UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Characterization of embryonic development of European cuttlefish (*Sepia officinalis*)

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Dissertação
Mestrado de Aquacultura e Pescas
(Especialização em Aquacultura)

Trabalho efectuado sob a orientação de:
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2014

Characterization of embryonic development of European cuttlefish (*Sepia officinalis*)

Autoria de trabalho

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Acknowledgements

Embora esta tese esteja escrita em inglês de modo a facilitar uma possível publicação em revistas da especialidade, optei por fazer os agradecimentos na língua materna.

Quero primeiramente agradecer ao Doutor António Sykes por ter dado a oportunidade de realizar este tópico, aceitando o meu trabalho. Pelo esforço e paciência que fez para garantir que eu tivesse realizado esta tese até o fim. É importante sentir que o interesse é mútuo, pois aumentou a confiança de que teria tempo para realizar todo o projeto. Obrigada pela ajuda e principalmente pela paciência em momento de dúvidas.

Queria agradecer também ao Mestre Rui Gonçalves pela formação, ajuda e confiança na parte prática. Não é todos os dias que encontramos alguém sem problemas alguns em transmitir tudo o que sabe, acreditando nas nossas capacidades sem conhecer os nossos trabalhos prévios.

Ao pessoal do laboratório 1.19, pelo incentivo e companhia nos últimos meses. E acima de tudo pela amizade criada. Ana, Céline e Cátia muito obrigada.

Agradeço amavelmente, ainda ao pessoal do Ramalhete pelas boleias e companheirismo num mês intenso de amostragem.

Aos meus colegas que ajudaram me a sair do estado de preguiça, passando horas na biblioteca, mergulhados em livros e artigos. Pelos incentivos nos momentos altos e principalmente nos baixos. Márcia Santos, Luís Negrão, Luís Calisto, Tânia Lourenço, Tania Gonzalez, e ainda Elena Alvarez e Maurício Namora. Vocês foram a família que escolhi nesta etapa, e agora tenho-vos para o resto da vida.

E finalmente mas não por último, à minha família que apesar de estarem a mais de 6000km de distância tem sido as minhas âncoras. Ajudando me a nível emocional e financeiro durante estes 9 anos fora de casa. Dedico-vos este trabalho.

Abstract

The increasing cephalopod human seeking as a source of food have been defined it as an aquaculture candidate in the past 20 years. Such have been propelling the cephalopods culturing, such of the European cuttlefish *Sepia officinalis*. However there are bottlenecks for this species, for instance, at nutritional level. It has been difficult to define a diet with high nutritional value for the cuttlefish and with reasonable costs for the producer. A variety of alternative types of foods have been tried, but these have been showed to be costly and not as good as natural diets. Artificial diets had been unsuccessful within the culturing of cuttlefish

This study aimed to characterize the development of digestive tract during embryogenesis of European cuttlefish *Sepia officinalis.* The external and internal yolk sacs were mainly focused, as well as their nutritional content (using specific stains) and function. Analysis at macroscopical scale (weight and measurements) and microscopical scale (photographs, histology and image analysis) was performed.

Eggs were weighed and measured in width and length. Then, these were cut open and embryos were removed from capsule and photographed. The embryos were then kept in formaldehyde. Images were treated and the area of the external yolk sac and the embryo was measured using image J. Histological techniques were performed to characterize the different embryonic stages and external and internal yolk sacs.

Changes in the eggs sizes were observed. An initial reduction of the weigh, width and length during the first 10 dpp (days post posture) was observed. Followed by a rapid increase until hatching ($p < 0.001$). A strong correlation between the weight and width of eggs ($R^2 = 0.92$) was observed. Being suggested as the most viable form of measurements for different studies. The evolution of egg weight was compared with the study of Sykes, *et al.* (2009). Similar variation was observed ($P < 0.05$), however, in the present study the weight of the eggs was higher. Females at the present study were bigger and laid larger eggs.

Both, external yolk sac and the embryo areas, measured with the image J were correlated. As the external yolk sac decreased, and thus consumed, the embryo increased its size. During embryonic development (from 12 day post posture, dpp) there was an inverse relationship between these two factors ($p < 0.001$, $R^2 = 0.91$).

The total area of the embryo (TEA, sum of the yolk sac area and embryo area) was estimated and compared with the weight of the eggs during the same time period. TEA had an almost linear evolution while the weight of the eggs was exponential during embryonic development.

Despite the high correlation between these two factors ($R^2 = 0.68$), it was suggested that the water content may be also relevant in this period.

