass (11.7 – 87.9 mg AFDW m^{-3}) and copepods' densities (3.2 – 1028.1 ind.

100 m^{-3}) but were independent of the abundances of potential predators (mysids and decapods).

The average nutritional condition of (only) nineteen shad larvae, expressed in terms of RNA/DNA ratio, ranged from 1.19 (±0.33 SD, *n*=3, Station 30 on 22 June 1998) to 2.4 (Station 21 on 31 May 2000). Average nutritional condition of these larvae agreed with the values found for *A. fallax fallax* in the River Mira (*cf.* Chapter 5). Nucleic acids contents were related to larval size and a significant relationship existed between size and condition. Using the residuals from the regression of larval RNA- to DNA-content as an index of nutritional condition, because they were independent of larval size, shad larvae were in better condition earlier in the spawning season (day of year=152 *i.e.* 31 May) than in later collections (day of year≥158 *i.e.* 6 June). Higher RNA/DNA ratios were found for increased levels of water turbidity and microplankton biomass as well as higher abundance of both copepods and mysids. Nevertheless, these findings were derived from pooling data from different years and stations and their inclusion in this study serves informative purposes.

7.2. Future research

Diel patterns in abundance and nutritional condition of larval Twaite shad in the River Mira generally support the choice of daytime sampling, but could require further confirmation under controlled conditions, *e.g.* experiments with constant light, temperature and prey densities. It was not possible to strictly confirm the daily deposition of growth increments on *sagittae* of larval Twaite shad, but the results suggest that increment's deposition be completed by the end of the day. Geffen (1992) provides a review of method(s) to validate the timing of increment formation.

On the other hand, the low catches of early life-history stages of *Alosa* spp. in the River Guadiana and tributary Ribeira de Odeleite might be increased if sampling frequency is intensified, particularly during peak spawning season, and/or if other more appropriate sampling gears are used, *e.g.* dip net, fixed (channel) net, sampling (light) traps or by electrofishing, particularly in the Ribeira de Odeleite and near Mértola.

Non-linear modelling methods, such as GAM, were useful to define Twaite shad eggs, embryos and larvae microhabitat precisely and to provide insight into early life-history stages distribution

in the upper River Mira. The results showed that the microhabitat used is influenced by a complex combination of several environmental variables mainly in a non-linear way. The additive models are less restrictive in what concerns the statistical distribution of the data. However, the error and link distribution applied have to be chosen beforehand (Hastie & Tibshirani, 1990). At present, methods of statistical inference and formal goodness-of-fit tests are not available for GAM (Bellido *et al.*, 2001). Thus interpretation of results is more complicated and relies mainly upon visual examination of effects and residuals. The large proportion of zeros in the response variable (shad abundance), which sometimes seems to show the density as a point process rather than a continuous process, poses further constraints to data analysis. In addition to increasing the sample size, modelling first the presence/absence as a binomial distribution and then modelling density solely when and where embryos or larvae occurred could overcome the difficulties mentioned above.

The fraction of starving larvae, *i.e*. those more likely to die of inanition or through an increased exposure to predation pressure, was estimated based upon threshold values derived for other clupeid species. It would be advisable to determine the starvation threshold in terms of RNA/DNA ratio under controlled laboratory conditions or *in situ*. The nutritional condition might have little to do with survival if predators take good- and poor-condition larvae indiscriminately, *i.e.* remove larvae with values in the lower- and upper-tail of the probability distribution. The ecological effect of interest concerns the possible change in scatter (say the difference between the 10^{th} and 90^{th} percentile in the probability distribution of RNA/DNA ratios) with age (or another covariate), and not the mean ('the average larva is dead') (Evans, 2000). If condition is important for survival, then a removal of the lowest tail of the probability distribution is expected as larval age (or other co-variate) increases. A randomisation test of significance is used to find evidence of change in scatter. This approach to data analysis could be employed to further study the relationship(s) between shad larvae condition and environmental factors.

The contribution of early life stages to inter-annual variability of recruitment may have to rely not only on data on eggs and larvae, but especially on data on abundances (and other life-history traits) closer to the age of recruitment (*e.g.* juvenile stage) (Peterman *et al.*, 1988). Furthermore, the combination of environmental factors, having known biological effects on recruitment, with stock-recruitment data (see Crecco *et al.*, 1986) provides a unifying approach to better understanding the various pathways through which climatic and density-dependent factors affect

recruitment. This approach is superior to using regression methods to describe the relationships between recruitment and lagged environmental data in the hope of finding significant statistical fits (Crecco *et al.*, 1986). Unfortunately, there is no available stock-recruitment data that would be useful to complement the analyses performed in this study.

The anadromous *A. alosa* and *A. fallax* (as most members of the Genus *Alosa*) are of ecological and patrimonial relevance, since they periodically migrate large distances, from marine to freshwater habitats, and exhibit a pronounced homing behaviour, similar to that of migratory salmonids (Baglinière & Elie, 2000). This stock-river relationship is important considering that environmental conditions during the embryo-larval period in the freshwater reaches of rivers play an important role in the future of populations (Baglinière, 2000). In contrast to salmonids, shads are more sensible to the presence of obstacles (dams, weirs, etc.) in their migratory pathways because shads are incapable of jumping and cannot swim for long periods. This poses a threat to the survival of shad populations (Assis, 1990). In recent years, adequate and more efficient fish passes have been developed and implemented (cf. Larinier *et al.*, 2000) but there has been very limited success in the attempts of artificial propagation, either to restore or augment population's abundance and distribution, of *Alosa* spp. in Europe (e.g. Quignard & Douchement, 1991a; Quignard & Douchement, 1991b) whereas in North America (especially in the USA) culture, stocking and translocation of *Alosa* spp. is a commonplace and successful practice (cf. Hendricks *et al.*, 2003). This might be considered in future project proposals to, first, produce, mark and stock cultured Twaite and Allis shad both as larvae and early juveniles, and secondly to monitor the abundance and mortality of larval and juvenile shad using marked the hatchery-produced fish and to assess the contribution of hatchery-produced fish on the resident/pre-migratory stock in the River Mira.

Chapter 8. References

Acolas, M.L., Bégout Anras, M.L., Véron, V., Jourdan, H., Sabatié, M.R., & Baglinière, J.-L. (2004) An assessment of the upstream migration and reproductive behaviour of allis shad (*Alosa alosa* L.) using acoustic tracking. *ICES Journal of Marine Science*, **61**, 1291-1304.

Adlandsvik, B., Gundersen, A.C., Nedreaas, K.H., Stene, A., & Albert, O.T. (1999) Modelling the advection and diffusion of eggs and larae of northeast Arctic Grrenland halibut. *ICES Council Meeting*, **1999/K**, 20 p.

Alexandrino, P. (1996) *Estudo de populações de sável (Alosa alosa L.) e savelha (Alosa fallax Lacépède). Análise da diferenciação interespecífica, subestruturação e hibridação*. Dissertação apresentada para a obtenção do grau de Doutor, Universidade do Porto, Porto, Portugal.

Alexandrino, P. & Boisneau, P. (2000). Diversité génétique. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 179-196. INRA Editions et Cemagref Editions, Paris.

