FELIPPE ALDERT POSTUMA

Population biology and behavioural aspects of the squid *Doryteuthis plei* (Blainville, 1823) in the northern coast of São Paulo, with emphasis on reproduction and feeding

Thesis submitted to the Oceanographic Institute of the University of São Paulo, in partial fulfilment of the requirements for the degree of Doctor of Sciences in Biological Oceanography

Supervisor: Prof. Dr. Maria de los Angeles Gasalla

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SUMMARY

Figure index

Reproductive activity of the tropical arrow squid *Doryteuthis plei* around São Sebastião Island (SE Brazil) based on a 10-year fisheries monitoring

Ethogram analysis reveals new body patterning behaviour of the tropical arrow squid *Doryteuthis plei* off the São Paulo coast

Ontogenetic, sexual, and spatial-temporal shifts in the diet of the squid *Doryteuthis plei* off São Sebastião region, SE Brazil

Hunting behaviours in newly-hatched paralarvae of *Doryteuthis plei* in experimental conditions

Table index

Reproductive activity of the tropical arrow squid *Doryteuthis plei* around São Sebastião Island (SE Brazil) based on a 10-year fisheries monitoring.

Ethogram analysis reveals new body patterning behaviour of the tropical arrow squid *Doryteuthis plei* off the São Paulo coast

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À minha família, em especial ao meu pai Eelco, e
amigo e pescador Maurão (in memorian)

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(Acknowledgments, in portuguese)

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Abstract

This PhD thesis addresses several aspects of the biology and ecology of the squid *Doryteuthis plei* in the coast of São Paulo, SE Brazil, at both the population and individual levels. On the population structure of the exploited stock, it includes the patterns of reproductive activity, size-at-maturity, and biometrics of reproductive organs associated with squid size, as well as feeding habits and ontogenetic, sexual, and spatial-temporal shifts. Generalized Additive Models for Location, Scale, and Shape (GAMLSS) were used to identify such population patterns and spatial and temporal factors based on the particular small-scale fishery that targets the species around the São Sebastião Island. At the individual level, the study focused on behavioural traits in two periods of the life-cycle: (1) the reproductive phase, where the body patterns were described, generating a comprehensive ethogram for the species, and (2) the paralarval phase, where the development of swimming and predatory behaviours was detailed. Size-at-maturity was found to be 151-175 mm of mantle length (ML) for females, and 187-190 mm ML for males, that also showed the co-occurrence of two distinct maturation groups (Chapter 1). A significant peak of females gonad weight occurs in February and March especially in the island's South and Southeastern areas, and of males in March. An ethogram analysis showed 19 chromatic, 5 locomotor, and 12 postural components and behavioural sequences for the species, including agonistic, courtship, and mating behaviour (Chapter 2). Light chromatic components (clear and iridophore splotches) showed a longer duration than dark chromatic components, especially those associated with calm behaviour, more frequent in females. Males appeared more dedicated to disputes over resources and used fast miscellaneous visual signalling. In respect to feeding habits (Chapter 3), prey composition differed among sexes, size, maturity, and spatio-temporal pattern. Females do not stop feeding during sexual maturation and the amount of food increases with size. Cephalopods were significant preys for mature females, as well as fish and crustaceans to the largest females, while pelagic polychaetes were dominant prey for largest males. Observations and filming of *D. plei* paralarvae (Chapter 4) show a complex predatory behaviour on live preys, with different hunting types differing in speed and position. Aggressive intra-specific behaviour was found in the absence of prey when paralarvae show both a pursuit strategy and a rapid backward escape after attack. These findings contribute to better understand the population biology and behavior of the species and also in the future definition and guidance of fisheries management plans.

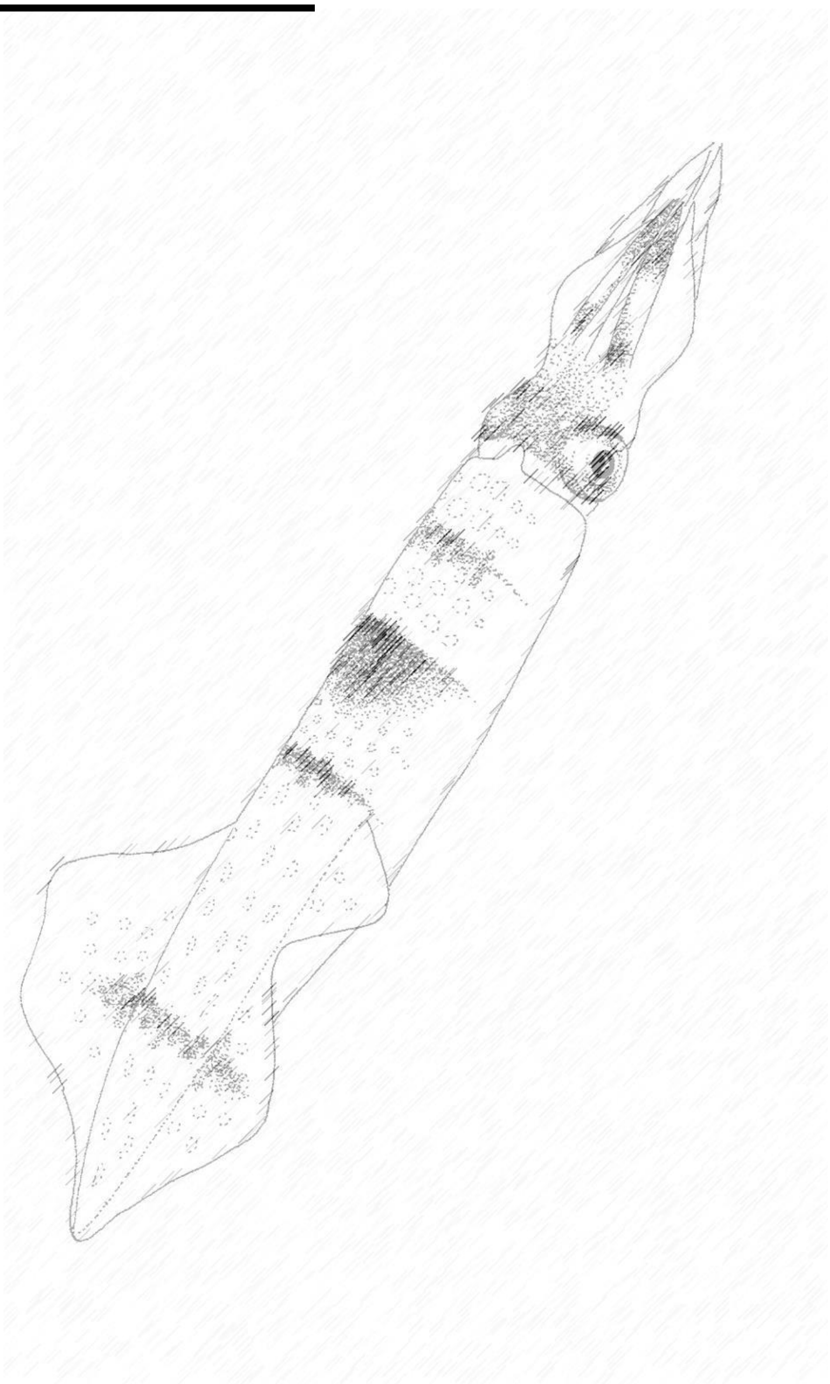
Keywords: Cephalopods, Life-cycle, Chromatophores, Mating, Reproductive gonads, Feeding habits.

Resumo

A presente tese aborda diferentes aspectos da biologia e ecologia da lula *Doryteuthis plei* na costa de São Paulo, SE do Brasil, tanto em nível individual quanto populacional. Com relação à estrutura populacional do estoque explorado, foram analisados padrões da atividade reprodutiva, tamanho de primeira maturação, morfometria dos órgãos reprodutivos associados às fases de desenvolvimento e também hábitos alimentares e mudanças ontogenéticas, sexuais e espaço-temporais. Modelos aditivos generalizados para localização, escala e forma (Generalized Additive Models for Location, Scale, and Shape - GAMLSS) foram utilizados com intuito de identificar padrões populacionais, espaciais e temporais baseados em desembarques da pesca de pequena escala em torno da Ilha de São Sebastiao. Em nível individual, o estudo abordou padrões comportamentais em 2 diferentes períodos do ciclo de vida: (1) fase de reprodução, onde padrões corporais foram descritos, tendo como resultado um abrangente etograma (2) fase paralarval, onde o desenvolvimento de comportamento natatório e predatório foram detalhados. O tamanho de primeira maturação foi estimado entre 151 e 175 mm de comprimento do manto (ML) para as fêmeas e entre 187 e 190 mm ML para os machos, também foi identificada a co-ocorrência de 2 grupos de maturação para os machos. (Capitulo 1). Um pico significativo com relação ao peso das gônadas foi identificado entre os meses de Fevereiro e Março para as fêmeas e em Março para os machos, especialmente nas áreas Sul e Sudeste da ilha. Uma análise dos comportamentos reprodutivos e dos padrões corporais, resultando em um amplo etograma, revelou 19 componentes cromáticos, 5 locomotores e 12 posturais, como também, sequências comportamentais para a espécie, incluindo comportamentos agonístico, de corte e acasalamento (Capitulo 2). Componentes cromáticos claros (manchas claras e irridóforos) apresentaram maior duração do que os componentes cromáticos escuros, particularmente aqueles relacionados ao comportamento calmo, mais frequente nas fêmeas. Machos aparentemente foram mais propensos a disputas por recursos usando sinalizações visuais rápidas e variadas. Em relação aos hábitos alimentares (Capítulo 3), a composição de presas diferiu entre sexos, tamanhos, maturidade e padrões espaço-temporais. As fêmeas não deixaram de se alimentar durante a maturação e foi observada uma relação positiva entre a quantidade de alimentos e o tamanho dos indivíduos. Pequenos cefalópodes, assim como peixes e crustáceos foram às presas mais significativas entre as fêmeas maduras, enquanto que para os machos foram os poliquetas. Através de observações e filmagens de paralarvas de *D. plei* (Capitulo 4), identificou-se um comportamento predatório complexo sobre presas vivas, com diferentes tipos de caça que diferem em velocidade e posição. Comportamentos agressivos entre indivíduos da mesma espécie (estratégias de ataque e fuga) foram identificados quando na ausência de presas. Estes achados contribuem para melhor compreender a biologia populacional, comportamento da espécie, e também na futura definição e orientação nos planos do manejo pesqueiro.

Palavras-chave: Cefalópode, Ciclo de vida, Cromatóforos, Gônadas reprodutivas, Acasalamento, Hábitos alimentares

General introduction



General introduction

Cephalopods (octopuses, squid, cuttlefish and nautiluses) are highly evolved marine molluscs with complex life histories and a varied range of habitat preferences (Robin et al., 2014). They are monocyclic, single-season breeders, and may produce their eggs in successive batches within one season. They have short lives 1–3 years in most cases, with some exceptional cold-water or large specimens living longer. The squids group (order Teuthida) can be divided into suborders Ogeopsina and Myopsida, together coating 28 families. The myopsid squid occur only on continental shelves and comprises two families: the commercially important Loliginidae and the recently described Australiteuthidae (Boyle and Rodhouse, 2005; Lu, 2005, Hoving, 2008). Squids throughout the lifespan undertake a succession of brief transitions from one stage to the next. In particular, squid's species release their eggs on the seabed, mainly sand and gravel bottoms. Embryogenesis may be relatively quick in some species, ranging from 5–10 days for small, unprotected eggs, to 50–100 days for large yolky-egged species. The paralarvae born with planktonic lifestyle and reach juvenile phase quickly. When they reach the adult stage, squids show a wide reproductive strategy (Boyle and Rodhouse, 2005; Vidal et al., 2014; Robin et al., 2014, Rodhouse et al., 2014).

The loliginid squid *Doryteuthis plei* (Blainville, 1823) inhabits coastal waters (from the sea surface to 370 meters depth) of the western Atlantic Ocean, from Cape Hatteras, USA (36ºN) to Northern Argentina (35ºS), being more abundant in shallow surface waters during the night and in the water column during the day (Jereb and Roper, 2010). In Brazil, the species is commonly found as an important fishery resource off the Southern and Southeastern coasts (Perez et al., 2002; Gasalla et al., 2005; Rodrigues and Gasalla, 2008; Postuma and Gasalla, 2010; Rodhouse, et al., 2014) being associated with the Brazil Current (Haimovici and Perez, 1991) and with South Atlantic Central Waters (SACW) bottom intrusions (Martins et al., 2006). In the Northern coast of the state of São Paulo, and particularly around the São Sebastião Island (24ºS), they tend to concentrate in the summer season from February to April (Postuma and Gasalla, 2010) when a small-scale fisheries targets the stock. Such fisheries operates under a wide range of environmental conditions, but especially in warm sea surface temperature, low wind and translucent waters conditions, and just after the summer rains (Postuma and Gasalla, 2010).

This PhD thesis is a result of my own original study on several aspects of the biology and ecology of the loliginid squid *D. plei* at both the population and the individual level. It was based on data collected across 10 years monitoring the small-scale fishing around the São Sebastião Island (2002-2012) and also during laboratorial observations (2011-2015) at the northern coast of São Paulo, Brazil. On the population structure of the exploited stock, it includes the patterns of reproductive activity, size-at-maturity, and biometrics of reproductive organs associated with squid size, as well as feeding habits and ontogenetic, sexual, and spatial-temporal shifts. At the individual level, the study focused on behavioural traits in two periods of the life-cycle: (1) the reproductive phase, where the body patterns were described, generating a comprehensive ethogram for the species, and (2) the paralarval phase, where the development of swimming and predatory behaviours was detailed. Overall, the data collection included: (1) sampling biological material from the fisheries in order to monitor the population biology of the exploited stock, (2) field work collecting and maintaining live squids in captivity and aquaria, and (3) observations and filming on the body patterns, reproductive, and feeding behaviour of adult specimens, as well as the swimming and hunting of their early life-stage.

The first Chapter will approach the structure of the exploited population of *D. plei* around the São Sebastião Island (SSI), with an emphasis on the reproductive activity, *i.e.* relevant aspects for the fisheries management perspectives. By means of monthly biological samples (n=139) from the small-scale hand-jigging fishery, the aim of this chapter is to identify the reproductive patterns in the region and the characteristics of the exploited population strata, including the use of non-linear statistical models. In this Chapter the hypothesis was tested that SSI is an important spawning ground for *D. plei* showing a peak reproductive activity in terms gonadal development during the warm summer months. This chapter was published in the international journal '*Fisheries Research' (2014), vol* 152, p *45-54.*

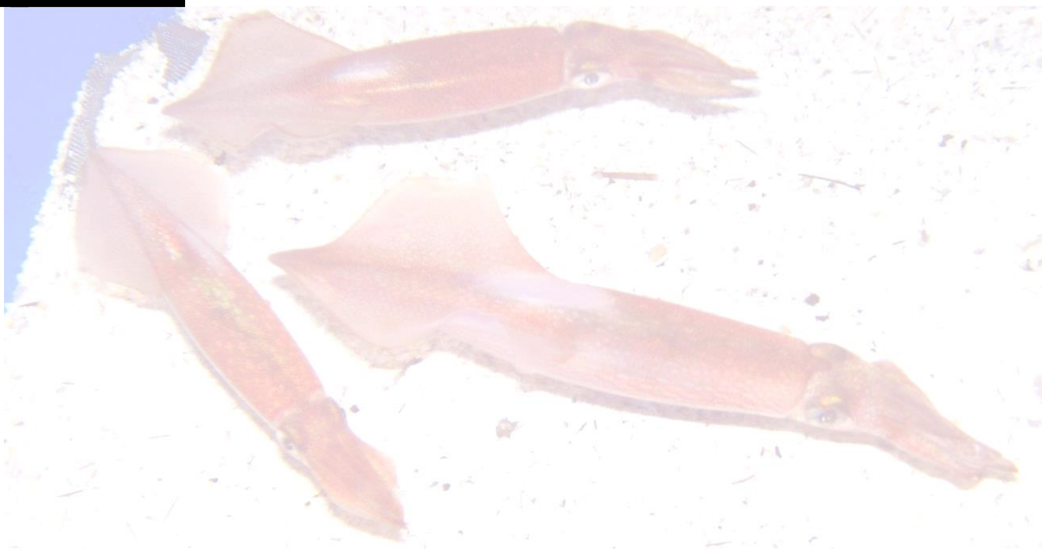
In Chapter 2, some behavioural aspects of reproduction will be covered. Body patterns of *D. plei* including many combinations of skin coloration, body postures, swimming movements and reproductive behaviour will be presented. The behavioural and body patterns have rarely been described in Brazilian waters for squids. To test the hypothesis that distinct chromatic components appear at different durations on the skin of the tropical arrow squid in the Southern Hemisphere, were identified and described its body patterning behavior. The major aim of the chapter was to describe the components and the body patterns found in laboratorial conditions at the northern coast of São Paulo, and a new ethogram of the signals especially related to reproductive behaviour. This chapter was accepted for publication on 7th May 2015 in the Woods Hole journal "*The Biological Bulletin".*

In Chapter 3, new aspects of the species feeding habits, including differences in the diet of males and females will be described. Prey composition variations among sexes, size, maturity and spatio-temporal patterns have not been detailed to any great degree for *D. plei* near the coast of the São Paulo shelf. To test the hypothesis that feeding habits are different regarding to ontogenetic, sexual, and spatial-temporal shifts. The aim of this chapter was to identify particular aspects of the feeding habits, such as ontogenetic, sexual, and spatial-temporal shifts for *D. plei* in shallow waters around São Sebastião Island.

The last chapter (four) will present behavioural aspects of the early stage of the species life-cycle. Based on the footage of newly-hatched paralarvae, the swimming and feeding behaviour will be described. The knowledge about the behaviour of *D. plei* early-stages is almost completely unknown in South Brazil, possibly because of some difficulties in collecting egg mops and maintaining live specimens under laboratorial conditions. To test the hypothesis that the paralarvae born with innate abilities, such as hunt and swimming strategies, including small crustaceans in their diet, the aim of this chapter was to examine the swimming speed, the early development of the hunting behaviours and the movements of the fins, arms, siphon, and mantle of *D. plei* paralarvae in first day of life.

Finally, the following sections will address particular scientific issues on the biology and ecology of the squid *Doryteuthis plei* in inshore waters of São Paulo, SE Brazil that will help to better understand the population biology and behavioural aspects and may also be useful in the definition of future regional fisheries management plans.

Chapter 1



Reproductive activity of the tropical arrow squid *Doryteuthis plei* around São Sebastião Island (SE Brazil) based on a 10-year fisheries monitoring

Chapter 1 - Reproductive activity of the tropical arrow squid *Doryteuthis plei* around São Sebastião Island (SE Brazil) based on a 10-year fisheries monitoring.

This chapter was published in the international journal '*Fisheries Research 152' (2014) 45-54.* doi:10.1016/j.fishres.2013.12.011

Abstract: Monthly samples of *Doryteuthis plei* were obtained from the small-scale hand-jigging fishery around São Sebastião Island (24ºS), Brazil, across seven fishing seasons (November to April) during the period 2002-2012, with the aim of identifying the exploited population patterns with emphasis on the reproductive activity. In order to explore the process of development of reproductive organs, the size-at-maturity, and the spatial-temporal factors explaining maturation, an analysis of the sex-ratio, maturity, gonado-somatic index (GSI), concentration of spermatophores, biometric relationships, and two GAMLSS (Generalized Additive Model for Location Scale and Shape) was applied. *D. plei* recruited to local fisheries at around 161 mm mantle length (ML), males being, on average, 1.6 times larger than females. Females appeared more frequently during the first three months of the fishing season while males tended to be more frequent at the end of the season, confirming the existence of female post-spawning mortality. The presence of high proportions of mature squid was found throughout the fishing seasons for both sexes. The size-at-maturity was estimated at 143.3mm ML in females, and 210 mm ML, in males. Both statistical models indicated robust non-linear correlation between gonad weight, biometric variables, and spatial-temporal factors ($p<0.001$). Ovary weight mostly correlated with accessory glands weight, while testis weight correlated with ML. In both sexes, depth was found to be a significant variable explaining maturation, with higher gonads weight between 15m and 20m, and especially in the island's South and Southeastern areas. Statistical models also evidenced a significant peak of gonad weight in February and March, for females, and in March, for males. Inter-annual variation showed a peak of gonad weight in the fishing season 2011-2012. Overall, findings based on long-term monitoring should assist resource management (*e.g.* for seasonal, size, and spawning area protection) which seems potentially valuable for long-term sustainability of local fishing communities.

Keywords: GAMLSS, small-scale fisheries, marine fisheries, life cycle, Cephalopoda

1. Introduction

The loliginid squid *Doryteuthis plei* (Blainville, 1823) is a semelparous species, *i.e*. it breeds only once in a lifetime (Boyle and Rodhouse, 2005). The species comprises an important fishery resource off the southern and southeastern coast of Brazil (Perez et al., 2002; Gasalla et al., 2005; Perez et al., 2005; Rodrigues and Gasalla, 2008; Postuma and Gasalla, 2010; Gasalla et al., 2010). Its distribution ranges from the coast of Florida, USA (Hixon et al., 1980) to Rio Grande do Sul, Brazil (Perez et al., 2005), and in the southernmost portion its presence has been associated with the Brazil Current (Haimovici and Perez, 1991) and with South Atlantic Central Waters (SACW) summer bottom intrusions (Martins et al., 2006).

In the South Brazil Bight (SBB) area (*sensu* Matsuura, 1989), the species is more abundant during the summer, with males often larger than females showing a polymodal size structure, in contrast to females that show unimodal size structure. *D. plei* appears to spawn throughout the year, more intensely in summer, when the fisheries seem to take advantage of the spawning concentrations (Perez et al., 2002; Rodrigues and Gasalla, 2008). In this regard, both artisanal (hand-jigging) and industrial (shrimp-trawling) fisheries have exploited the stock in the whole area (Perez, 2002; Gasalla et al., 2005; Postuma and Gasalla, 2010). Some differences on spatial patterns in size-at-maturity in respect to season, depth ranges, and inshore-offshore migration have been found along the region, converging that summer coastal spawning squid tend to mature at larger sizes than their offshore counterparts in winter (Perez et al., 2002; Rodrigues and Gasalla, 2008).

Overall, *D. plei* has been more studied on the shelf region (40-100 meters deep) of the SBB while little is known on their reproductive activity in coastal waters (< 30 m), where the small-scale fisheries occurs. There are two main islands within the bight, where squid hand-jigging has been reported: the Santa Catarina Island, at latitude 27.5°S (Martins and Perez, 2007), and the São Sebastião Island, at 24°S (Postuma and Gasalla, 2010). In the São Sebastião Island (SSI), squids have been caught especially in sheltered bays ~ 20 m deep and during both daylight hours and at night. The catch per unit effort (CPUE) tends to peak in the period from February to April, and the fisheries seems to operate under a wide range of environmental conditions, but especially in settings of warm sea surface temperature, low wind and translucent waters, and just after summer rains (Postuma and Gasalla, 2010).

Previous studies on the reproductive activity of *D. plei* in Brazil have described spatial and seasonal patterns of maturity by using the gonado-somatic index (GSI) (Perez et al., 2002; Rodrigues and Gasalla, 2008), as has been occurring for other loliginids around the world (*e.g., Loligo reynaudii* in South Africa, Olyott et al., 2006; *Loligo forbesi* in European waters, Porteiro and Martins,1994; Guerra and Rocha, 1994; Rocha and Guerra, 1999, Pierce et al., 1994; Coelho et al., 1994; Pham et al., 2009; and *Doryteuthis sibogae* off the southeast coast of India, Neethiselvan et al., 2001). However, more recently, some generalized additive models (GAMs), based on the gonad weight, have been proposed to better understand the reproductive activity of certain European loliginid's stocks (*e.g.* Smith et al., 2005; Sanchez and Demestre, 2010), showing that more robust fits to data can be obtained to explain the main factors affecting reproduction, as one of the main advantages of the method.

Within this context, the main objective of the present study is to identify the exploited population patterns of *D. plei* around São Sebastião Island with emphasis on the reproductive activity monitored during a 10-year period, including the use of non-linear statistical models. Specifically, it is intended to explore the process of development of males and female reproductive organs, the main biometric variables that better translate the reproductive activity, the size at maturity, and the spatial and temporal factors that influence the reproduction of the species locally.

2. Materials and Methods

2.1. Study area

São Sebastião Island (SSI) is located between 23º70 and 24º00S and 45º50 and 45º00W in the South Brazil Bight coast (Figure 1.1). Small-scale hand-jigging fishery operates at a depth of between 5 and 30 m depth, mainly close to the shore and in the island's sheltered bays, but also close to smaller islands such as Búzios and Vitória (Figure 1.1). The structure and dynamics of water masses occurring around SSI seem to be typical of the adjacent continental shelf, with a predominance of Coastal Waters

(CW) (Pires-Vanin, 2008). Summer wind-driven bottom intrusions of the nutrient-rich South Atlantic Central Waters (SACW) seem to be the most relevant physical process for the ecosystem, promoting stratification of the water column and enhancing biological productivity (Pires-Vanin, 2008).

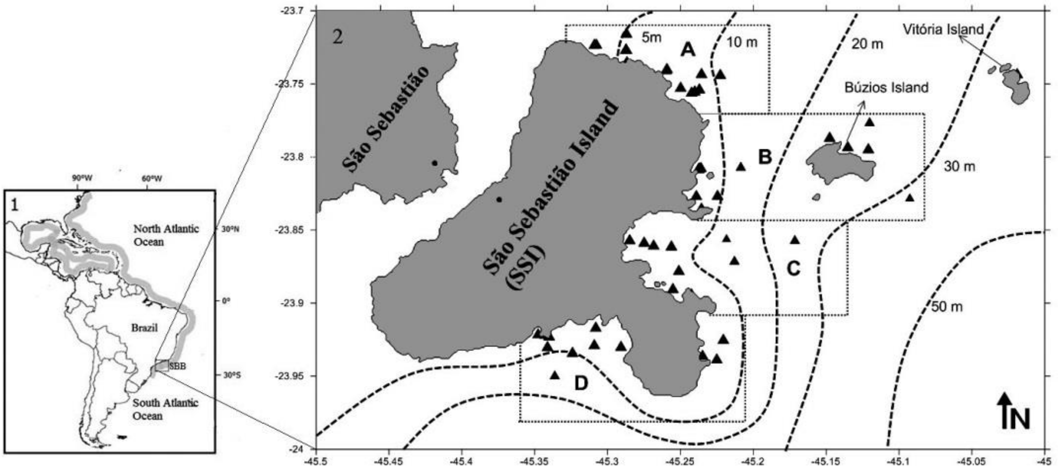


Figure 1.1: Geographic distribution of the tropical arrow squid *Doryteuthis plei* (1) in the Western Atlantic Ocean (shadowed area) and (2) in detail shows the area of operation of the small-scale squid hand-jigging fishery around São Sebastião Island (SSI) in the South Brazil Bight (SBB), the triangles representing the fishing spots in Latitude and Longitude. Depth ranges at 5, 10, 15, 20 and 30 in meters (m) and the four fishing areas: A) North, B) Northeast, C) Southeast and D) South.

2.2.Squid sampling

During a 10-year period, samples were collected weekly from the landings of hand-jigging fishery operating around SSI, across seven fishing seasons (2002-03, 2003-04, 2004-05, 2006-07, 2008-09, 2010-2011 and 2011-2012) between November and April. All squid were caught at depths of less than 30m. A total of 182 landings in the municipalities of São Sebastião (92%) and Ilhabela (8%) were monitored, from which 139 biological samples were collected, totalling 200 kg of squid. Information collected from the fishers included: fishing area (Fa), fishing depth (Fd) and fishing date, which allowed the identification of both fishing month (Fm) and fishing season (Fs).

2.3.Processing of samples

A total of 2 351 specimens of *D. plei* caught by the small-scale hand-jigging fishery were sampled. The dorsal mantle length (ML) in mm and total body weight (BW) in g were recorded for each squid with an ichthyometer and a precision scale respectively. Squid dissection was conducted by cutting longitudinally the ventral size of the mantle to expose of the mantle cavity and visceral mass. The squid were sexed and the sexual maturity stage (I-immature, II-in maturation, III-mature and IV-spent) were determined macroscopically according to Juanicó (1983) and modified by Perez et al., (2002). Spermatophore concentration in the male Needham's sac was scored as (i) empty, (ii) few, (iii) many. Females were assessed for the presence (p) or absence (a) of spermatophores in the buccal receptacle (Roper, 1965; Porteiro and Martins, 1994). Table 1.1 summarizes the total sample and subsamples information during the fishing seasons that were investigated.

Table 1.1: Number of specimens in the total sample and subsamples, for both sexes, obtained from fisheries landings across seven fishing seasons during the period 2002-2012.

	Females		Males	
Fishing season	Total Sample	Sub-sample	Total Sample	Sub-sample
2002-2003	148	0	119	0
2003-2004	143	0	170	0
2004-2005	188	28	117	28
2006-2007	206	44	113	70
2008-2009	182	141	149	149
2010-2011	211	141	205	164
2011-2012	211	182	189	101
Total	1289	536	1062	512

2.4.Reproductive structures

The following data were collected from reproductive organs: nidamental gland length (NiGL), oviducal gland length (OvGL), and accessory glands weight (nidamental + oviducal glands) (AGW), ovary (and proximal oviduct) weight (OW) of females;

testis length (TL) and weight (TW), and spermatophoric complex weight (SW) (spermatophoric organ + Needham's sac) of males. The weight of the reproductive organs was measured to an accuracy of 0.001 g and their length to the nearest 0.1 mm (Portero and Martins, 1994, Perez et al., 2002; Rodrigues and Gasalla, 2008).

Based on previous CPUE analysis of the local fisheries (Postuma and Gasalla, 2003), the study area was divided in five depth strata (5 m, 10 m, 15 m, 20 m and 30 m) and four fishing areas: A) North, B) Northeast, C) Southeast and D) South (Figure 1.1). The samples were grouped monthly and into the six months that comprise one fishing season (November to April) (Fs). A chi-square non-parametric test was used to determine significant differences between the sex-ratio among depth strata, fishing areas, and months; the null hypothesis (Ho) being the sex ratio 1:1, and the alternative hypothesis (Ha) the ratio being different from the parity.

ML-BW relationships were fitted using the power model ($ML = aBW^b$). The ML and BW were log-transformed and subsequently tested with covariance analysis ANCOVA to investigate the effect of the sex and maturity stages on the exponent “b” of the ML-BW relationship, where log BW was the dependent variable, sex and maturity stages, the factors of ANCOVA, and log ML, the covariable exponent (Zar, 1996). Squids at stages III and IV were pooled in the analysis because of the low frequency of spent squid in the samples. Initially, the gonado-somatic index (GSI), being defined as GSI = 100 × (OW + AGW)/BW for females, and GSI = 100 × (TW + SW)/BW, in males, was used to observe spatial and temporal variations on reproductive activity. Both GSIs for males and females were tested for normality using a Shapiro–Wilk test. Because data were found to violate the criteria for normality, the non-parametric Kruskal-Wallis test (Hollander and Wolfe, 1973; Zar, 1996) was applied to test the influence of fisheries-related factors (*i.e.* area, month, and depth) on GSI. A post-hoc pairwise comparison test was applied to determine which factor was significantly different from the average (Siegel and Castellan, 1988). All statistical tests were considered at a 0.05 level of significance.

The size at maturity (ML50) was estimated by fitting a sigmoid model P = 1/ (1+ exp (a + b*ML)), where variable P is the relative frequency of mature individuals at each ML class, a and b are parameters, and ML is the mantle length of mature squid. ML50 was estimated as being a/b of the fitted model.

Generalized Additive Models

GAMLSS were implemented using a series of packages in R (R Development Core Team, 2013) downloaded from http//www.gamlss.org. The GAMLSS procedure was used with a cubic spline smoothing function (cs) (Stasinopoulos and Rigby, 2007). Models were applied to analyze the correlations between body measurements and reproductive structures and with the spatial and temporal factors of the small-scale fisheries. Thus, two models were analyzed separately: the first one considering reproductive and biometric variables, and the second exploring the factors that better explain maturation expressed by gonad weight in terms of fisheries-related variables such as depth, area, month, and fishing season. The data were analyzed separately for males and females. The first model was defined: OW ~ ML + BW + AGW + OvGL + NiGL+ Mat, for females, where Mat is the factor maturity stages, and TW ~ ML + BW + SW + TL+ Mat, for males.

Moreover, the second model was used to determine the level of significance of the response variables (OW) and (TW) with the spatial (fishing area [Fa] and fishing depth [Fd]) and temporal (fishing month [Fm] and fishing season [Fs]) factors of the fisheries variability. Thus, the model used was: (OW/TW)~Fd + Fa + Fm + Fs.

For both models, a weibull’s probability distribution was selected for the examination of the response variable for males, and a normal probability distribution for females. In both cases, a logarithmic function was used to link the mean distribution of the variable with the linear model. The variance inflator factor (VIF) was used to test co-linearity between variables in the GAMLSS (Montgomery and Peck, 1992). VIF values greater than 3 indicated collinearity, following the recommendation made by Zuur et al., (2010).

The best fitted models were selected based on the Akaike Information Criterion (AIC), Schwarz's Bayesian criterion (SBC) and Global Deviation (GD). The significance of each term was assessed using the “drop1” function, and their relative importance assessed accordingly to the AIC, likelihood-ratio test (LRT) and probability of the Chi-squared test criteria (PrChi) obtained (Stasinopoulos and Rigby, 2007).

3. Results

3.1. Population structure of squid *D. plei* caught by the São Sebastião Island small-scale fishery

From the total of 2 351 *D. plei* specimens sampled, 1 289 were females and 1062 males. Females were more frequent than males at depths ranging from 5 m to 10 m, in the South and Southeast areas, and mainly during the first three months of the fishing season (November, December, and January) where statistical difference was found significant (Table 1.2). The mean ML for females was 136.86mm ML (+ /- 34.17 s.d.; range 30 mm 231 mm), while in males it was 198.05 mm ML (+ /- 62.46 s. d.; range 49 mm 390 mm). Females showed a unimodal distribution peaking at 140 mm ML, while males showed a multi-modal distribution (modes at 190 mm and 230 mm). Regarding the distribution of BW in specimens of *D. plei*, females tended to peak around 50 g, reaching a maximum of 162 g, while males peaked around 90 g, reaching a maximum of 297 g (Figure 1.2).