Different stages were identified according to the description of Naef (1928). Throughout embryogenesis it was observed the development of various rudimentary organs, such as eyes, arms and tentacles, mantle and its pigmentation. From stages X-XI onwards, the enclosure of the external yolk sac was observed. From the 13 dpp onwards histological images were collected and allowed a more detailed evolution of both yolk sacs. The contents of the sacs were PAS positive, suggesting the presence of polysaccharides. The extra-ectoderm layer (exe) was observed surrounding the external yolk sac, which was positive for Toluidine-O. The outer side of this layer presented cilia that are responsible for molecules diffusion present in the perivitelline fluid (pf). The inner side of the exe was in contact with the perivitelline sinus (ps), which cells appeared to be absorbed or pinocytosis to the yolk sinus (ys). The sacs content was composed of yolk platelets and empty spaces that may be related to the presence of lipids. Similar spaces were also observed in the internal yolk sac but in this structure, agglomeration of yolk droplets were mostly observed. Such droplets could be the early stages of the digestive gland cells of the embryo. A darker layer around the internal yolk sac was observed, which might be its epithelium, where it was noticed some triangular cells. It was suggested that, similarly to other cephalopods, these cells could either be hemocytes reacting to bacteria or either cellular replacement or detoxification. Near hatching, the animals showed some resemblance with the adults and the external yolk sac was greatly reduced.

Keywords: Cuttlefish *Sepia officinalis*, embryology, histology, external yolk sac, internal yolk sac.

Resumo

A procura por cefalópodes como fonte de alimento tem vindo a definir, nos últimos 20 anos, o seu potencial como candidatos para a aquacultura, propulsionando o cultivo de espécies como o choco (*Sepia officinalis*). Esta espécie apresenta um ciclo de vida curto e rápido crescimento, o que permite rápidos ciclos de produção a reduzido custo de manutenção. Têm altas taxas de sobrevivência e resistência a elevadas densidades e patologias. Contudo existem ainda alguns obstáculos à sua produção em grande escala, nomeadamente a nível nutricional. Apesar de diferentes tipos de alimentos terem sido testados em chocos, ainda não se definiu uma dieta com elevado valor nutricional e simultaneamente com custos reduzidos para o produtor.

Este trabalho teve como objetivo caracterizar o desenvolvimento do trato digestivo durante a embriogénese de *S. officinalis*. Os sacos vitelinos externo e interno foram o foco principal desta dissertação, onde foi estudado não só o seu conteúdo nutricional (com recurso a colorações específicas), como também as suas funções. Foram realizadas análises a nível macroscópico (peso e medidas) e microscópico (fotografia, histologia e análise de imagem).

Ao longo de 31 dias de amostragem, no Centro de Ciências do Mar (Estação Experimental do Ramalhete), foram recolhidos um total de 364 ovos de choco. Estes foram colocados em cestos de incubação, num tanque de 1500 L, com temperatura (19.4 ± 0.95 ºC), salinidade (34.5 ± 0.63 ‰) e oxigénio (93.7 ± 3.58 %) constantes. Foram recolhidos, aleatoriamente, 5 ovos por dia para pesagem e medição de largura e comprimento. Estes ovos foram abertos e cada embrião foi retirado da cápsula e córion, fotografado e de seguida preservado em formaldeído.

Foi realizado o protocolo padrão de histologia para caracterizar os sacos vitelinos externo, interno e os diferentes estágios embrionários. Os chocos foram inicialmente desidratados através de lavagens em etanol de percentagens crescentes por períodos de tempo estabelecidos. Realizaram-se pré-infiltrações, infiltrações e polimerizações de acordo com as instruções em Heraeus Technovit 7100 (Heraeus Kulzer GmbH, Germany). Depois da solução de resina ter sido preparada, cerca de 3 ml foram colocado em moldes e os embriões cuidadosamente posicionados. Após 48 h de secagem, cortes longitudinais de 3 µm foram obtidos, colocados em lâminas de microscópio (3 a 5 cortes por lâmina) e corados com azul de tolueno e ácido periódico de Schiff (PAS – *periodic acid-Schiff*). Após a secagem, as lâminas foram observadas sob uma lupa Zeiss STEMI 2000-C e para maiores amplificações foi utilizado um microscópio Leica DM200 com câmara Leica SFC480 incorporada.