Almeida, P.M. (1996) *Biologia e Ecologia de Liza ramada (Risso, 1826), Chelon labrosus (Risso, 1826) (Pisces, Mugilidae) no Estuário do Mira (Portugal). Inter-relações com o Ecossistema Marinho*. Dissertação apresentada para a obtenção do grau de Doutor, Universidade de Lisboa, Lisboa.

Alzola, C. & Harrell, F. (2002). An introduction to S and the Hmisc and Design libraries. University of Virginia School of Medicine, Charlottesville.

Anderson, D.R., Burnham, K.P., & Thompson, W.L. (2000) Null hypothesis testing: problems, prevalence, and an alternative. *Journal of Wildlife Management*, **64**, 912-923.

Andrade, F. (1986) *O estuário do Mira: caracterização geral e análise quantitativa da estrutura dos macropovoamentos bentónicos*. Dissertação apresentada para a obtenção do grau de Doutor, Universidade Clássica de Lisboa, Lisboa.

Aprahamian, M.W. (1981) Aspects of the biology of the twaite shad (*Alosa fallax*) in the rivers Severn and Wye. In Proceedings of the 2nd British Freshwater Fisheries Conference, pp. 111-119.

Aprahamian, M.W. (1982) *Aspects of the biology of the twaite shad, Alosa fallax fallax (Lacépède), in the Rivers Severn and Wye*. Doctoral Dissertation, University of Liverpool, Liverpool, UK.

Aprahamian, M.W. (1988) The biology of the twaite shad, *Alosa fallax fallax* (Lacépède), in the Severn Estuary. *Journal of Fish Biology*, **33 (Suppl. A)**, 141-152.

Aprahamian, M.W. (1989) The diet of juvenile and adult twaite shad *Alosa fallax fallax* (Lacépède) from the Rivers Severn and Wye (Britain). *Hydrobiologia*, **179**, 173-182.

Aprahamian, M.W., Baglinière, J.-L., Sabatié, M.R., Alexandrino, P., Thiel, R., & Aprahamian, C.D. (2003) Biology, status, and conservation of the anadromous Atlantic Twaite shad *Alosa fallax fallax*. *American Fisheries Society Symposium*, **35**, 103-124.

Assis, C.A. (1990) Threats to the survival of anadromous fishes in the River Tagus, Portugal. *Journal of Fish Biology*, **37 (Suppl. A)**, 225-226.

Assis, C.A., Almeida, P.R., Moreira, F.M., Costa, J.L., & Costa, M.J. (1992) Diet of the twaite shad *Alosa fallax* (Lacépède) (Clupeidae) in the River Tagus estuary. *Journal of Fish Biology*, **41**, 1049-1050.

Baglinière, J.-L. (2000). Le genre *Alosa* sp. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 3-30. INRA Editions et Cemagref Editions, Paris.

Baglinière, J.-L. & Elie, P. (2000) *Les aloses (Alosa alosa et Alosa fallax spp.)* Cemagref Editions et INRA Editions, Paris.

Baglinière, J.-L., Sabatié, M.R., Alexandrino, P., Aprahamian, M.W., & Elie, P. (2000). Les aloses: une richesse patrimoniale à conserver et à valoriser. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 263-275. INRA Editions et Cemagref Editions, Paris.

Baglinière, J.-L., Sabatié, M.R., Rochard, E., Alexandrino, P., & Aprahamian, M.W. (2003) The Allis shad *Alosa alosa*: biology, ecology, range, and status of populations. *American Fisheries Society Symposium*, **35**, 85-102.

Bailey, K.M., Canino, M.F., Napp, J.M., Spring, S.M., & Brown, A.L. (1995) Contrasting years of prey levels, feeding conditions and mortality of larval walleye pollock *Theragra chalcogramma* in the western Gulf of Alaska. *Marine Ecology Progress Series*, **119**, 11-23.

Bailey, K.M. & Houde, E.D. (1989) Predation on eggs and larvae of marine fishes and the recruitment problem. *Advances in Marine Biology*, **25**, 1-83.

Bain, M.B., Finn, J.T., & Booke, H.E. (1988) Streamflow regulation and fish community structure. *Ecology*, **69**, 382-392.

Balon, E.K. (1990) Epigenesis of an epigeneticist: the development of some alternative concepts on the early ontogeny and evolution of fishes. *Guelph Ichthyology Reviews*, **1**, 1-48.

Barron, M.G. & Adelman, I.R. (1984) Nucleic acid, protein content, and growth of larval fish sublethally exposed to various toxicants. *Canadian Journal of Fisheries and Aquatic Sciences*, **41**, 141-150.

Battini, M., Alonso, M.F., & Cussac, V.E. (1995) Growth and nutritional condition of the larvae of *Odontesthes microlepidotus* (Atherinidae): an experimental approach. *Environmental Biology of Fishes*, **42**, 391-399.

Baumann, H., Peck, M.A., & Herrmann, J.-P. (2005) Short-term decoupling of otolith and

somatic growth induced by food level changes in postlarval Baltic sprat, *Sprattus sprattus*. *Marine and Freshwater Research*, **56**, 539-547.

Bellido, J.M., Pierce, G.J., & Wang, J. (2001) Modelling intra-annual variation in abundance of squid *Loligo forbesi* in Scotish waters using generalised additive models. *Fisheries Research*, **52**, 23-39.

Bergeron, J.-P. (1997) Nucleic acids in ichthyoplankton ecology: a review, with emphasis on recent advances for new perspectives. *Journal of Fish Biology*, **51 (Suppl. A)**, 284-302.

Bergeron, J.-P. & Boulhic, M. (1994) Rapport ARN/ADN et évaluation de l'état nutritionel et de la croissance des larves de poissons marins: un essai de mise au point expérimentale chez la sole (*Solea solea* L.). *ICES Journal of Marine Science*, **51**, 181-190.

Betsill, R.K. & Avyle, M.J.v.d. (1997) Effect of temperature and zooplankton abundance on growth and survival of larval threadfin shad. *Transactions of the American Fisheries Society*, **126**, 999-1011.

Blaxter, J.H.S. & Hunter, J.R. (1982) The biology of clupeoid fishes. *Marine Biology*, **20**, 1-223.

Boehlert, G.W. & Morgan, J.B. (1985) Turbidity enhances feeding abilities of larval Pacific herring, *Clupea harengus pallasi*. *Hydrobiologia*, **123**, 161-170.

Boisneau, P., Mennesson-Boisneau, C., & Baglinière, J.-L. (1990) Description d'une frayère et comportement de reproduction de la grande alose (*Alosa alosa* L.) dans le cours supèrieur de la Loire. *Bulletin Français de Pêche et Pisciculture*, **316**, 15-23.

Brosse, S. & Lek, S. (2000) Modelling roach (*Rutilus rutilus*) microhabitat using linear and nonlinear techniques. *Freshwater Biology*, **44**, 441-452.

Buckley, L.J. (1979) Relationship between RNA-DNA ratio, prey density, and growth rate in Atlantic cod (*Gadus morhua*) larvae. *Journal of the Fisheries Research Board of Canada*, **361**, 1497-1502.