The relationship between the ML/BW was obtained by non-linear fits (power function), as being $BW=2\text{x}10^{-2}ML2.06$ ($r^2=0.87$; n=1283) and $BW=4.9\text{x}10^{-2}ML1.86$ ($r^2=0.86$; n=1056), respectively for females and males. The exponent "b" values were significantly higher in females than in males (Figure 1.3), and in immature and in-maturation individuals of both sexes, suggesting somatic growth during these stages. The lower value in mature and spent (stages III and IV) suggests reproductive growth (Table 1.3 and 1.4).

Table 1.2: Statistical analysis (X^2) chi-square of differences between the sex ratio, depth, month and area. Differences between values were significant $X^2 > 3.48$ with 95% confidence ($p < 0.05$).

Variables	Factors	Females	Males	X^2	*P value*
Depth (in meters)	5	294	178	28.5	<0.05
	10	593	511	6.09	<0.05
	15	242	231	0.25	0.61
	20	98	74	3.34	0.06
	30	8	5	0.69	0.40
Fishing month	November	355	193	47.90	<0.05
	December	121	102	1.61	<0.05
	January	273	210	8.21	<0.05
	February	297	276	0.76	0.38
	March	135	158	1.80	0.17
	April	96	100	0.88	0.77
Fishing area	North	496	428	5.00	0.02
	Northeast	142	148	0.12	0.72
	Southeast	299	228	9.56	<0.05
	South	352	258	14.50	<0.05
	Total	1289	1062		

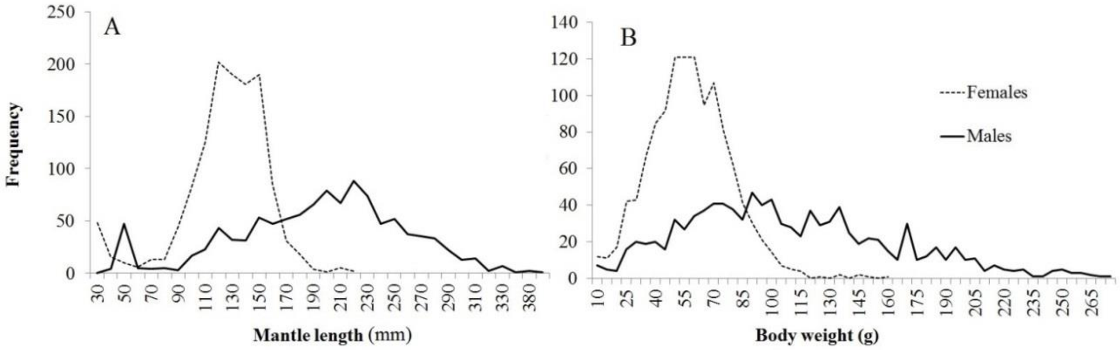


Figure 1.2: The frequency of individuals A) per mantle length class (in mm) and B) body weight (in g) for males and females of *Doryteuthis plei* caught by the hand-jigging fishery around São Sebastião Island between 2002 and 2012.

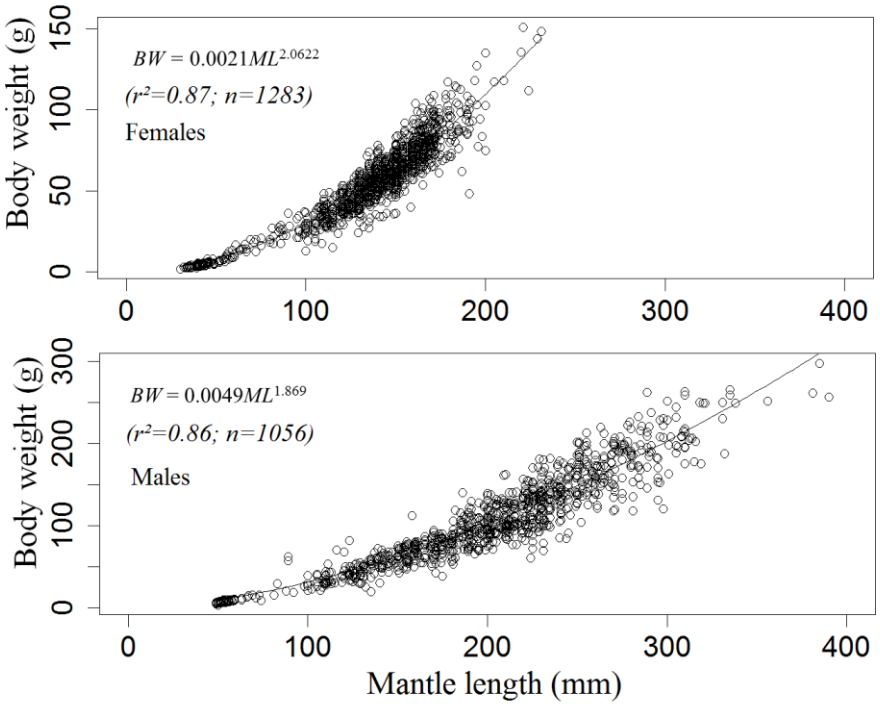


Figure 1.3: ML/weight relationships for A) females and B) males of *Doryteuthis plei* caught by the small-scale squid hand-jigging fishery around São Sebastião Island (SSI) (24ºS) during the period November 2002 – April 2012.

Table 1.3: Coefficients of the model fitted to the mantle length–weight relationship, BW=aML^b, of males and females of *Doryteuthis plei* for maturity and sex.

	Females			Males		
Maturity scale	*a*	*b*	*r*	*a*	*b*	*r*
(I) immature	9×10^{-3}	2.27	0.95	1.6×10^{-2}	2.06	0.95
(II) in maturation	5.4×10^{-2}	1.85	0.85	2.3×10^{-2}	2.02	0.96
(III) matures + (IV) spent	4.9×10^{-2}	1.88	0.86	5.1×10^{-2}	1.86	0.84

Table 1.4: Results of the covariance analysis ANCOVA to test the effect of sex and maturity (factors) on body weight (dependent variable) of female and male *Doryteuthis plei*, using ML as a covariate *d.f.:* degrees of freedom; MS: mean squares. All variables were log transformed. The effect of the interaction between the factors and the covariate.

Factors	*df*	MS	*F*	*p*
log ML	1	234.55	37123.50	<0.001
Sex	1	0.451	71.41	<0.001
Sex × log ML	1	0.562	88.96	<0.001
Males				
log ML	1	75.191	8996.171	<0.001
Scale maturity	2	0.091	10.945	<0.001
Scale maturity × log ML	2	0.092	11.057	<0.001
Females				
log ML	1	32.443	5636.569	<0.001
Scale maturity	2	0.135	23.502	<0.001
Scale maturity × log ML	2	0.117	20.416	<0.001

3.2.Reproductive activity.

3.2.1. Females

Approximately 69% of the females were mature (stages III and IV) with a peak of maturity observed in February (Table 1.5). Both proportions of mature females and females with the presence of spermatophores in the buccal receptacle were present in all samples with the highest frequencies found at 10 m depth and in the fishing area Northeast (Table 1.5). The ML50 of squid caught by the local squid fishery was estimated at 143.3 mm (Figure 1.4). The sum of the ovary weight + oviducal and nidamental glands represented a mean of 12.23% of total body weight with a range between 3.71 to 34.52%. The mean GSI was significantly different among areas (Kruskal-Wallis test, $p<0.05$). The pairwise comparison test showed that the mean GSI were significantly higher in the south and southeast areas (Table 1.5) in relation to the others ($p <0.05$).

In the first GAMLSS model for females, the residuals appear random, although the normal Q-Q plot shows a possible single outlier in the upper tail and a slightly longer extreme lower tail than the Box–Cox t-distribution. However, all GAMLSS models showed a good fit to the data.

Table 1.5: Mean of gonado-somatic index (GSI), percentage of mature individuals (% Mat), presence of spermatophores in the buccal membrane in females and concentration of spermatophore in Needham's sac in males (% Sperm) of *Doryteuthis plei* caught by the small-scale squid hand-jigging fishery around São Sebastião Island (SSI) during the period November 2002 – April 2012.

		Females			Males		
Variables	Factors	Mean GSI	% Mat.	% Sperm.	Mean GSI	% Mat.	% Sperm.
	5	12.36	25.61	24.65	1.59	17.81	17.80
	10	11.86	41.09	42.09	1.65	45.55	46.13
Depth in (m)	15	12.45	20.71	20.69	1.62	23.63	22.77
	20	13.13	8.13	7.40	1.86	7.08	7.51
	30	11.92	4.45	5.17	1.93	5.94	5.20
	November	12.53	27.56	24.16	1.72	12.79	13.76
	December	12.97	13.92	8.73	1.85	10.27	10.17
Fishing month	January	11.77	26.99	22.94	1.80	21.12	20.12
	February	11.63	31.96	24.97	1.74	26.48	26.47
	Mach	12.35	16.05	11.17	1.41*	17.24	17.23
	April	12.98	11.08	8.02	1.58	12.10	12.25
	North	11.67	11.58	12.58	1.71	15.07	15.14
Fishing area	Northeast	8.93	33.74	31.74	1.51	37.21	36.99
	Southeast	12.82*	26.50	25.25	1.73*	25.00	25.09
	South	13.34*	28.17	30.43	1.89*	22.72	22.77

*significant difference ($p < 0{,}05$), Kruskal-Wallis test.

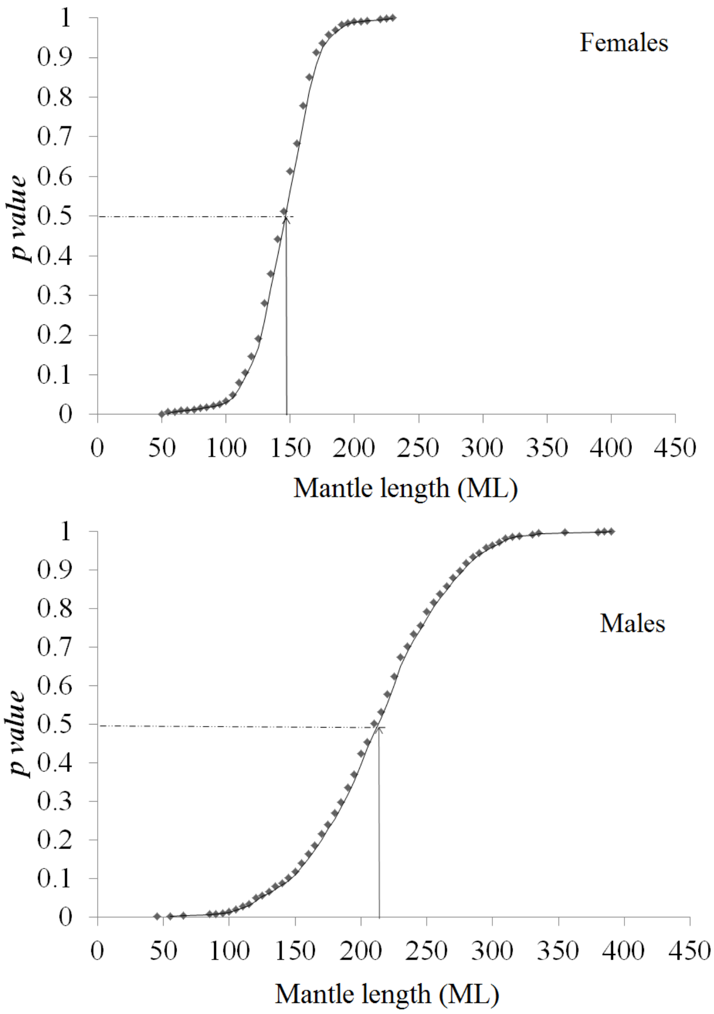


Figure1.4 : Cumulative percentage of mature (stages III and IV) female (A) and male (B) of *Doryteuthis plei* per ML category (solid line). ML of 50% of mature squid is indicated.

Considering the biometric relationships, Table 1.6 shows the best-fitted GAMLSS model (AIC=1738.295; SBC= 1853.975; GD=1684.294) indicating that ML was the most significant explanatory variable according to the AIC criterion. A partial plot of such model showed a positive relationship with OW from 131 mm ML onward, peaking at 151mm ML, with a slight decrease from 175 mm on (Figure 1.5A). During maturation, the accessory gland weight (AGW) showed a high correlation with OW, increasing monotonically (LRT and Chi-squared test in Table 1.6 and Figure 1.5B). The NiGL also correlated positively to OW, increasing monotonically, with an inflection point at 31.5 mm (Figure 1.5C). The OW increases significantly with maturity (Mat), showing a negative relationship with immature and in maturation squid, peaking in mature squids and decreasing slightly in spent (Figure 1.5D).

Table 1.6: Summary of optimal GAMLSS models fitted to the ovary weight (OW) where explanatory variables are mantle length (ML), body weight (BW), accessory glands weight (AGW), oviducal gland length (OvGL), maturity (Mat) and nidamental gland length (NiGL) in females, while in males, testis weight (TW) and explanatory variables are spermatophoric complex weight (SW) and testis length (TL) from *Doryteuthis plei* caught by the small-scale squid hand-jigging fishery around São Sebastião Island (SSI) during the period November 2004 – April 2012. (AIC = Akaike Information Criterion, LRT = Likelihood-ratio test, Pr(Chi) = probability of Chi squared test, and cs() = cubic spline).

Selected model (females): *cs(OW) ~ cs(ML) + cs(BW) + cs(NiGL) + cs(AGW) + Mat*

	Variables	Estimate	Std. Error	t value	Pr(>|t|)	AIC	LRT	Pr(Chi)
	(Intercept)	-0.108	0.146	-0.739	$4.602e^{-01}$			
	ML (df = 4)	0.001	0.001	-0.334	$7.385e^{-01}$	1711.67	5.25	0.386
	BW (df = 4)	0.007	0.001	5.084	$5.219e^{-07}$	1734.55	28.14	$3.421e^{-05}$
	NiGL (df = 4)	0.018	0.004	4.946	$1.032e^{-06}$	1733.72	27.30	$4.98e^{-05}$
	AGW (df = 6)	0.158	0.012	13.413	$2.635e^{-35}$	1836.85	134.44	$7.431e^{-26}$
	Factors							
Mat	in maturation	0.176	0.100	1.756	$7.973e^{-02}$	1743.71	33.30	$2.778e^{-07}$
	mature	0.340	0.098	3.448	$6.117e^{-04}$			
	spent	0.111	0.125	0.885	$3.764e^{-01}$			
Selected model (males): cs(*TW*) ~*cs(ML) + cs(SW) + cs(TL) + Mat*								
	(Intercept)	-2.061	0.104	-19.77	$1.99e^{-64}$			
	ML (df = 4)	0.003	0.000	6.914	$1.47e^{-11}$	456.37	66.46	$7.646e^{-12}$
	SW (df = 6)	0.171	0.045	3.762	$1.89e^{-04}$	416.71	22.79	0.0003
	TL (df = 4)	0.016	0.002	7.697	$7.73e^{-14}$	441.26	47.35	$4.816e^{-09}$
	Factors							
Mat	in maturation	0.291	0.111	2.63	$8.80e^{-03}$	417.47	19.55	0.0002
	mature	0.506	0.105	4.839	$1.75e^{-06}$			
	spent	0.481	0.131	3.685	$2.54e^{-04}$			

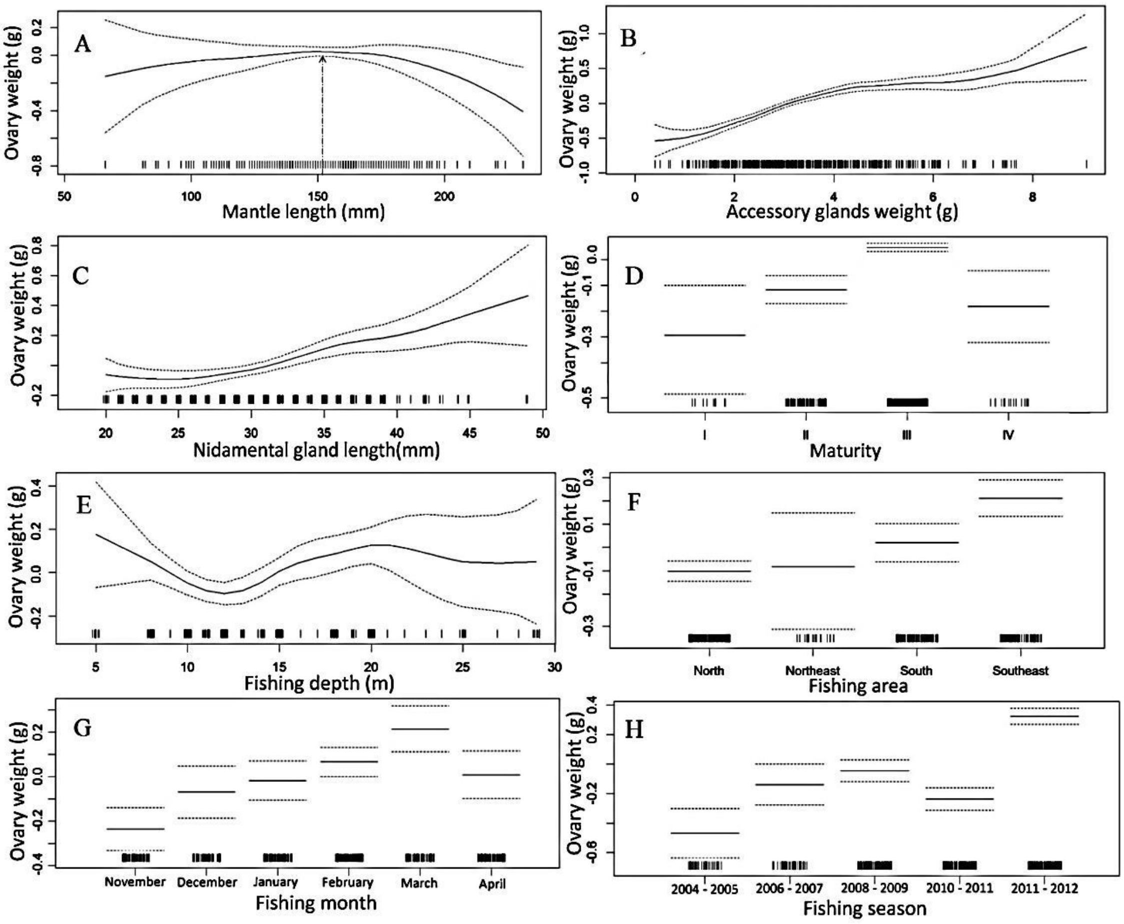


Figure 1.5: GAMLSS smoothing curves fitted to partial effects of explanatory variables on reproductive and temporal variables, in females of *Doryteuthis plei*. OW is represented as a function of (A) ML, (B) AGW, (C) NiGL, (D) Mat, (E) Fd, (F) Fa, (G) Fm and (H) Fs. Dashed lines represent 95% confidence intervals around the main effects. The arrow in 5A shows the correlation peaks in relation to the ML.

In the second GAMLSS model (AIC=2120.389,SBC=2201.791,GD=2088.388), fishing depth (Fd) was the most important variable explaining the maturity in females (Table 1.7).The partial plot showed a positive correlation with OW between 15 m and 23 m, with a peak at depth of 20 m (Figure 1.5E), and in the Southeast fishing area (Figure 1.5F). The OW was positively correlated to the months February and March, while was negatively in other months (Figure 1.5G). The inter-annual variation of OW also proved to be significant (LRT in Table 1.7), with positive correlation in the fishing season 2011 – 2012 (Figure 1.5H).

Table 1.7: Summary of optimal GAMLSS models fitted to the weight of the ovary weight (OW) and testis weights (TW) of the *Doryteuthis plei*, and the explanatory variable with the spatial (fishing area [Fa], fishing depth [Fd], temporal (fishing months [Fm] and the fishing season [Fs] of the small-scale squid hand-jigging fishery around São Sebastião Island (SSI) during the period November 2004 – April 2012, where AIC = Akaike Information Criterion, LRT = Likelihood-ratio test, Pr(Chi) = probability of Chi squared test, and cs( ) = cubic spline

	Selected model (females): ***OW ~ cs(Fd) + Fa + Fm + Fs***							
Variables	**Factors**	Estimate	Std. Error	t value	Pr(>|t|)	AIC	LRT	Pr(Chi)
	(Intercept)	0.449	0.125	8.421	$3.44e^{-04}$			
Fd	Fd (df = 4)	0.009	0.094	1.928	$5.44e^{-02}$	2125.67	15.28	0.009
Fa	Northeast	0.020	0.120	0.169	$8.66e^{-01}$	2142.33	27.94	3.73^{e-06}
	South	0.790	0.057	2.153	$3.71e^{-16}$			
	Southeast	0.314	0.056	5.615	$3.21e^{-08}$			
Fm	December	0.166	0.079	2.090	$3.71e^{-02}$	2143.06	32.67	4.37^{e-06}
	January	0.217	0.071	3.053	$2.38e^{-03}$			
	February	0.301	0.068	4.452	$1.04e^{-05}$			
	March	0.449	0.076	5.873	$7.67e^{-09}$			
	April	0.243	0.080	3.049	$2.42e^{-03}$			
Fs	2006 - 2007	0.328	0.112	2.939	$3.44e^{-03}$	2236.51	124.12	7.02^{e-26}
	2008 - 2009	0.423	0.098	4.326	$1.83e^{-05}$			
	2011 - 2012	0.231	0.093	2.470	$1.38e^{-02}$			
	2010 - 2011	0.122	0.004	3.604	$3.18e^{-02}$			
	Selected model (males): ***TW ~ cs(Fd) + Fa + Fm + Fs***							
	(Intercept)	-0.638	0.125	-5.103	$4.78e^{-07}$			
Fd	Fd (df = 4)	0.004	0.005	0.740	$4.60e^{-01}$	651.91	7.90	0.094
Fa	Northeast	0.179	0.078	2.295	$2.22e^{-02}$	655.47	9.44	0.023
	South	0.081	0.055	1.469	$1.42e^{-01}$			
	Southeast	0.136	0.060	8.413	$4.34e^{-16}$			
Fm	December	-0.001	0.088	-0.011	$9.92e^{-01}$	676.17	34.15	2.22^{e-06}
	January	0.787	0.094	0.608	$5.44e^{-01}$			
	February	-0.001	0.080	-0.008	$9.93e^{-01}$			
	March	0.291	0.077	3.793	$1.67e^{-04}$			
	April	0.117	0.083	1.401	$1.62e^{-01}$			
Fs	2006 - 2007	0.493	0.094	5.231	$2.50e^{-07}$	711.87	67.85	6.45^{e-14}
	2008 - 2009	0.726	0.094	7.713	6.81^{e-14}			
	2011 - 2012	0.503	0.092	5.447	8.09^{e-08}			
	2010 - 2011	0.051	0.084	2.272	2.35^{e-02}			

3.2.2. Males

A high percentage of mature males (82%) were observed towards the end of all fishing seasons (February-March). Mature males occurred more frequently in the Northeast fishing area, and also at 10 m depth, decreasing occurrence stepwise until 30 m (Table 1.5). The ML50 of males was estimated at 210 mm (Figure 1.4).The spermatophoric complex (SW) represented on average 1.66% of body weight, ranging from 0.38% to 7.82%. The mean GSI was significantly different between areas (Fa) and months (Fm) (Kruskal-Wallis test, $p<0.001$). The pairwise comparison test shows that the mean GSI was significantly higher in the Southern fishing area (Table 1.5). During March, mean GSI was significantly lower compared with other months ($p <0.05$).

The first GAMLSS model for males (AIC=403.914, SBC=497.169; GD=359.909) indicated co-linearity between TW and BW (VIF=3.05), so BW was excluded from the analysis. The best fitted model is shown in Table 1.6, being the spermatophoric complex weight (SW), the most significant explanatory variable, with high correlation during maturation, and increasing monotonically with TW (Figure 1.6B). On the other hand, the ML was the most likely variable in the model (LRT and Chi-squared test, Table 1.6). The partial plot showed a positive correlation between TW and ML at 187.77 mm ML with a second peak at 281.01 mm ML (Figure 1.6A). It also showed a positive relationship between TW and TL, from 42 mm TL onward, peaking at 51 mm TL (Figure 1.6C). In relation to the maturity stages, the same pattern found in females was found in males, where the gonad weight correlated significantly to maturity stage III (Figure 1.6D).

In the second model (AIC=652.0224, SBC=728.314,GD=616.012), depth was also found to be a significant factor for males (Table 1.7). A positive correlation was found between TW and depths from 13 m to 18 m, peaking at 15 m depth (Figure 1.6E). No significant relationship was found between TW and fishing areas (Figure 1.6F). However, gonad weight peaked in the fishing month March (Figure 1.6G). The inter-annual variation was also significant in males, with a positive correlation with TW observed in the 2011-2012 season (Figure 1.6H).

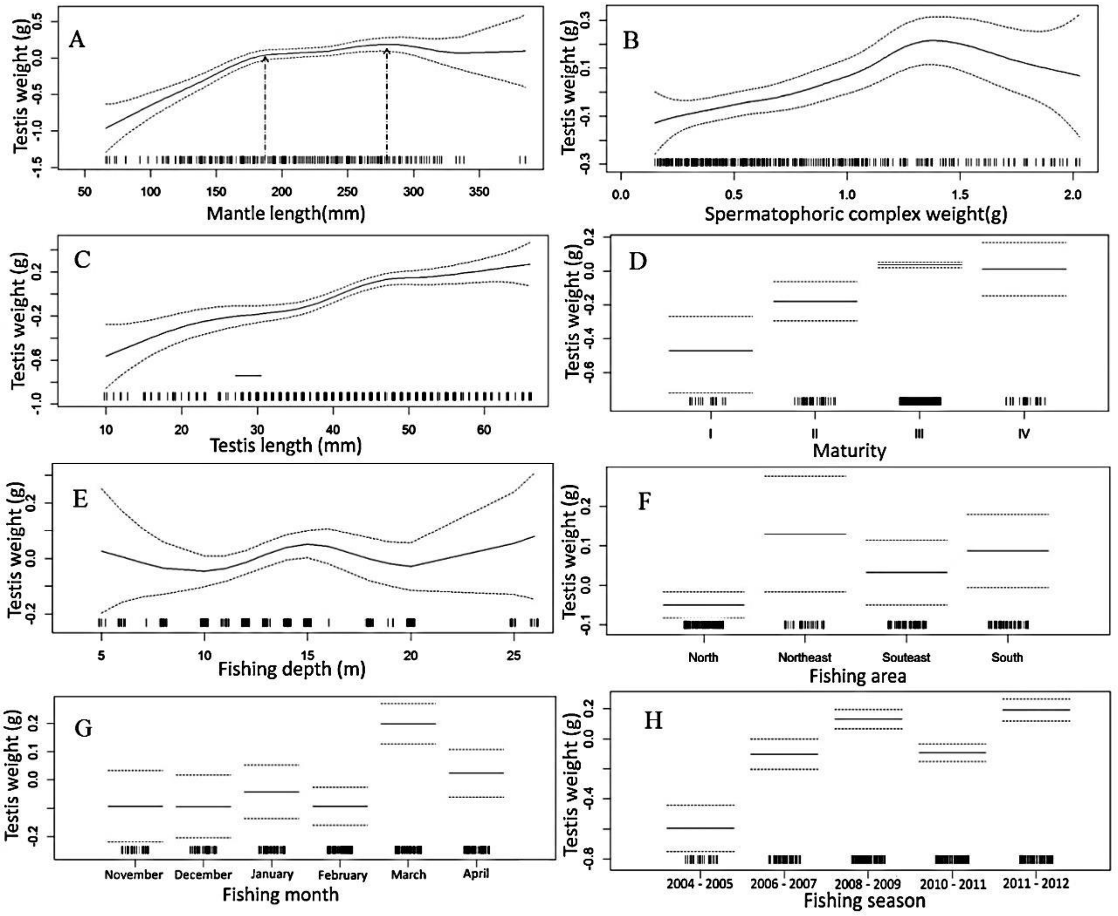


Figure 1.6: GAMLSS smoothing curves fitted to partial effects of explanatory variables on reproductive and temporal variables, in males of *Doryteuthis plei*. *TW* is represented as a function of (A) *ML*, (B) *SW*, (C) *TW*, (D) *Mat*, (E) *Fd*, (F) *Fa*, (G) *Fm* and (H) *Fs*. Dashed lines represent 95% confidence intervals around the main effects. The arrow in 6A shows the correlation peaks in relation to the ML.

4. Discussion

Some characteristics of *D. plei* population structure exploited by the small-scale fisheries of SSI seemed to be very similar to those described from the shelf trawling fishery off the South Brazil Bight (Perez et al., 2002; Rodrigues and Gasalla, 2008), *i.e.* females being smaller and more robust than the males (which on average, were 1.6 times bigger than females), and more abundant during the summer months. This pattern was also observed for other loliginids caught elsewhere, *e.g. L. forbesi* and *L. vulgaris* off the European coast (Pierce et al., 1994; Moreno et al., 1994, 2007; Rocha and Guerra, 1999); *L. reynaudii* in South Africa (Olyott et al., 2006) and *D. pealei* in the North Atlantic Ocean (Dawe et al., 1990). However, in contrast to the shelf fisheries, the hand-jigging around the island catches squid with a larger size as a whole (*e.g.* maximum ML and higher values of the ML/BW relationship's exponent "*b*"), which is associated to gear selectivity and to the local occurrence of spawning concentrations. The fact that females were more frequent than males during the first three months of the fishing season, while males appeared more frequently at the end of the season, confirms the existence of female post-spawning mortality or migration. Regarding the ML/BW relationship, since exponent "b" values were significantly higher in females than in males, and in immature and in-maturation individuals (stages I and II) of both sexes, findings suggest somatic growth during these stages, and reproductive growth in mature and spent squid (stages III and IV).

Our results show that the local small-scale fishery seasonally removes part of the reproductive stock of the squid *D. plei* in shallow waters around the island, as confirmed by the high proportion of mature squid in all samples, with a peak in February. In this sense, all findings refer to the portion of the squid population which is vulnerable to the local hand-jigging fishery, thus, it is not intended to represent the whole population. Also, mated females were present throughout the fishing seasons, as confirmed by the presence of spermatophores in the females' buccal receptacle in all samples.

Previous studies on loliginids reported that size-at-maturity in squid can vary dramatically, depending on the location and time of collection of samples (Raya et al., 1999; Perez et al., 2002; Gasalla and Rodrigues, 2008). The size-at-maturity of squid caught by the shrimp trawling off the coast of São Paulo and Santa Catarina, was relatively smaller than the ones found in SSI by the present study. This may be due to the effect of fishing gear selectivity, as suggested by Martins and Perez (2007) that compared values obtained from squid caught by hand-jigging and fish-traps in Santa Catarina Island.

Postuma and Gasalla (2010) showed that the hand-jigging of *D. plei* squid targets dense concentrations of squid in austral-summer months (November to April), with CPUE peaks between February and March, where sea surface temperature were warmer. Present study confirms that these squid were in reproductive activity, as indicated by the positive correlation between gonad weight and month, for both sexes. According to the hypothesis of Forsythe (2004), warmer temperatures in surface waters during the summer would stimulate somatic and reproductive growth and the development of gonads and gametes in females, which could explain the great lengths and the greater concentration of mature individuals in this coastal region.

In relation to gonadal development, it was found that the ovary enlarges as females grow up to 151–175 mm ML. In females larger than this, the ovary loses weight possibly due to spawning activity. In males, the GAMLSS suggested the co-occurrence of two different groups, one that matures at 187 mm ML and another that matures at 290 mm ML. This kind of pattern among males seems to have been observed in *Loligo vulgaris* caught off southern Portuguese waters (Coelho et al., 1994), *L. vulgaris* and *L. forbesi* males in Galician waters (Guerra and Rocha, 1994) and *D. plei* caught by the industrial trawling off Brazil (Marques and Gasalla, 2002; Perez et al., 2002). Hanlon (1998) and Hanlon et al., (2002) studying the behaviour of *Loligo* sp. and *Loligo vulgaris reynaldii* at field in South Africa, used the term "sneakers" for the smaller males and "consorts" for the larger males that formed different reproductive rows during spawning in the seafloor. One could speculate that the small mature males found in present study could correspond to a “sneakers” group and that the group of larger males could correspond to a “consorts” group. Anyway, the fact that the population could have one stratum in males that matures earlier than the other could represent an interesting reproductive strategy for increasing fertilization.

The first GAMLSS model showed a relationship between gonads length and weight in both sexes. In females, the accessory gland weight and nidamental gland length were found to be positively correlated to ovary weight. This was also reported in Sanchez and Demestre (2010), which suggested that nidamental glands could be used as a signal of maturity in females, since the development of the gland, is well connected to the ovary development, probably linked to the production of gametes. In males, the ML was the variable that better and positively correlated to testis weight. This suggests that the female invest in maximizing egg production to ensure successful successfully spawning, whereas males invest more in somatic growth due the characteristic sexual dimorphism in this genus (*i.e.* males larger than females), in order to optimize the chances of effective mating with females. However, a peak of testis weight was found when the spermatophoric complex attained 1.3 g of spermatophoric complex, after this the testis loses weight, suggesting the transfer of spermatophores to the females during mating.

Based on the above mentioned model, it may be recommended that for future optimized fishery monitoring initiatives where only length data may be obtained, the

NiGL (females) and TL (males) should be the most important biometric variables to monitor the reproductive activity of *D. plei*.

The second GAMLSS model showed that depth (Fd) was the most significant factor influencing the reproductive activity in both sexes, also indicating that it intensifies between 13m to 23m, peaking at 15m depth in males and 20m in females. The strong correlation found between the gonads weight (OW-TW) and the fishing areas (Fa) indicated a peak occurring in the South and Southeastern areas of the island, coinciding also with the highest mean GSIs, where females were more abundant. However, the lower values of OW in females found in the North and Northeastern areas, from 5 m to 10 m depth, could also indicate an evacuation of the ovary during/after the spawning process. Considering local oceanographic studies, the South and Southeastern areas of the SSI seem to be relatively biologically poorer if compared with the other areas (Pires-Vanin, 2008).Thus, it may be suggested that the sheltered bays of the South and Southeastern areas of SSI could actually be a breeding ground for mating, while the North and Northeast areas may correspond to spawning grounds.

The inter-annual and intra-annual variation of gonadal weight showed a significant peak during February and March and in the 2011-2012 fishing season. The reason why that particular year showed higher values of maturation in comparison to other years should be further investigated by using environmental data series in future studies.