As medições efetuadas permitiram detetar alterações no crescimento dos ovos. Foi observada uma redução inicial do peso, largura e comprimento durante os primeiros 10 dias após a postura (dpp - *days post posture*). Houve desidratação dos ovos endurecendo a cápsula gelatinosa exterior, o que permitiu a retenção dos nutrientes necessários ao desenvolvimento embrionário e ainda proteção contra agentes patológicos. De seguida, observou-se o aumento rápido das três variáveis (peso, largura e comprimento) até à eclosão ($p < 0.001$). A reabsorção de água facilitou a eclosão. Há uma maior correlação entre o peso e a largura dos ovos ($R^2 = 0.92$) do que entre as restantes variáveis. O peso e a largura do ovo foram então sugeridas como as mais viáveis para diferentes estudos. O crescimento em peso dos ovos teve uma variação semelhante à obtida por Sykes, *et al.* (2009a) ($p < 0.05$), no entanto, o peso dos ovos apresentou valores superiores no presente estudo. Uma possível explicação é o facto de as fêmeas utilizadas neste estudo serem maiores, produzindo ovos de maiores dimensões. Fez-se uma correlação entre a dimensão do saco vitelino externo e do embrião. O saco vitelino externo diminui à medida que foi consumido, o que se traduziu no aumento de tamanho do embrião. Assim, estas variáveis estão inversamente relacionadas ($p < 0.001$, $R^2 = 0.91$). A área total do embrião (TEA – *total embryo area*, área do saco vitelino mais a área do embrião) foi estimada e comparada com o peso dos ovos durante o desenvolvimento embrionário. A TEA apresentou uma evolução quase linear, enquanto o peso dos ovos aumentou exponencialmente. Apesar de a correlação entre as duas variáveis ter sido elevada ($R^2 = 0.68$), o teor de água poderá ser o aspeto mais relevante neste período.

Os diferentes estágios de desenvolvimento embrionário foram identificados de acordo com a descrição de Naef (1928). Foi observado o desenvolvimento de órgãos rudimentares como olhos, braços e tentáculos, manto e sua pigmentação. A partir do estágio X-XI foi observada a inclusão do saco vitelino externo. De 13 dpp (estágio XIII-XIV) em diante, foi possível reunir imagens histológicas que permitiram uma observação detalhada de ambos os sacos vitelinos. Os conteúdos dos sacos tiveram resultados positivos para PAS, o que sugere a presença de polissacarídeos, enquanto que órgãos rudimentares e membranas dos sacos vitelinos tiveram resultados positivos para azul de tolueno. Foi observada a camada extra-ectodérmica (exe), positiva para tolueno. O lado exterior de exe é composto por cilia que é responsável pela difusão de moléculas presentes no fluido perivitelino. O lado interno de exe está em contato com o sinus perivitelino onde foram absorvidas e pinocitadas células para o sinus do vitelo. Os sacos eram constituídos por várias plaquetas de vitelo e por espaços, relacionados com a presença de lípidos. No saco vitelino interno foram também observados pontualmente os mesmos espaços, mas na maior parte dos casos foi observado um aglomerado de gotículas,

que poderão ser fases iniciais de células da glândula digestiva do embrião. Foi observada uma camada mais escura em torno do saco vitelino interno, correspondendo possivelmente ao epitélio no qual existiam algumas células de formas triangulares. Estas células poderão advir do processo de substituição ou desintoxicação celular, ou poderão ser ainda hemócitos, que reagem à presença de bactérias, como observado anteriormente noutros cefalópodes.

O volume do saco vitelino externo foi idêntico ao volume do embrião no fim do estágio XVI, podendo ser comparado com os resultados da correlação anteriormente efetuada entre a área do saco vitelino externo e a área do embrião. Nos estágios XVII-XVIII, o volume do saco vitelino externo e interno apresentaram semelhantes proporções. Próximo da eclosão os animais eram morfologicamente semelhantes aos adultos e o saco vitelino externo era bastante reduzido.

Palavras-chaves: Choco *Sepia officinalis*, embriologia, histologia, saco vitelino externo, saco vitelino interno.

Abbreviations and Acronyms

ARA	Arachidonic Acid (20:4n-6)
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic Acid (20:5n-3)
EUROSTAT	European statistics
EU	European Union
INE	Instituto Nacional de Estatística (Portuguese Nacional Institute of Statistics)
FAO	Food and Agriculture Organization of the United Nations
FIGIS	Fisheries Global Information System
PUFA	Polyunsaturated fatty acids
UK	United Kingdom
a	arm
ac	arm crown
amm	annular mouth muscle
Bm	Buccal mass
br	basal ring
cb	cuttlebone
ch	Chorion
chu	cheek hump
Cm	Cephalic mass
dda	digestive duct appendages
dg	digestive gland
dgd	digestive gland duct
dpp	days post posture
e	eye
ec	external capsule
eg	egg
exe	extra-embryonic ectoderm
eys	external yolk sac
f	fins
FT	Funnel Tube
g	gills