Buckley, L.J. (1982) Effects of temperature on growth and biochemical composition of larval winter flounder *Pseudopleuronectes americanus*. *Marine Ecology Progress Series*, **8**, 181-186.

Buckley, L.J. (1984) RNA-DNA ratio: an index of larval fish growth in the sea. *Marine Biology*, **80**, 291-298.

Buckley, L.J., Caldarone, E.M., & Ong, T.L. (1999) RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. *Hydrobiologia*, **401**, 265-277.

Buckley, L.J., Turner, S.I., Halavik, T.A., Smigielski, A.S., Drew, S.M., & Laurence, G.C. (1984) Effects of temperature and food availability on growth, survival, and RNA-DNA ratio of larval sand lance (*Ammodytes americanus*). *Marine Ecology Progress Series*, **15**, 91-97.

Burnham, K.P. & Anderson, D.R. (1998) *Model selection and inference: a pratical information-theoretic approach. Chapters 2-3* Springer-Verlag, New York.

Cabral , M.J., (coord.) , Queiroz, A.I., Palmeirim, J., Almeida, J., Rogado, L., Santos-Reis, M., Oliveira, M.E., Ferrand Almeida, N., Almeida, P.R., & Dellinger , T., (eds.) (2006) *Livro vermelho dos vertebrados de Portugal*, 2ª edn. Instituto de Conservação da Natureza, Lisboa.

Campana, S.E. (1992). Measurement and interpretation of the microstructure of fish otoliths. In *Otolith microstructure examination and analysis* (eds D.K. Stevenson & S.E. Campana), Vol. 117, pp. 59-71. Canadian Special Publications on Fisheries and Aquatic Sciences, Ottawa.

Campana, S.E. & Neilson, J.D. (1985) Microstructure of fish otoliths. *Canadian Journal of Fisheries and Aquatic Sciences*, **42**, 1014-1032.

Canino, M.F. (1994) Effects of temperature and food availability on growth and RNA/DNA ratios of walleye pollock *Theragra chalcogramma* (Pallas) eggs and larvae. *Journal of Experimental Marine Biology and Ecology*, **175**, 1-16.

Canino, M.F., Bailey, K.M., & Incze, L.S. (1991) Temporal and geogrpahic differences in feeding and nutritional condition of walleye pollock larvae *Theragra chalcogramma* in Shelikof Strait, Gulf of Alaska. *Marine Ecology Progress Series*, **79**, 27-35.

Cartaxana, A. (1994) Distribution and migrations of the prawn *Palaemon longirostris* in the Mira River Estuary (Southwest Portugal). *Estuaries*, **17**, 685-694.

Cassou-Leins, F. & Cassou-Leins, J.-J. (1981) *Recherches sur la biologie et l'haliêutique des migrateurs de la Garonne et principalement de l'alose Alosa alosa L.* Thèse presentée pour obtenir le grade de Docteur de Troisieme Cycle, Institut National Polytechnique de Toulose, Toulouse.

Cassou-Leins, J.-J., Cassou-Leins, F., Boisneau, P., & Baglinière, J.-L. (2000). La reproduction. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 73-92. Cemagref Editions et INRA Editions, Paris.

Caswell, P.A. & Aprahamian, M.W. (2001) Use of river habitat survey to determine the spawning habitat characteristics of twaite shad (*Alosa fallax fallax*). *Bulletin Français de Pêche et Pisciculture*, **362/363**, 919-929.

Chang, W.Y.B. (1982) A statistical method for evaluating the reproducibility of age determination. *Canadian Journal of Fisheries and Aquatic Sciences*, **39**, 1208-1210.

Chícharo, L., Chícharo, M.A., Esteves, E., Andrade, P., & Morais, P. (2001a) Effects of alterations in freshwater supply on the abundance and distribution of *Engraulis encrasicolus* in the Guadiana Estuary and adjacent coastal areas of south Portugal. *Ecohydrology & Hydrobiology*, **1**, 195-200.

Chícharo, M.A. (1997) Starvation percentages in field caught *Sardina pilchardus* larvae off southern Portugal. *Scientia Marina*, **61**, 507-516.

Chícharo, M.A. (1998) Nutritional condition and starvation in *Sardina pilchardus* (L.) larvae off southern Portugal compared with some environmental factors. *Journal of Experimental Marine Biology and Ecology*, **225**, 123-137.

Chícharo, M.A., Chícharo, L., Valdez, L., López-Jamar, E., & Ré, P. (1998a) Does the nutritional condition limit survival potential of sardine *Sardina pilchardus* (Walbaum, 1792) larvae off the north coast of Spain? RNA/DNA ratios and their variability. *Fisheries Research*, **790**, 1-12.

Chícharo, M.A., Chícharo, L., Valdez, L., López-Jamar, E., & Ré, P. (1998b) Estimation of starvation and diel variation of the RNA/DNA ratios in field-caught *Sardine pilchardus* larvae off the north of Spain. *Marine Ecology Progress Series*, **164**, 273-283.

Chícharo, M.A., Chícharo, L.M., Galvão, H., Barbosa, A., Marques, M.H., Andrade, J.P., Esteves, E., Miguel, C., & Gouveia, C. (2001b) Status of the Guadiana Estuary (south Portugal) during 1996-1998: An ecohydrological approach. *Aquatic Ecosystem Health and Management*, **4**, 73-98.

Clemmesen, C. (1989) RNA/DNA ratios of laboratory-reared and wild herring larvae determined with a highly sensitive fluorescence method. *Journal of Fish Biology*, **35 (Suppl. A)**, 331-333.

Clemmesen, C. (1994) The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. *Marine Biology*, **118**, 377-382.

Clemmesen, C. (1996). Importance and limits of RNA/DNA ratios as a measure of nutritional condition in fish larvae. In *Survival strategies in early life stages of marine resources* (eds Y. Watanabe, Y. Yamashita & Y. Ooseki), pp. 67-82. A.A. Balkema, Roterdam.

Clemmesen, C. & Doan, T. (1996) Does otolith structure reflect the nutritional condition of a fish larva? Comparison of otolith structure and biochemical index (RNA/DNA ratio) determined on cod larvae. *Marine Ecology Progress Series*, **138**, 33-39.

Clemmesen, C., Sanchez, R., & Wongtschowski, C. (1997) A regional comparison of the nutritional condition of SW Atlantic anchovy larvae, *Engraulis anchoita*, based on RNA/NDA ratios. *Archives of Fisheries and Marine Research*, **45**, 17-43.

Collares-Pereira, M.J., Cowx, I.G., Ribeiro, F., Rodrigues, J.A., & Rogado, L. (2000) Threats imposed by water resource development schemes on the observation of endangered fish species in the Guadiana River Basin in Portugal. *Fisheries Management and Ecology*, **7**, 167-178.

Collares-Pereira, M.J., Cowx, I.G., Sales Luis, T., Pedrosa, N., & Santos-Reis, M. (1999) Observations on the ecology of a landlocked population of allis shad in Aguieira Reservoir, Portugal. *Journal of Fish Biology*, **55**, 658-664.

Costa, M.J., Bruxelas, A., & Rosado, D. (1987) Ictiofauna do estuário do Mira, abundância e diversidade. In 2º Congresso sobre o Alentejo, Vol. I, pp. 225-230, Beja.