Overall, some further speculation on the aggregation of mature squid in the study area may link the combined effect of the following factors: (i) the wind-driven coastal upwelling events, with intrusion of the SACW stratifying water column in summer (Martins and Perez, 2007), (ii) changes in environmental variables in the inshore region (Pecl and Jackson, 2008), (iii) the favourable condition of sea surface water temperatures (Postuma and Gasalla, 2010), (iv) food availability (Gasalla et al., 2010), and (v) reproductive strategies (Rocha et al., 2001) somehow favoured by island's environment.

Finally, the spatial-temporal pattern of reproductive activity found here may be useful in drafting specific studies to detect spawning grounds of *D. plei* in the region, as well as in the definition of future fisheries management plans.

5. Conclusions and summary

The SSI was evidenced as an important reproductive area for *D. plei* in the South Brazil Bight area. The combination of spatial-temporal factors (depth, area, month, and year) helped understanding the pattern of squid aggregation for reproductive activity around the island, while non-linear correlations between reproductive structures were useful to identify the biometric variables that better described maturation. The GAMLSS showed advantages to identify long-term reproductive patterns, which would be unlikely possible by using the gonado-somatic index alone.

In summary, the following spawning scenario was proposed for the species in the region, with a peak of reproductive activity during February and March. The concentration of mature organisms predominated in the Southern and Southeastern coasts of SSI, where the largest sheltered bays are located. It suggests that favourable breeding grounds for mating may be located in these areas, while the North and Northeast ones seem to represent spawning grounds. Females were more frequent at the beginning of the fishing season (November and December), concentrating in shallow waters to spawn. During the reproductive activity peak in February, the sex ratio tended to equalize (1:1) at depths of 10-20m, where squid would most likely be mating. Local fishers seem to track this trend of aggregation. Lastly, present findings should assist the future development of management measures (*e.g.* for seasonal, size, and spawning area protection) aiming the sustainable use of the resource.

Chapter 2

Ethogram analysis reveals new body patterning behaviour of the tropical arrow squid *Doryteuthis* plei off the São Paulo coast

Chapter 2 - Ethogram analysis reveals new body patterning behaviour of the tropical arrow squid *Doryteuthis plei* off the São Paulo coast

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Abstract:

Squids can express several body patterns, aided by a variety of visual signals that are produced by chromatophore organs. However, for several squid species, body patterning behavior during reproductive activity is still not completely understood. For example, what are the specific patterning changes and other visual signals, how do they appear, and how long do they last? To test the hypothesis that distinct chromatic components appear at different durations on the skin of the tropical arrow squid *Doryteuthis plei* in the Southern Hemisphere, we identified and described its body patterning behavior. Specimen squids were obtained from off the South Brazil Bight, near the coast of the São Paulo shelf. Animals were maintained and monitored in circular tanks for 62d over six observation periods, from 2011 through 2013. An ethogram was constructed showing 19 chromatic, 5 locomotor, and 12 postural components, or body patterns, associated with reproductive behavior. New chromatic components (*i.e.*, those not yet reported in the North Atlantic *D. plei* species), particularly those linked to female sexual maturity, were observed. A postural component, the "J-Posture", linked to defenses and alarm, also was noted. The average time spent for "light" components was 32s. The corresponding "dark" components had an average duration of 28s. Females displayed the chromatic components related to calm behavior longer than males. However, males appeared to be more dedicated to disputes over resources, and used rapid, miscellaneous visual signaling. In conclusion, new basic types of body patterns are described for *D. plei*. The repertoire of chromatic components reported in the ethogram is, to our knowledge, the first record for *D. plei* of the Southern Hemisphere.

Chromatophores, Mating, Reproductive behaviour, Agonistic behaviour, Cephalopods

1. Introduction

Squids can express multiple body patterns, each associated with specific behavioral responses. These visual signals are produced by chromatophore organs in the skin that are controlled by hormones and neurotransmitters through specific structures (Hanlon and Messenger, 1996; Messenger, 2001). In most cephalopods, body patterns are created by the simultaneous occurrence of chromatic, postural, and locomotor components. These affect the appearance of the animal and may be acute, with a duration lasting seconds, or chronic, when extending for minutes. The composite is produced by groups of the chromatic units constructed from different elements (Hanlon, 1982; Hanlon et al., 1994, 1999; DiMarco and Hanlon, 1997). The variety of body patterns of each individual is used both for crypsis and communication.

The body pattern outline and the spread of chromatophores in the body are important tools for the study of ethology of cephalopods. These characteristics have been used to compare behavioral variation among loliginid squid species (Hanlon, 1982; Hanlon et al., 1994; Hanlon and Messenger, 1996). The pigmentation of cephalopod skin is contained within unique cellular chromatophore organs (Cloney and Florey, 1968); chromatophores have a unique ability to rapidly change their shape through a specialized neuromuscular control system (Hanlon and Messenger, 1996).

The body pattern dynamics in loliginids have been investigated using captive or field studies in several parts of the world. The patterns are linked predominantly to courtship and mating during reproductive behavior, interspecific association, and competition for resources (Hanlon and Messenger, 1996). The following species have been investigated in previous behavioral studies: *Loligo reynaudii* Orbigny, 1841 (Sauer and Smale, 1993; Sauer et al., 1997; Hanlon et al., 2002), *Doryteuthis pealeii* Lesueur, 1821 (Griswold and Prezioso, 1981; Hanlon et al., 1999; Sharsha and Hanlon, 2013), *Doryteuthis opalescens* Berry, 1911 (Hurley, 1977; Hunt et al., 2000; Hanlon et al., 2004), *Loligo* spp. (Hanlon, 1998), *Sepioteuthis australis* Quoy and Gaimard, 1832 (Jantzen and Havenhand, 2003), and *Sepioteuthis sepioidea* Blainville, 1823 (Arnold, 1965). There were also behavioral studies of *Doryteuthis plei* Blainville, 1823 in captivity in the North Atlantic (Hanlon, 1982; Hanlon et al., 1983) and in the wild, *i.e.*, from a research submersible during night dives (Waller and Wicklund, 1968).

The number of chromatic components described for the genus *Loligo* is large and complex. *Loligo vulgaris reynaudii* demonstrates 23 chromatic signals (Hanlon et al., 1994), *Loligo forbesi* Steenstrup, 1856 shows 17 signals (Porteiro et al., 1990), and *D. pealeii* shows 34 signals (Hanlon et al., 1999). Studies focused on *D. plei* have reported that the organization of chromatophores and iridophores is not constant, differing and specific in certain regions of the body. For example, larger brown chromatophores are located on the dorsal mantle while small yellow chromatophores appear on the arms or tentacles (Hanlon, 1982). Therefore, the final appearance of a certain chromatic component is not only the result of neural excitation of colored chromatophores, but is also due to the size and distribution (vertical and horizontal) of chromatophores in different parts of the body. According to Hanlon (1982), *D. plei* displayed 16 chromatic components that were produced through specific static, morphological, chromatic units.

D. plei inhabits coastal and shelf waters in the Western Atlantic Ocean, from the coast of Florida in the United States (Hixon et al., 1980) to Rio Grande do Sul, Brazil (Perez et al., 2005). This species is an important fishing resource off the São Paulo coast and is mostly found at shallow depths (< 30 m) in coastal waters (Gasalla et al., 2005). The squid spawns throughout the year, but peaks during the summer months (Rodrigues and Gasalla, 2008; Postuma and Gasalla, 2010, 2014). In the South Brazil Bight (SBB), many studies have addressed the population biology of *D. plei*, including growth, reproduction, feeding, and fisheries oceanography (Martins et al., 2006; Martins and Perez, 2007; Gasalla et al., 2010; Postuma and Gasalla, 2010, 2014). However, the behavioral and body patterns have rarely been described. In this study, we present illustrations and descriptions of behavioral and body patterning. We also provide details of the variety of patterns and duration of each chromatic component observed. It is noteworthy that recent phylogeographic studies suggest that the Brazilian population of *D. plei* is genetically distinct from *D. plei* in North and Central America (Sales et al., 2013).

The aim of the study was to describe the components and body patterns of the squid *Doryteuthis plei*, which may aid in distinguishing species in the North Atlantic Ocean based on previous work by Hanlon (1982) and Hanlon et al. (1983). To this end, an ethogram of the signals of *D. plei*, especially those related to reproductive behavior, was constructed. The ethogram is based on quantification of the time and duration of each chromatic component, gender differences, and types of chromatic components (light or dark).

2. Materials and Methods

2.1. Animal capture

Seventy-eight specimens of the tropical arrow squid *Doryteuthis plei* were obtained using hand jigs and Japanese-style pound nets (“kaku-ami”) off the Ubatuba coast (lat 23° 51’ S; long 45° 08’ W) in marine waters less than 10.9 m deep. Additional samples were collected in São Sebastião (23° 83’ S; 45° 44’ W) in 6 m depth. Animals were immediately transported to the laboratories of the research station at the northern coast of São Paulo, Brazil, using the research vessel, *Veliger II,* and the small boat, *Nautilus*. During transport, animals were held in a 300-l tank containing local seawater that was constantly aerated by a submersible pump capable of pumping 432 l h^{-1}. Transport to the laboratory required 5–40 min after each sampling, as the distance ranged from 1.3 to 3.5 nautical miles. All of these steps were taken to minimize the animals’ stress and damage during collection and transport (Aguiar et al., 2012; Marian, 2012).

2.2. Experimental tank setup

In the laboratory, animals were held in two indoor circular tanks: a 2.3-m diameter, 3000-l tank with a closed seawater system, and a 1.8-m diameter, 1000-l flow-through system tank (Figure 2.1). Both tanks contained gravel and sand substrates (Figure 2.1a). The closed seawater system provided a continuous flow of seawater. A pump with a capacity of 10,000 l h^{-1} (Figure 2.1b) was used to circulate water through the sand filter (Figure 2.1c) and a UV sterilizing filter (Figure 2.1d). Squids were exposed to ambient light during a 12:12 light: dark photoperiod. During the night periods, observations were aided by a low-intensity LED light (approximately 50 lumens/watt). Water quality, *i.e.*, temperature, salinity (ppt), and dissolved oxygen were measured daily with a multi-parameter probe. Temperatures ranged from 21.86 to 28.81 °C, salinity ranged from 34.71–35.83 ppt, and dissolved oxygen was no greater than 5.00 mg.L^{-1}. The mean level of toxic ammonia was 0.020 ppm (range, 0.012–0.035, n = 15), and the mean level of total nitrite-nitrogen (NO2) measured 0.5 mg.L^{-1} (range, 0.02–3.05, n = 15).

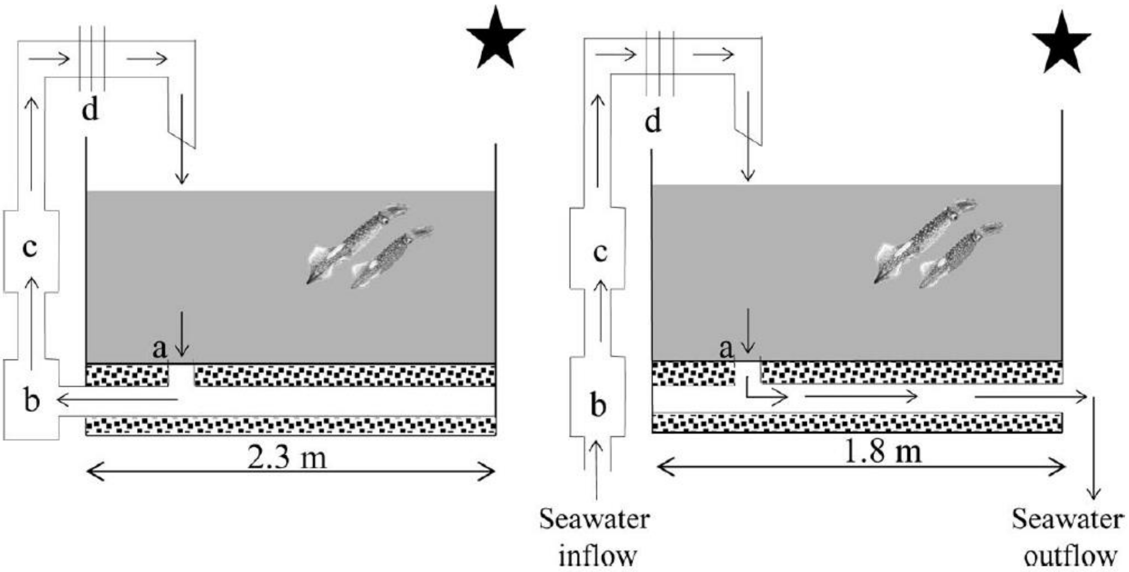


Figure 2.1: Two circular tank systems: left, a 2.3-m diameter, 3000-l tank with closed seawater system; right, a 1.8-m diameter, 1000-l flow-through system tank. a) Substrate bottom of the tank; b) pump; c) sand filter; d) UV sterilizing filter. Star shows observer site and camera location.

2.3.Animal care and husbandry

Specimens were monitored for 62d over six observation periods conducted in November 2011 (15d), February 2012 (7d), and March 2012 (14d) with the closed seawater system; then in November 2012 (13d), February 2013 (19d), and November 2013 (10d) using the flow-through system tank.

The squids’ survival period in both systems averaged 7 d; some animals survived up to 19 d in February 2013. Of the population of 78 *Doryteuthis plei* specimens maintained in captivity, 46 were females and 32, males. Females were more frequent than males only during February 2012 (19 females, 0 males). Multiple combinations of male/female pairs were acclimated in the tanks (Table 2.1) for observation and identification of the components of the body patterns related to behaviors. Based on previous studies, a components checklist was devised, including calm and reproductive behaviors (*e.g.,* agonistic (such as males' dominance over females), courtship with displays of reproductive gonads, mating types, and egg-directed behaviors), was used to categorize behavioral context (Roper, 1965, Hanlon et al., 1983, 1994, 1999, 2002, 2004; Hanlon and Messenger, 1996; DiMarco and Hanlon, 1997; Shashar and Hanlon, 2013).

Table 2.1: Number of females and males of *Doryteuthis plei* with mean mantle length in millimetres (between bars), sex-ratio with *Chi-square* test: results during the six laboratory maintenance periods from 2011 through 2013.

	Females	Males	Sex-ratio	Chi-square	*df*	*P*-value
November 2011	3 (144)	7 (209)	0.4286	1.600	1	0.21
February 2011	19 (140)	0	19.000	19.000	1	$1.30E^{-05}$
March 2011	6 (162)	5 (194)	1.200	0.090	1	0.76
November 2012	6 (143)	5 (210)	1.200	0.090	1	0.76
February 2013	8 (160)	7 (281)	1.142	0.060	1	0.79
November 2013	4 (145)	8 (210)	0.555	1.330	1	0.24
Total	46 (144)	32 (227)	1.516	2.510	1	0.11

During the observation period, a male in the tank was considered "dominant" when, having been paired with a female for 10 min, he won the disputes with other males considered "intruders." The definition of these terms and of the period were chosen based on a study of fighting tactics for *D. plei* in the Gulf of Mexico (DiMarco and Hanlon, 1997).

Mean mantle length (ML) for females was 144.23 mm (± 26.55 mm, s.d.; range 65–243 mm), and mean ML for males was 227.84 mm (± 45.73 mm, s.d.; range 99– 299 mm). Food was offered ad libitum one to two times per day, and consisted of small fish, frozen or live, plus live crustaceans when available. The species offered during feeding were *Sardinella brasiliensis*, *Anchoa tricolor*, and *Anchoa sp.* measuring 45–89 mm. However, species not described as prey in the diet of *D. plei* also were offered, e.g., the crustacean *Callinectes danae* and fish, the barred grunt *Conodon nobilis.* Observation periods at the 3000-l tank with the closed seawater system showed a mean daily mortality rate of 22.02% (range, 0–33%); observation times at the tank with the 1000-l, flow-through seawater system had a mean daily mortality rate of 21.05% (range, 0–47%). A number of factors, including water quality, space confinement, live feed, exposure to light, and low noise and stress, were monitored to ensure the welfare of the study squids, as recommended for cephalopods by Moltschaniwskyj et al. (2007), and the ethical use of animals in applied ethology studies (Sherwin et al., 2003).

2.4.Experimental methods

To describe the organization of body patterns, we used the hierarchical classification developed for octopus by Packard and Sanders (1971) and Packard and Hochberg (1977), and reviewed by Hanlon and Messenger (1996). The classification hierarchy follows a top-down flow: (i) body patterns; (ii) components; (iii) units; and (iv) elements.

The chromatic, postural, and locomotor components were described and tabulated in a spreadsheet and compared according to time, gender, and chromatic component (light and dark). The body patterns were classified into two categories: (1) chronic patterns and (2) acute patterns. The terminology for names of the components and body patterns were based on studies conducted on the behavior of other loliginids around the world, *e.g.*, *Loligo vulgaris reynaudii* (Hanlon et al., 1994) and *Doryteuthis pealeii* (Hanlon et al., 1999), and on previous studies, particularly of *Doryteuthis plei* in the North Atlantic (Hanlon, 1982; Hanlon et al., 1983).

Overall, 96 observations were made from the top of the tank (Figure 2.1), 3 h each on average, three observations per day, and totaling 530 h within six observation periods. In addition, 1056 video frames were recorded at an average rate of 15 per day, totaling 28 h, 40 min of video recording time, with a mean filming time of 30 min each (range, 1–45 min) (Table 2.2). The videos were focused on male and female pairs of *D. plei*, and especially on those that exhibited reproductive behavior. The videos were reviewed six times each, two times looking at one component category, following the methodology described by Hanlon et al. (1999). Observations started with the chromatic component (looking only for chromatic signals), followed by the postural components, and, finally, the locomotor components. Then each component of the body patterns was observed and noted for any squid at a given time during the observation periods. With respect to egg capsule deposition, the first hours of the day (00:00 to 06:00 am) were monitored and observations of egg deposition were made. Therefore, the observations were focused on a single female, and we conducted ‘‘focal animal sampling’’ by following that female as long as possible, filming continuously to record the sequence of behaviors that preceded egg laying, after the methodology of Hanlon et al. (2004).

Table 2.2: Video recordings (n) during the six laboratory maintenance periods, with total video time footage and mean, minimum, maximum, and standard deviation (h:min:s) of each video

	n	Total video time	mean	min	max	s.d.
November 2011	462	13:27:20	0:55:55	0:01:26	0:28:33	0:35:21
February 2012	47	1:26:31	0:12:22	0:05:12	0:20:10	0:05:44
March 2012	197	8:53:14	0:38:05	0:05:42	0:38:49	0:56:07
November 2012	189	2:42:02	0:25:32	0:01:12	0:12:10	0:06:34
February 2013	159	1:12:06	0:29:51	0:03:45	0:45:22	0:21:54
November 2013	36	1:02:06	0:19:51	0:02:46	0:35:22	0:19:05
Total	1090	28:43:19	0:30:16	0:01:12	0:45:22	0:19:07

In analyzing the 1056 videos, we calculated in seconds the mean duration of each chromatic component. The duration of these components did not have a normal distribution (Shapiro-Wilk test); in fact, the duration in seconds was found to violate the criteria for normality. Therefore, the non-parametric Kruskal-Wallis test and post-hoc pairwise comparison tests (Siegel and Castellan, 1988) were applied to assess the influence of gender and type of chromatic component (light or dark) on the duration of each chromatic component. All statistical tests were considered significant when $P <$ 0.05.

3. Results

3.1.*Ethogram*

An ethogram for *Doryteuthis plei*, based on our observations and the videos, is shown in Figures 2.2 and 2.3. The chromatic, locomotor, and postural components found in this study were used to build up body patterns and represent a segment of the behaviors for this species, especially those related to reproduction. The variation of each chromatic component is shown in Figure 2.2.

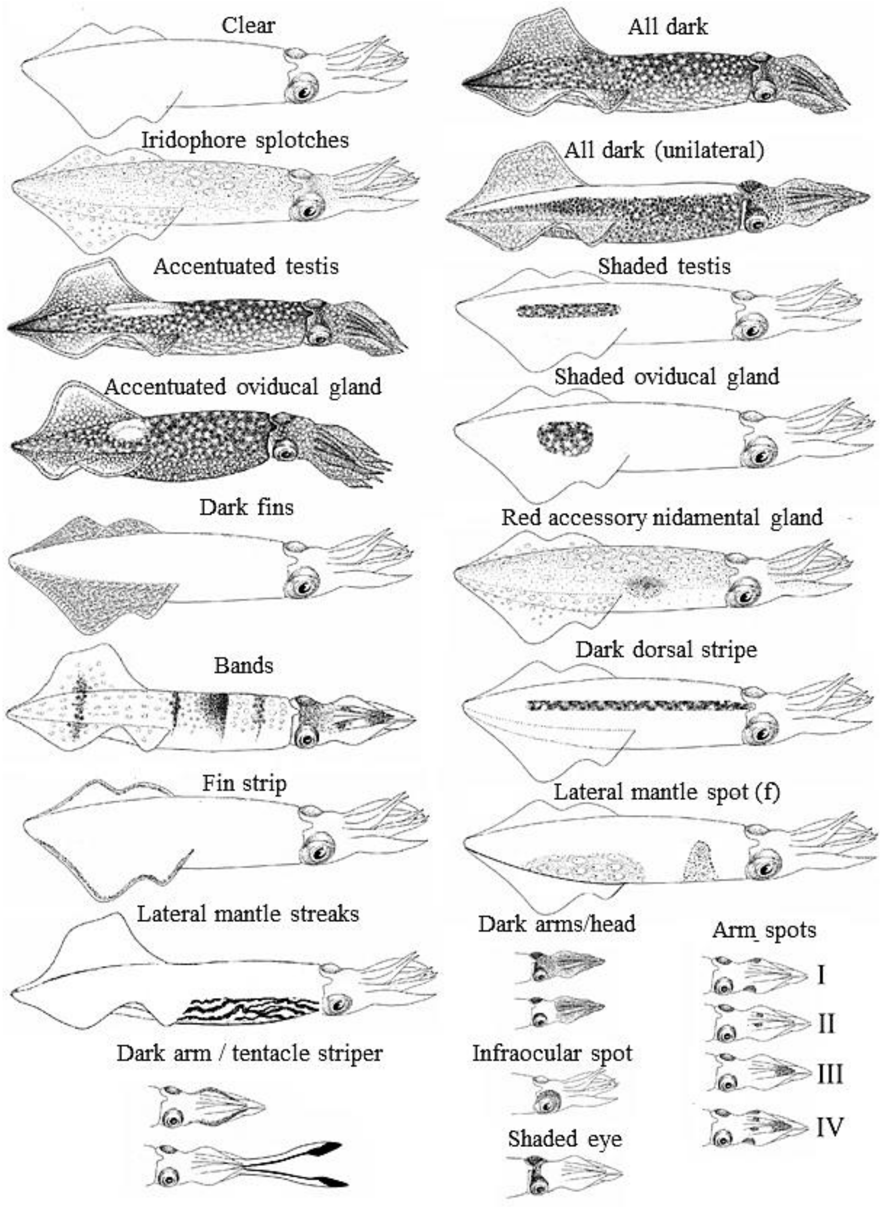


Figure 2.2: The chromatic components of body patterning found in the squid *Doryteuthis plei*.

3.1.1. Locomotors components

The hovering in the water position was caused by the interaction of fin movements and siphon jetting. This component was observed in solitary squids or in groups when they were calm. Free swimming occurred when the squid moved forward, with the head backward and raised a bit, and the arms slightly depressed. During this component, the siphon and fins steered the animal during swimming. This posture was observed in calm squids preparing to mate.

Parallel positioning involved two animals hovering or swimming parallel to one another in the same direction, within one body length of the other. Fin beating, a male-specific behavior, occurred during parallel positioning, when two males maneuvered themselves into position to beat their fins against each other. This was a physical and escalating stage with an agonistic context, but it resulted in no obvious physical damage. These encounters lasted up to 10s in disputes for females during mating.

The chase occurred when one squid (of either sex) vigorously pursued another squid by intraspecific competition or cannibalism in the tank. It occurred especially in moribund squid. This component was often observed during agonistic behavior in males. During the contests, a winner could chase the loser several times, and each chase could last up to 40s. During feeding, squids also chased their prey; chases lasted from a few seconds to minutes, *i.e.*, when the squid’s chase was failing. Escape and fleeing occurred during intraspecific competition between winners and losers, especially among males during mating.

Jetting consisted of rapid body movements causing the expulsion of water from the siphon, producing rapid jet propulsion. Jetting usually stretched from 0.5 to 1 m in distance and was always performed backwards. Jetting was combined in escapes used to avoid both predators and conspecifics over spawning among females after mating and deposition of an egg capsule into the egg mop (*i.e.*, an assembly of egg capsules on the substrate).

The courting pair’s component occurred when a male initiated a parallel position to a mature female; agonistic encounters also began with this movement. However, when squids were placed in the tank, there was no synchronic, parallel swimming. At first, pairs did not swim in the same direction. However, within one day (24 h) of the experiment, they began to do so. Swimming upwards was observed in females when they swam up the tank. This movement occurred before male-parallel mating. The female drew the male to mating by swimming upwards. This movement was observed eight times in the total video footage, lasted 15 s per episode, on average.

Head-to-head mating occurred when a male and female faced each other and the male grasped the female's arms. Spermatophores were placed in a seminal receptacle below the mouth. Male-parallel mating involved a male positioning himself under the female, then grasping her anterior mantle to pass spermatophores into her mantle cavity. (Appendix 2.3).

Oviposition was a female taking a single, extruded egg capsule and affixing it to the substrate or to the existing communal egg mop. The female did not hold the egg mop for long periods. A female with a 180-mm mantle was observed sometimes depositing an egg capsule on the substrate. She then positioned herself vertically, *i.e.*, at 90 degrees to the substrate, and affixed the egg to it. Afterward, this squid performed a fast backwards movement similar to jetting, together with an all-dark chromatic component.

Bottom sitting occurred when a squid rested on the substrate. This posture was observed in tired squids and was accompanied by the chromatic component of arm spots and bands (see *Chromatic components* below). This component preceded the death of a squid and could last up to 1 min.

Egg touching consisted of contacts with an egg mop by both males and females. Contact ranged from a brief exploratory touch to an embrace of the egg capsule with all of the squid’s arms. Females usually laid eggs on the existing egg mop; touching may have been a way of assessing the egg-laying substrate. Males commonly touched eggs; however, touching was often followed by highly aggressive agonistic bouts, suggesting that the eggs provided a visual, tactile, or perhaps chemosensory stimulus.

3.1.2. Chromatic components

*Light chromatic components.*The clear chromatic component was the most common behavior, observed 176 times during the entire video footage (Table 2.3). This component is caused by a retraction of all or a majority of the chromatophores. As a result, the squid's mantle appeared to be almost transparent (Figure 2.3A). In clear waters, when the squid was observed against a gravel background, translucency camouflaged the animal. The internal organs, such as the testis in males and the oviducal and nidamental glands in females, were readily visible. During feeding, both the stomach and the entire digestive system also were visible.

Iridophore splotches appeared on the dorsal mantle and the head. They were a distinctive yellow or golden color, and they helped to produce general camouflage. Three iridescent components are thought to aid in crypsis: (i) iridescent arm stripes; (ii) dorsal mantle splotches; and (iii) dorsal iridophore sheen. This component was observed especially when the squid was calm and floating in the tank. This component occurred 61 times during the total video footage period, lasting from 2 s to 3 min, with an average of 46 ± 36 s (s.d.) for the dorsal iridophore splotches (Figure 2.3B).

Dark chromatic components. The all-dark chromatic component usually occurred at night, together with agonistic behavior in males. This coloring is created by expansion of all of the chromatophore cells across the entire mantle, turning the squid entirely dark. Expansion of all of the chromatophore cells produced a brown and red coloring (Figure 2.3C). All-dark (unilateral) coloring occurred six times and was used during agonistic encounters between males and females (Figure 2.3D). This coloring appeared on one side of the mantle, showing the chromatophores expanded in perfect lateral symmetry.

A dark dorsal stripe was observed in calm squids. This stripe is thought to aid in crypsis through counter-shading when viewed laterally, and through disruptive coloration when viewed from above, by covering some of the bright organs, such as the testis, oviducal glands, and ink sac (Figure 2.3E). The transverse bands component was observed frequently in groups of large males (Figure 2.3F), and appeared in four varieties. The component was found in crypsis behavior through disruptive coloring, as a warning sign when a squid moved close to a possible predator, or when the prey that were offered for feeding were equal to or greater than 50% of the mantle length of the squid. It also occurred in females at the bottom of the tank (Figure 2.3G). The most commonly observed pattern was one band (n= 37), followed by a variation that included four bands (n=31) during the total video footage (Table 2.3).

The arm spots component was observed at the base of the third arms, the second arms, or both sets of arms. This component was common and had several variations, but we can only highlight points on the arms on one side of the body (Figure 2.3H). The arm and tentacular stripes components were most readily observed when the tentacles were extended. In most animals, the first or third pair of arms was darkened and the average duration was 55 s (Figure 2.3I).

The infraocular spot appeared near the eyes in a circular shape and occurred roughly between the eye and the arm spots component. Both components were sometimes expressed simultaneously during alarm situations when coloring is rarely observed. The shaded eye component is a transverse head band of expanded chromatophores that may aid crypsis by covering the bright iridescent sclera of the eyes. The fin stripe component expressed during agonistic contests was also observed when the squid was transported to captivity. It was also noted in alarmed squids and especially in large males during fights. Dark arm and head were observed during intraspecific agonistic encounters, but were also noted during mating and the care of the egg mop.

The dark fins component was caused by expanded chromatophores in the region above the fins, which made them darker. This component lasted for an average of 2 s and was rarely observed. The component was most commonly observed in females when displaying alarm behavior.

Females

Females displayed the light chromatic components for longer periods than males, possibly the result of their calm or courtship behaviors. The white accentuated oviducal gland of females was similar in appearance to the males’ accentuated testis (see *Males* below), but it differed in shape, position, and frequency of expression (Figure 2.3J and Table 2.3). The accentuated area was an ovate shape on the dorsal lateral region of the mantle. The oviducal glands were observed on the mantle in quick flashes for 2 s or, rarely, for a longer duration, *i.e.,* 1 min.

The lateral mantle spot was expressed as a bold mantle side area of dark chromatophores near the head or in the middle of the mantle. The mantle side spot was observed only when the female was paired with the male during mating, and could indicate receptivity. This component occurred several times and passed relatively quickly after approximately 2 s. This pattern may also indicate the maturity of the animal (Figure 2.3K). The shaded oviducal gland component preceded mating and occurred during parallel positioning. It was caused by the selective expansion of chromatophores over the oviducal gland (Figure 2.3L).

The red accessory nidamental gland occurred more than 10 times during the entire video footage (Table 2.3), usually during the daytime, when the females positioned themselves parallel to the males. In *Doryteuthis plei,* this gland is large and bright, and it can be observed through the mantle either laterally or from below (Figure 2.3M). It may also signal female sexual maturity, because it turns red only upon attainment of full maturation.

Males

The accentuated testis component occurred 20 times during the total video footage (Table 2.3) in mature, mating males with mantle lengths of 200–299 mm (Figure 2.3N). It occurred during courtship and parallel swimming immediately prior to or during mating. The accentuated testis appeared when the chromatophores directly above the testis were retracted and the mantle darkened completely. When the mantle is entirely dark, the sexual organ whitens laterally to the mantle and assumes an elongated shape.

The lateral mantle streaks component is produced by longitudinally oriented rows of partly expanded chromatophores (Figure 2.3O). This phenomenon was observed during agonistic behavior. Shaded testis, caused by the selective expansion of chromatophores over the testis, preceded mating and occurred during parallel positioning.