HO	Hoyle’s organ
i	iris
Is	Ink sac
iys	internal yolk sac
l	lens
ma	mantle
mp	mantle primordium
ol	optic lobe
pf	perivitelline fluid
ps	perivitelline sinus
psg	shell gland primordium*
psp	perivitelline space
sh	shell
sg	salivary glands*
sk	suckers
sp	shell pouch*
st	statocysts
T	Tentacle
Tp	Tentacle pouch*
vm	vitelline membrane
ye	yolk epithelium
ys	yolk syncitium
ym	yolk membrane
yp	yolk platelets

* abbreviations used in-text only

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1. Introduction

1.1. The importance of cephalopods as food: share and market value

The world capture production of seafood was estimated to be about 97 million tonnes live weight in 2011. Being China (26.7%), followed by Europe (13.8%), Peru (8.6%) and Indonesia (5.4%) the main world producers (catches and aquaculture) (Eurostat, 2012; FAO, 2012). Aquaculture is a growing industry worldwide (FAO, 2012). In 2011, aquaculture production contributed with almost 16% for the world's seafood production, being China the greatest producer (Figure 1.1.1). In the European Union (EU), seafood aquaculture represents 6% of the total volume of EU fisheries production and 4.8% of the world aquaculture production (Eurostat, 2012).

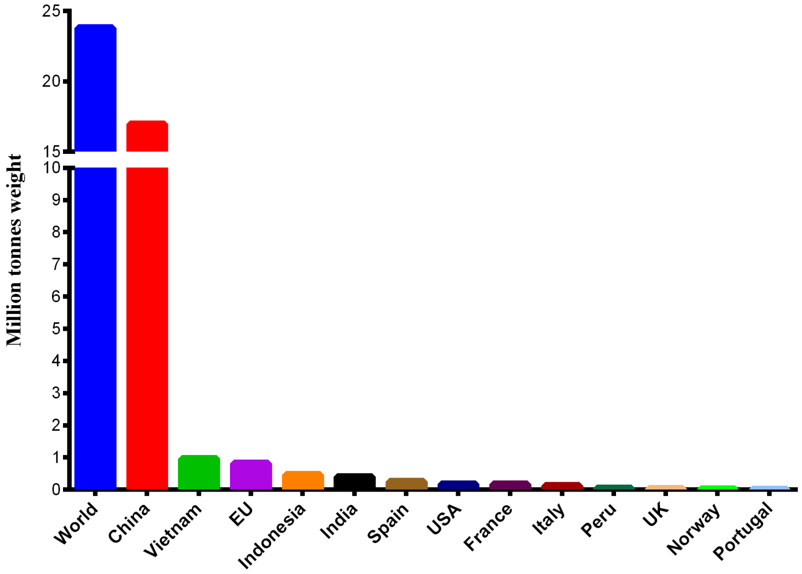

Figure 1.1.1 The world aquaculture production and the contributors for its growth (FAO 2012)

Worldwide cephalopod products (raw, fresh, fresh-cooked or processed) have been extensively consumed due to its high protein and low fat nutritional content (Sykes*, et al.*, 2009b). This has resulted in great demand for cephalopods worldwide, increasing its share as fishery products and promoting its potential for culture (Almansa*, et al.*, 2006; FAO, 2012). The high cephalopod demand in the Mediterranean and Asian markets greatly contributes for the world production (Almansa, *et al.*, 2006). According to FAO (2012), cephalopods contributes to 14% of the world fisheries. In 2011, the production of squid, cuttlefish and octopus reached 3.7 million tonnes weight. Countries, such as Spain, Italy and Japan, are the largest world's cephalopods consumers and consequently the largest importers. Nonetheless, in the EU, Spain represented the biggest cephalopods trade (figure 1.1.2), being the biggest importer (221,721 t) and exporter (109,871 t) of squids, cuttlefish and octopuses in 2011 (FAO 2012).