Crawley, M.J. (2002) *Statistical computing. An introduction to data analysis using S-Plus* John Wiley & Sons Ltd, Chichester, England.

Crecco, V. & Blake, M.M. (1983) Feeding ecology of coexisting larvae of American shad and blueback herring in the Connecticut River. *Transactions of the American Fisheries Society*, **112**, 498-507.

Crecco, V. & Savoy, T. (1984) Effects of fluctuations in hydrographic conditions on year-class strength of American shad (*Alosa sapidissima*) in the Connecticut river. *Canadian Journal of Fisheries and Aquatic Sciences*, **41**, 1216-1233.

Crecco, V. & Savoy, T. (1985) Effects of biotic and abiotic factors on growth and relative survival of young American shad, *Alosa sapidissima*, in the Connecticut River. *Canadian Journal of Fisheries and Aquatic Sciences*, **42**, 1640-1648.

Crecco, V. & Savoy, T. (1987a) Effects of climatic and density-dependent factors on intra-annual mortality of larval American shad. *American Fisheries Society Symposium*, **2**, 69-81.

Crecco, V. & Savoy, T. (1987b) Review of recruitment mechanisms of the American shad: the critical period and match-mismatch hypothesis reexamined. *American Fisheries Society Symposium*, **1**, 455-468.

Crecco, V., Savoy, T., & Gunn, L. (1983) Daily mortality rates of larval and juvenile American shad (*Alosa sapidissima*) in the Connecticut River with changes in year-class strength. *Canadian Journal of Fisheries and Aquatic Sciences*, **40**, 1719-1728.

Crecco, V., Savoy, T., & Whitworth, W. (1986) Effects of density-dependent and climatic factors on American shad, *Alosa sapidissima*, recruitment: a predictive approach. *Canadian Journal of Fisheries and Aquatic Sciences*, **43**, 457-463.

Crump, B.C. & Baross, J.A. (1996) Particle-attached bacteria and heterotrophic plankton associated with Columbia River estuarine turbidity maxima. *Marine Ecology Progress Series*, **138**, 265-273.

Cushing, D.H. (1975) *Marine ecology and fisheries* Cambridge University Press, Cambridge.

DeVries, D.R. & Stein, R.A. (1992) Complex interactions between fish and zooplankton: quantifying the role of an open-water planktivore. *Canadian Journal of Fisheries and Aquatic Sciences*, **49**, 1216-1227.

Eiras, J. (1977) Algumas medidas de protecção ao sável (*Alosa alosa* L.) do Rio Douro. *Publicações do Instituto de Zoologia "Dr. Augusto Nobre", Faculdade de Ciências do Porto*, **134**, 11-16.

Eiras, J. (1981) Sur une population d'*Alosa alosa* L., poisson migrateur amphibiotique, thalassotrophe, bloquée en eau douce au Portugal. *Cybium*, **5**, 69-73.

Elie, P., Taverny, C., Mennesson-Boisneau, C., & Sabatié, M.R. (2000). L'exploitation halieutique. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 199-226. INRA Editions et Cemagref Editions, Paris.

Emerson, J.D. & Hoaglin, D.C. (1992). Capítulo 6. Análise de tabelas bidimensionais através de medianas. In *Análise exploratória de dados. Técnicas robustas. Um guia* (eds D.C. Hoaglin, F. Mosteller & J.W. Tukey), pp. 167-212. Edições Salamandra, Lisboa.

Esteves, E. (1999) *Factores que influenciam a abundância e a condição nutricional de larvas de peixe dos rios Mira e Guadiana*. Dissertação apresentada para a obtenção do grau de Mestre, Universidade de Coimbra, Coimbra, Portugal.

Esteves, E., Chícharo, M.A., Pina, T., Coelho, M.L., & Andrade, J.P. (2000a) Comparison of RNA/DNA ratios obtained with two methods for nucleic acid quantification in gobiid larvae. *Journal of Experimental Marine Biology and Ecology*, **245**, 43-55.

Esteves, E., Pina, T., & Andrade, J.P. (2000b) The distribution of estuarine fish larvae: nutritional condition and co-occurrence with predatrs and prey. *Acta Oecologica*, **21**, 161-173.

Evans, G.T. (2000) Local estimation of probability distribution and how it depends on covariates. *Canadian Stock Assessment Research Document*, **120**, 11 p.

Ferron, A. & Leggett, W.C. (1994) An appraisal of condition measures for marine fish larvae. *Advances in Marine Biology*, **30**, 217-303.

Fey, D.P. (2005) Is the marginal otolith increment width a reliable recent growth index for larval and juvenile herring? *Journal of Fish Biology*, **66**, 1692-1703.

Fogarty, M.J., Sissenwine, M.P., & Cohen, E.B. (1991) Recruitment variability and the dynamics of of exploited marine populations. *Trends in Ecology and Evolution*, **6**, 241-246.

Folkvord, A., Ystanes, L., Johannessen, A., & Moksness, E. (1996) RNA:DNA ratios and growth of herring (*Clupea harengus*) larvae reared in mesocosms. *Marine Biology*, **126**, 591-602.

Francis, R.I.C.C. (1990) Back-calculation of fish length: a critical review. *Journal of Fish Biology*, **36**, 883-902.

Frank, K.T. & Leggett, W.C. (1985) Reciprocal oscillations in densities of larval fish and potential predators: a reflection of present or past predation? *Canadian Journal of Fisheries and Aquatic Sciences*, **42**, 1841-1849.

Fry, J.E.J. (1971). The effects of enviornmental factors on the physiology of fish. In *Fish physiology* (eds W.S. Hoar & D.J. Randall), Vol. 6, pp. 1-98. Academic Press, New York.

Fuiman, L.A. & Higgs, D.M. (1997). Chapter 8. Ontogeny, growth and recruitment processes. In *Early life history and recruitment in fish populations* (eds R.C. Chambers & E.A. Trippel), pp. 224-249. Chapman and Hall, London.

Gadomski, D.M. & Barfoot, C.A. (1998) Diel and distributional abundance patterns of fish embryos and larvae in the lower Columbia and Deschutes rivers. *Environmental Biology of Fishes*, **51**, 353-368.

García, A., Cortés, D., & Ramirez, T. (1998) Daily larval growth and RNA and DNA content of the NW Mediterranean anchovy *Engraulis encrasicolus* and their relations to the environment. *Marine Ecology Progress Series*, **166**, 237-245.

Geffen, A.J. (1992). Validation of otolith increment deposition rate. In *Otolith microstructure examination and analysis* (eds D.K. Stevenson & S.E. Campana), Vol. 117, pp. 101-113. Canadian Special Publications on Fisheries and Aquatic Sciences, Ottawa.

Gerkens, M. & Thiel, R. (2001) Habitat use of age-0 twaite shad (*Alosa fallax* Lacépède, 1803) in the tidal freshwater region of the Elbe River, Germany. *Bulletin Français de Pêche et Pisciculture*, **362/363**, 773-784.

Gilliers, C., Amara, R., Bergeron, J.-P., & Le Pape, O. (2004) Comparison of growth and condition indices of juvenile flatfish in different coastal areas. *Environmental Biology of Fishes*, **71**, 189-198.