Table 2.3: Time (h:min:s) of expression and frequency of the chromatic components observed in the video footage of *Doryteuthis plei* over the six observation periods from 2011 through 2013

Chromatic components	**Observation frequency**	**Component variations**		**Time**			
Light chromatic components				Mean	Min	Max	s.d
Clear	176		-	00:00:32	00:00:01	00:03:07	00:00:32
Iridophore splotches	61	*Dorsal iridophore sheen*	8	00:00:47	00:00:02	00:01:31	00:00:25
		Dorsal iridophore splotches	44	00:00:46	00:00:02	00:03:00	00:00:36
		Iridescent arm stripes	9	00:01:00	00:00:03	00:03:00	00:00:48
Accentuated oviducal gland	51		-	00:00:16	00:00:01	00:01:31	00:00:21
Accentuated testis	20		-	00:00:28	00:00:05	00:01:30	00:00:30
Dark chromatic components							
All dark	94	*All dark*	88	00:00:34	00:00:01	00:03:00	00:00:32
		All dark (unilateral)	6	00:00:46	00:00:05	00:02:25	00:00:53
Arm spots	155	*Arm spots I*	66	00:00:27	00:00:01	00:01:31	00:00:28
		Arm spots II	11	00:00:12	00:00:01	00:00:55	00:00:15
		Arm spots III	13	00:00:21	00:00:01	00:01:31	00:00:27
		Arm spots IV	65	00:00:27	00:00:01	00:02:25	00:00:29
Bands	75	*Bands I*	37	00:01:11	00:00:02	00:03:00	00:00:56
		Bands II	2	00:01:33	00:00:05	00:03:00	00:02:04
		Bands III	5	00:00:17	00:00:01	00:00:55	00:00:22
		Bands IV	31	00:00:39	00:00:01	00:02:11	00:00:34
Dark dorsal stripe	62	*Dark dorsal stripe*	42	00:00:26	00:00:01	00:02:10	00:00:30
		Dark dorsal stripe II	20	00:00:30	00:00:01	00:01:31	00:00:26
Lateral mantle streaks	54		-	00:00:31	00:00:01	00:02:41	00:00:38
Shaded oviducal gland	40		-	00:00:34	00:00:02	00:02:25	00:00:35
Shaded testis	32		-	00:00:35	00:00:01	00:02:25	00:00:39
Infraocular spot	21		-	00:00:41	00:00:03	00:02:05	00:00:28
Shaded eye	14		-	00:00:09	00:00:03	00:00:18	00:00:05
Fin strip	14		-	00:00:41	00:00:05	00:01:30	00:00:44
Dark arms/head	18		-	00:00:35	00:00:02	00:01:31	00:00:35
Dark arm stripes / Tentacle stripes	10		-	00:00:39	00:00:01	00:02:55	00:00:52
Lateral mantle spot (f)	11		-	00:00:19	00:00:03	00:00:55	00:00:23
Dark fins	8		-	00:00:50	00:00:02	00:02:00	00:00:38
Red accessory nidamental gland	7		-	00:00:08	00:00:05	00:00:31	00:00:08

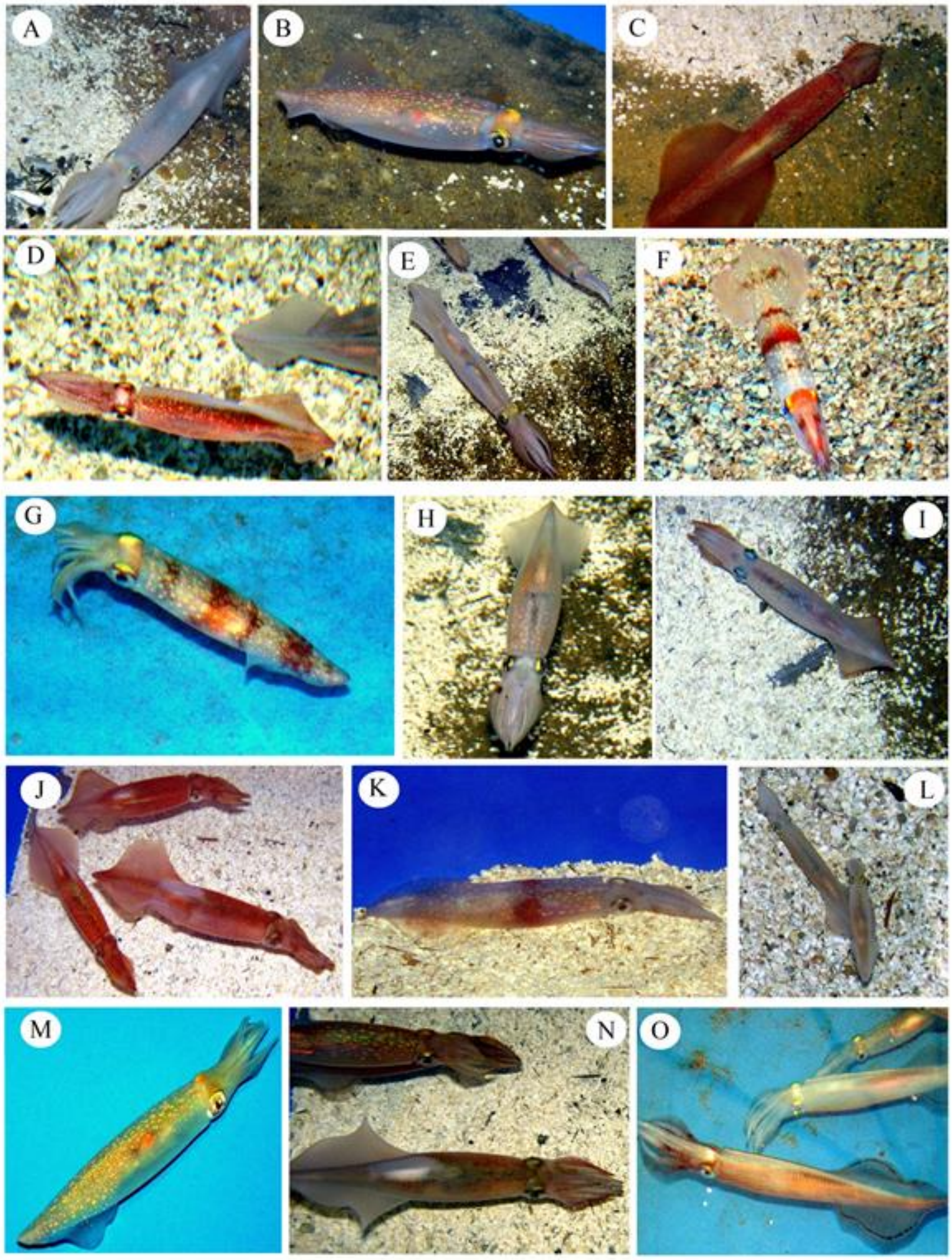


Figure 2.3: Underwater photographs of the components of *Doryteuthis plei* in captivity. (A) The chronic clear body pattern during the free swimming locomotor component. (B) Yellow iridophore splotches seen on the dorsal mantle and head. (C) All dark coloration, and (D) all dark (unilateral). (E) Dark dorsal stripe with arm stripes. (F) Male with four bands, arm spots, and dark head, and (G) downward curl with three bands in females. (H) Arm spots with one side at the base of the third arms and small spot in second and iridophore splotches sheen. (I) Arm stripes. (J) Arm stripes, noted only in females during courtship behavior, and accentuated oviducal glands, with the chromatophores directly above the retracted glands. (K) Lateral mantle spot. (L) Shaded oviducal gland. (M) Red accessory nidamental gland. (N) Accentuated testis in males. (O) Fin stripe, lateral mantle streaks, and arm spots during agonistic behavior.

Frequency and duration of the expression of chromatic components

A total of 19 chromatic (4 light and 15 dark), 5 postural, and 12 locomotor components were identified in our compiled videos and correlated with different body patterns. We observed 923 displays of chromatic components in the skin of squids during the total video footage (Table 2.3). Most chromatic signals were significantly more frequent during the day than at night (73% of observations; Chi square test, $\chi 2 = 3.38$, $P = 0.05$, $df = 1$). At night, the all-dark chromatic components were observed more frequently (Table 2.3). Data in Figure 2.4 show the frequency of the chromatic components identified. The clear chromatic component was observed most often (> 13%), and it occurred together with the arm spots (11%) and dark dorsal stripe (7%) components. All-dark occurred in 9% of the observations during total video footage, followed by bands (8.5%). The chromatic components were more evident in the body patterns. However, some components were rarely observed (< 2%). For example, the red accessory nidamental gland, lateral mantle spots, infraocular spots, dark fins, and shaded eye were rare in females.

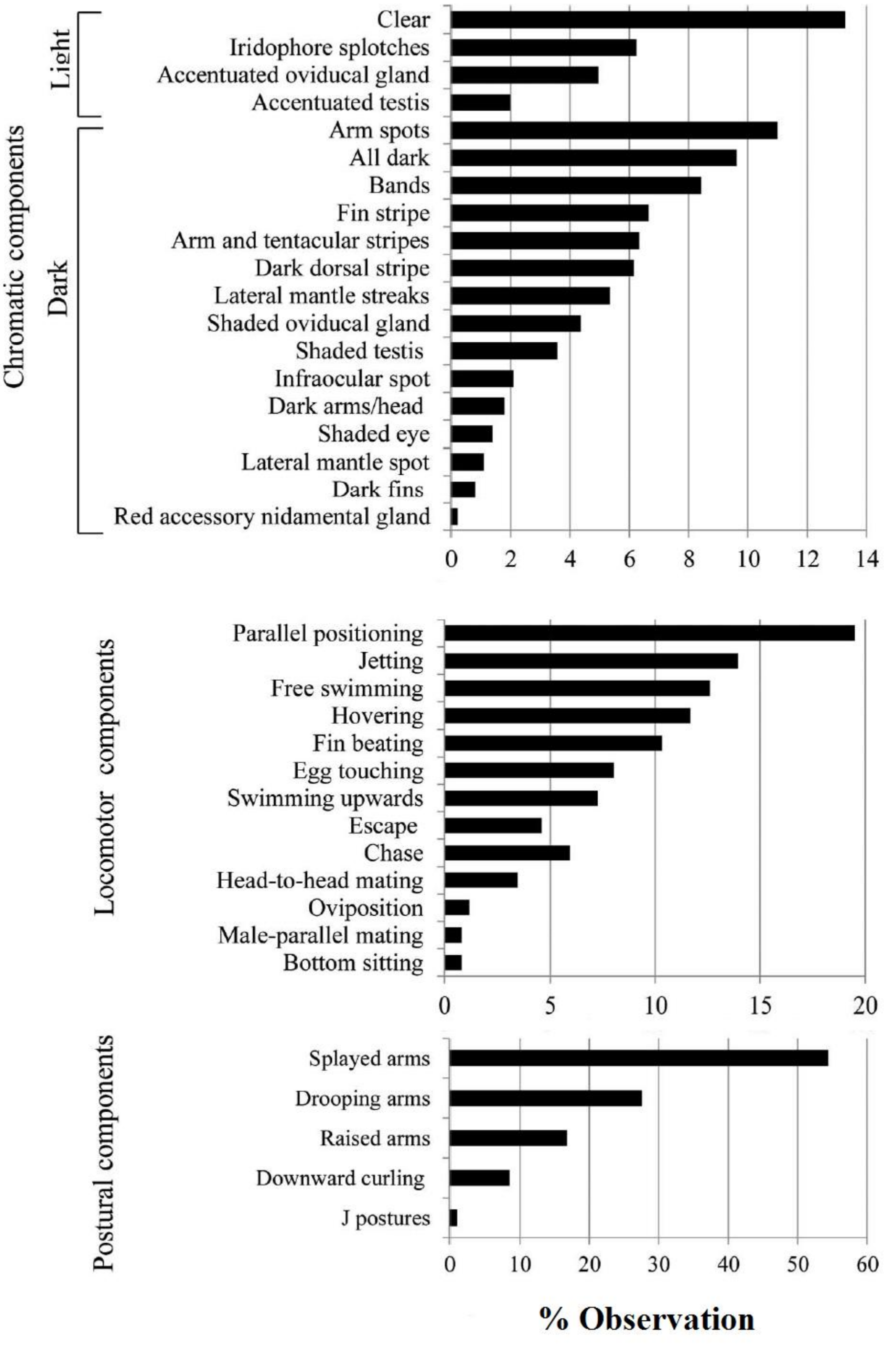


Figure 2.4: Percentage of observations of each chromatic (light and dark), postural, and locomotor component and body pattern observed in *Doryteuthis plei*.

The average durations of the chromatic components differed significantly (Kruskal-Wallis test, $P < 0.05$). A pair-wise comparison test showed that mean durations were significantly higher in the most lasting components, such as clear, iridophore splotches, and bands ($P < 0.05$), than the shortest components, such as arm spots, lateral mantle spot in females, fin stripe, accentuated oviducal gland, lateral mantle streaks, and dark dorsal stripe (Figure 2.5 and Table 2.4). The infraocular spot component was seldom displayed; however, it showed a significant difference in time from the accentuated oviducal gland, a fast component. The average time of the chromatic components of light (*e.g.*, clear and iridophore splotches) and dark (*e.g.*, arm spots, lateral mantle spot; see Table 2.4) also showed a significant difference (χ^2 = 13.55, $df = 1$, $P < 0.05$). The light components had a longer duration (mean = 32 ± 25.2 s, s.d.) than the dark components (mean = 28 ± 32 s, s.d.). Significant differences based on gender also were noted ($P < 0.05$, Table 2.4 and Figure 2.6).

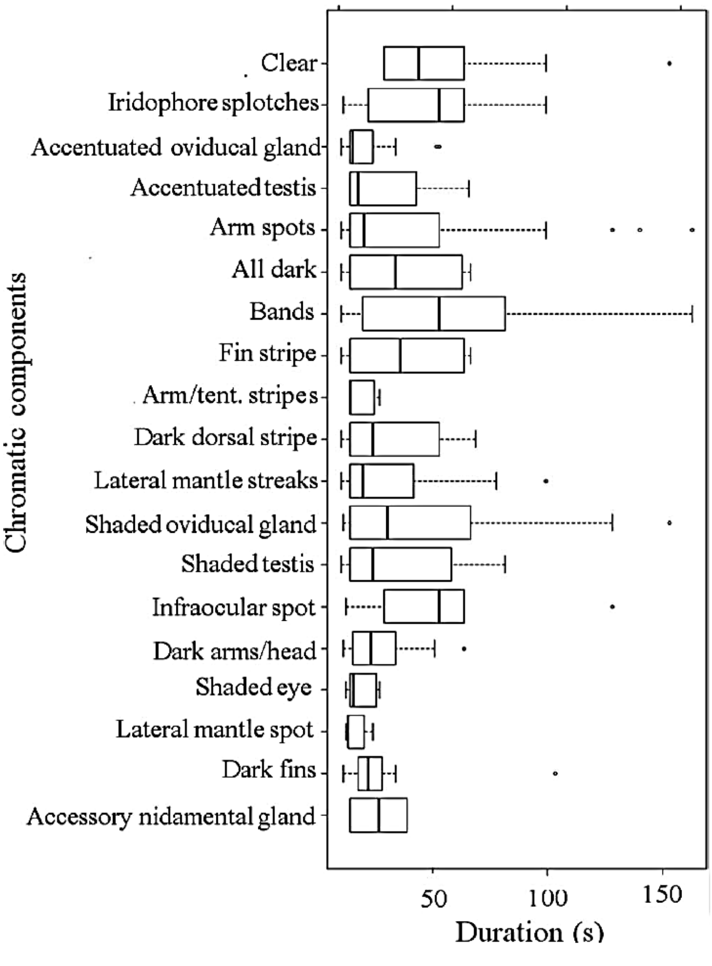


Figure 2.5: Duration of chromatic components identified for *Doryteuthis plei*. The solid black line represents medians; open boxes are 25% and 75% of the observations, and bars indicate the range of durations. Dots show moderate outliers.

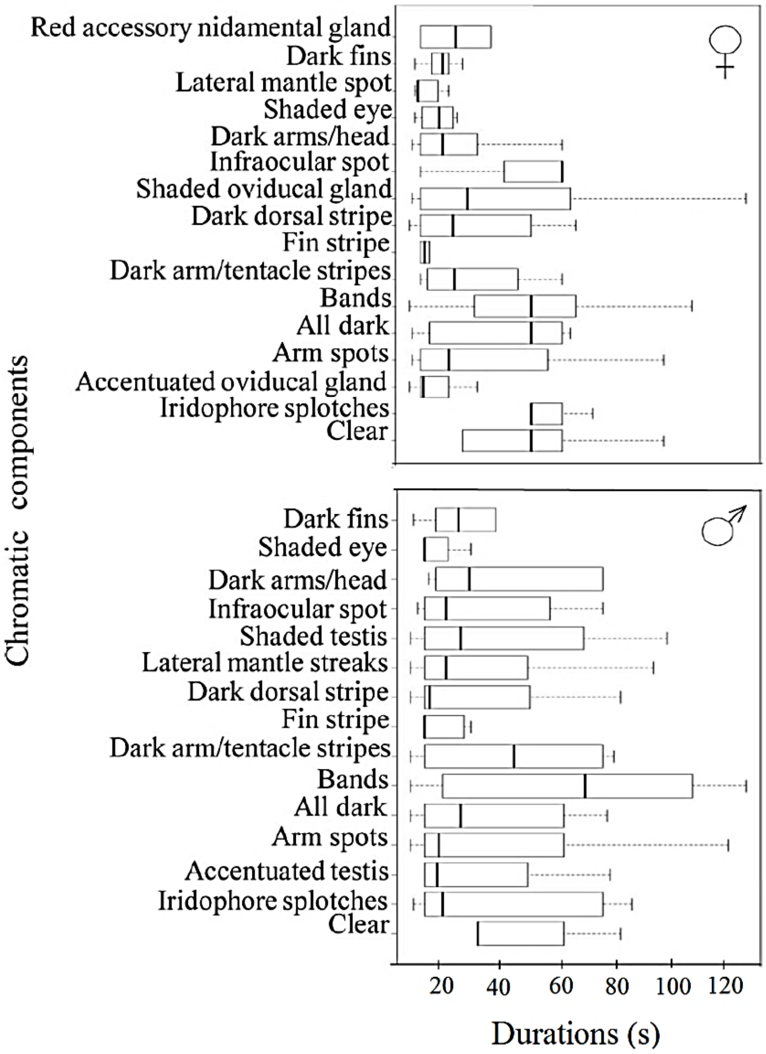


Figure 2.6: Duration of chromatic components identified for males and females of *Doryteuthis plei*. The solid black line represents medians; open boxes are 25% and 75% of the observations, and bars indicate the intervals of durations range.

Table 2.4: The pairwise comparison test for the significance difference in the average time [in seconds] of the expression of chromatic components between the factors: females and males, light and dark and among 19 components observed from 2011 to 2013 for *Doryteuthis plei* in South Brazil Bight. The observed differences higher than a critical value are considered statistically different at a given significance level ($P<0.05$).

Factors		Observed difference	Critical difference	P value
Females	Males	59.830	29.110	<0.05
Light component	Dark component	69.7656	31.272	<0.05
Clear	Accentuated Oviducal gland	311.958	163.567	<0.05
Clear	Arm spots	183.532	114.425	<0.05
Clear	Fin stripe	337.264	292.921	<0.05
Clear	Dark dorsal stripe	183.417	155.506	<0.05
Clear	Lateral mantle streaks	211.561	159.289	<0.05
Clear	Lateral mantle spot	384.121	304.739	<0.05
Iridophore splotches	Accentuated Oviducal gland	276.297	167.525	<0.05
Iridophore splotches	Arm spots	147.871	120.014	<0.05
Iridophore splotches	Fin stripe	301.604	295.149	<0.05
Iridophore splotches	Lateral mantle streaks	175.900	163.350	<0.05
Iridophore splotches	Lateral mantle spot	348.460	306.881	<0.05
Bands	Accentuated Oviducal gland	320.248	174.072	<0.05
Bands	Arm spots	191.822	128.996	<0.05
Bands	Fin stripe	345.554	298.914	<0.05
Bands	Dark dorsal stripe	191.706	166.521	<0.05
Bands	Lateral mantle streaks	219.850	170.059	<0.05
Bands	Lateral mantle spot	392.410	310.504	<0.05
Infraocular spot	Accentuated Oviducal gland	284.110	251.086	<0.05

3.1.3. Postural component

Drooping arms in a swimming squid is a posture in which all of the arms appear relaxed and hang downwards. This component preceded catching prey and occurred soon after relaxation. The squid maintains rigid arms, pointing at prey before capturing it. A splayed arm is a posture in which all eight arms are spread and flattened on the horizontal plane. This posture was expressed by both sexes when the chromatophores on the sides of the arms could also be observed. It occurred when a squid was defending a resource such as an egg mop, or when in the presence of another male during courtship or mating. The raised-arms posture was a strong signal of alarm, and was used when a rival male was near. It also occurred when the animal was detected by a predator; it assumed a threatening posture by raising its arms to another animal. It was rarely observed in females.

The downward curling posture consists of all of the arms and tentacles curled slightly downward at 90 degrees. It is also accompanied by four transverse bands on the mantle (Figure 2.3G). This posture was observed in aggressive encounters and in courtship, and it was usually assumed at the bottom of the tank, next to the substrate. Females were more likely to use this posture. The J-posture is characterized by raised arms at an angle of approximately 45 degrees, resembling the letter “J”, such that the tips were close to the anterior dorsal margin of the mantle. This posture relates to defense and alarm, it was observed in both sexes, and it lasted 5–7 s.

3.2.Body patterning

3.2.1. Chronic patterns

Patterns in this category can last for seconds or minutes. For example, when squids were calm, they had a clear body pattern and the chromatophores were retracted over the mantle. This patterning leads to chromatic components such as the dorsal stripe, arm spots, and the iridophore splotches located on the mantle, fin, and head. These patterns were observed frequently in normal laboratory conditions. Calm animals were usually swimming forwards and backwards, or swimming in place. This is described by the locomotor components, free swimming and hovering, and is when the postural component of drooping arms occurred.

The bands body pattern was associated with alert or alarmed behavior and can be considered a chronic pattern because it occurred frequently for period ranging from 20 s to 1 min. This pattern can take place when prey or predators are near or when the squid is alone. This body pattern occurred together with other chromatic components (see the male in Figure 2.3F, showing a band with arm spots; and females in Figure 2.3G, with bands, dorsal iridophore sheen, and downward curling).

The all-dark body pattern was considered chronic because, during the nighttime observation periods, the squids were mostly totally dark or brown (90% of the night observations). In two situations during the daytime observation period, an entire school of squids appeared all dark. Video analysis showed that one squid darkened when it detected a predator, prey, or observer above the tank. This event caused all of the squids in the school to become all dark for 20 s to 2 min. At night, the squids were all dark and moved fast together, using the jetting locomotor component. Jetting occurred when the squid was alone or when prey in a tank was larger than 50% the size of the mantle; at these times, the squid likely believed the prey to be a potential predator. The all-dark component was also used for hunting live prey (fish) or as camouflage at night.

3.3.Reproductive behaviour

3.3.1. Acute patterns

These patterns occurred quickly and were linked to intra- and interspecific interactions during reproductive behavior, such as agonistic behavior during fights for a mate or during courtship, mating, spawning, and egg touching. These body patterns occur for seconds only.

Figure 2.7 summarizes the behavioral sequence observed during the study. The dashed arrows represent the flow of behaviors over time. After the animals’ acclimation period (1 or 2 d), the observed behaviors became more complex. During the reproductive behaviors, combination of chromatic components was noted in agonistic activity, including arm spots and lateral mantle streaks (only males). Mating activity included accentuated testis and oviducal gland, red accessory nidamental gland, mantle spot in females, and shaded testis, observed together with the locomotor components of parallel positioning, fin beating, courting pairs, and oviposition (during the spawning process).

Agonistic behaviour

In the first days of observation, a male established dominance and then protected the females from other males in disputes. During agonistic behavior displays, multiple chromatic components occurred, such as arm spots, lateral mantle streaks (only males), infraocular spot, dark fins, and arms/tentacular stripes together with fin beating, chase (winner), and flee (loser) accompanied by raised arms or splayed arms (Figure 2.3O). The lateral display patterns were easily observed immediately after the clear body patterning. The displays occurred in a fraction of a second and were repeated two or three times.

The dark flashing pattern occurred in a situation of high stress or an alarm signal during spawning and egg touching, and in response to non-specific threats such as the presence of people around the tank or noises made nearby. A strong alarm signals the squids to darken completely and to assume the J-posture component in both sexes. After mating, the females swim using jet propulsion; their mantle color is completely dark. The jetting is of short duration.

Courtship behaviour

The display of gonads body pattern was the most often shown pattern for reproduction during courtship behavior. Males and females formed pairs, during which the male pursued the female and protected her from other males. Video analysis showed that this behavior occurred 159 times, at an average of two events per day. In most cases (90%), the male began courtship by pairing with a female and moving forwards and backwards in parallel positioning for a lengthy period (> 3 min); the reproductive gonads are highlighted between the animals, accompanied by slight touching. However, less frequently, females initiate courting by swimming upward.

Mating behaviour

Two types of mating were observed in our study, and each differed with regard to animal positioning, duration, and frequency (Table 2.5). Head-to-head mating was most commonly observed (*n* = 18), lasting 5–41 s (mean = 17 ± 10.24 s, s.d.). In this position, the body pattern was dark, the mate category was “sneakers”, and females showed faster displays. Male-parallel mating occurred less often ($n = 4$), and it lasted 10–15 s (mean = 12.2 ± 2.06 s, s.d.). During this type of mating, the fourth pair of male arms was totally dark. The mating category was “consorts”.

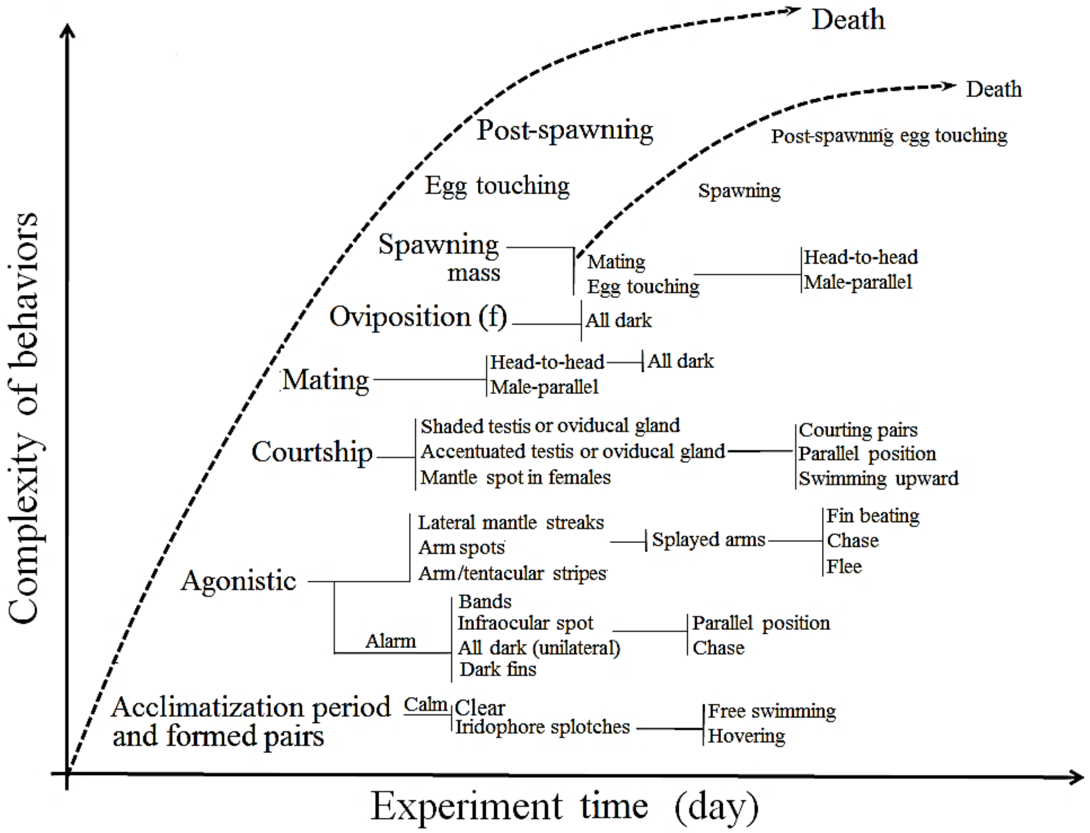


Figure 2.7: Schematic representation of the observed behaviors of *Doryteuthis plei*. Dashed arrows represent the flow of behaviors over time.

Table 2.5: Types of mating and their characteristics observed for *Doryteuthis plei*: day period (daytime, nightly), presence of light (yes, Y; no, N), temperature (T, °C), salinity (sal, ppt), percentage of dissolved oxygen (% DO), duration of mating (s), squid in the tank (n), number of females (f), and number of males (m)

Data	Period	Light	T °C	Sal ppt	% DO	n (f - m)	Mating type	During of mating (s)	Mate category
13/11/2011	Daytime	N	27.2	35.2	81.9	8 (4 - 4)	Head-to-head	15	Sneakers
13/11/2011	Daytime	N	27.4	35.2	77.3	8 (4 - 4)	Head-to-head	25	Sneakers
13/11/2011	Daytime	N	27.4	35.2	77.3	8 (4 - 4)	Head-to-head	06	Sneakers
13/11/2011	Nightly	Y	27.1	35.1	77.4	8 (4 - 4)	Male-parallel	15	Consort
13/11/2011	Nightly	Y	27.1	35.1	77.4	8 (4 - 4)	Male-parallel	12	Consort
13/11/2011	Nightly	Y	27.1	35.1	77.4	8 (4 - 4)	Head-to-head	13	Sneakers
13/11/2011	Nightly	Y	27.1	35.1	77.4	8 (4 - 4)	Head-to-head	14	Sneakers
13/11/2011	Nightly	Y	27.1	35.1	77.4	8 (4 - 4)	Head-to-head	08	Sneakers
13/11/2011	Nightly	Y	27.1	35.1	77.4	8 (4 - 4)	Head-to-head	09	Sneakers
14/11/2011	Daytime	Y	26.0	35.4	83.2	7 (3 - 4)	Head-to-head	17	Sneakers
17/11/2011	Daytime	Y	24.4	35.5	86.1	4 (2 - 2)	Head-to-head	20	Sneakers
17/11/2011	Nightly	N	24.4	35.5	86.1	4 (2 - 2)	Male-parallel	10	Consort
17/11/2011	Nightly	N	24.4	35.5	86.1	4 (2 - 2)	Male-parallel	12	Consort
16/03/2012	Nightly	N	27.50	35.00	87.6	10 (5 - 5)	Head-to-head	05	Sneakers
17/03/2012	Daytime	N	26.70	34.80	93.3	10 (5 - 5)	Head-to-head	10	Sneakers
18/03/2012	Nightly	Y	24.90	34.90	95.1	7 (5 - 2)	Head-to-head	11	Sneakers
19/03/2012	Daytime	N	24.90	34.70	97.4	6 (4 - 2)	Head-to-head	25	Sneakers
19/03/2012	Nightly	Y	25.90	34.70	90.5	6 (4 - 2)	Head-to-head	41	Sneakers
19/03/2012	Nightly	N	25.90	34.80	90.1	6 (4 - 2)	Head-to-head	37	Sneakers
20/03/2012	Daytime	N	26.30	34.90	87.3	5 (3 - 2)	Head-to-head	12	Sneakers
20/03/2012	Nightly	N	27.00	34.80	90.1	5 (3 - 2)	Head-to-head	21	Sneakers

4. Discussion

The reduction and internalization of the shell is a key trait in the evolution of cephalopods. It has allowed for an active life in the water column and an ability to compete with the vertebrates (Packard, 1972). But this lifestyle has also made these animals more exposed and vulnerable to predatory attacks. In response, they have developed sophisticated mechanisms for camouflage that include the use of chromatophores (Messenger, 2001). A system of neurally controlled chromatophores is supremely well adapted for signaling, and many shallow-water cephalopods also use the chromatophores to make visual signals, both inter- and intraspecific (Hanlon and Messenger, 1996; Messenger, 2001).

This study is the first concerted effort to analyze the behavioral components of *Doryteuthis plei* in the South Brazil Bight and in the Southern Hemisphere. The behavior of *D. plei* is complex and presents a variety of chromatic, postural, and locomotor components. Chromatophores can alter visual appearance in response to stimuli (Hanlon and Messenger, 1996). For example, color changes occurred when a male intruder approached a dominant male to fight for a mating female. During the study, it was possible to observe that the light chromatic components (clear and iridophore splotches) had a longer duration than the dark chromatic components, especially those associated with calm behavior. Squid chromatophores are neurally controlled, allowing the animal quickly to select and demonstrate various body patterns. With this quick polymorphism, squids can rapidly hide from predators.

The locomotor components include a variety of movements using the siphon, arms, and fins. However, more attention is required to observe the postural components because they involve the body's position and arms. The locomotor and postural components found in this study were earlier described for other squids (Hanlon, 1978; Hanlon et al., 1983, 1994, 1999, 2000, 2002; Hanlon and Messenger, 1996; Jantzen and Havenhand, 2003; Buresch et al., 2004; Pham et al., 2009; Shashar and Hanlon, 2013).

Most of the chromatic components observed in this study occurred during diurnal periods, which can be explained by the difficulty of night observations due to the lack of light in the tank. When the LED light was turned on in the tank, the squids kept to the periphery of the light and no deaths were observed. Several of the 19 chromatic components identified in our study of *D. plei* in the Southern Hemisphere were identical to those previously described for other loliginids, including *Loligo vulgaris* (Hanlon et al., 1994), *Doryteuthis pealeii* (Hanlon et al., 1999), and *Doryteuthis opalescens* (Hunt et al., 2000). However, in comparison to *D. plei* in the North Atlantic (see Hanlon, 1982, 1988; Hanlon et al., 1983), a greater variety of chromatic components was observed during our study. Hanlon (1982) describes only 16 chromatic components in *D. plei* from the North Atlantic (USA). However, the chromatic components that were observed in our study in relation to the sexual maturity of females were not reported by the author studying *D. plei* of the North Atlantic (*e.g.*, accentuated oviducal gland; (Figure 2.3J)); dark arms/head, dark fins, and infraocular spot components also were not mentioned. Possibly, maintenance conditions, such as color, depth, and bottom type of the tank used in this study, influenced the observed patterns of skin coloration of the squids.

Body patterns and behaviour

Calm behaviour

Calm behavior in this species was scored when squids did not haphazardly strike the tank walls, avoiding significant damage to skin and fin (Hanlon et al., 1983). Through video analysis, we observed that at the beginning of each maintenance period, squids adapted to the tank conditions and showed calm behavior. This state was also easily identified through the clear chromatic component and free swimming. During parallel positioning, a calm state was noted when the postural component was relaxed, with drooping arms; squids appeared to be at ease, and there was no threat in the tank. The clear chromatic component was the most frequently observed display in this study (see Table 2.3). Our observations support the notion that the *D. plei* squid mantle is transparent or has a pale coloration. Unlike the all-dark pattern, this pattern (*i.e.,* clear pattern = calm and all-dark pattern = alarmed) has been found for this species (Boycott, 1965; Hanlon et al., 1983) and other loliginids around the world (*e.g., Loligo forbesi* in Europe, Porteiro et al., 1990; *Loligo vulgaris reynaudii* in South Africa, Hanlon et al., 1994; *Doryteuthis pealeii* in Massachusetts, Hanlon et al., 1999; *Doryteuthis opalescens* on the California coast (USA), Hunt et al., 2000; and *Sepioteuthis australis* in Australia, Jantzen and Havenhand, 2003).

Alarm behaviours

During the dark observation period, alarm and jetting behaviors were observed through the expression of all chromatophores on the mantle. This component can also be used as camouflage to catch prey at night. When large or uncommon prey was placed in the tank, it caused a rapid expansion of chromatophores, turning the squid all dark during the day period. However, this pattern is also used intraspecifically during agonistic encounters and between males and females when one squid is alarmed (Boycott, 1965; Hanlon, 1978, 1982; Hanlon et al., 1983, 1994, 1999). Downward curling and J-posture were less frequently observed postures, but downward curling (Figure 2.3H) was noted more often than the J-posture. Both postures were observed in males and females, and were related to aggressive behavior or alarm. These two postural components are commonly observed among other squids. The J-posture was reported in *D. opalescens* (Hunt et al., 2000) and *Lolliguncula brevis* Blainville, 1823 (Martins and Perez, 2006). Other equivalent components are found in the following squids: *Ornithoteuthis antillarum* Adam, 1957 (Vecchione and Roper, 1991); the J-curl in *Gonatus onyx* Young, 1972 (Hunt and Seibel, 2000); arms flexed dorsally in *Octopoteuthis megaptera* Verrill, 1885 (Vecchione et al., 2002); upward curl in *Sepioteuthis australis* (Jantzen and Havenhand, 2003); and dorsal arm curl in the deep-sea squid *Octopoteuthis deletron* Young, 1972 (Bush et al., 2009). This posture was related to deimatic behavior in cephalopods (Hanlon and Messenger, 1996). Downward curling has been reported for *D. plei* (Hanlon, 1978) and other loliginids (Hanlon et al., 1994, 1999; Hunt et al., 2000; Jantzen and Havenhand, 2003).

Reproductive behaviour in D. plei

The reproductive behavior in *D. plei* includes a variety of skin colors, movements, and postures (Hanlon et al., 1983; DiMarco and Hanlon, 1997). Shoaling squids have ample opportunity for social communication with conspecifics throughout most of their lives, and some species have established elaborate behavioral sequences, including agonistic, courtship, and mating behaviors (Hanlon and Messenger, 1996).

Agonistic behaviour

In our study, the male initiated courtship and immediately established and maintained a dominant relationship over females. The fighting between large males was a conspicuous event during their reproductive behavior. The behaviors also included threats, chases, and fleeing during fin beating; together with the presence of lateral mantle streaks and arm splotch, these behaviors are easily identified. The most noted posture that we observed is splayed arms during agonistic behavior. The locomotor component of fin beating is also easily recognized and represents the escalation of an agonistic encounter by involving physical contact (Porteiro et al., 1990; Hanlon and Messenger, 1996; DiMarco and Hanlon, 1997; Hanlon et al., 2002; Jantzen and Havenhand, 2003; Pham et al., 2009; Shashar and Hanlon, 2013). The courtships are interrupted by large, lone males or intruders, as previously reported (DiMarco and Hanlon, 1997; Hanlon et al., 2002), that engage the paired consorts in agonistic contests, often resulting in successful takeovers. The agonistic behavior of this species was described in detail by DiMarco and Hanlon (1997), who observed various aspects of the behavior mainly in laboratory studies. This behavior occurred similarly in other loliginids (Hanlon et al., 1994, 1999; Hunt et al., 2000).

Courtship behaviour

In this study, most squids formed mate pairs (females and males), and the duration of mate pairing lasted for a long period. For example in the experiment of November 2011, we observed that in the first two d of maintenance, the squids performed free swimming inside the tank. However, they formed pairs by Day 3. The pairs were generally formed after the agonistic contests. In females, the red accessory nidamental gland and oviducal gland were often visible, as in some species of *Loligo* (Hanlon et al., 1994, 1999, 2002; Hunt et al., 2000).

Mating behaviour

Doryteuthis plei mating in this study occurred in two positions and was similar in time and positioning to that reported for other loliginids (Hanlon et al., 1994, 1999; Sauer et al., 1997; Jantzen and Havenhand, 2003; Zeidberg, 2007; Sharsha and Hanlon, 2013). The first position was head-to-head mating with a sneaker male, which was observed more often than the male parallel position with a large consort male. Hanlon (1998) and Hanlon et al. (2002) examined the behavior of *Loligo* sp. and *Loligo vulgaris reynaudii* in South Africa. They used the term “sneakers” for the smaller males and consorts for the larger males that formed different reproductive disputes during spawning on the seafloor. The male-parallel mating always occurred soon after eggs were deposited on sand at the bottom of the tank. Waller and Wicklund (1968) observed a larger natural spawning shoal in the sea, noting that nearly all squids were paired and mating in the male-parallel position was followed almost immediately by egg laying (oviposition). Shashar and Hanlon (2013) detailed the multiple mating tactics during copulation for *Doryteuthis pealeii.*

Egg-directed behaviour

The oviposition component was rarely observed in the filming (*i.e.,* only twice). Egg deposition of females occurred in a completely darkened lab or in the early hours of the day. Egg depositions that occurred during the night were filmed with the aid of an LED light. The egg mop developed rapidly, and the first paralarvae appeared 10 d after eggs were deposited at the bottom of the tank. This result is similar to findings reported by Roper (1965) for this species. Oviposition was also observed in shallow waters during field studies of this species (Waller and Wicklund, 1968), for *D. opalescens* in Monterey Bay California USA (Hanlon et al., 2004), and for *Loligo vulgaris reynaudii* in South Africa (Sauer and Smale, 1993; Hanlon et al., 1994). However, egg touching was observed for long periods among *D. plei* males and females. Egg touching is common in captivity and can be artificially stimulated by inserting an egg capsule, as described for *D. pealeii* by Arnold (1962). This action was most common in males guarding an egg mop.

5. Concluding remarks

This study showed the first findings about the body patterning behavior of *Doryteuthis plei* in the Southern Hemisphere. Currently, it represents the only ethogram with quantitative analysis for a myopsid cephalopod in South America. Our results showed that most behaviors observed for *D. plei* are similar to those of other squids around the world, both in captivity and in the field. However, some differences were found between *D. plei* investigated here and previous studies in the North Atlantic. For example, the chromatic component of the female accentuated oviducal glands was readily observed during pairing or courtship in our study (Figure 2.3J). However, the glands were not observed by Boycott (1965) in Bermuda, in natural habitats in the Bahamas (Waller and Wicklund, 1968), or in captivity in Massachusetts (Hanlon, 1982, 1988; Hanlon et al., 1983). We did not observe chromatic components such as infraocular spots or dark arms and head for long periods, as was reported only for *Loligo vulgaris* (Hanlon et al., 1994) and *Doryteuthis pealeii* (Hanlon et al., 1999).

Recent phylogeographical studies of *D. plei* have suggested that the Brazilian population is genetically distinct from *D. plei* in North and Central America (Sales et al., 2013). For this reason, behavior is another attribute that can assist in the taxonomic identification and phylogenetic analyses (Hanlon, 1988; Hanlon et al.,1999). The genetic description, coupled with detailed behavioral aspects such as those reported in this study, should provide insights into the variability of reproductive behaviors and the potential for differences between the various populations of *D. plei* in the Atlantic Ocean.

In summary, the squid *D. plei* has a large repertoire of body patterns, including many combinations of skin coloration, body postures, and swimming movements, that are used specifically for communication during reproductive behavior. Our results showed that the duration of each chromatic component differed significantly, suggesting that these components are connected to behaviors performed during the short life cycle, *e.g.*, agonistic behavior or mating. Females displayed the chromatic components for longer durations than the males, which may have been the result of their calm behavior or display of their reproductive gonads. The males were dedicated to winning the females in the first days of the observation periods. Head-to-head mating was more frequent and longer lasting than male-parallel mating, and egg-directed behaviors occurred during night periods only.

Our evidence supports the theory that the elaborate sensorial system in cephalopods, allowing for rapid chromatophore activity and skin-based communication skills for intra- and interspecific relationships, is complex and highly evolved, even in small-size nektonic species. The particularly ritualized reproductive behavior found in *D. plei*, with the gonadal displays during courtship and the immediate expansion of some groups of chromatophores and retraction of others, seems to be one of the most complex and interesting body patterning behaviors noted in the marine realm.

Chapter 3

Ontogenetic, sexual, and spatial-temporal shifts in the diet of the squid *Doryteuthis plei* off São Sebastião region, SE Brazil.

Chapter 3 - Ontogenetic, sexual, and spatial-temporal shifts in the diet of the squid *Doryteuthis plei* off São Sebastião region, SE Brazil.

Abstract: *Doryteuthis plei* has been considered an active predator on several fish, squid, and crustaceans' species in Southeastern Brazil, but details about ontogenetic shifts by sex and spatio-temporal variations of diet composition have not been studied to any great degree. With the aim of identifying those particular aspects of feeding habits, a robust statistical analysis using a Generalized Additive Model for Location Scale and Shape (GAMLSS) was applied to a set of stomach contents data obtained from squids sampled from 2003 to 2012. Complementary observations from adult squid were obtained from laboratorial conditions. Diet differed in prey composition among sexes, size, maturity, and spatio-temporal pattern. A high non-linear correlation between full stomach weight and maturity was found for females, especially in mature individuals. Females do not stop feeding during sexual maturation and the amount of food increases with size. The models evidence that squids were significant prey for mature females from 142 to 213mm of mantle length (ML), as well as fish and crustaceans to the largest females up 195mm ML, while pelagic polychaetes were dominant prey for largest males (222-299 mm ML). In general, polychaetes and crustaceans were most found in squids from the Southern portion of the São Sebastião Island, in January and February, while crustaceans were more consumed in the Northern side, in November. Teleost fishes were the main prey in both day and night samples and cephalopods were more frequent than crustaceans in stomach contents collected at night. Piscivory on small pelagics fishes, cannibalism, and ontogenetic shifts found here corroborate the experimental observations in captivity. Also these findings highlight differences between squid males and females not evidenced before. The analysis of a long dataset with robust statistics allowed to show yet unrevealed patterns of the tropical arrow squid feeding habits.

Keywords: Cephalopoda, feeding habits, GAMLSS, food, predation

1. Introduction

Squids are voracious predators, characterised by rapid growth rates, being also important prey found in the stomachs of a wide variety of fishes, marine mammals and seabirds in ocean and coastal environments (Clarke, 1996; Jereb and Roper, 2010). They may also play a crucial role in food webs (Boyle and Rodhouse, 2005) and as a keystone marine species (Gasalla et al., 2010). This was the case in the South Brazil Bight (SBB) marine ecosystem where the loliginid squid *Doryteuthis plei* (Blainville, 1823) was found to play such a keystone local role (Gasalla et al., 2010). This species is also one of the main cephalopod currently targeted by some regional fisheries (Perez, 2002; Gasalla et al., 2005; Postuma and Gasalla, 2010).

Squids seem to perform changes in dietary habits, especially during the transition from subadult (maturity scale I and II) to adult (III and IV), and also when they change their habitat (Sauer and Lipiński, 1991; Robin et al., 2014; Vidal et al., 2014). Some loliginid diets reported around the world show that these species may be piscivorous and opportunistic, including crustaceans and polychaetes in their diet (Fields, 1965; Karpov and Calliet, 1978; Yang et al., 1983; Sauer and Lipiński, 1991; Pierce et al., 1994; Hunsicker and Essington, 2006; Gasalla et al., 2010), however, some species like *Doryteuthis gahi* prey principally on crustaceans (*e.g.* amphipod and euphausiid), fish being of secondary importance (Guerra et al., 1991). Some studies have also reported ontogenetic shifts in the diets of squids. For example, in *Loligo forbesi* and *Loligo vulgaris* in Scottish waters, crustaceans and teleost occurred together more often in the stomachs of smaller squid, whilst in contrast, only teleost became increasingly important in the diets of larger squid (Pierce et al., 1994) where cannibalism was also found (Sauer and Lipiński, 1991; Yang et al., 1983; Ivanovic and Brunetti, 1994; Collins and Pierce, 1996; Ibánez and Keyl, 2010; Wangvoralak et al., 2011). In general, feeding in several species of the Family is well documented and indicate that coastal areas are of the used as feeding grounds by mature loliginid squids (*e.g.* Martins, 1982; Sauer and Lipiński, 1991; Santos and Haimovici, 1998; Martins and Perez, 2006; Gasalla et al., 2010). The digestion process of the loliginid squid seems very rapid, being completed in as little as 4 hours in the *Sepioteuthis lessoniana* (Semmens, 2002) for example, and also Bidder (1950) mentioned that *Loligo vulgaris* and *Loligo forbesii* showed that the exceptionally speedy and efficient digestive mechanism, well fitted to the life of a perpetually swimming, active predator.

Studies on feeding of *D. plei* in Brazil have described some spatial and seasonal patterns in diet through some feeding indices (Juanicó, 1979; Martins and Perez, 2007; Gasalla et al., 2010). However, the diet differing in prey composition among sexes, size, maturity and spatio-temporal patterns have not been studied to any great degree. The aim of the present study was to identify particular aspects of feeding habits, such as ontogenetic, sexual, and spatial-temporal shifts, based on the following questions: What is the relationship between prey type and the size-structure of females and males squid? What are the ontogenetic shifts in diet compositions during maturation and maturity? What is the spatio-temporal pattern of prey dominance during the fishing season in the study area?

2. Material and methods

2.1. Study area

The São Sebastião Island (SSI) is a sub-tropical island located between latitudes 23°70 -24°00S and longitudes 45°50 and 45°00W in the SBB marine coast. Over the region, small-scale hand-jigging fishing operations for squid occur at a depth of between 5 m and 30 m, mainly close to the shore and in the SSI's sheltered bays, but also close to smaller islands such as Búzios and Vitória (Figure 3.1).

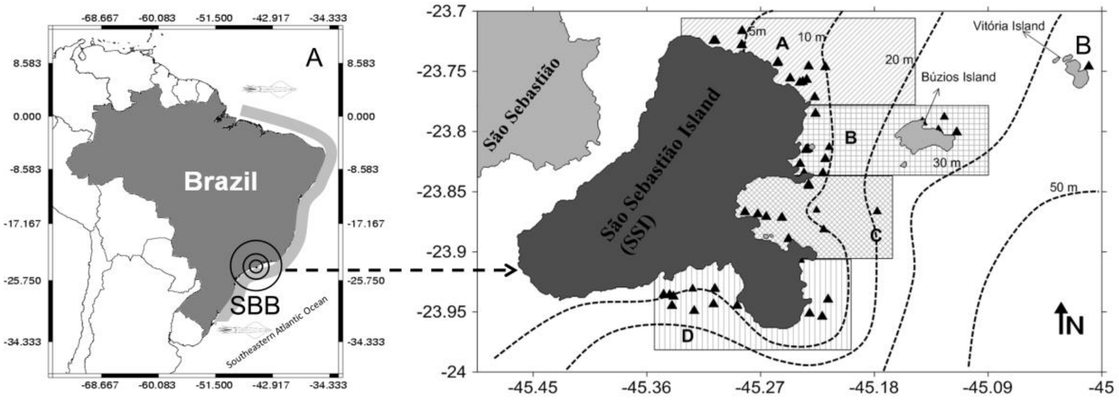


Figure 3.1: Study area. São Sebastião Island, on the South Brazil Bight marine coast, in Western Atlantic Ocean. The stars represent the fishing spots in four typically fishing areas: A) North, B) Northeast, C) Southeast and D) South

2.2. Squid sampling

Samples were collected weekly from the landings of the hand-jigging fishery operating around São Sebastião Island, between November and April across six fishing seasons from 2003 to 2012. A total of 182 landings in the municipalities of São Sebastião (92%) and Ilhabela (8%) were monitored, from which 139 biological samples were collected, totalling 200 kg of squid. The dorsal mantle length (ML) in mm and total body weight (BW) in g were recorded for each squid. Squid dissection was conducted by cutting longitudinally the ventral part of the mantle to expose the visceral mass, and the stomach contents were removed. Table 3.1 summarizes the total sample of squid and the sum of the stomachs from the fishing seasons that were investigated.

Table 3.1: Number of specimens and stomachs collected between 2003-2012, per sex and fishing season.

	Females		Males	
Fishing season	Specimens	Stomachs	Specimens	Stomachs
2003-2004	143	15	170	38
2004-2005	188	3	117	7
2006-2007	206	13	113	8
2008-2009	182	22	149	23
2010-2011	211	46	205	38
2011-2012	211	46	189	37
Total	1289	145	1062	151

The diet composition of *D. plei* was identified by means of stomach-content analysis. Fisheries information related was obtain through interviews to covering fishing areas (Fa), fishing depths (Fd) and fishing dates, which allowed for the identification of both fishing months (Fm) and fishing seasons (Fs). Stomachs containing food remains were examined under a stereomicroscope. The contents were sorted into four biological prey groups (teleost, cephalopods, crustaceans, and polychaetes), and the weight (g) and frequency of occurrence in each prey were recorded. Because of the level of digestion of the prey, teleost were identified mainly by the presence of otoliths, scales, vertebrae, and ocular globes in the stomach contents. Teleosts were identified to the lowest taxon possible, consulting local unpublished identification guides and an otolith reference collection (Rossi-Wongtschowski et al., 2014). The otoliths were sorted into left and right otoliths, and mostly the left otoliths were counted (Kubodera et al., 2007). The number of otoliths (right or left) was used to represent the number of fish prey. The maximum diameters of fish otoliths were also measured to estimate body sizes (Standard Length [SL] for fish), using relationships generated from intact specimens of the prey items (Rossi-Wongtschowski et al., 2014). Crustaceans were identified from eyes, exoskeletal remains and appendages, and cephalopods from beaks, ocular globes, statoliths, arms, and sucker rings. The statoliths found in stomach contents were measured to obtain the size of the prey consumed, using the log-equation (Barcellos and Gasalla, 2014) for *D. plei* in the study area, ($y = -0.4842 + 0.4049*\log(x)$), where y = Statolith length (mm) and x = mantle length (mm).

2.3. Data analysis

Stomach content data were spatialized and mapped with Surfer 8.0 software, using kriging as the geostatistical gridding method selected. In the laboratory, the squid were sexed and the sexual maturity-scale (I-immature, II-in maturing, III-mature and IV-spent) was determined macroscopically according to Juanicó, (1983) and modified by Perez et al., (2002), whilst the mantle length (ML, mm) was also recorded.

The frequency of occurrence of prey was adopted for the analysis of stomach contents (Hyslop, 1980) $\underline{\%FO}=(Ni/Nt) \times 100$, where *Ni* is the number of stomachs containing a prey type and *Nt* the total number of non-empty stomachs and percentage of total prey weight $\underline{\%W}=(Wi/ Wt) \times 100$, where *Wi* is the weight of a certain individual prey and *Wt* the total weight of all the prey. The percentage of prey total number $\%N=(Nj/N) \times 100$, where *Nj* is the total number of prey item *j* and *N* the total number of prey. The finally, the index of relative importance (*IRI*) (Pinkas et al., 1971), which measures the importance of each prey item relative to other prey by taking into account the weight and the number of each prey item and the frequency at which each is found in the diet. *IRI* was calculated for prey items i using the equation: $IRIi = (\%Ni + \%Wi) \times \%Fi$. The *IRIi* of each major group was then standardized to $\%IRIi=(IRIi/\sum_{i}^{n} IRI)\times 100$ (Hacunda, 1981), where n is the total number of groups identified.

Generalized Additive Model for Location Scale and Shape (GAMLSS) were implemented using a series of packages in R (R team, 2013). Two set of models were applied to analyse the correlations of the response variables, such as "stomach content weight" (Scw) and the "weight of each prey type" [being teleost (T), crustacean (Cr), cephalopod (Ceph), and polychaetes (Polq)]. In the first models the exploratory variables were: mantle length (ML in mm) and maturity-scale (Mat), and in the second models the spatial and temporal factors of the small-scale fishing, such as fishing area (Fa), fishing month (Fm), fishing season (Fs) and daylight/night fishing were used. Thus, the first models were defined: (Sc/T/Cr/Ceph/Polq ~ cs (ML mm; *df*=4) + (Mat), where Mat is the factor. The data were analyzed separately for males and females. Moreover, the second models were used to determine the level of significance of the response variables with the spatial and temporal factors of the fishing variability. Thus, the model used was: (Sc/T/Cr/Ceph/Polq ~ cs(Fd m; *df*=4) + (Fa) + (Fm) + (Fs) + (daylight/night). In these last models the data from male and females was pooled. For a partial plot of the GAMLSS we use the following function to find the intersection point of the curve: $f=(coef\ (x)*\ x-mean(x))\ +mu.s$, where *(coef)* represents the coefficient of the exploratory variable *(x)*, and (mu.s) is the vector of initial values for the location parameter in the model.

The distribution used was the Zero Adjusted Inverse Gaussian (ZAIG) for the model fitting. The variance inflator factor (VIF) was used to test co-linearity between variables in the GAMLSS (Montgomery and Peck, 1992). VIF values greater than 3 indicated co-linearity, following the recommendation made by (Zuur et al., 2010). To determine significance differences in diet of males and females the Chi-square test was used.

The best fitted models were selected based on the Akaike Information Criterion (AIC), Schwarz's Bayesian criterion (SBC) and Global Deviation (GD), and was also based on a distribution of residuals. The significance of each term was assessed using the "drop1" function, and their relative importance was assessed according to the AIC, likelihood-ratio test (LRT) and probability of the Chi-squared test criteria (PrChi) obtained (Moltschaniwskyj et al., 2010).

2.4. Maintenance and observations of squid in laboratory.

Foraging behaviour was obtained from direct observations and video recordings of squid under laboratory conditions. Seventy-eight specimens of *D. plei* were obtained using hand-jigs and Japanese-style pound nets (“Kaku-ami”) off the Ubatuba coast (latitude 23°51’S, longitude 45°08’W) in a depth of < 10.9 m. Additional samples were collected in São Sebastião (23°83’S, 45°44’W) in a depth of 6 m. The specimens were monitored for 62 days in five observation period conducted during the following times: November 2011 (15 days), February (7 days), March (14 days), November 2012 (13 days) and February 2013 (19 days). The observations were conducted with the aid of two circular tanks: a 2.3m diameter, 3000l tank with a closed seawater system, and a 1000l flow-through system tank with a diameter of 1.8m. The temperatures ranged from 21.86 to 28.81°C. The salinity ranged from 34.71 to 35.83 ppt and dissolved oxygen was no greater than 5.00 mg^3/l. The mean level of the total toxic ammonia from detailed chemical tests was 0.020 ppm, with a range of 0.012 to .0.035 ppm (n=15). Mean level of the total nitrite-nitrogen (NO_2) was 0.5 mg/l with a range of 0.02 to 3.05 mg/l (n= 15).

The squids were fed *ad libitum* twice daily with a variety of species of fresh or frozen fish, squid and crustaceans. The prey offered to squids as life food was from samples taken from the beaches (tidal pools) and near the sites of the tanks every day and in the early hours of the day (for six hours through until ten o'clock in the morning). The prey collected was transported to 300 litres tanks, where they were kept until they were offered squid.

The observations were made by viewing the tank from the top. The squid survival period in both systems averaged seven days and some animals survived for up to 19 days in February 2013. The prey offered during feeding were *Sardinella brasiliensis, Anchoa tricolor, Anchoa sp and Conodon nobilis* measuring from 45 mm to 89 mm. During the experiments, a number of factors, including water quality, space confinement, live feed, exposure to light and low noise and stress, were monitored to ensure the welfare of the squid used in this study, as recommended for cephalopods by (Moltschaniwskyj et al., 2010).

3. Results

3.1. Diet composition of *Doryteuthis plei*

In the total of 2 351 specimens of *D. plei* sampled, only 10% showed full stomach (n=298), 49% in females and 51% in males. The mean ML for females was 136.86 mm ML (+ /- 34.17 s.d.; range 30 mm-231 mm), while in males it was 198.05 mm ML (+ /- 62.46 s. d.; range 49 mm-390 mm). The BW in females tended to peak around 50 g, reaching a maximum of 162 g, while males peaked around 90 g, reaching a maximum of 297 g.

Of the 298 analyzed stomachs, 64% had some type with food remains. Overall, only 16% of the stomachs (n=48) had more than one type of prey, 41% with fish (used food remains vertebrae, otoliths, scales and ocular globes), and cephalopods (beaks, statolith, suchers ocular globes), 31% fish and crustaceans (eyes, exoskeletal remains and appendages), 16% fish and polychaetes (bristles), 6% fish, cephalopod and crustaceans, and 4% fish, crustaceans and polychaetes. Prey identified in the diet of *D. plei* is listed in Table 3.2, along with estimates of their relative importance (%IRI). Initially, the Chi-square test showed no significant differences between the of food indices between males and females. The frequency of occurrence (*%F*), number (*%N*), weight (*%W*), and the index of relative importance (*%IRI*) of each prey item in the diet of *D. plei* are listed in Table 3.2. According to the indices and %IRI the teleost fish was the most important prey, followed by crustaceans, cephalopods and pelagic polychaetes.

Stomach weight ranged from 0.04% to 17.3% of body weight, with the highest recorded weight being 6.13 g (85% of the stomach content) in a mature female with 47.64 g of body weight and 130 mm ML.

Overall, 57 otoliths were found in squid stomach contents from 2003 to 2012. However, only the 52 identifiable and 29 intact otoliths were used to estimate the size of fish prey and five otoliths were found in the same stomach. The lengths of otoliths ranged from 0.44 mm to 4.10 mm with the average of 1.53 mm, while the estimated fish prey sizes were 9.6 –76.3 mm for squid sizing 90 –184 mm ML. The smallest teleost fish was identified as *Trachurus lathami* (ranging from 9.6 mm to 51.8 mm) being found in a squid with 90 mm ML. The largest fish was identified as *Sardinella brasiliensis* in a squid with 184 mm ML (Figure 3.2).

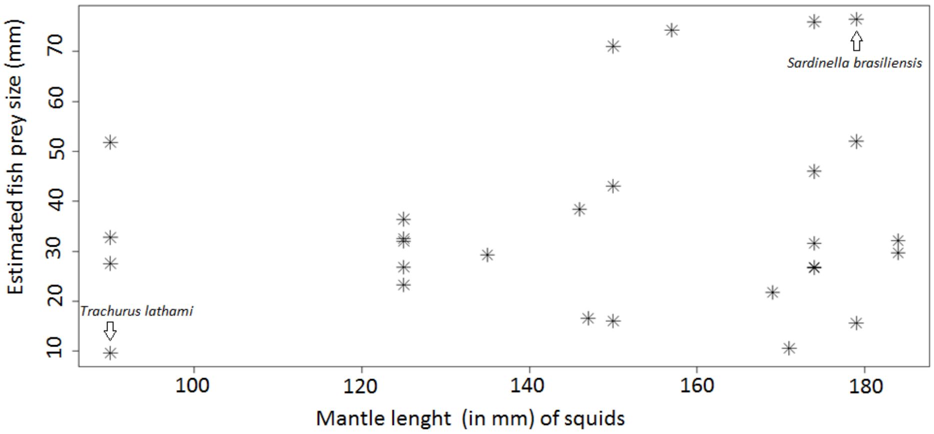


Figure 3.2: Relationship between the estimated size of the prey (in mm) and the size of squid (in mm) sampled in shallow waters of the São Sebastião Island.

Otoliths found in squid stomachs mostly belonged to Carangidae (*Trachurus lathami)* with *% IRI* of 0.129, followed by Engraulid (*Anchoa spp*) with 0.091 and Clupeidae (*Sardinella brasiliensis*) with 0.025. Otolith occurrence appeared to vary with each month, with the highest occurrence of otoliths being found in November, December and April. The otoliths were more frequent in the South and North area around the island. Two statoliths belonging to *Doryteuthis plei* were found. The length of statoliths so 1 mm, while the estimated prey sizes were between 35 mm and 50 mm ML found on stomach of a squid with 169 mm ML.

Table 3.2: Percentages of weight (*%W*), frequency of occurrence (*%FO*), (%N) number of prey type and (%IRI) and the index of relative importanceof the prey of the squid *Doryteuthis plei* identified from stomach contents.

Prey type	Dietary groups	Species	%W	%FO	%N	%IRI
(Family)						
Total Fish	Teleost		81.082	71.044	67.282	55.373
Fish unidentified	Teleost		71.150	54.209	62.154	37.962
(Sparidae)	Teleost	*Pagrus pagrus*	0.200	0.673	0.205	0.001
(Carangidae)	Teleost	*Decapterus punctatus*	0.117	0.673	0.205	0.001
(Sciaenidae)	Teleost	*Cynoscion jamaicensis*	0.170	0.673	0.205	0.001
(Sciaenidae)	Teleost	*Ctenosciaena gracilicirrhus*	0.603	1.010	0.308	0.005
(Carangidae)	Teleost	*Trachurus lathami*	3.321	5.051	1.538	0.129
(Engraulididae)	Teleost	*Anchoa* sp	2.633	4.377	1.333	0.091
(Carangidae)	Teleost	*Selene setapinnis*	0.765	1.347	0.410	0.008
(Clupeid)	Teleost	*Sardinella brasiliensis*	1.767	2.020	0.615	0.025
(Centropomidae)	Teleost	*Centropomidae*	0.356	1.010	0.308	0.004
Crustacea unidentified	Crustacean		11.098	22.222	23.077	3.990
Total Cephalopoda	Cephalopod		6.331	18.182	6.051	1.183
Cephalopoda unidentified	Cephalopod		5.839	16.835	5.949	1.043
(Loliginidae)	Cephalopod	*Doryteuthis plei*	0.492	1.347	0.103	0.004
Polychaeta unidentified	Polychaete		1.488	6.734	3.590	0.180

3.1.1. Feeding model of females

Approximately 11% of females were collected with full stomachs with mean ML of 142.48 mm (±25.21 s.d.; range: 90–230 mm). The mean stomach weight was 0.66 g (± 0.79 s.d; ranging 0.03 to 5.22 g) representing an average of 1.19% of body weight, ranging from 0.06% to 14.6%.

For the females, the ZAIG distribution provided the best fit for GAMLSS models in three biological prey groups (cephalopods, crustaceans and pelagic polychaetes). Residuals from the ZAIG distribution showed a better fit suggesting no evidence of heteroscedasticity (Appendix 3.2). Considering the full stomach content weight, the maturity-scale was the most significant explanatory variable according to the AIC and likelihood-ratio test (LRT value, Table 3.3). The results showed a positive relationship with from 151 mm onward, peaking at 231 mm ML (Figure 3.3A) and showing a high non-linear correlation in mature squid, decreasing slightly in spent individuals (Figure 3.3B and Table 3.4).

Table 3.3: The significance of each term of optimal GAMLSS models fitted to the full stomach weight, to four prey types: teleost, crustacean, cephalopods and pelagic polychaetes and the explanatory variable ML and maturity-scale of *Doryteuthis plei* around São Sebastião Island during the period November 2003 – April 2012. AIC = Akaike Information Criterion, LRT = Likelihood-ratio test and Pr(Chi) = probability of Chi squared test

		Females			Males		
Response variables	***Terms***	***AIC***	***LRT***	***Pr(Chi)***	***AIC***	***LRT***	***Pr(Chi)***
Full stomach weight (g)	*ML(mm)*	158.37	4.579	0.469	152.36	9.607	0.087
	Maturity-scale	174.86	17.066	**0.001***	147.82	1.07	0.784
Teleost weight (g)	*ML(mm)*	300.05	9.371	0.095	340.07	6.734	0.241
	Maturity-scale	308.61	13.056	**0.004***	337.73	0.389	0.942
Crustacean weight (g)	*ML(mm)*	2.459	11.881	**0.036***	-2.168	7.561	0.182
	Maturity-scale	-3.173	2.249	0.522	-5.089	0.643	0.886
Cephalopods weight (g)	*ML(mm)*	-68.442	9.545	0.092	-255.401	6.733	0.245
	Maturity-scale	-73.472	2.249	0.808	-257.51	0.62	0.891
Pelagic polychaetes weight (g)	*ML(mm)*	-788.83	9.092	0.105	271.6	9.395	0.094
	Maturity-scale	-787.05	6.875	0.076	267.52	1.313	0.725

**p value* <0.05

Table 3.4: Summary of optimal GAMLSS models for females fitted to the to the full stomach weight, to four prey types: teleost, crustacean, cephalopods and pelagic polychaetes and the explanatory variable ML and maturity-scale of *Doryteuthis plei* caught around São Sebastião Island during the period November 2003 – April 2012. Pr(>|t|)= probability of Student test.

	Terms	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
Full stomach weight (g)	***(Intercept)***	-0.914	0.626	-1.461	0.146
	ML (df=4)	0.006	0.004	1.575	0.118
	(II) in maturation	-1.373	0.540	-2.543	**0.012**
	(III) matures	-0.661	0.543	-1.217	0.226
	(IV) spent	-1.216	0.714	-1.703	**0.091**
Teleost weight (g)	***(Intercept)***	-0.577	1.012	-0.570	0.569
	ML (df=4)	0.002	0.005	-0.060	0.952
	(II) in maturation	-0.569	0.912	-0.623	0.533
	(III) matures	0.468	0.918	0.510	0.610
	(IV) spent	-1.336	0.974	-1.371	0.172
Crustacean weight (g)	***(Intercept)***	-0.080	0.139	-0.575	0.566
	ML (df=4)	0.001	0.001	0.927	0.355
	(II) in maturation	0.062	0.123	0.506	0.614
	(III) matures	0.050	0.123	0.405	0.686
	(IV) spent	-0.096	0.161	-0.597	0.551
Cephalopods weight (g)	***(Intercept)***	-0.143	0.127	-1.303	0.195
	ML (df=4)	0.003	0.001	2.089	**0.039**
	(II) in maturation	0.009	0.097	0.090	0.929
	(III) matures	-0.011	0.097	-0.113	0.911
	(IV) spent	-0.074	0.110	-0.586	0.559
Pelagic polychaetes weight (g)	***(Intercept)***	-0.011	0.009	-1.164	0.247
	ML (df=4)	0.006	0.002	1.943	0.540
	(II) in maturation	0.003	0.008	0.324	0.747
	(III) matures	-0.005	0.008	-0.607	0.545
	(IV) spent	-0.007	0.011	-0.701	0.485

The ML was also the most important variable to models with teleost, cephalopods and crustaceans as prey (Table 3.4). Teleost prey, a negative relationship was observed from 142 mm to 195 mm, and then increasing up to 231 mm ML (Figure 3.3C) whilst the maturity-scale was also the most significant variable with the highest correlation in mature females (Figure 3.3D). Crustaceans’ prey showed a positive correlation starting from 193 mm ML (Figure 3.3E). However, the cephalopod was also an important prey in the diet of females in the sizes from 142 mm to 213 mm, peaking at 172 mm ML (Figure 3.3F).