González, J.A.M. (1995) *Sedimentología del Estuario del Río Guadiana (S.O. España-Portugal)* CEP Universidad de Huelva, Huelva.

Hastie, T. & Tibshirani, R. (1990) *Generalized Additive Models* Chapman and Hall, London.

Henderson, P.A. (1987) The vertical and transverse distribution of larval herring in the River Blackwater estuary, Essex. *Journal of Fish Biology*, **31**, 281-290.

Hendricks, M.L., Hoopes, R.L., Arnold, D.A., & Kaufman, M.L. (2002) Homing of hatchery-reared American shad to the Lehigh River, a tributary to the Delaware River. *North American Journal of Fisheries Management*, **22**, 243-248.

Hendricks, M.L., Hoopes, R.L., Arnold, D.A., & Kaufman, M.L. (2003) Culture and transplant of alosines in North America. *American Fisheries Society Symposium*, **35**, 303-312.

Hjort, J. (1914) Fluctuations in the great fisheries of northern Europe. *Rapports des Proces-verbaux des Réunions du Conseil International pour l'Exploration du Mer*, **20**, 1-228.

Houde, E.D. (1987) Fish early life dynamics and recruitment variability. *American Fisheries Society Symposium*, **2**, 17-29.

Houde, E.D. (1996). Evaluating stage-specific survival during the early life of fish. In *Survival strategies in early life stages of marine resources* (eds Y. Watanabe, Y. Yamashita & Y. Ooseki), pp. 51-66. A.A. Balkema, Roterdam.

Houde, E.D. (1997a). Chapter 6. Patterns and consequences of selective processes in teleost early life histories. In *Early life history and recruitment in fish populations* (eds R.C. Chambers & E.A. Trippel), pp. 173-196. Cahpman and Hall, London.

Houde, E.D. (1997b) Patterns and trends in larval-stage growth and mortality of teleost fish. *Journal of Fish Biology*, **51 (Suppl. A)**, 52-83.

Hovenkamp, F. (1990) Growth differences in larval plaice *Pleuronectes platessa* in the Southern Bight of the North Sea as indicated by otolith increments and RNA/DNA ratios. *Marine Ecology Progress Series*, **58**, 205-215.

Hovenkamp, F. & Witte, J.I. (1991) Growth, otolith growth and RNA/DNA ratios of larval plaice *Pleuronectes platessa* in the North Sea 1987 to 1989. *Marine Ecology Progress Series*, **70**, 105-116.

Hugie, D.M. & Dill, L.M. (1994) Fish and game: a game theoretic approach to habitat selection by predators and prey. *Journal of Fish Biology*, **45 (Suppl. 1)**, 151-169.

Hunter, J.R. (1981). Feeding ecology and predation of marine fish larvae. In *Marine fish larvae.*

Morphology, ecology and relation to fisheries (ed R. Lasker), pp. 33-77. Washington Sea Grant Program, Washington.

Hunter, J.R. (1982). Predation and Recruitment. In *Fish Ecology III. A foundation for REX - a Recruitment EXperiment* (eds B.J. Rothschild & C.G.H. Rooth), pp. 172-210. University of Miami, Rosenstiel School of Marine and Atmospheric Science and The National Oceanic and Atmospheric Admnistration Cooperative Institute for Marine and Atmospheric Studies, Miami, Florida.

Hunter, J.R. (1984). Feeding ecology and predation of marine fish larvae. In *Marine fish larvae. Morphology, ecology, and relation to fisheries* (ed R. Lasker), pp. 33-79. Washington Sea Grant Program, Seattle.

I.H.M. (1997-1999) *Tabela de Marés 1998-2000* Ministério da Defesa Nacional, Instituto Hidrográfico da Marinha, Lisboa.

International Union for Conservation of Nature (1994) *IUCN red list categories.* IUCN Species Survival Commission, Gland, Switzerland.

Johnson, J.H. & Dropkin, D.S. (1995) Effects of prey density and short term food deprivation on the growth and survival of American shad larvae. *Journal of Fish Biology*, **46**, 872-879.

Johnson, J.H. & Ringler, N.H. (1998) Predator response to releases of American shad larvae in the Susquehanna River basin. *Ecology of Freshwater Fish*, **7**, 192-199.

Johnson, J.R. & Loesch, J.G. (1983) Morphology and development of hatchery-cultured American shad, *Alosa sapidissima* (Wilson). *Fishery Bulletin*, **81**, 323-339.

Jones, C.M. (1992). Chapter 1. Dvelopment and application of the otolith increment technique. In *Otolith microstructure examination and analysis* (eds D.K. Stevenson & S.E. Campana), Vol. 117, pp. 1-11. Canadian Special Publication on Fisheries and Aquatic Sciences, Ottawa.

Kashuba, S.A. & Matthews, W.J. (1984) Physical condition of larval shad during Spring-Summer in a Southwestern reservoir. *Transactions of the American Fisheries Society*, **113**, 119-204.

Kimmerer, W.J., Burau, J.R., & Bennett, W.A. (1998) Tidally oriented vertical migration and position maintenance of zooplankton in a temperate esturay. *Limnology and Oceanography*, **43**, 1697-1709.

Larinier, M., Travade, F., & Dartiguelongue, J. (2000). La conception des dispositifs de franchissement. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 249-259. INRA Editions et Cemagref Editions, Paris.

Lasker, R. (1975) Field criteria for survival of anchovy larvae: the relation between inshore chlorophyl maximum layers and successful first feeding. *Fishery Bulletin*, **73**, 453-461.

Lasker, R. (1978) The relation between oceanographic conditions and larval anchovy food in the California current: identification of factors contributing to recruitment failure. *Rapports des Proces-verbaux des Réunions du Conseil International pour l'Exploration du Mer*, **173**, 212-230.

Laurence, G.C. (1982). Nutrition and trophodynamics of larval fish - review, concepts, strategic recommmendations and opinions. In *Fish Ecology III. A foundation for REX - a Recruitment EXperiment* (eds B.J. Rothschild & C.G.H. Rooth), pp. 123-171. University of Miami, Rosenstiel School of Marine and Atmospheric Science and The National Oceanic and Atmospheric Admnistration Cooperative Institute for Marine and Atmospheric Studies, Miami, Florida.

Leach, S.D. & Houde, E.D. (1999) Effects of environmental factors on survival, growth, and production of American shad larvae. *Journal of Fish Biology*, **54**, 767-786.

Legall, O. (2000). Origine et histoire des aloses. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 127-136. INRA Editions et Cemagref Editions, Paris.

Leggett, W.C. & DeBlois, E. (1994) Recruitment in marine fishes: is it regulated by starvation and predation in the egg and larval stages? *Netherlands Journal of Sea Research*, **32**, 119-134.

Leggett, W.C. & Whitney, R.R. (1972) Water temperature and the migrations of American shad. *Fishery Bulletin*, **70**, 659-670.

Letcher, B.H. & Rice, J.A. (1997) Prey patchiness and larval fish growth and survival: inferences from an individual-based model. *Ecological Modelling*, **95**, 29-43.