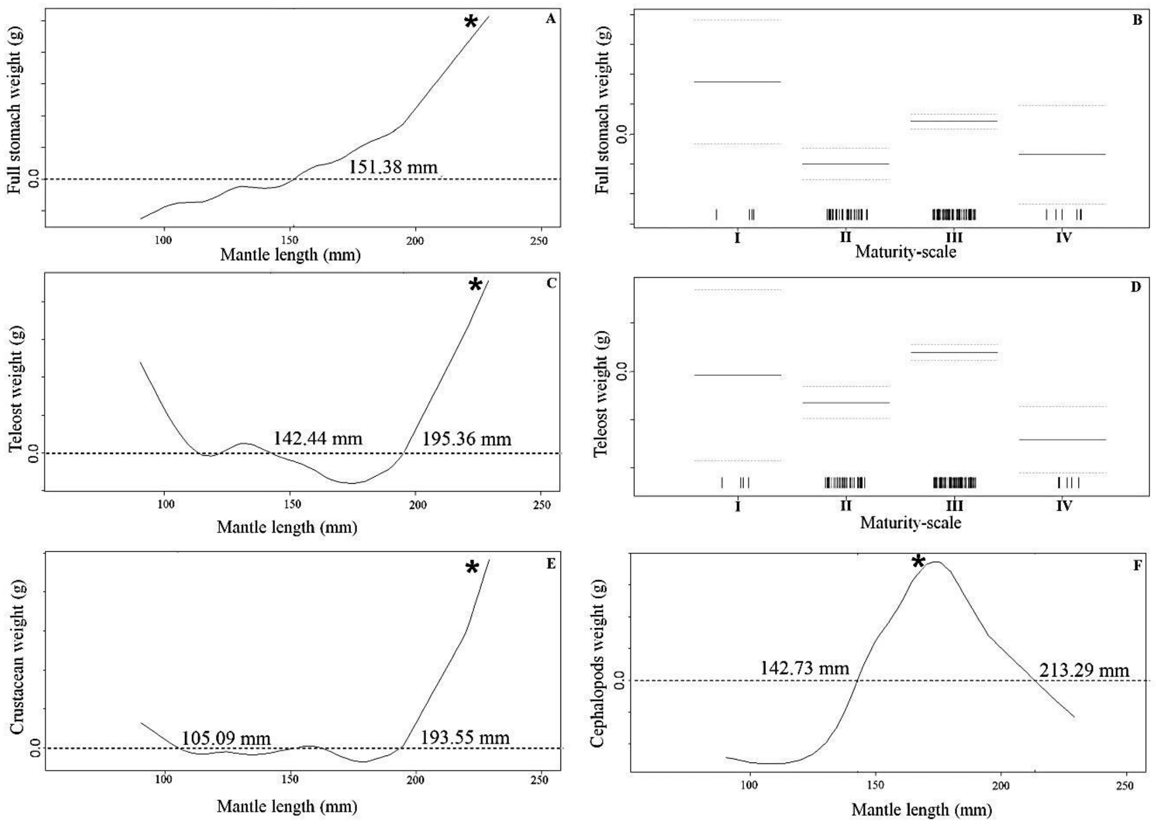


Figure 3.3: GAMLSS smoothing curves fitted to partial effects of explanatory variables on feeding for females of *Doryteuthis plei*. Considering the full stomach weight is represented as a function of (A) mantle length and (B) maturity-scale. For the four prey types: for teleost weight as a function of (C) mantle length and (D) maturity-scale, for crustacean as a function of (E) mantle length and for cephalopods weight as a function of (F) mantle length. The asterisk shows the correlation peaks in relation to the ML.

3.1.2. Feeding of males

Approximately 14% of males were collected with full stomachs. Their mean ML was 170.83 mm (±49.90 s.d.; range: 80–355 mm). The mean stomach weight was 0.97 g (± 0.93 s.d; ranging from 0.09 to 4.75 g) representing an average of 1.17% of body weight, ranging from 0.04 to 17.32 %. For the males, the ZAIG distribution also provided the best fit for models only for polychaetes. Residuals from the ZAIG distribution showed a better fit suggesting no evidence of heteroscedasticity (Appendix 3.2).

Unlike females, where the maturity-scale was the most significant exploratory variable in models, in males the ML was the most significant according to the AIC and LRT (Table 3.3). The full stomach content weight (in g) increases progressively with an inflection point at 162 mm, with a slight decrease to 258 mm ML and increasing until maximum size (355 mm ML; Figure 3.4A). The ML was the most important variable to model with polychaetes weight (Table 3.3 and Table 3.5). For pelagic polychaetes a positive relationship was found between in males from 222 mm and 299 mm ML (Figure 3.4B).

Table 3.5: Summary of optimal GAMLSS models for males fitted to the to the full stomach weight, and to four prey types: teleost, crustacean, cephalopods and pelagic polychaetes and the explanatory variables ML and maturity-scale of *Doryteuthis plei* around São Sebastião Island (SSI) during the period November 2003 – April 2013. Pr(>|t|)= probability of Student test.

	Terms	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
Full stomach weight (g)	**(Intercept)**	-1.394	0.541	-2.793	0.006
	ML (df=4)	0.003	0.005	0.646	0.520
	(II) in maturation	0.082	0.499	0.152	0.879
	(III) matures	0.262	0.575	0.456	0.649
	(IV) spent	-0.366	1.198	-0.306	0.760
Teleost weight (g)	**(Intercept)**	-0.962	0.006	-0.971	0.333
	ML (df=4)	0.005	0.991	0.766	0.445
	(II) in maturation	-0.428	1.044	-0.410	0.683
	(III) matures	-0.303	1.066	-0.285	0.777
	(IV) spent	-0.514	2.064	-0.249	0.804
Crustacean weight (g)	**(Intercept)**	0.092	0.186	1.072	0.285
	ML (df=4)	0.000	0.000	0.245	0.807
	(II) in maturation	-0.064	0.091	-0.706	0.481
	(III) matures	-0.038	0.086	-0.725	0.661
	(IV) spent	-0.135	0.086	-0.440	0.470
Cephalopods weight (g)	**(Intercept)**	0.025	0.037	0.663	0.508
	ML (df=4)	0.000	0.000	-0.299	0.765
	(II) in maturation	-0.010	0.039	-0.257	0.797
	(III) matures	0.011	0.081	0.284	0.777
	(IV) spent	0.002	0.037	0.021	0.983
Pelagic polychaetes weight (g)	**(Intercept)**	-0.129	0.231	-0.560	0.577
	ML (df=4)	0.001	0.001	0.746	0.057
	(II) in maturation	-0.009	0.232	-0.040	0.968
	(III) matures	0.089	0.228	0.389	0.698
	(IV) spent	-0.246	0.487	-0.506	0.614

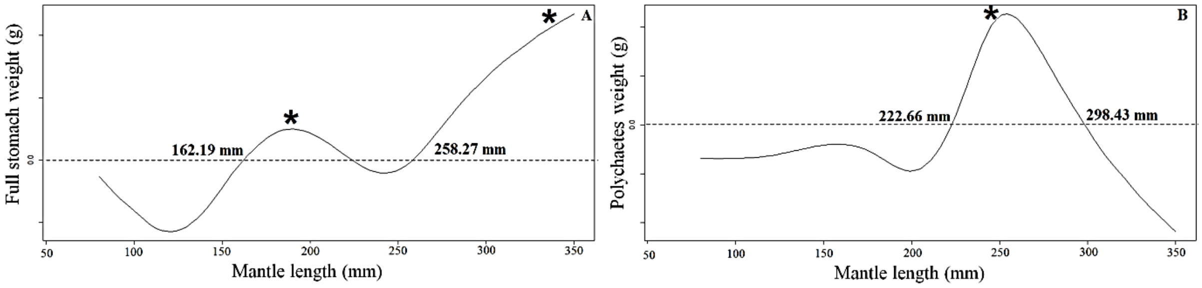


Figure 3.4: GAMLSS smoothing curves fitted to partial effects of explanatory variables on feeding in males of *Doryteuthis plei*. Considering the full stomach weight is represented as a function of (A) mantle length and for poliquaets weight as a function of (B) mantle length. The asterisk shows the correlation peaks in relation to the ML.

3.2. Spatial and temporal patterns in the diet

Frequency of prey and geostatistical gridding results indicates that the Southern island area was the main feeding ground, where the highest values of full stomach weights were found (Figure 3.5A). The teleost was frequently consumed in all fishing areas, but the highest values were found in the Southern area (Figure 3.5B and Figure 3.5F). Crustaceans were more frequent in the North (Figure 3.5C), while cephalopods were most consumed in the South and North areas of the island (Figure 3.5D) and pelagic polychaetes in the South and Southeast areas (Figure 3.5E).

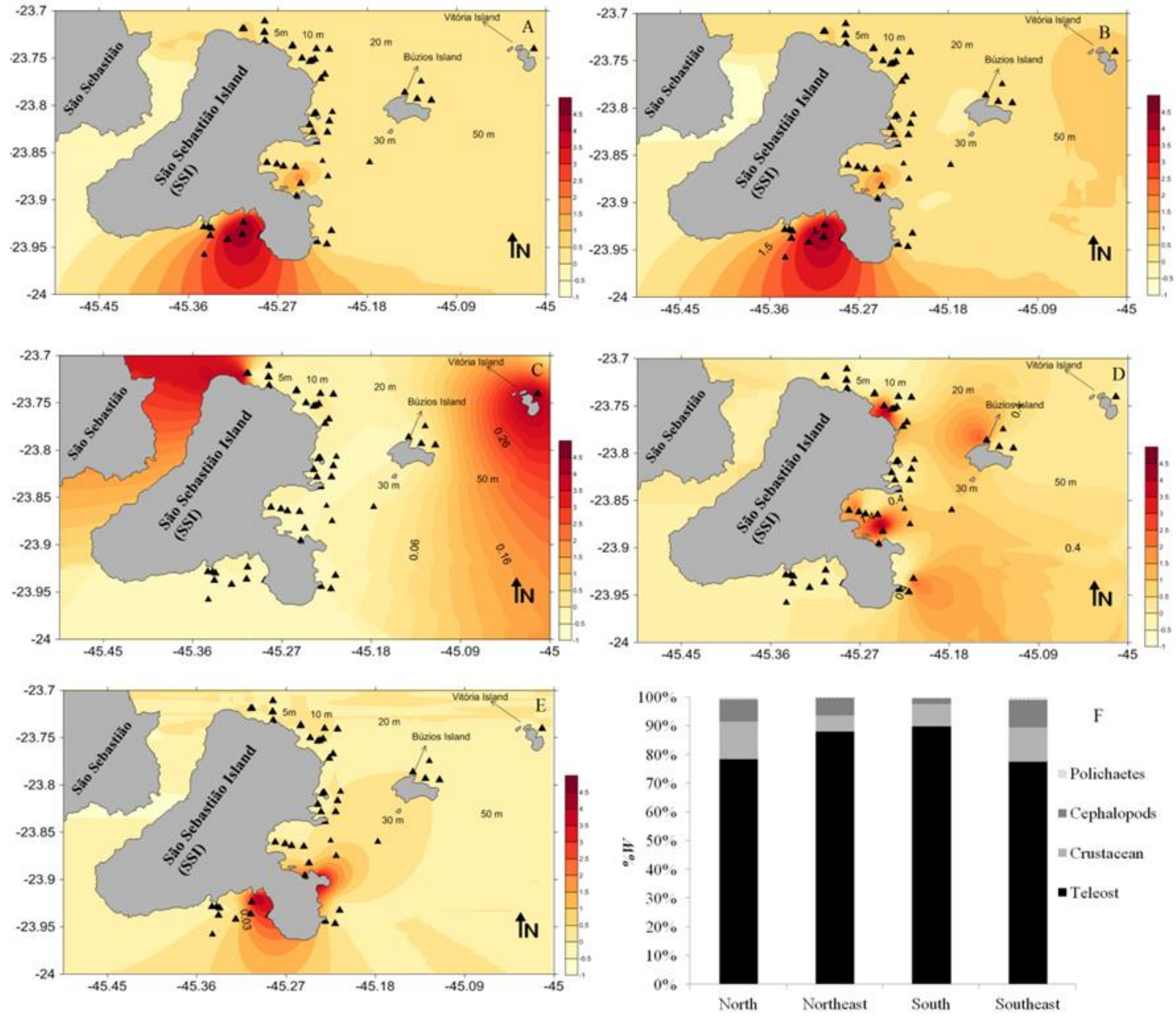


Figure 3.5: Spatial distribution of weight in (g) of stomach contents found in *Doryteuthis plei* caught in fishing areas surrounding the São Sebastião Island (SE) of Brazil during the period 2003 to 2012. A) full stomach weight; B) teleosts; C) crustaceans; D) cephalopods, E) pelagic polychaetes and F) Percentage of total prey weight (%W).

Regarding temporal variability of the prey type between 2003 and 2012, teleost showed an increase into fishing season, the lowest values being observed in November (40%) and increasing gradually through until 70% in April. The highest occurrences of cephalopods and crustaceans were observed at the beginning of the fishing season between November and January (Figure 3.6A). Percentage of total prey weight showed a decline of teleost importance during the period 2003 to 2012 (Figure 3.6). At the beginning of the study period, from the seasons 2003-2004 to 2006-2007, the teleost accounted for 80% of the prey, however in recent seasons these amounts dropped to 50%. In contrast, the crustaceans and cephalopods were more frequent in more recent seasons (from 2008-2009 to 2011-2012; Figure 3.6B). The weight of stomachs sampled at night accounted for 59% of the total. The teleost is also the main prey in both day and night; on the other hand the cephalopods were more frequent than crustaceans in stomach contents collected at night (Figure 3.6C).

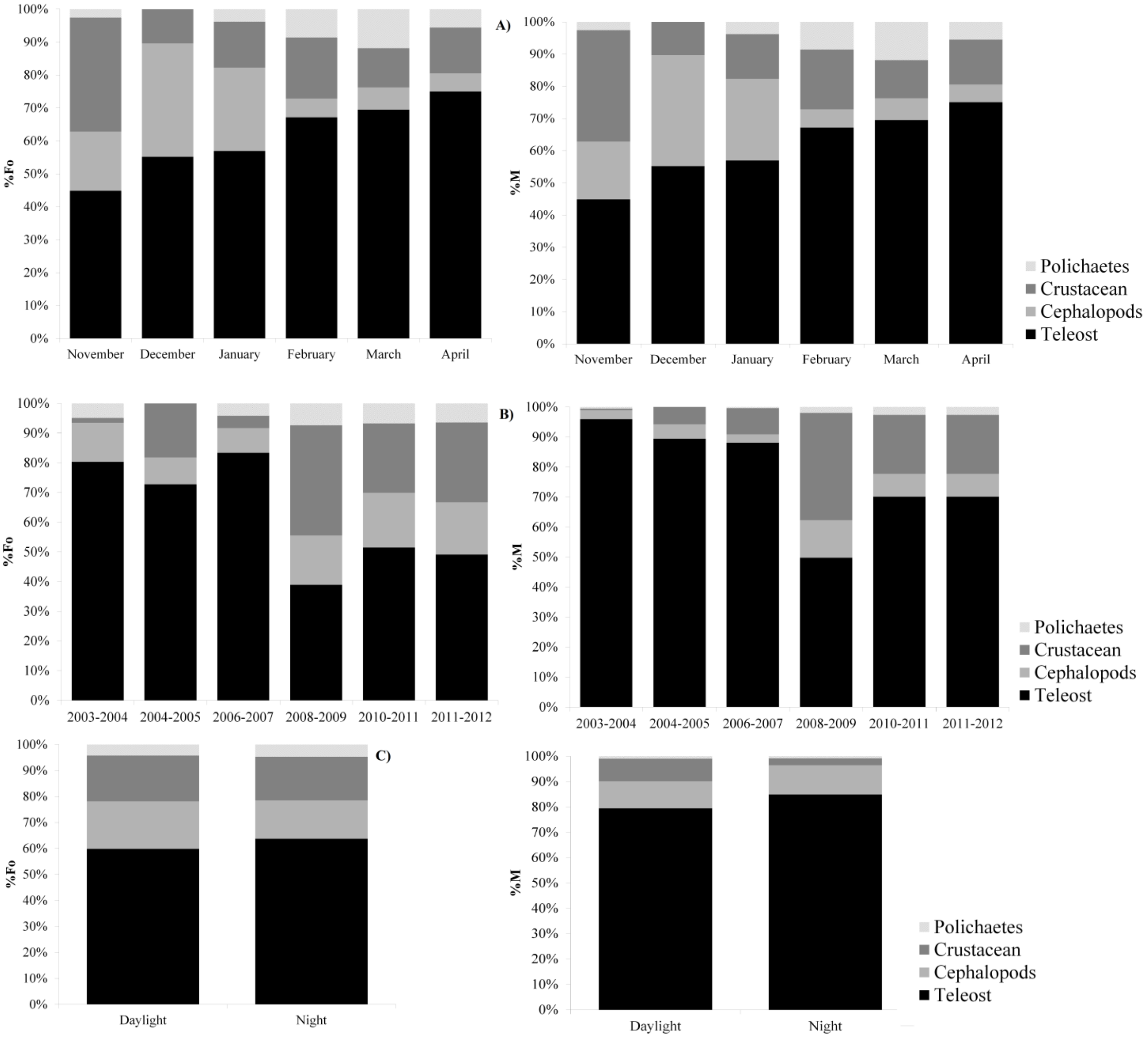


Figure 3.6: Temporal distribution (A) Fishing month; (B) Fishing season and (C) Fishing period of the frequency of occurrence of food items identified in the stomachs of *Doryteuthis plei* caught by squid fishing around São Sebastião Island in the period between 2003 and 2009.

GAMLSS models demonstrate that all variables are significantly correlated with the full stomach weight variable (Table 3.6). A positive correlation was found between full stomach weight and North and South fishing areas (Figure 3.7A) and in depths from 15 m to 25 m, peaking at a depth of 20 m (Figure 3.7B). The inter-annual variation was found to be a significant factor and a positive correlation was observed in the 2011-

2012 season (Figure 3.7C). Full stomach weight peaked in the month of March (Figure 3.7D), and then during the night fishing period (Figure 3.7E).

Table 3.6: The significance of each term of optimal GAMLSS models fitted to the full stomach weight, and to four prey types: teleost, crustacean, cephalopods and polychaetes and the explanatory variable the fishing season, fishing area, fishing month, diel vertical migration, fishing depth of the small-scale squid hand-jigging fishery around São Sebastião Island (SSI) during the period November 2003 – April 2013, where AIC = Akaike Information Criterion, LRT = Likelihood-ratio test, Pr(Chi) = probability of *Chi squared* test

Response variables	**Terms**	**AIC**	**LRT**	**Pr(Chi)**
Full stomach weight (g)	Fishing season	314.65	24.210	0.001
	Fishing area	305.73	11.291	0.010
	Fishing month	301.5	11.057	0.050
	Daylight/night	305.23	6.795	0.009
	Fishing depth df=4	305.81	13.374	0.009
Teleost weight (g)	Fishing season	670.81	16.132	0.006
	Fishing depth df=4	670.81	14.449	0.005
Crustacean weight (g)	Fishing month	-18.96	26.502	7.13^{e-05}
Cephalopods weight (g)	Fishing month	-245.99	21.868	0.001
Polychaetes weight (g)	Fishing area	-817.74	8.652	0.034

The Fs variable was the most important for the model with teleost as prey, essentially during 2008-2009 (see LRT in Table 3.6 and Figure 3.7F), and teleost showed significant correlation in depths from 15 m to 25 m, peaking at a depth of 20 m (Figure 3.7G). The crustaceans was more consumed during March (Figure 3.7H), the cephalopods in November and December (Figure 3.7I). The polychaetes in the South and Northeast island region (Figure 3.7J).

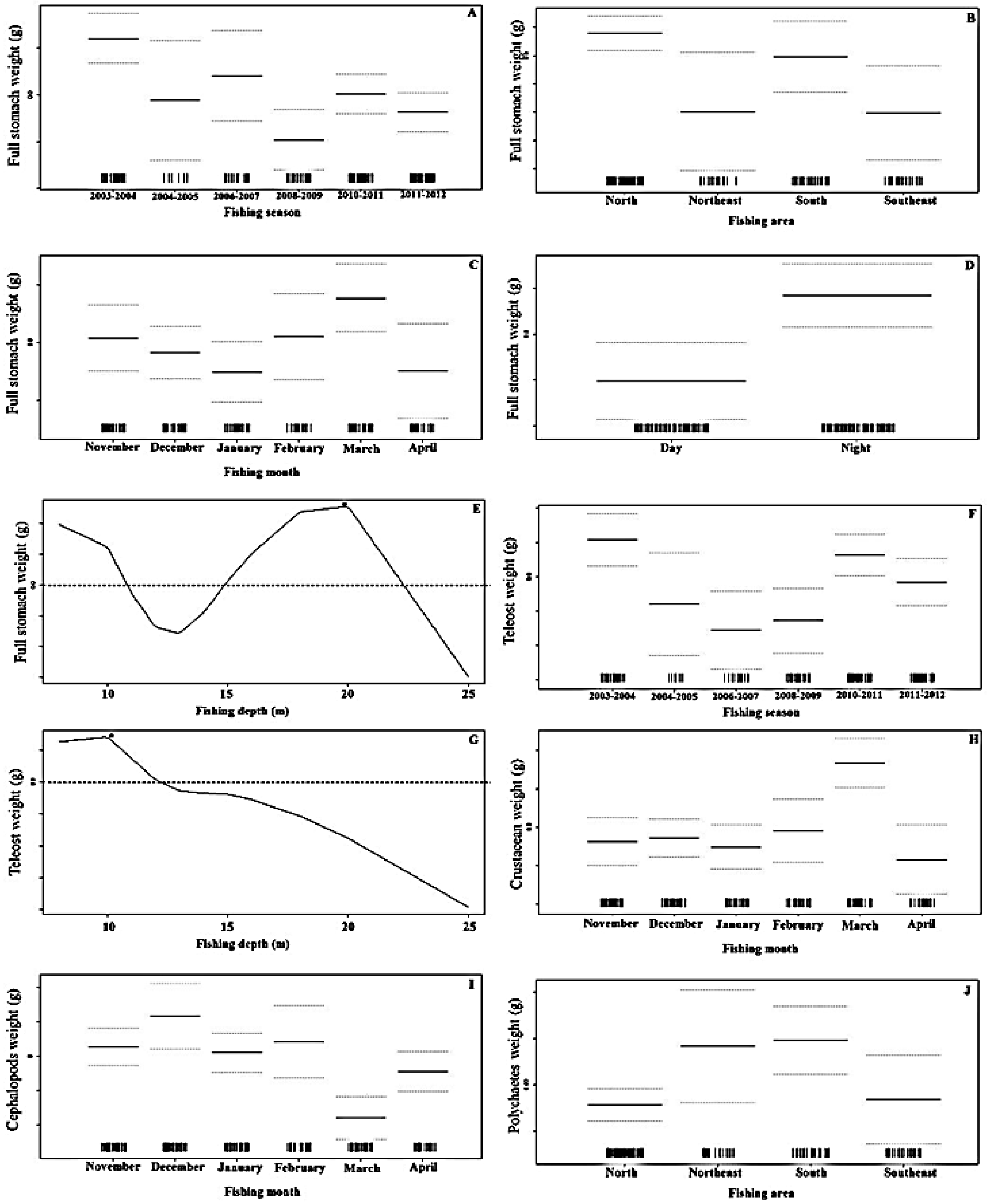


Figure 3.7: GAMLSS smoothing curves fitted to partial effects of explanatory variables of explanatory variables on feeding of *Doryteuthis plei*. Considering the full stomach weight (Scw) is represented as a function of (A) Fishing season, (B) Fishing area, (C) Fishing month, (D) Fishing depth in (m) and E) Fishing period. For teleost weight as a function of (F) Fishing season and (G) Fishing depth in (m). For crustacean weight as a function of (H) Fishing month. For cephalopods as a function of (I) Fishing month. For poliquaets weight as a function of (J) Fishing area. The asterisk shows the correlation peaks in relation to the fishing depth.

3.3. Feeding behaviour of *Doryteuthis plei* in laboratorial condition

Of the seventy-eight squid maintained in captivity, 46 were females and 32 were males. The mean ML for females was 144.23 mm (+/- 26.55 s.d; range 65 mm – 243 mm), while in males the mean ML was 227.84 mm (+/- 45.73 s. d.; range 99 mm – 299 mm). During the period of laboratorial conditions it was possible to observe that *D. plei* are voracious carnivores and feed during the daylight and at night from various live or frozen preys. Overall 96 live preys were offered, of which 70 were fish, 19 squid and 7 crustaceans (Appendix_Table_3.1). The first squid detected prey using their eyes. Overall, the feeding involves four stages: attention, positioning, attack and bite prey. In the first stage, the squid generally approaches the prey from underneath or laterally, always following it with the eyes (see in Appendix 3.3). For fast swimming prey, squid use the tentacles to capture it.

A squid can eat four fish at the same time. Large squids (males and females) can capture large fish. During feeding the large males tried to steal prey from other squid inside the tank. During February 2012, aggressive disputes were observed for food, the squids being fed frozen fish, and the competition for the prey was so strong, that squid would jump out of the tank; it was observed that one fish can be divided by 3 or 4 squid at the same time. Males captured most of the crustaceans offered during the feeding and the squid swam in the background surrounding the shrimp during feeding using the tentacles. Some species of fish offered, such as *Conodon nobilis,* were rejected for being quick and aggressive to the squid.

Cannibalism has often been observed, mainly in two situations: 1) when large squid invested in small squid, and 2) when a moribund squid was attacked by other males before death. Body colour varies the intensity of the pigment, and in this case is generally smooth whilst, during feeding, other organs can be observed such as, for example, the stomach and the entire digestive system.

Table 3.7: Findings in the observations stomach contents and feeding behaviour in captivity for *Doryteuthis plei.*

Topic	**Stomach contents analysis (Field data)**	**Feeding behaviour (experimental observation)**
Types of prey	Teleost (*Anchoa spp, Sardinella brasiliensis, Selene spp and Trachurus lathami*), Crustacean (Decapods), Cephalopods (*Doryteuthis plei*) Pelagic polychaetes	Teleost (*Anchoa spp, Sardinella brasiliensis,* Crustacean (*Xiphopenaeus kroyeri*) and Cephalopods (*Doryteuthis plei, Lolliguncula brevis*).
Size of prey	Size of the prey was estimated at 74 mm for *Sardinella brasiliensis.*	*Sardinella brasiliensis* : 96 mm, 84 mm and 72 mm; and *Anchoa tricolor*: 34 mm, 46 mm, *L. brevis*: 80 mm and 95 mm ML and *D. plei*: 100 mm ML.
Ontogenetic shifts	Teleost and crustaceans - were significantly related to squid up 195 mm and 193 mm ML, respectively. Cephalopods seem to be eaten by larger squid of up to 213mm ML.	Large males can capture large fish measuring 98 mm in length and the largest squid consumed the smallest *D. plei.* The largest squid eat shrimp.
Cannibalism	Two statoliths belonging to *Doryteuthis plei* with 1 mm in length were found. The estimated prey sizes were 35 mm and 50 mm ML.	The largest squid practiced cannibalism in the absence of other prey and invested in small squid, and a moribund squid was attacked by other males before being killed.
Number of prey by stomach	Five otoliths were found in the same stomach.	A squid can capture four fish at the same time.

4. Discussion

Most cephalopods actively catch and eat live prey (except *Vampyroteuthis* squid), and a wide variety of prey has been identified around the world, mainly including a variety of teleost species, crustaceans, cephalopods and pelagic polychaetes (Boyle and Rodhouse, 2005). In this study, dietary ontogenetic and spatio-temporal shifts, not evidenced before were detailed with greater precision.

Evidenced by the hard structures found in the stomachs, including statoliths and otoliths, the horse mackerel *Trachurus lathami* was found to be present, and cannibalism was evident. However, by otoliths other species such as *Sardinella brasiliensis*, *Anchoa ssp* and *Selene* spp, were also identify in average proportions well as has been reported by other studies (Juanicó, 1979; Martins and Perez, 2007; Gasalla et al., 2010).

Some feeding patterns of the squid *D. plei* in shallow-waters around SSI seemed to be very similar to those previously described by previous studies off the in Brazilian waters (Juanicó, 1979; Martins and Perez, 2007; Gasalla et al., 2010), however this study found differences in the prey composition among sexes, size, maturity, and spatio-temporal pattern using a robust statistical analysis little reported. For instance, the teleost was the most important prey found in stomach contents, which also included small pelagic fish as the main food item, whilst only 16% of stomachs in this study were observed to contain more than one type of prey, and fish were mostly found added to another type of prey. This pattern (fish as the main food item and a low frequency of mixed prey) was also observed amongst other loliginids caught elsewhere, *e.g. L. forbesi* and *L. vulgaris* off the European coast (Pierce et al., 1994; Collins and Pierce, 1996; Rocha and Guerra, 1999); *L. reynaudii* in South Africa (Sauer and Lipiński, 1991; Olyott et al., 2006) and *D. pealei* in the North Atlantic Ocean (Hunsicker and Essington, 2006) and *D. opalescens* (Fields, 1965; Karpov and Calliet, 1978; Yang et al., 1983).

Dietary ontogenetic shifts

It was possible to observe non-linear relationships between the diet of the *D. plei* and both size structure (ML) and the factor maturity-scale were possible by means of the models providing greater precision and most robust dietary ontogenetic shifts. These findings highlight differences between the feeding habits of males and females. In the case of the females, the relations were more evident than amongst the males, with the weight of the full stomach weight positively correlating with mature females. The relations with the ML were also evident, with prey such as teleost's, crustaceans and cephalopods being found amongst females. The models suggest dietary ontogenetic shifts in feeding habits; for example, teleost's are mostly preyed upon by large squid, with the same occurring with crustaceans with 195 and 193 mm ML, respectively. In counterpart, cephalopods are consumed by small squid starting at 142 mm and going up to 213 mm, with a peak at 172 mm. According to research into the reproductive activity, of this species in the study area performed by (Postuma and Gasalla, 2014), the size-at-maturity of females occurs from 131 mm up to 175 mm ML.

In relation to the males, the ML is the most important variable, with the stomach fullness weight correlating positively in squid of more than 162 mm ML. Contrary to that which was found amongst the females, the males showed a slight reduction from 162 to 285 mm ML, and the only prey that significantly correlated with the males was polychaetes, that represented positive correlations in large squid starting at 299 mm ML. These results suggest that the males invest their feed in the growth of the ML, whilst just as there is no evidence of changes in the feeding habits, neither is there a preference for polychaetes amongst large males. On the other hand, the females seem to invest their feed in the maturing process, principally through the preying on cephalopods and teleost's. The slight reduction of the full stomach weight found in males may be linked to somatic growth due to the characteristic sexual dimorphism in this genus (*i.e.* males larger than females), in order to optimize the chances of effective mating with females during their life-cycle. Ontogenetic shift in the diet of squid is often published (Boyle and Rodhouse, 2005; Hunsicker and Essington, 2006; Uchikawa et al., 2007; Pethybridge et al., 2007; Wangvoralak et al., 2011; Alegre et al., 2014) and with the feeding indices for *D. plei* (Martins and Perez, 2006; Gasalla et al., 2010).

The highest *%W* values of cephalopod prey observed during the night period in this study may also be related to this pattern. Cannibalism is thought to be an important mechanism for density-dependent regulation in cephalopods. It produces feedback mechanisms that can lead the population towards a determined density and as such can be an important component of natural mortality (Ibánez and keyl, 2010; Caddy, 1983; Pierce and Guerra, 1994). Over the last few years, several works have shown that the frequency of the occurrence of cannibalism in the diets of squid can be overestimated depending upon the fishing equipment used for capture, as reported for Jumbo squid Ommasthrephidae *Dosidicus gigas* caught by jigging at night using artificial light (Ibánez and keyl, 2010, Alegre et al., 2014) and similar methods used by small-scale hand-jigging fishery around SSI (Postuma and Gasalla, 2010).

Spatial and temporal variation in diet

The occurrence of full stomachs in *D. plei* indicates that this species utilizes the coastal area as a feeding ground during grown and reproduction (Martins and Perez, 2006; Martins and Perez, 2007; Gasalla et al., 2010, Postuma and Gasalla, 2014; Barcellos and Gasalla, 2014). By means of an analysis of the dataset over a lengthy time period, it was possible to describe the foraging behaviour of *D. plei*, around SSI; for example the strong correlation found between the full stomachs weight and the Fa by means of robust estimates using GAMLSS models, indicated a peak occurring in the Southern area of the island in depths from 15 m to 25 m, peaking at a depth of 20 m, coinciding also with the highest *W%* and geostatistical gridding approach , where teleost's and cephalopods are frequently consumed by squid. However, the crustacean prey was important prey in the North, and polychaetes were consumed in the Southeast.

Compared to the recent study of reproductive activity performed by Postuma and Gasalla, (2014), where it was suggested that sheltered bays in the Southern and Southeastern areas of SSI could actually be the breeding ground for mating in the water column, where the mainly small pelagic fish and cephalopods were found in the stomachs of squid, and the North are used as spawning grounds on the sand bottom, exactly where the crustaceans were more frequent in feeding, we suggest that changes in the composition of the squid's diet of *D. plei* in relation to the size structure could be more related to the availability of this prey in the spawning area, than to food preference.

This pattern of abundance of types of prey of squids also overlap with the studies on the abundance of ichthyofauna larvae around SSI reported that the larvae of Engraulididae, *Sardinella brasiliensis*, *Trachurus lathami* and *Decapterus puncatus* species are considered the most abundant, also carrying great importance for fishing in the Northeast and Southeast Island and the biomass of larvae of crustaceans tends to be higher in the North of the island (Katsuragawa et al., 2008; Pires-Vanin, 2008).

The inter-annual and intra-annual variation of full stomach weight showed a significant peak during February and March and in the 2011–2012 fishing season. The earlier results (*e.g.* %W) indicate a substitution of the type of prey in the diet over time, for example, in the absence of fish observed in the stomachs there was an increase of crustaceans and cephalopods from 2008-2009 to 2011-2012 (Figure 3.7A). This substitution in the feeding was also observed in fishing month, with records of the teleost showing the lowest values being observed in November, when the cephalopods and crustaceans are more abundant in the stomachs of squid. These results also coincide with studies of temporal abundance of the larval stages of fish and crustaceans in the study area (Pires-Vanin, 2008).

Daily vertical migrations

The results showed that the most active feeding occurred during night time near surface layers, but squid were also likely to feed during the day when their main prey migrates to depths, thus performing daily vertical migrations throughout the water column in search of food; for example, the prey *Thachurus lathami* performed diel feeding migrations in the water column (Carvalho and Soares, 2006), commonly being found on the sea's surface at night and in deep waters during the day. Squid in their spawning grounds feed mainly at night, as observed in other studies for *L. forbesi* and *L.vulgaris,* and this may signify a forced resting phase for spawning females, (Macy, 1982; Sauer and Lipiński, 1991; Ibánez and keyl, 2010).

The main findings of this study by means of analysis of stomach contents can define issues of feeding behaviour, where little known about the subject for this species in the area, highlighting, for example, a preference for small, engrailed and carangid. pelagic fish; the predator-prey size relationship with large squid consuming large prey; a diel vertical migration in the water column in search for food; cannibalism; and consumption of crustaceans on sandy bottoms; whilst these results were also confirmed in experiments conducted in tanks. The squid showed a wide variety of behavioural adaptations for feeding from the highly active hunting and are can be considered a versatile opportunistic predators. We suggest should be made direct observations in the field to better understand how foraging take place in the selection of the prey type between the sexes in spawning areas.

5. Conclusion

This study showed that *Doryteuthis plei* is an active predator of fish, squid, and crustaceans' species in shallow waters of Southeastern Brazil. Ontogenetic shifts in the feeding habits of *D. plei* during the summer months were found exactly when intense reproductive activity occurs, with teleosts being the main prey, followed by crustaceans and cephalopods. It was evident that dietary habits differ among sexes and depending size of the predator, maturity scale, and spatial and temporal pattern. Data suggested that females were more selective in feeding than males. The high correlation found between full stomach weight and maturity, especially in mature females, suggests feeding and breeding overlapping. Regarding to size structure, the females do not stop feeding during growth and reproduction and their amount of food increases with size (from 90 mm to 231 mm) while males appear to take a “short break” in feeding upon reaching 162 mm ML. Squids were important preys in females from 142 to 213mm ML, fish and crustaceans in females around 195 mm ML, while polychaetes were dominant prey for the largest males (222-299 mm ML).

Moreover, the combination of spatial–temporal factors assisted in understanding the pattern of the feeding habits of *D. plei* around the island. A scenario was proposed indicating a peak in feeding activity during February and March, mainly in the Southern areas of the island, and on the Southeastern coasts, where cephalopod was the main prey group. Indeed, it coincides with the development of breeding areas identified for the species. On the other hand, around the North island, crustaceans were predominant in the stomachs, also overlapping with the spawning area proposed by previous studies. The results also suggested that there is a vertical migration in the search of food, with the heaviest stomachs appearing at night when cephalopods are the most important prey rather than crustaceans. This suggests that squid have different feeding habits in the breeding and spawning grounds during their lifecycle around SSI. The statistical models (GAMLSS) together with the observation in captivity showed advantages in the identification of patterns of feeding habits, which is unlikely to have been possible by using feeding indexes alone. Lastly, the analysis of a relatively long dataset allowed to evidence yet unrevealed patterns of the tropical arrow squid feeding habits.

Chapter 4

Hunting behaviours in newly-hatched paralarvae of *Doryteuthis plei* in experimental conditions.

Chapter 4 - Hunting behaviours in newly-hatched paralarvae of *Doryteuthis plei* in experimental conditions.

Abstract: Cephalopods assume several forms of hunting behaviour according to their ecological, morphological, and cognitive features, and their specific hunting skills are developed ontogenetically in relation to these features. However, the behaviour of loliginid early life-stage has been poorly investigated especially in regard to first hunt and swimming strategies. This study aims to examine the early development of the swimming and hunting behaviour focusing on the movements of the fins, arms, siphon, and mantle of *Doryteuthis plei* paralarvae. Specimens were obtained by egg mops collected by scuba diving in inshore waters off the northern coast of São Paulo, SE Brazil, and recorded in aquaria with both high-frequency horizontal optical system and hand cameras. In the first day of life, the paralarvae occupied the surface of the water column, moving fast and laterally. When they began swimming upwards their bodies decreased in volume, with a synchronized motion between the siphon, mantle and fins, reaching an average rise speed of 11.10 mm s^{-1}. Unsuccessful attacks on copepods (erratically moving organisms) were observed in 4-days old paralarvae. However, successfully attacks on mysidaceans occurred, showing an elaborate pursuit strategy with use particularly of the fins and eyes. In the absence of prey, paralarvae exhibited aggressive intra-specific behaviour, when they showed both the pursuit strategy and a rapid backward escape after attack. Size and fast escape behaviour of the prey seem to be limiting factors for the survival in early-stages. The newly-hatched squid seems to behave as miniatures of the adults, and born with a highly developed nervous system and innate hunting skills, being active swimmers and predators..

Keywords: Feeding, swimming, pursuit strategy, predator/prey interactions, squid, paralarvae, food

1. Introduction

The loliginid family contains a number of known squid species and their small hatchings are immediately planktonic, conventionally called “paralarvae” (Young and Harman, 1988). Paralarvae are active swimmers using jet propulsion; they show high growth rates as active predators feeding on live zooplankton with a complex hunting behaviour (Villanueva et al. 1996; Bartol et al., 2001, 2008, 2009a, 2009b; Vidal et al., 2002, 2006; Thompson et al., 2010; Stewart et al., 2010; Sugimoto and Ikeda, 2013). The availability of food and temperature are considered the main factors regulating the survival and growth of hatching squid (Vidal et al., 2002, 2006; Forsythe, 2004). Among the main prey organisms identified under laboratory conditions are for example, enriched *Artemia* nauplii, wild zooplankton (mainly copepods) and juveniles of the mysid *Metamysidopsis elongata atlantica* in *Loligo vulgaris reynaudii* (Vidal et al., 2005) and *Doryteuthis opalescens* (Chen et al., 1996, Vidal et al., 2006), as well mysids in *Sepioteuthis lessoniana* (Sugimoto and Ikeda, 2013).

Planktonic paralarvae do not show any morphological differences compared to adults and in the first days of life. They seem to use fins and siphons for swimming and exhibit a unique hunting behaviour using eight arms and two elongate tentacles equipped with suckers specialized to capture prey (Boucher-Radoni et al., 1897). Squids adopt various forms of hunting according to their ecological, morphological and cognitive features. The only difference from adults is the planktonic life-style (Baron, 2003), meaning they can be transported by ocean currents. The distribution of some paralarvae species have been shown to be dependent on oceans currents, water column properties (*e.g.*, thermohaline stratification) and plankton productivity (Bakun and Csirke, 1998; Jackson et al., 2008; Vidal et al., 2010; Xavier et al., 2014).

Paralarvae rearing is relatively difficult due to its delicate body structure, short arms for food catch, limited swimming ability with small fins and the availability of live food (Villanueva, 1995; Vidal et al., 2014). In laboratory conditions, the photoperiod is important, and the majority of hatching occurs during the hours of darkness. Upon hatching, the paralarvae retains the yolk in the stomach, as an endogenous food source, and that provides the energy source for the first few days of active life until the transition to dependence on prey capture (Chen et al., 1996; Vidal et al., 2002, 2006, 2014).

Hunting behaviour in cephalopods consists of three phases: attention, positioning, and seizure (Messenger, 1968; Hurley, 1976; Chen et al., 1996; Sugimoto and Ikeda, 2013). Differences in cephalopod morphological features and ecological characteristics influence the development of hunting behaviour. Few studies have investigated the process and timing of ontogenetic changes in squid hunting modes (Chen et al., 1996; Sugimoto and Ikeda, 2013). Chen et al. (1996) provided some information about the ontogeny of hunting behaviour in the California market squid *D. opalescens*. According to the authors, in the early stages the squid catching the copepods using the arms and tentacles, however the juveniles only use tentacles and when the juveniles have not had experiences with copepods, the attacks is failed.

Evidence suggest that all stages of *Doryteuthis plei* can be found in the São Sebastião Island region (Gasalla et al., 2012) from egg mops (Gasalla et al., 2011), paralarvae (Araujo, 2012), juveniles (Gasalla et al., 2011), and mature and spent individuals (Postuma and Gasalla, 2014). The seasonal presence of this squid in the area seems to be influenced by favourable environmental conditions (*e.g.* SST (°C) and chlorophyll-a concentration (Postuma and Gasalla, 2010) and current patterns (Martins et al., 2013). The knowledge about the early-stages behaviour of this species is almost completely unknown in South Brazil. The aims this study is examine the swimming speed, and the early development of the hunting behaviours and the movements of the fins, arms, siphon, and mantle of *D. plei* paralarvae.

2. Materials and Methods

2.1. Collection and rearing

The paralarvae used in the study were obtained by means of some egg mops of the tropical arrow squid *D. plei* collected by scuba diving in the inshore waters off the Northern coast of São Paulo, Brazil, close to rocky shores and sandy bottoms, at 6- and 8-m depth. During transport to the laboratory, the egg mops were held in a 300-L tank containing local seawater that was constantly aerated by a submersible pump. The identification of the paralarvae was based on the head chromatophores pattern in two areas located in the lower half of its ventral surface, separated by funnel areas called '*cheek patched'* (Hanlon et al., 1992, Araujo, 2012). *D. plei* has four chromatophores arranged in the quadrangular *'cheek patches'* area (Hanlon 1978; McConathy et al., 1980).

The paralarvae were transported to the tanks. The period of the two experimental groups defined, together characteristics of the water abiotic data (mean temperature [T°C] and oxygen [O²]), are shown in Table 4.1. The first group of squid hatchings was kept in a flow-through system consisting of three 1 m^3 cylindrical rearing tanks (a control tank and two replicas), and they were offered *Artemia* and copepods (Calanoides) as food. The second group was maintained in a 100-L tank and the paralarvae survived for up to 12 days feeding on *Artemia* and mysids.

After hatching, the density of paralarvae in the tanks was estimated once a day (relative to paralarvae numbers) by three samples with the help of a 200 ml *becker*. Water quality such as, water temperature and dissolved oxygen were measured daily with a multi-parameter probe. The temperature ranged from 26°C to 27.1°C and dissolved oxygen ranged from 3.76 to 6.23 $mg^{\cdot} L^{-1}$. The mean level of ammonia was 0.018 ppm (range: 0.013 to 0.036; n = 15), and the mean level of total nitrite-nitrogen (NO_2) was 0.5 mg. L^{-1} (range: 0.02 to 3.05; n = 15).

Table 4.1: Water quality information mean (minimum - maximum; standard deviation) of (T°C) temperature and dissolved oxygen (DO^2) on the two paralarvae groups and food, volume and maintenance periods, of *Doryteuthis plei* paralarvae.

Group	Sampling period	Volume (L)	T°C Mean (min.-max; s.d)	DO² Mean (min.-max; s.d)	Food
1	26/02 - 14/03/2013	1000	27 (22.1- 29.8; 0.83)	5.30 (3.76 - 6.26; 0.63)	*Artemia* and Copepods
2	20/11 - 05/12/2013	100	24 (22.1 - 24.8; 0.85)	6.01 (5.98 - 6.06; 0.02)	*Artemia* and Mysids

2.2.Food of *Doryteuthis plei*

Newly-hatched paralarvae fed on their yolk reserves during the first three days. After this time interval, *Artemia* nauplii (sized 0.3 mm) was offered as food, followed in subsequent 4 days by *Artemia* metanauplii (sized 0.8 mm) enriched with RotiGrow (Tetraselmis 3600) and RotiGreen (*Nannochloropsis* and *Tetraselmis*). Live copepods in experimental Group 1 with 7 day of experiment and mysidaceans in experimental Group 2 with 4 days of experiment were also available to a variable extent.

2.3.Record and analysis of behaviours

The swimming behaviour of *D. plei* paralarvae, including individual displacements and motions of the body, fins and siphon, was recorded with a horizontal optical system (Figure 4.1). The imaging technique was that of a matched spatial filter system (Strickler & Hwang (1998) consisting of a laser (657.5 nm), objective (20 x), a pinhole (10 µm), a dotted spatial filter, collimator and focus lenses, and a high-speed digital camera (*Photron Fastcam SA2 86K C3* (ranging from 60 to 1080 frames per second). Twenty-five scenes, totalling 1 min 50 s of slow motion footage were recorded. For each filming, five paralarvae were placed in a small-volume aquaria (~50 mL; measuring 6.0 x 5.5 x 1.5 cm) filled with filtered seawater. Video images were analyzed using the *ImageJ* software. Dorsal mantle length (ML) was measured from images of *D. plei* individuals.

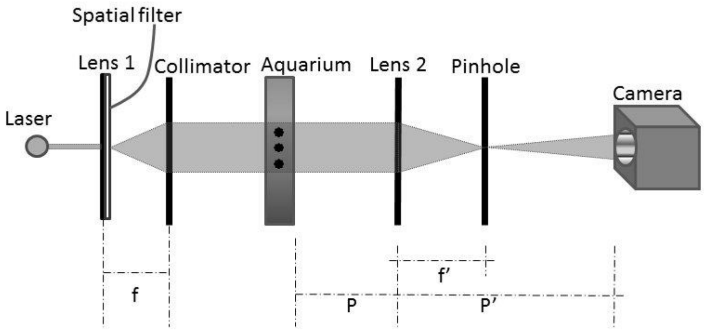


Figure 4.1: Diagram of the horizontal optical system used for image acquisition, where (f) in the distance between lens 1 and collimator; (*P*) is distance between the aquarium and lens 2 (f') the spot size of the lens 2, and (*P'*) the distance between the lens 2 and the camera. The dots represent the paralarvae

Additionally, the hunting behaviour were recorded during 10 min sessions with a high definition hand-can camera mounted on a tripod, operating at 60 frames per second and with a low intensity light mounted 10 cm overhead providing supplemental lighting (Figure 4.2). Overall, 271 films were recorded with video camera, totalling 8 h 53 min. The videos were recorded once a day. The frames were focused on a single paralarvae and conducted ''*focal animal sampling*'' filming constantly to acquire the sequence of behaviours. Attack speed was computed as the greatest distance travelled by the squid between two consecutive frames during the attack lunge and is expressed in millimetres per seconds (mm s^{-1}).

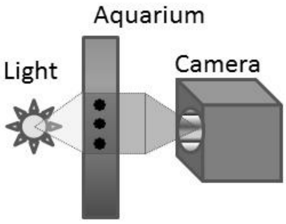


Figure 4.2: Diagram of the horizontal high definition camera set up used for image acquisition. The dots represent the paralarvae.

The behaviours of *D. plei* were described and tabulated in a spreadsheet and compared according to time and the attack distance between the tip of tentacles and prey. The terminology used for the type of behaviour was based on studies conducted on other newly-hatched loliginids around the world, *e.g.*, *D. opalescens* (Chen et al., 1994), *D. pealei* (Vidal, 2000; Bartol et al., 2008, 2009a, 2009b) and *Sepioteuthis lessoniana* (Sugimoto and Ikeda, 2013).

3. Results

3.1.Swimming behaviour of *Doryteuthis plei* paralarvae

The results show that the first body movements were linked to swimming and feeding behaviour of newly-hatched *D. plei* squid. The video recording analysis showed that the mantle siphon and fins performed a synchronized motion (Figure 4.3). Most paralarvae (29 individuals) filmed in Group 1 immediately after hatching swam in the water column for 60% of the total footage period. However, 6 paralarvae remained on the surface of the aquarium for the entire footage time. The paralarvae performed vertical swimming, ascending during mantle contraction and descending during mantle refilling. Paralarvae with 1.37 mm ML occupied the surface of the water aquarium just after hatching, and then, at 3-day-old, they moved fast with lateral manoeuvres. When paralarvae swam upwards (64.28% of a total 79 swimming events) their bodies decreased in volume and reached an average rise speed of 11.10 mms^{-1} (range: 0.85 - 48.00; s. d. 10.90 mms^{-1}) with a synchronized motion of siphon and fins (Figure 4.3A).

During downward swimming (8.57%) the mantle showed the highest volume and retained water in its cavity. The average descent speed was 10.79 mm s^{-1} (range 3.79 - 25.29; s.d. 8.90 mm s^{-1}) (Figure 4.3B), with the fin being used less frequently and more strongly. After 4 days upon hatching paralarvae performed lateral movements with the aid of fins (Figure 4.3C).

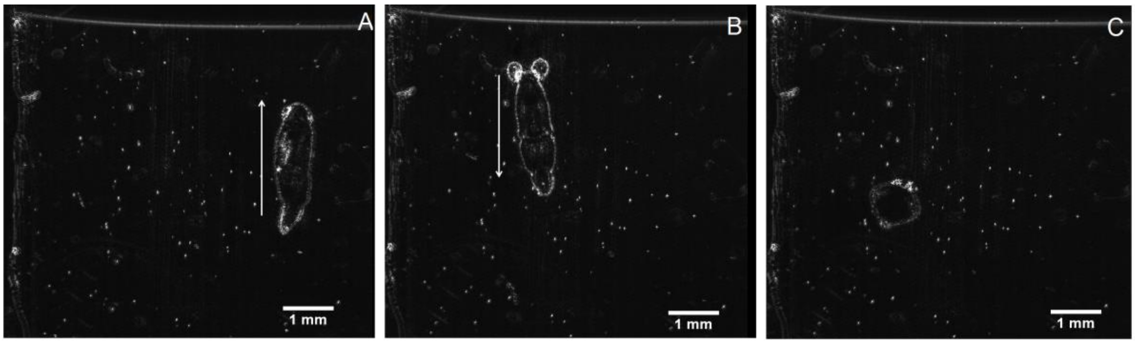


Figure 4.3: First swimming behaviour of *Doryteuthis plei*: (A) Upward swimming of a 1-day-old larva; B) Downward swimming of a 3-day-old larva; (C) Lateral swimming 7-day-old.

The average distance of upward swimming was 5.42 mm, three times greater than the distance covered in downward swimming (1.91 mm), and the paralarvae used the siphon more powerfully in upwards swimming. The swimming speed and the distance covered are shown in figure 4.4.

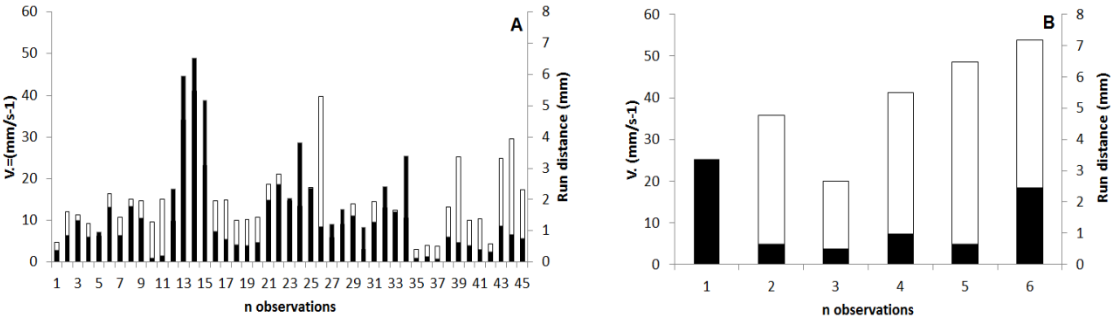


Figure 4.4: Run distance (white bars) and swimming speed (black bars) of *Doryteuthis plei* paralarvae. (A) Upward swimming; (B) Downward swimming.

3.2.Feeding of *Doryteuthis plei* paralarvae

Figure 4.5 shows the survival rates for both experimental Group 1 and Group 2, and shows that the number of paralarvae decreased during the transition from prey composition offered. Seven-day-old paralarvae of *D. plei* showed hunting behaviour was clearly divided in attention, positioning and seizure.

In the attention phase, the paralarvae (7-day-old) detected prey with their eyes and turned quickly to the prey pointing arms and tentacles in the form of a triangle. In the positioning phase, they threw their tentacles gradually from the centre of the triangle-shaped arms as they moved quickly toward to capture the prey. In the seizure phase, they moved forward with lunged rapidly to capture the prey with their arms and tentacles. In Group 1, around 7 days of experimentation, there was a severe decreased, in survival rate just after copepods addition, while filming the copepods showed erratically moving to escape. In Group 2 from 4 day of experimentation, the reduction of the number of paralarvae in the tank occurred after the mysidaceans addition. However unlike the copepods, the attack amongst mysidaceans was recorded successfully using an elaborate pursuit strategy.

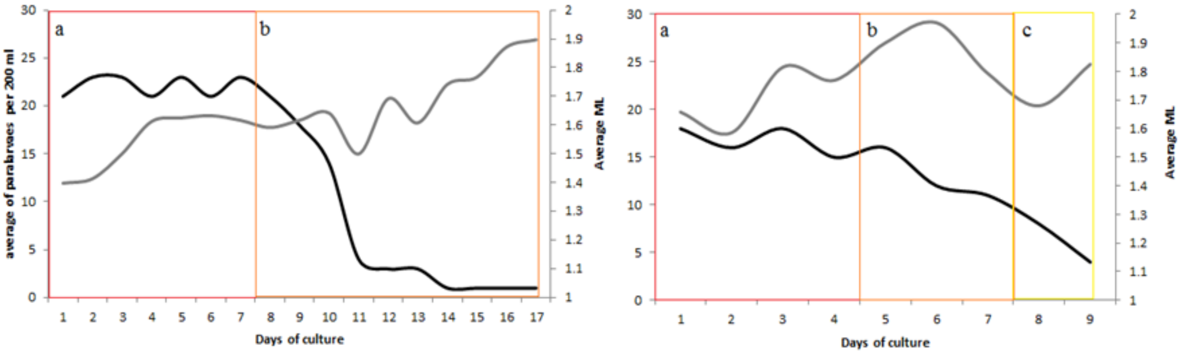


Figure 4. 5: The average paralarvae per 200 ml (line black) and the average of ML mm (gray line) in Group 1 (right panel) with a) *Artemia*; b) copepods and *Artemia*, and then in Group 2 (left panel) with a) *Artemia*; b) mysidacean and *Artemia*; c) *Artemia*.

Three basic types of hunting behaviour were identified: *i*) downward attacks when the paralarvae was positioned above the prey (Figure 4.6); *ii*) upward attacks when the paralarvae was positioned below the prey (Figure 4.7) and *iii*) head-on attacks when the paralarvae was positioned in the same line as the prey (Figure 4.8). From hatching to 3 days of age, the paralarvae showed, the first attacks on nauplius of *Artemia* (slow-moving organisms) were performed by paralarvae measuring 1.56 mm. First, the paralarvae initiate the attack using their eyes, and keeping attention focused on prey (Figure 4.6). Using the fins and siphon together, paralarvae approached the prey with quick, repeated movements, maintaining a fixed position relative to its prey. The first hunts were made at a speed of 20.16 mm/s^{-1}. Shortly afterwards, an attack was carried out with speedy backward jets. Other attacks observed were upwards, reaching a speed of 2 mm s^{-1}.

In Group 1, attacks on copepods were not observed and this prey showed greater escape rapid behaviour than the hunting behaviour of paralarvae. The figure 4.9 shows the escape behaviour of the copepods. In contrast, in Group 2 the mysidaceans were larger, and the paralarvae caught them relatively easily, this prey showed regular fast movements, unlike copepods showing irregularly movements. However pursuit strategy and successful attacks on mysidaceans were observed (Figure 4.10). From 12 to 17-day-old, the paralarvae, repeated attacks occurred between paralarvae, culminating in cannibalism between paralarvae in the absence of other prey types (Figure 4.11). During the hunting on mysidaceans and paralarvae, elaborate strategy was observed, unlike amongst the *Artemia* where paralarvae momentarily stopped in front of their prey.

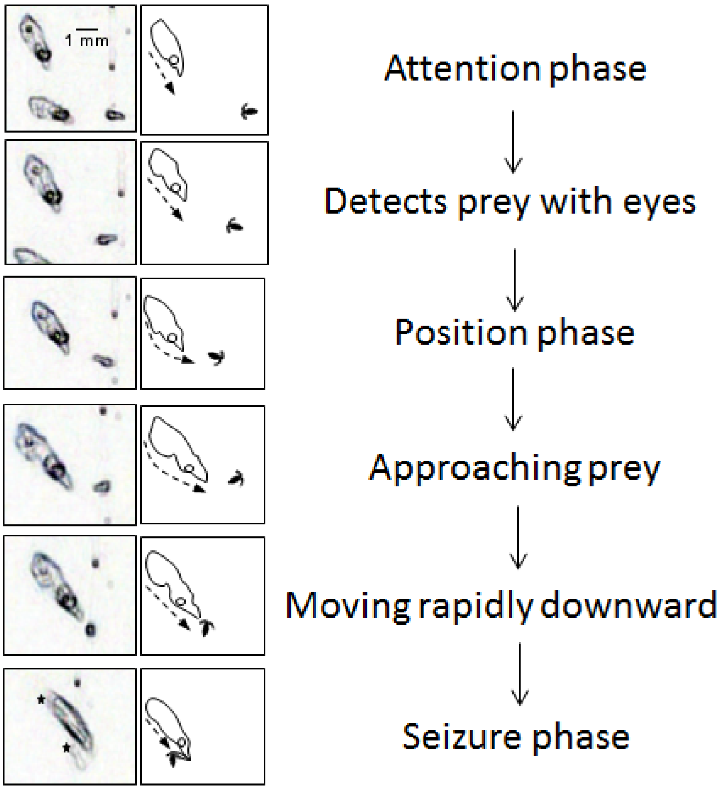


Figure 4.6: Sequential video frame and drawings of downward attack to *Artemia* prey in the experimental Group 1, by a paralarvae of *Doryteuthis plei* of 1. 82 mm ML.

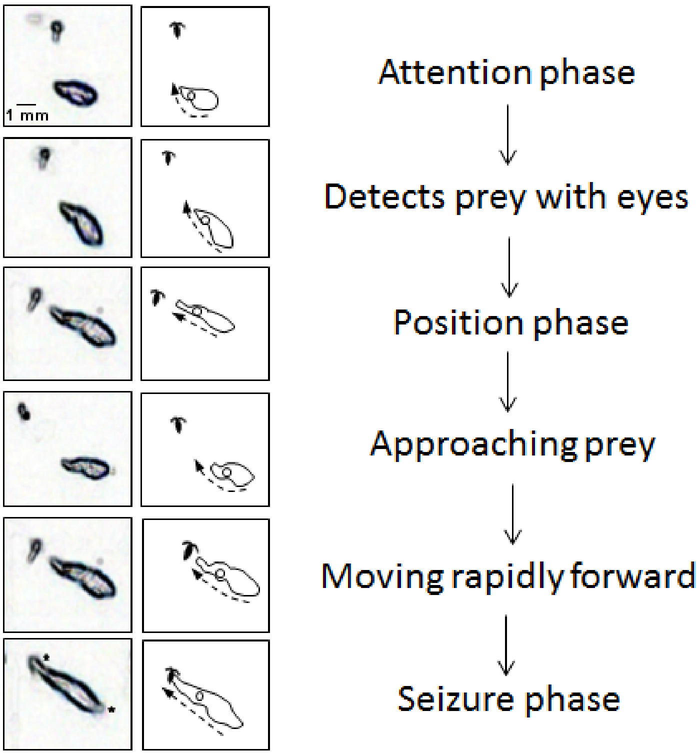


Figure 4.7: Sequential video frame and drawings of upwards attack sequence to *Artemia* prey in the experimental Group 1, by a paralarvae of *Doryteuthis plei* of 1.73 mm ML.

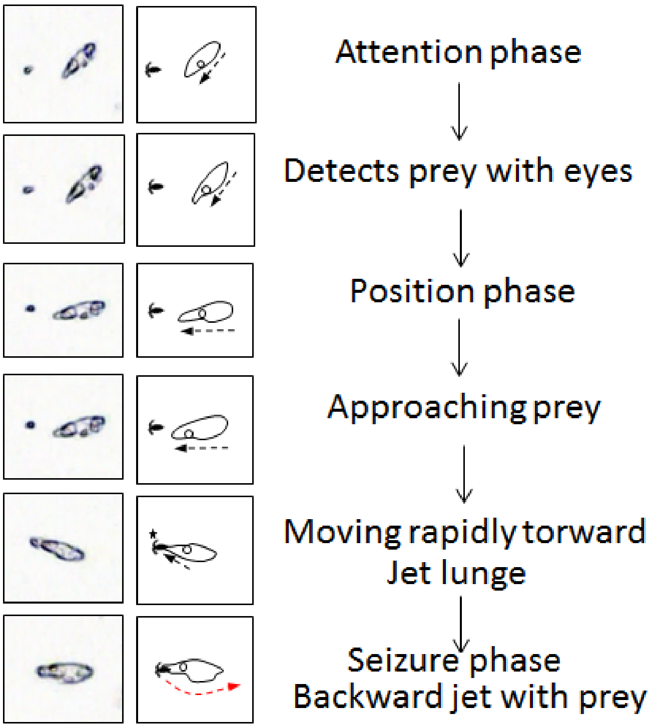


Figure 4.8: Sequential video frame and drawings of head-on attacks sequence on *Artemia* prey in the experimental Group 2, by a paralarvae of *Doryteuthis plei* of 1.85 mm ML. Attack speed was calculated: 16.16 mm/s^{-1}.

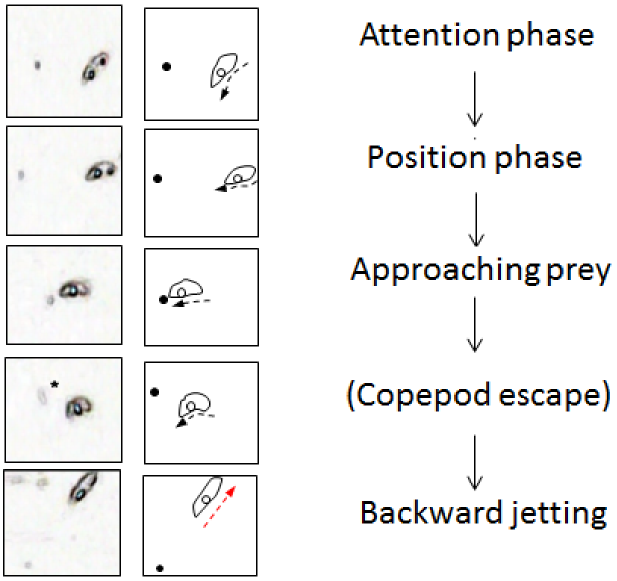


Figure 4.9: Sequential video frame and drawings of pursuit and failed attack sequence on copepods prey in the experimental Group 1, by a paralarvae of *Doryteuthis plei* with 1.87 mm ML.

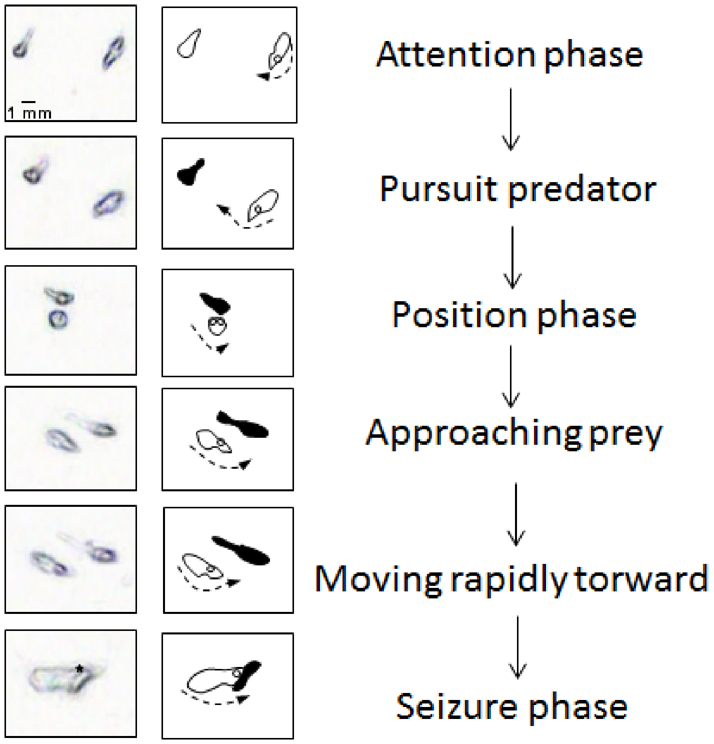


Figure 4.10: Sequential video frame and drawings of pursuit and hunting behaviour sequence on mysidaceans prey in the experimental Group 2, by a paralarvae of *Doryteuthis plei* with 1.89 mm ML.

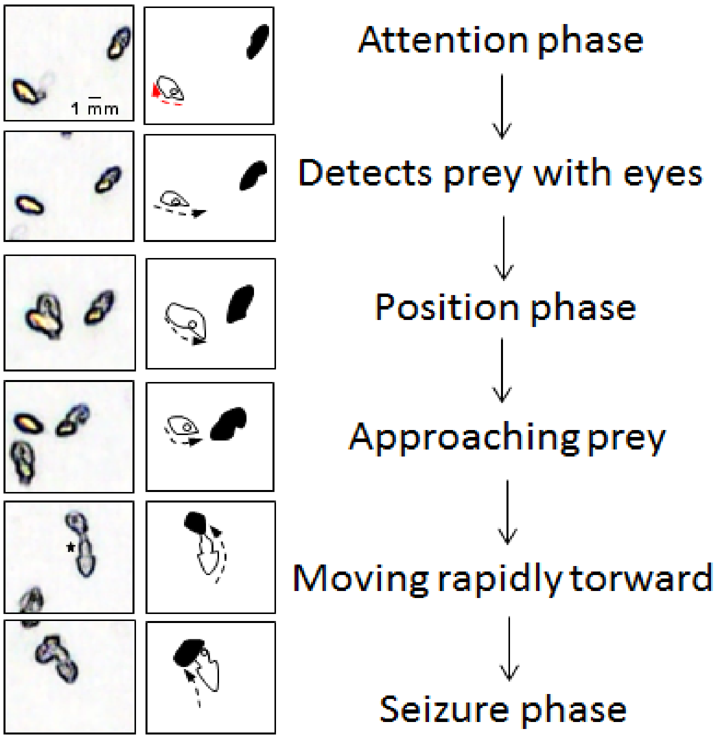


Figure 4.11: Sequential video frame and drawings of pursuit and hunting behaviour sequence between the paralarvae in the experimental Group 2

4. Discussion

Cephalopods have a large specialised central nervous system (Hanlon and Messenger, 1996) but a very different neural anatomy from those of vertebrates, reflecting their molluscan heritage. For these reason cephalopods has been used in neurosciences research in the last century (Fiorito et al., 2014). The in central specialized nervous system is an important feature for learning in early life to acquire abilities for hunting prey.

This study provides the first records on the swimming and hunting behaviour of *Doryteuthis plei* paralarvae in experimental observation conditions. From hatching to 3 days of age, the newly-hatched squid spend most of the time performing vertical swimming in the water column, being upwards swimming faster than downward swimming. It was observed that the fins and siphon perform synchronous movements during swimming. However, the fins in upward swimming were more active, whilst on the other hand; in downward swimming the siphon performed higher power together with the fins. This type of behaviour (vertical swimming) has been described for other hatched squid in the species *Doryteuthis pealei* (Bartol et al., 2008, 2009a, 2009b; Thompson et al., 2010), *Lolliguncula brevis* (Stewart, et al., 2010) and *Loligo vulgaris reynaudii* (Vidal et al., 2005).

Swimming motions (*e.g.* vertical swimming directions) performed by *D. plei* paralarvae observed herein, can be in fact influenced by the seawater physic-chemical properties that such as is the seawater viscosity (*e.g.,* Reynolds number [*Re*]) which acts over the mantle (Bartol et al., 2008). The newly-hatched squid need to adapt to the environment, especially with the viscosity of the seawater and they encounter vastly different flow regimes throughout ontogeny as they undergo critical morphological changes to their two locomotive systems: the fins and jet (Bartol, et al., 2008; Vidal et al 2014; Robin et al., 2014).