Letcher, B.H., Rice, J.A., Crowder, L.B., & Binkowski, F.P. (1997) Size- and species-dependent variability in consumption and growth rates of larvae and juveniles of three freshwater fishes. *Canadian Journal of Fisheries and Aquatic Sciences*, **54**, 405-414.

Limburg, K.E. (1996a) Growth and migration of 0-year American shad (*Alosa sapidissima*) in the Hudson River estuary: otolith microstructural analysis. *Canadian Journal of Fisheries and Aquatic Sciences*, **53**, 220-238.

Limburg, K.E. (1996b) Modelling the ecological constraints on growth and movement of juvenile American shad (*Alosa sapidissima*) in the Hudson River Estuary. *Estuaries*, **19**, 794-813.

Lorda, E. & Crecco, V. (1987) Stock-recruitment relationship and compensatory mortality of American shad in the Connecticut River. *American Fisheries Society Symposium*, **1**, 469-482.

Loureiro, J.J., Nunes, M.N., & Botelho, O.F. (1984) *Monografia hidrológica do rio Mira* Direcção-Geral dos Recursos e Aproveitamentos Hidraúlicos, Comissão de Coordenação da Região do Alentejo, Évora.

Maitland, P.S. & Hatton-Hellis, T.W. (2003). Ecology of the Allis and Twaite shad. English Nature, Peterborough.

Maitland, P.S. & Lyle, A.A. (2005) Ecology of Allis shad *Alosa alosa* and Twaite shad *Alosa fallax* in the Solway Firth, Scotland. *Hydrobiologia*, **534**, 205-211.

Mallin, M.A., Cahoon, L.B., McIver, M.R., Parsons, D.C., & Shank, G.C. (1999) Alternation of factors limiting phytoplankton production in the Cape Fear River Estuary. *Estuaries*, **22**, 825-836.

Maniukas, Y.L. (1989) Biology of the Atlantic shad, *Alosa fallax fallax*, in Kurshskiy Bay. *Journal of Ichthyology*, **29**, 125-128.

Marker, A.F.H. & Collett, G.D. (1997) Spatial and temporal characteristics of algae in the River Great Ouse. I. Phytoplankton. *Regulated Rivers: Research & Management*, **13**, 219-233.

Martin, F.D., Wright, D.A., Means, J.C., & Setzler-Hamilton, E.M. (1985) Importance of food supply to nutritional state of larval striped bass in the Potomac river estuary. *Transactions of the American Fisheries Society*, **114**, 137-145.

Mathers, E.M., Houlihan, D.F., & Burren, L.J. (1994) RNA, DNA and protein concentration in fed and starved herring *Clupea harengus* larvae. *Marine Ecology Progress Series*, **107**, 223-231.

Mattos, M.A.R. (1995) Distribuição espacio-temporal do zooplâncton no estuário do rio Mira. *Relatórios Técnico-Científicos do Instituto Português de Investigação Marítima*, **11**, 29.

McGurk, M.D., Warburton, H.D., Galbraith, M., & Kusser, W.C. (1992) RNA-DNA ratio of herring and sand lance larvae from Port Moller, Alaska: Comparison with prey concentration and temperature. *Fisheries Oceanography*, **1:3**, 193-207.

Mennesson-Boisneau, C., Aprahamian, C.D., Sabatié, M.R., & Cassou-Leins, J.J. (2000a). Caractéristiques des adultes. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 33-53. INRA Editions et Cemagref Editions, Paris.

Mennesson-Boisneau, C., Aprahamian, C.D., Sabatié, M.R., & Cassou-Leins, J.J. (2000b). Remontée migratoire des adultes. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 55-72. INRA Editions et Cemagref Editions, Paris.

Mennesson-Boisneau, C., Boisneau, P., & Baglinière, J.-L. (1986) Premières observations sur les caractéristiques biologiques des adultes de grande alose (*Alosa alosa* L.) dans le cours moyen de la Loire. *Acta Oecologica*, **7**, 337-353.

Moller, H. (1984) Reduction of a larval herring population by jellyfish predator. *Science*, **224**, 621-622.

Moller, H. & Dieckwisch, B. (1991) Larval fish production in the tidal River Elbe 1985-1986. *Journal of Fish Biology*, **38**, 829-838.

Moser, M.L. & Ross, S.W. (1994). Effects of changing current regime and river discharge on the estuarine phase of anadromous fish migration. In *Changes in fluxes in estuaries: implications from science and management* (eds K.R. Dyer & R.J. Orth), pp. 343-347. Olsen & Olsen, Fredensborg.

Mueter, F.J. & Norcross, B.L. (2002) Spatial and temporal patterns in the demersal fish community on the shelf and upper slope regions of the Gulf of Alaska. *Fishery Bulletin*, **100**, 559-581.

Neilson, J.D. (1992). Sources of error in otolith microstructure examination. In *Otolith microstructure examination and analysis* (eds D.K. Stevenson & S.E. Campana), Vol. 117, pp. 115-126. Canadian Special Publications on Fisheries and Aquatic Sciences, Ottawa.

Nelson, J. (1994) *Fishes of the world*, 3rd Edition edn. John Wiley and Sons Inc., New York.

Newell, G.E. & Newell, R.C. (1963) *Marine plankton, a pratical guide* Hutchinson of London, London.

Odum, E.P. (1988) *Fundamentos de Ecologia*, 4ª edn. Fundação Calouste Gulbenkian, Lisboa.

O'Leary, J.A. & Kynard, B. (1986) Behavior, length, and sex ratio of seaward-migrating juvenile American shad and blueback herring in the Connecticut River. *Transactions of the American Fisheries Society*, **115**, 529-536.

Omori, M. & Ikeda, T. (1992) *Methods in marine zooplankton ecology* Krieger Publishing Company, Florida.

Pardue, G.B. (1983). Habitat suitability index models: alewife and blueback herring, Rep. No. FWS/OBS-82/10.58. U.S. Department of the Interior, Fish and Wildlife Service.

Paula, J.M.d. (1993) *Ecologia da fase larvar e recrutamento de crustáceos decápodes no estuário do rio Mira*. Dissertação apresentada para a obtenção do grau de Doutor, Universidade de Lisboa, Lisboa.

Pepin, P. (1989) Using growth histories to estimate larval fish mortality rates. *Rapports des Proces-verbaux des Réunions du Conseil International pour l'Exploration du Mer*, **191**, 324-329.

Pepin, P., Evans, G.T., & Shears, T.H. (1999) Patterns of RNA/DNA ratios in larval fish and their relationship to survival in the field. *ICES Journal of Marine Science*, **56**, 697-706.

Pepin, P., Shears, T.H., & Lafontaine, Y.d. (1992) Significance of body size to the interaction between a larval fish (*Mallotus villosus*) and a vertebrate predator (*Gasterosteus aculeatus*). *Marine Ecology Progress Series*, **81**, 1-12.

Peterman, R.M., Bradford, M.J., Lo, N.C.H., & Methot, R.D. (1988) Contribution of early li stages to interannual variability in recruitment of Northern anchovy (*Engraulis mordax*). *Canadian Journal of Fisheries and Aquatic Sciences*, **45**, 8-16.

Pina, T. (2000) *Reprodução de savelha Alosa fallax fallax (Lacépède, 1803) nos rios Mira e Guadiana*. Dissertação apresentada para a obtenção do grau de Mestre, Universidade do Algarve, Faro, Portugal.