In general, the paralarvae make use of pulse jet propulsion, with more rapid and frequent mantle contractions than those observed in adults (Bartol et al., 2008; Thompson and Kier, 2001). Bartol et al., (2008) also noted that squid paralarvae swim predominately along a vertical axis, likewise *D. plei* paralarvae in this study, with sinking occurring as the mantle is refilled. Backward swimming is considered routine swimming and has been observed in planktonic squid. Forward swimming forms part of predatory swimming behaviour (Villanueva et al., 1997).

Paralarvae rearing is at present one of the main bottlenecks for cephalopod culture (Vidal et al., 2014). Newly hatched planktonic paralarvae are delicate and have relatively short arms and limited swimming ability (Villanueva et al., 1995). During the first days after hatching, paralarvae use a combination of endogenous (yolk) and exogenous (prey) food sources (Vidal et al., 2002, Vidal et al., 2014). During this study a high mortality occurred just after the transition of the prey types, as occurred in *D. opalescens* (Chen et al., 1996), and this pattern (mortality after a change of prey) can be also occur particularly during the transition from yolk absorption to external feeding, due mainly to the high metabolic demands of paralarvae, their inability to resist even short periods of starvation (Vidal et al., 2006), and the difficulties of offering prey in adequate quantity and quality (Vidal et al., 2014).

It was shown in this study that *Artemias* is the best prey for newly hatched paralarvae in laboratorial conditions. In previous studies it was suggested that the best survival and growth rates are obtained when using live crustacean zoeae as single or complementary prey, but it is very difficult to obtain these in large quantities (Villanueva, 1994). *Artemia* is readily available but seems to have an unbalanced composition as prey for octopus and squid paralarvae. Nevertheless, apparently, *Artemia* is not limited in terms of vitamin A and E content (Guino et al., 2013).

At only 4-day-age, three sequences based hunting behaviours on paralarvae were observed (upward, downward and head-on attacks), each differing in speed and position. The upward attacks showed mean speed attacks of 1.4 times greater than the downward attack; however the head-on attacks were three times greater than the upward attacks. A correlation between attack distance and speed is evident in this study, as reported for *D. opalescens* by Chen et al., (1996), suggesting that newly hatched squid can accurately gauge the distance to their intended prey and adjust their attack speed accordingly. A sophisticated early development pursuit strategy was starting from 7 days of life, unlike other squids, where these strategies were observed from 30-day-old, for example, *D. opalescens* (Chen et al., 1996) and *S. lessoniana*(Sugimoto and Ikeda, 2013) and displayed variations in mantle musculature configuration (Thompson and Kier, 2001), and develop different jet escape responses (Thompson and Kier, 2001).

Sugimoto and Ikeda (2013) emphasizes that differences in the process of acquiring hunting behaviour in squid, such as observed in this study, can be result of both physical limitations and learning. Since *D. plei*, like other squids, hatch out as miniatures of adults, hunting strategy (pursuing) is basically the same in both the young and adults because of their physical limitations. Other cephalopods such as, cuttlefish (*Sepia officinalis*) particularly also shows a hunting behaviour and associative learning (Cole and Adamo, 2005) especially with ambushing hunting strategy (Sugimoto and Ikeda, 2013).

The unsuccessful attacks observed occurred when the distance between the prey and the paralarvae was great, while the successful attacks occurred at short distances. In the experimental Group 1 from 4 day of experimentation successful attacks on the copepod (erratically moving organisms) were not observed, and paralarvae survival rates decreased drastically in the tank. Studies reported that the copepods have very fast escape behaviour, common for this zooplankton in group (Gemmell and Buskey, 2011). However, studies conducted on newly-hatched squid *D. opalescens*, show multiple attack strategies for this zooplankton with fast escape behaviour and the positive impact of both failed and successful copepod attacks on the ontogeny of prey capture appears to be far stronger in early in life than at day 40 (Chen et al., 1996). More specialized attacks such as arm interceptions were observed only with *Artemia*. The mysidaceans were captured by paralarvae with pursuit strategy, and they also had rapid escape behaviour; likewise for the copepods, but they were easily caught by paralarvae because of their larger size.

In the absence of prey, paralarvae exhibited intra-specific aggressive behaviour, when they showed both a pursuit strategy and escape rapid backward after attack, something that has been rarely reported during this life stage for other paralarvae squid around the world.

Feeding conditions are of crucial importance for cephalopod population dynamics from the early life stages in spite the fact that they still have some endogenous yolk reserves during their first days after hatching while already hunting prey. Food availability can induce growth plasticity in paralarvae in very short time periods (Vidal et al., 2005). The rates of yolk utilisation in fed and starved laboratory-reared loliginid paralarvae suggest that yolk utilisation is dependent on feeding conditions, with fed paralarvae having lower utilisation rates (Vidal et al., 2002, 2005).

5. Concluding remarks

The newly-hatched paralarvae of *D. plei* were found to be active swimmers by the early use of jet propulsion, showing a complex predatory behaviour on live preys. Overall, upwards swimming was more frequent and rapid than downwards swimming. Jets forward were more observed in the attacks during food intake. Survival rates decreased drastically after the change in prey composition. Thus, the relationship between attack behaviour and prey composition was considered an important factor to the survival in the first days of paralarvae.

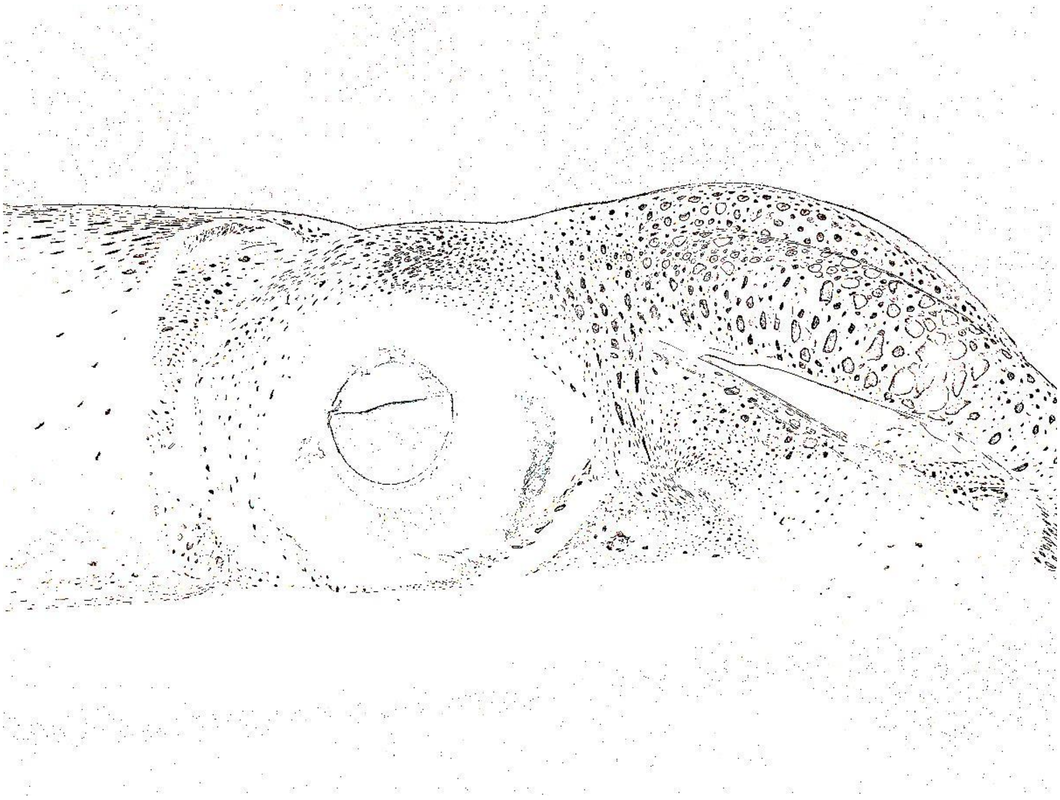
During the first stage of the hunting mode (attention phase), the squid eyes focused directly on the prey, appearing highly well-developed, as well as the sense organs responses. In the absence of prey, paralarvae exhibited aggressive intra-specific behaviour, when they showed both a pursuit strategy and a rapid backward escape after attack. The gradual development of sensory systems after hatching has certainly a direct impact on the ability of the paralarvae to perceive threats and to initiate evasive responses.

The observed hunting behaviours provide insights into the experience-dependent aspects of the ontogeny of prey capture, and were found to be similar to those reported to adult specimens. It includes using the eyes to the location and attention of prey, the fins and siphon for positioning before the attack, and finally the arms and tentacles to seize the prey.

Swimming performance and ontogeny of predator/prey interactions during early life appeared to be key factors to squid’s survival, when new capabilities arise and performance levels improve rapidly. Such improvement in swimming performance can be a significant factor, for example, in the vertical distribution pattern of squid paralarvae. The newly-hatched squid seems to be indeed miniatures of the adults, and born with a highly developed nervous system and innate hunting skills, being active swimmers and predators which likely allows some advantages over other organisms with planktonic lifestyle.

Lastly, the swimming and hunting performance in the first days of life observed in laboratorial condition can help to understand how the paralarvae confront ecological challenges in the wild, considering their abilities to obtaining food, evading predators, and locating and remaining in a suitable habitat. It may help to elucidate on the evolutionary advantages and plasticity of these animals in the marine environment.

General conclusion



General conclusions

This thesis provided new detailed information on several aspects of the biology and ecology of the squid *Doryteuthis plei* around the São Sebastião Island, in the coast of São Paulo, SE Brazil. It included the population structure of the exploited stock, reproductive activity, feeding habits, and particular behavioural traits in two crucial periods of the life-cycle: the planktonic phase in early life (paralarvae), and the reproductive phase (adults).

In the first days of life, newly-hatched squids do not show morphological differences compared to adults, they were found to be active swimmers by an early use of jet propulsion, showing a complex predatory behaviour. From hatching to the age of 3 days, they spend most of the time performing vertical swimming in the water column, for which the fins and the siphon showed an important role in the guidance and body control during locomotion and feeding-related pursuit strategies. This likely allows them some advantages over other organisms with planktonic lifestyle.

Squid paralarvae at the age of 4-days in aquaria showed to be active predators, developing numerous hunting strategies on mysids and *Artemia* prey. A sophisticated early development of pursuit strategies started from the 7th day of life, and 15-days old paralarvae exhibited intra-specific aggressive behaviour, showing both a pursuit strategy and a rapid backward escape after attack. The observed hunting behaviours provide insights into the experience-dependent aspects of the ontogeny of prey capture, and were found to be similar to those reported to adult specimens. It included using the eyes, for the location and attention of prey, the fins and siphon, for positioning before the attack, and, finally, the arms and tentacles to seize the prey.

In the adult phase, *D. plei* showed an amazing reproductive strategy from November to April in shallow waters around the São Sebastião Island. Firstly, mature females between 151–175 mm ML were more frequent in November and December, concentrating in shallow waters in the Southern and Southeastern areas of the island to spawn. Secondly, in January and February, the sex-ratio tended to equalize at depths of 10-20 m, where squid would most likely be mating.

Size-at-maturity was found to be from 151 to 175 mm ML for females, and between 187 mm and 190 mm ML for males. Males showed the co-occurrence of two distinct maturation groups, a population strategy that increases species' fertilization opportunities.

During the mating period in the tanks, the squid showed complex behaviours, *e.g.* a gonadal display body pattern in both nidamental and oviducal glands in females and testis in males during courtship, or lateral mantle streaks in large males during fights for mating. In this study, *D. plei* mating occurred in two positions. The first position was the "head-to-head" mating with female and a sneaker male, which was observed more frequently. The second was the "male-parallel position" occurred with a large consort male.

During the reproductive phase, mature females fed mainly on cephalopods and small pelagic fish. They appear to spawn on sandy bottoms in the North and Northeast areas of the island, confirmed by the occurrence of spent individuals. Crustaceans as prey were more frequent in the stomachs of females and in those areas. The squids showed a vast repertoire of body patterns and behaviours linked to intra and interspecific interactions during reproductive behaviour. During the spawning process, egg-directed behaviours were observed in captivity, with the females extruding a single egg capsule and affixing it to the substrate or to existing communal egg mops. The eggs capsule deposition occurred in the darkness period. At the population level, the males appeared more frequently at the end of the season (March), confirming the existence of female post-spawning mortality.

The results indicated that the local small-scale fishery seasonally removes part of the reproductive stock of the squid *D. plei* in shallow waters around the island, as confirmed by the high proportion of mature squid in all samples. Though, the study highlights that such a valuable fishery resource urges a local fisheries management plan that would certainly benefit from the information obtained during the preparation of this thesis.

Squid feeding habits differed among sexes, also depending on the body size, the maturity scale, and the spatial-temporal pattern. Females do not stop feeding during sexual maturation and the amount of food increases with size. Cephalopods were significant prey for mature females from 142 to 213 mm ML, as well as fish and crustaceans to the largest up 195 mm ML, while polychaetes were dominant prey for largest males. The hunting strategy already reported for adult squid was also shown in the early days of life in *D. plei* paralarvae.

Lastly, the analysis of a long fisheries-related dataset along with robust statistical analysis (such as GAMLSS) together with observations on behavioural responses in laboratorial conditions, allowed to evidence yet unrevealed aspects of the tropical arrow squid in Southeastern Brazil. Aspects of the life-cycle observed herein may elucidate on the evolutionary advantages and plasticity of these squids in the marine environment.

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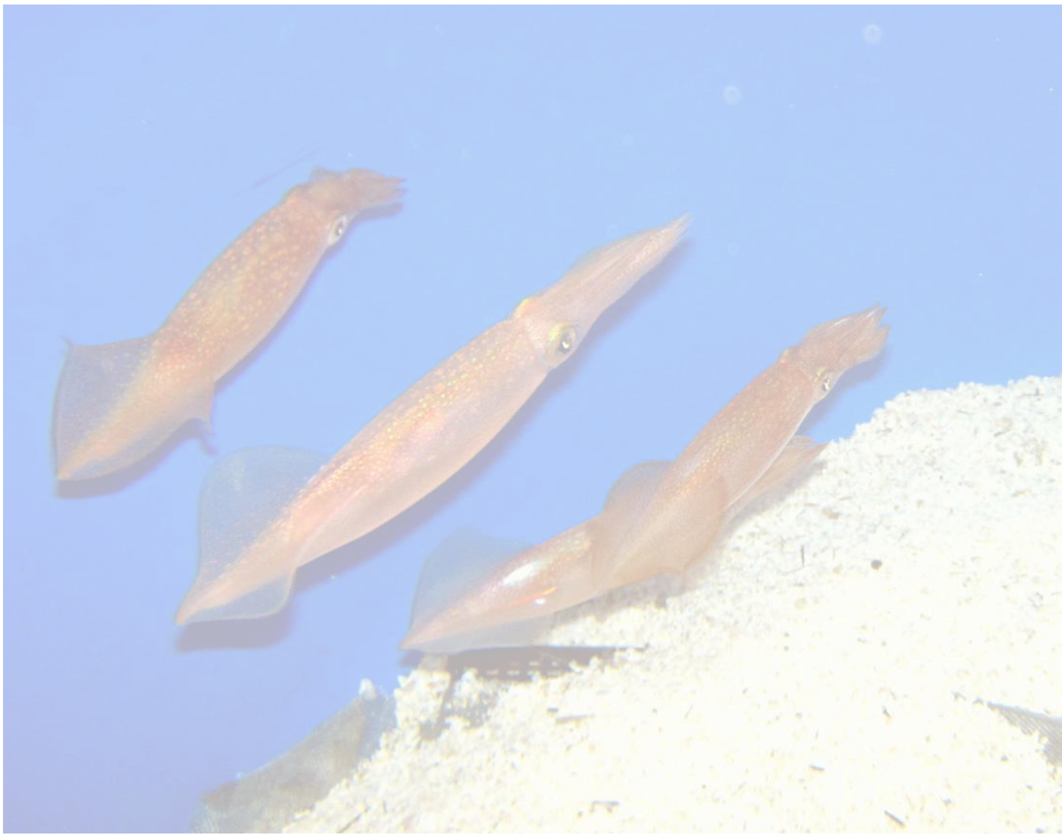

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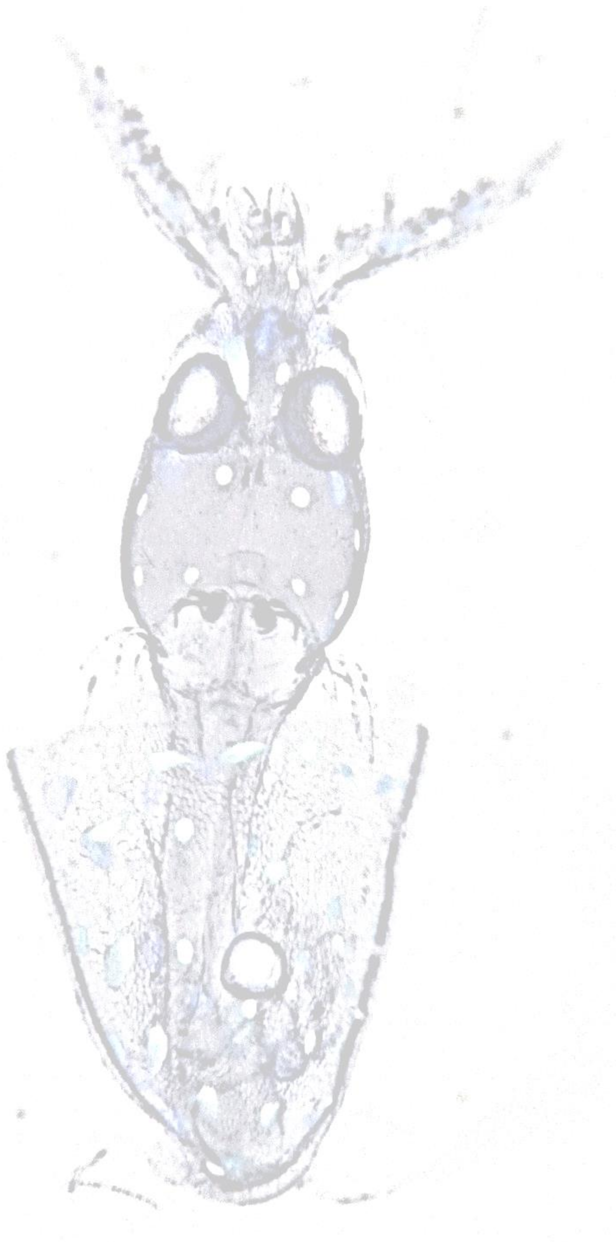
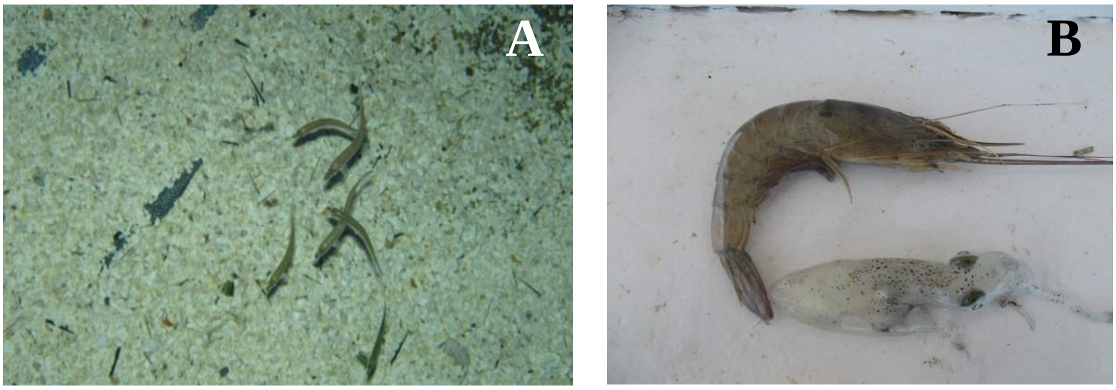
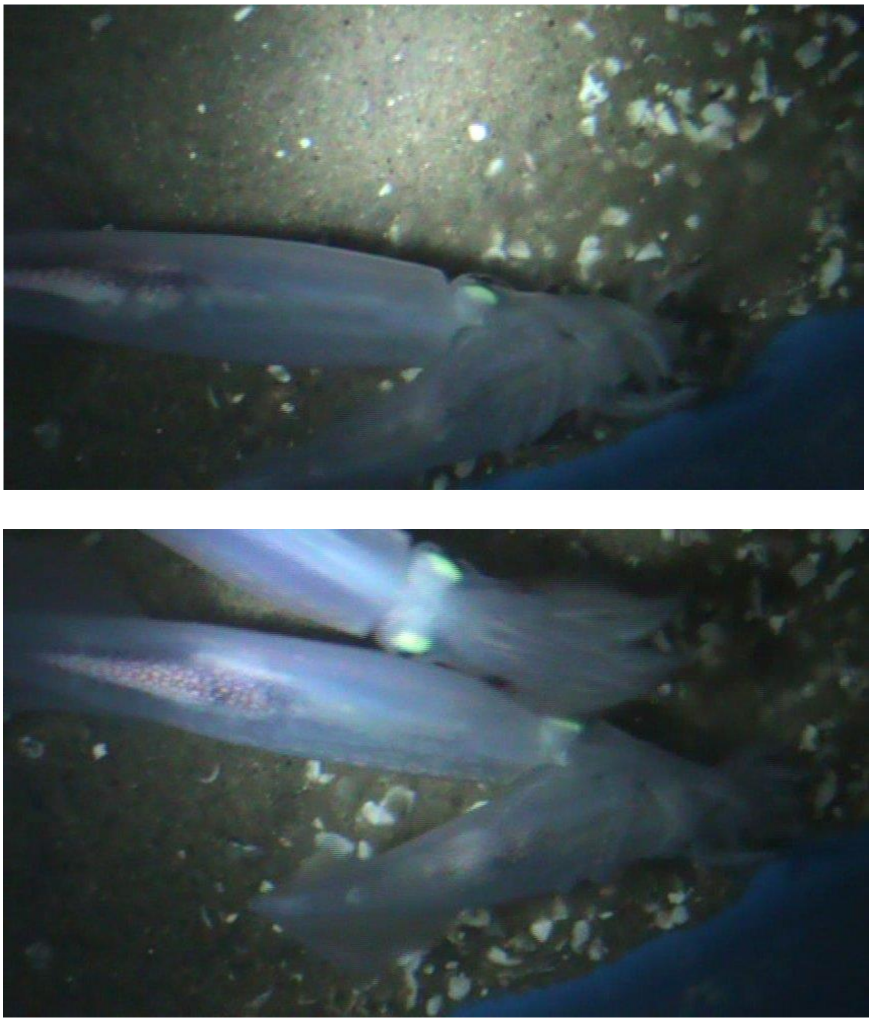
Appendix



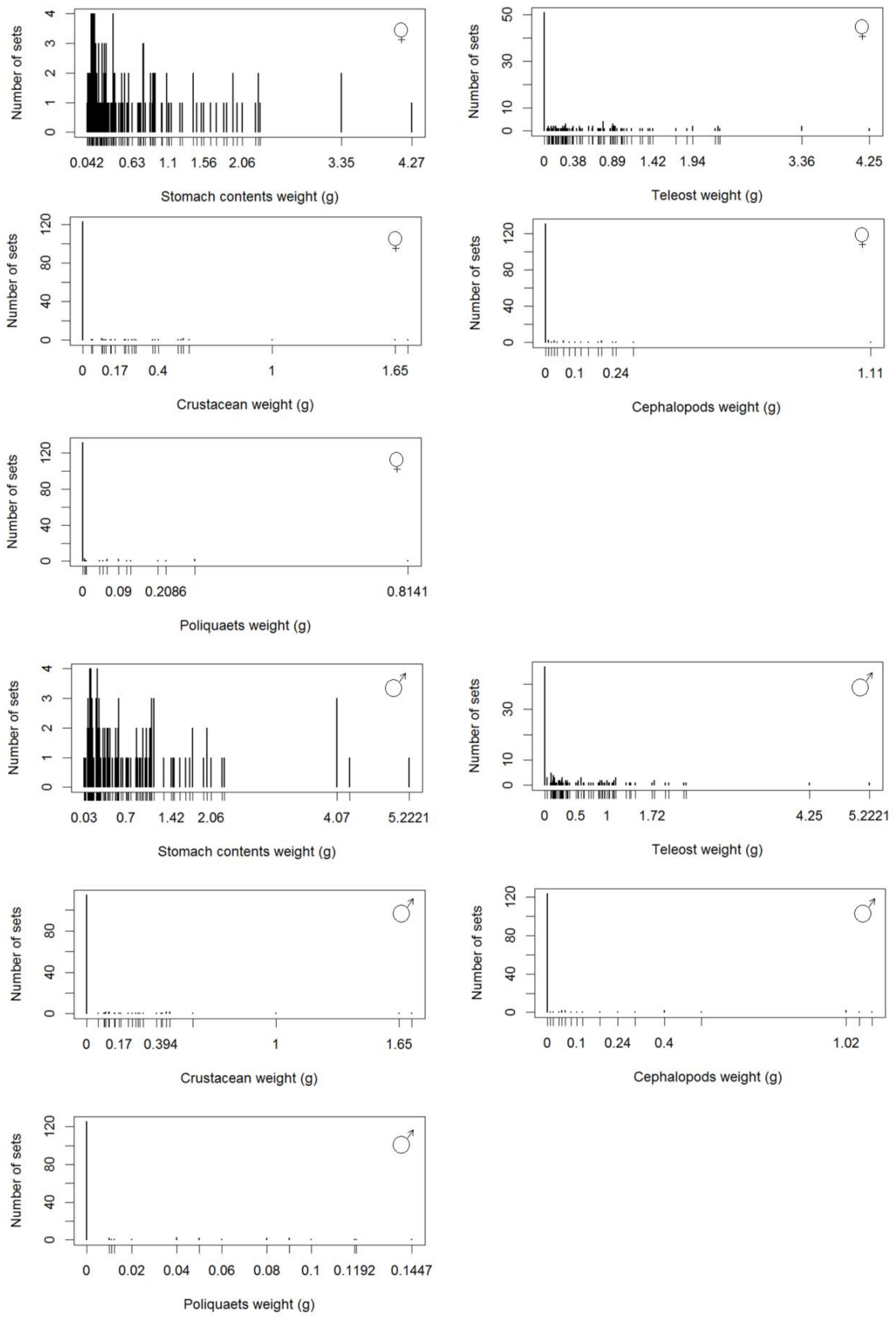
Appendix 2.1: (A) Tank (300-l) containing local seawater used to transport the squid and (B) research vessel *Veliger II* for sampling.



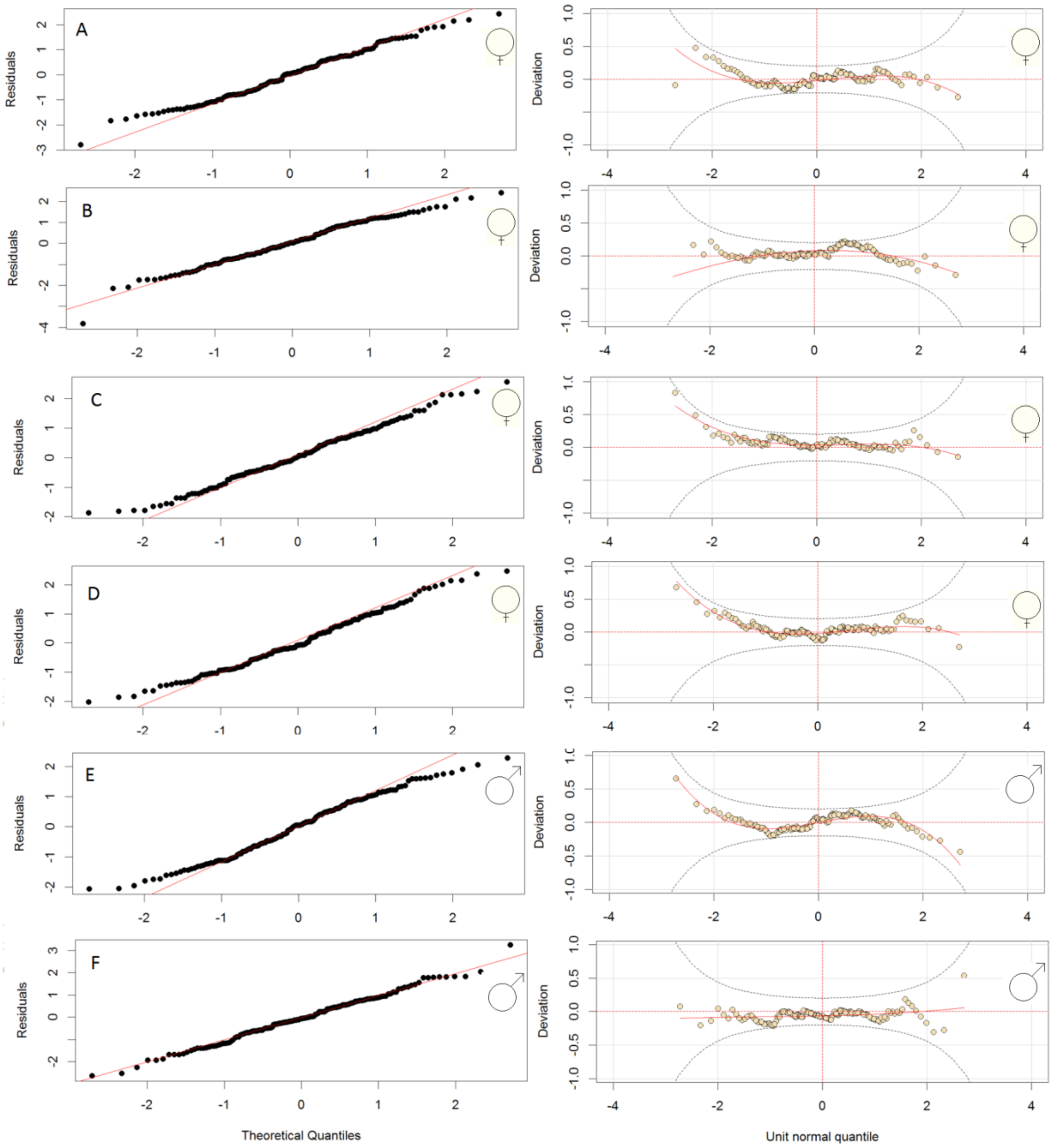
Appendix 2.2: Food of squid (A) live fish and (B) *Lolliguncula brevis* and seabob shrimp (*Xiphopenaeus kroyeri*).



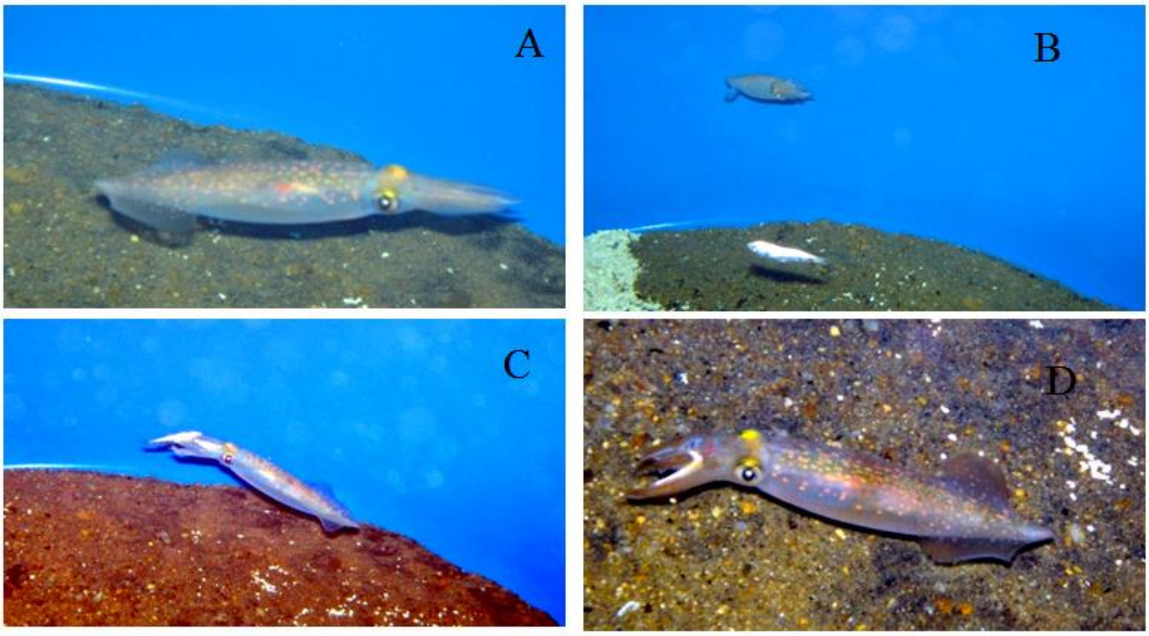
Appendix 2.3: Sequence of Male-parallel mating over the egg in captivity, with the approach of a male intruder.



Appendix 3.1: Fraction of the prey weight found in the stomach contents of the *Dorytheutis plei*. (A) Stomach contents weight; (B) Teleost weight; (C) Crustacean weight; (D) Cephalopods weight and (E) Pelagic polychaetes weight, in grams (g) for females.



Appendix 4.2: Quantile–quantile (QQ) plots of the deviance residuals (left panel) and worm plot (right panel) of the models fitting of *Doryteuthis plei*. Females (from A-D) and male (from E-F). (A) and (E) Lognormal distribution for full stomach contents; (B) “Zero Adjusted Inverse Gaussian” distribution for teleost weight, (C) same for crustaceans, (D) same for cephalopods and (F) same for poliquaete.



Appendix 3.3: Feeding a female *Doryteuthis plei* with 143 mm ML in captivity. Involving four stages: (A) Attention; (B) Position; (C) Attack and (D) Bite prey.

Appendix 4.2: Sampling of egg masses by scuba-diving in the inshore waters off the Northern coast of São Paulo, Brazil

Appendix 4.3: Structures of the flow-through system consisting of three cylindrical rearing tanks of paralarvae (a control tank and two replicas) each 1 000 L, kept at the University of São Paulo's Center for Marine Biology (CEBimar) near the São Sebastião Channel.

Appendix 4.4: (A) Newly-hatched squid *Doryteuthis plei* and *Artemia* prey. (B) Stomach contents of a paralarvae with 1.65 mm ML (100 x zoom).