Pina, T., Esteves, E., & Andrade, J.P. (2003) Gametogenesis in twaite shad, *Alosa fallax fallax* (Lacépède, 1803), from rivers Mira and Guadiana (Portugal). *Scientia Marina*, **67**, 313-322.

Powell, A.B., Cheshire, R.T., Laban, E.H., Colvocoresses, J., O'Donnell, P., & Davidian, M. (2004) Growth, mortality, and hatchdate distributions of larval and juvenile spotted seatrout (*Cynoscion nebulosus*) in Florida Bay, Everglades National Park. *Fishery Bulletin*, **102**, 142-155.

Purcell, J.E. & Grover, J.J. (1990) Predation and food limitation as causes of mortality in larval herring at a spawning ground in British Columbia. *Marine Ecology Progress Series*, **59**, 55-61.

Quignard, J.P. & Douchement, C. (1991a). *Alosa alosa* (Linnaeus, 1758). In *The freshwater fishes of Europe. Vol. 2. Clupeidae, Anguillidae* (ed H. Hoestland), pp. 89-126. AULA-Verlag Wiesbaden.

Quignard, J.P. & Douchement, C. (1991b). *Alosa fallax fallax* (Lacepède, 1803). In *The freshwater fishes of Europe. Vol. 2. Clupeidae, Anguillidae* (ed H. Hoestland), pp. 225-253. AULA-Verlag Wiesbaden.

Quintela, A.C. (1984). 9 - Hidrologia de águas superficiais. In *Curso Internacional de Hidrologia Operativa. Manual. Volume II*, Vol. II, pp. 508-731. Direcção-Geral dos Recursos e Aproveitamentos Hidráulicos e UNESCO, Lisboa.

R Development Core Team (2003) *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria.

Ramirez, T., Cortés, D., & García, A. (2001) Growth of North Alboran Sea sardine larvae estimated by otolith microstructure, nucleic acids and protein content. *Journal of Fish Biology*, **59**, 403-415.

Ramos, M.A. (1977) Reprodução artificial de *Alosa alosa* L. *Repositório de Trabalhos do Laboratório Nacional de Investigação Veterinária*, **IX**, 41-45.

Ré, P. (1994) Anchovy spawning in Mira estuary (1985/1992). *Arquivos do Museu Bocage, Nova Série*, **II**, 423-454.

Ré, P. & Gonçalves, E. (1993) Ecologia da fase planctónica da anchova (*Engraulis encrasicolus*) no estuário do rio Mira, resultados de oito anos de estudo (1985/1992). *Boletim UCA, U. Algarve*, **1**, 527-542.

Richard, P., Bergeron, J.-P., Boulhic, M., Galois, R., & Ruyet, J.P.-L. (1991) Effect of starvation on RNA, DNA and protein content of laboratory-reared larvae and juveniles of *Solea solea*. *Marine Ecology Progress Series*, **72**, 69-77.

Richkus, W.A. (1974) Factors influencing the seasonal and daily patterns of alewife (*Alosa pseudoharengus*) migration in a Rhode Island river. *Journal of the Fisheries Research Board of Canada*, **31**, 1485-1497.

Rilling, G.C. & Houde, E.D. (1999) Regional and temporal variability in growth and mortality of bay anchovy, *Anchoa mitchilli*, larvae in Chesapeake Bay. *Fishery Bulletin*, **97**, 555-569.

Robinson, S.M.C. & Ware, D.M. (1988) Ontogenic development of growth rates in larval Pacific herring, *Clupea harengus pallasi*, measured with RNA-DNA ratios in the Strait of Georgia, British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences*, **45**, 1422-1429.

Rodrigues da Costa, A.B. (1980) A navegabilidade do Rio Guadiana. *Anais do Clube Militar Naval*, **110**, 661-682.

Rooker, J.R. & Holt, G.J. (1996) Application of RNA:DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). *Marine and Freshwater Research*, **47**, 283-290.

Rooker, J.R., Holt, G.J., & Holt, S.A. (1997) Condition of larval and juvenile red drum (*Sciaenops ocellatus*) from estuarine nursery habitats. *Marine Biology*, **127**, 387-394.

Ross, R.M. & Backman, T.W.H. (1992) Larval American shad: effects of age and group size on swimming and feeding behavior. *Transactions of the American Fisheries Society*, **121**, 508-516.

Ross, R.M., Bennett, R.M., & Backman, T.W.H. (1993) Habitat use by spawning adult, egg, and larval American shad in the Delaware river. *Rivers*, **4**, 227-238.

Ross, R.M., Johnson, J.H., Bennett, R.M., & Dropkin, D.S. (1996) Behavioral changes associated with suboptimal prey densities for larval American shad. *Ecology of Freshwater Fish*, **5**, 163-168.

Russell, F.S. (1976) *The eggs and planktonic stages of British marine fishes* Academic Press, London.

Rutherford, E.S. & Houde, E.D. (1995) The influence of temperature on cohort-specific growth, survival, and recruitment of striped bass, *Morone saxatilis*, larvae in Chesapeake Bay. *Fishery Bulletin*, **93**, 315-332.

Sabatié, M.-R., Alexandrino, P., & Baglinière, J.-L. (1996) Comparaison des caratéristiques biologiques de la grande alose (*Alosa alosa*) dans l'Oued Sebou (façade nord-Atlantique du Maroc) et dans le fleuve Lima (Portugal). *Cybium*, **20**, 59-73.

Sabatié, M.R., Boisneau, P., & Alexandrino, P. (2000). Variabilité morphologique. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 137-178. INRA Editions et Cemagref Editions, Paris.

Sammons, S.M. & Bettoli, P.W. (2002) Spatial and diel variation in distribution of limnetic larvae of fishes in a Tennesse reservoir. *Journal of Freshwater Ecology*, **17**, 45-53.

Saville, A. (1964) Clupeiodae. Identification sheets on fish eggs and larvae. *ICES Identification sheets*, **1**, 5 p.

Savoy, T. & Crecco, V. (1987). Daily increments on the otoliths of larval American shad and their potential use in population dynamics studies. In *The age and growth of fish* (eds R.C. Summerfelt & G.E. Hall), pp. 413-431. The Iowa State University Press, Ames, Iowa.

Savoy, T. & Crecco, V. (1988) The timing and significance of density-dependent and density-independent mortality of American shad, *Alosa sapidissima*. *Fishery Bulletin*, **86**, 467-481.

Scheidegger, K.J. & Bain, M.B. (1995) Larval fish distribution and microhabitat use in free-flowing and regulated rivers. *Copeia*, **1995**, 125-135.

Secor, D.H. & Dean, J.M. (1989) Somatic growth effects on the otolith-fish size relaionship in young pond-reared striped bass, *Morone saxatilis*. *Canadian Journal of Fisheries and Aquatic Sciences*, **46**, 113-121.

Secor, D.H., Dean, J.M., & Laban, E.H. (1991) *Manual for otolith removal and preapration for*

microstructural examination Electric Power Research Institute and Belle W. Baruch Institute for Marine Biology and Coastal Research, Palo Alto.

Secor, D.H. & Houde, E.D. (1995) Temperature effects on the timing of striped bass egg production, larval viability, and recruitment potential in the Patuxent River (Chesapeake Bay). *Estuaries*, **18**, 527-544.

Sirois, P. & Dodson, J.J. (2000) Influence of turbidity, food density and parasites on the ingestion and growth of larval rainbow smelt *Osmerus mordax* in an estuarine turbidity maximum. *Marine Ecology Progress Series*, **193**, 167-179.

Smith, D.L. (1977) *A guide to marine and coastal plankton and marine invertebrate larvae* Kendall/Hunt Company, Dubuque, Iowa.

Sousa, L., Matos, J., Matono, P., Suaréz, S.d.S., Maxímino, P., & Bernardo, J.M. (2003). Monitorização de peixes migradores no rio Guadiana. Laboratório de Ecologia Aquática da Universidade de Évora e EDIA - Empresa de Desenvolvimento e Infra-Estruturas do Alqueva S.A., Évora.

Stein, R.A., DeVries, D.R., & Dettmers, J.M. (1995) Food-web regulation by a planktivore: exploring the generality of the thropic cascade hypothesis. *Canadian Journal of Fisheries and Aquatic Sciences*, **52**, 2518-2526.

Stevenson, D.K. & Campana, S.E. (1992) Otolith microstructure examination and analysis. *Canadian Special Publications of Fisheries and Aquatic Sciences*, **117**, 126 p.

Suneetha, K.-B., Folkvord, A., & Johannssen, A. (1999) Responsiveness of selected condition measures of herring, *Clupea harengus*, larvae to starvation in relation to ontogeny and temperature. *Environmental Biology of Fishes*, **54**, 191-204.

Suthers, I.M. (1996) Spatial variability of recent otolith growth and RNA indices in pelagic juvenile *Diaphus kapalae* (Myctophidae): an effect of flow disturbance near an island? *Marine and Freshwater Research*, **47**, 273-282.

Suthers, I.M., Clearly, J.J., Bataglene, S.C., & Evans, R. (1996) Relative RNA content as a measure of condition in larval and juvenile fish. *Marine and Freshwater Research*, **47**, 301-307.

Suthers, I.M. & Frank, K.T. (1990) Zooplankton biomass gradient off south-western Nova Scotia: nearshore ctenophore predation or hydrographic separation? *Journal of Plankton Research*, **12**, 831-850.

Swartzman, G., Huang, C., & Kaluzny, S. (1992) Spatial analysis of Bering Sea groundfish survey data using generalized additive models. *Canadian Journal of Fisheries and Aquatic Sciences*, **49**, 1366-1378.

Talbot, G.B. (1954) Factors associated with fluctuations in abundance of Hudson river shad. *Fishery Bulletin*, **56**, 373-413.

Taverny, C. (1991) *Contribution à la connaissance de la dynamique des populations d'aloses (Alosa alosa et Alosa fallax) dans le système fluvio-estuarien de la Gironde: Pêche, biologie et écologie. Étude particulière de la dévailason et de l'impact des activités*

humaines. Thèse presentée pour obtenir le grade de Docteur, Université de Bordeaux I, Bordeaux.

Taverny, C., Belaud, A., Elie, P., & Sabatié, M.R. (2000a). Influence des activités humaines. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 227-248. INRA Editions et Cemagref Editions, Paris.

Taverny, C., Cassou-Leins, J.J., Cassou-Leins, F., & Elie, P. (2000b). De l'oeuf à l'adulte en mer. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 93-124. Cemagref Editions et INRA Editions, Paris.

Taverny, C., Cassou-Leins, J.J., Cassou-Leins, F., & Elie, P. (2000c). De l'ouef à l'adulte en mer. In *Les aloses (Alosa alosa et Alosa fallax spp.)* (eds J.-L. Baglinière & P. Elie), pp. 93-124. INRA Editions et Cemagref Editions, Paris.

Theilacker, G.H. (1980) Changes in body measurements of larval northern anchovy, *Engraulis mordax*, and other fishes due to handling and preservation. *Fishery Bulletin*, **78**, 685-692.

Thiel, R., Sepúlveda, A., Kafemann, R., & Nellen, W. (1995) Environmental factors as forces structuring the fish community of the Elbe Estuary. *Journal of Fish Biology*, **46**, 47-69.

Thiel, R., Sepúlveda, A., & Oesmann, S. (1996). Occurrence and distribution of twaite shad (*Alosa fallax* Lacepède) in the lower Elbe River, Germany. In *Conservation of endangered freshwater fishes in Europe* (eds A. Kirchhoffer & D. Hefti), pp. 157-170. Birkhauser Verlag, Basel, Switzerland.

Todd, C.D. & Laverack, M.S. (1991) *Coastal marine zooplankton. A pratical manual for students* Cambridge University Press, Cambridge.

Tomás, J., Augagneur, S., & Rochard, E. (2005) Discrimination of the natal origin of young-of-the-year Allis shad (*Alosa alosa*) in the Garonne-Dordogne basin (south-west France) using otolith chemistry. *Ecology of Freshwater Fish*, **14**, 185-190.

Turner, T.F., Trexler, J.C., Miller, G.L., & Toyer, K.E. (1994) Temporal and spatial dynamics of larval and juvenile fish abundance in a temperate floodplain river. *Copeia*, **1994**, 174-185.

Utne-Palm, A.C. (2001) Visual feeding of fish in a turbid environment: physical and behavioural aspects. *Marine and Freshwater Behaviour and Physiology*, **35**, 111-128.

Venables, W.N. & Ripley, B.D. (1999) *Modern applied statistics with S-Plus*, 3rd edn. Springer-Verlag, New York.

Véron, V., Jatteau, P., & Bardonnet, A. (2003) First results on the behaviour of young stages of allis shad *Alosa alosa*. *American Fisheries Society Symposium*, **35**, 241-251.

Wagner, M., Durbin, E., & Buckley, L.J. (1998) RNA:DNA ratios as indicators of nutritional condition in the copepod *Calanus finmarchicus*. *Marine Ecology Progress Series*, **162**, 173-181.

Whitehead, P.J.P. (1985) FAO Species catalogue. Vol. 7. Clupeiod fishes of the world (suborder Clupeioiedei). *FAO Fisheries Synopsis*, **125**, 1-303.

Winkler, G., Dobson, J.J., Bertrand, N., Thivierge, D., & Vincent, W.F. (2003) Trophic coupling across the St. Lawrence River estuarine transition zone. *Marine Ecology Progress Series*, **251**, 59-73.

Wood, S. (2001) mgcv: GAMs and generalized ridge regression for R. *R news*, **1**, 20-25.

Wood, S.N. & Augustin, N.H. (2002) GAMs with integrated model selection using penalised regression splines and applications to environmental modelling. *Ecological Modelling*, **157**, 157-177.

Zar, J.H. (1996) *Biostatistical analysis*, 3rd edn. Prentice-Hall International (UK), London.

Zydlewski, J. & McCormick, S.D. (1997) The ontogeny of salinity tolerance in the American shad, *Alosa sapidissima*. *Canadian Journal of Fisheries and Aquatic Sciences*, **54**, 182-189.