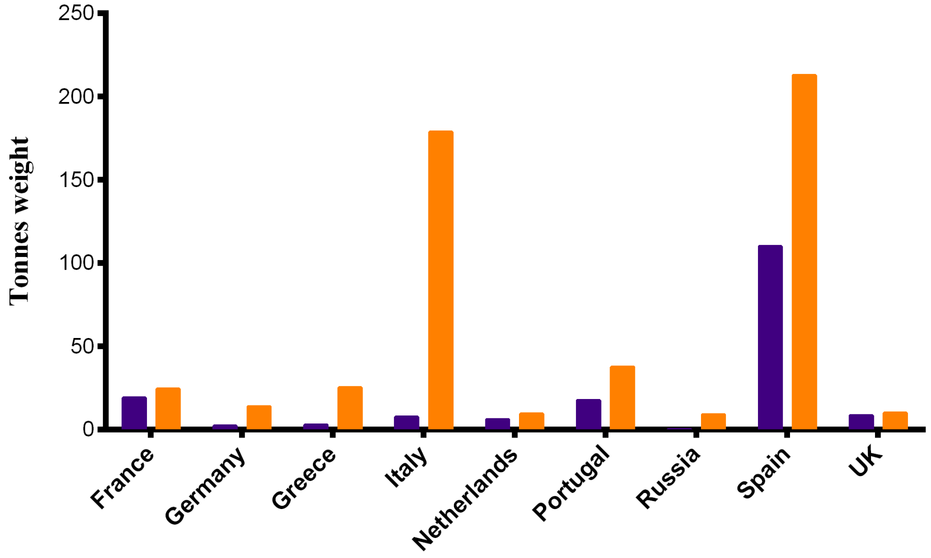

Figure 1.1.2 Main cephalopods commodities trade in 2011 within European countries, (■) exports (■) imports (FAO 2012)

In 2009, Portugal was ranked the third country in the world in seafood consumption and the first in the EU, with nearly 61.1 kg per capita per year (Eurostat, 2012; FAO-FIGIS, 2012). However, the Portuguese production is insufficient to supply the market demands. By 2012 Portugal had captured 151,343 tonnes of seafood (INE, 2013). According to FAO (2012), 0.7% of the Portuguese captures in 2011 were European cuttlefish (*Sepia officinalis*). Additionally, in the same year, the species captures in Portugal contributed for 8.7% of the EU total production (figure 1.1.3).

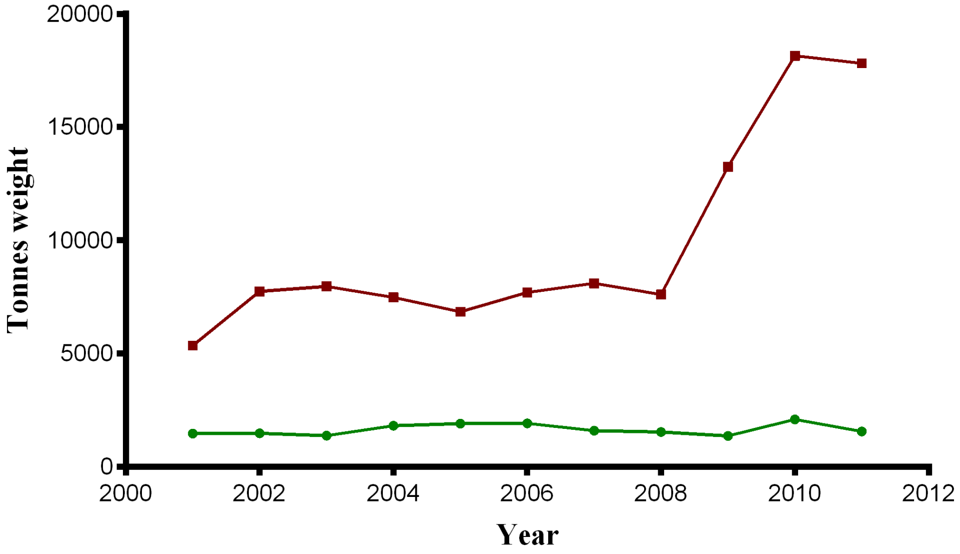

Figure 1.1.3 European cuttlefish production in the EU (■) and in Portugal (●), (FAO 2012)

In contrast to other cephalopods, in the last few years, the world's *S. officinalis* market has reached a plateau and stocks remain overexploited in the central eastern Atlantic (FAO, 2012). The scarcity of the species as well as its price in the markets has led its experimental culture. In fact, it has attained the status of candidate species for aquaculture in the past 20 years (Sykes*, et al.*, 2009a). The knowledge of biological and physiological aspects together with the above mentioned set of figures and features, and the high market demand, provides a solid background for the potential intensive or semi-intensive aquaculture of this species (Domingues*, et al.*, 2002; Sykes*, et al.*, 2006a).

1.2. Cuttlefish biology and aquaculture

About 100 species of cuttlefish have been found around the world (Domingues*, et al.*, 2003). The European cuttlefish, *S. officinalis* (Linnaeus 1758), which belong to the family *sepiidae*, can be mostly found in eastern Atlantic and Mediterranean Sea (Boyle, 1987). It has been within the most studied cephalopod in that region and it has several characteristic that makes one of the most promising species to culture (Sykes*, et al.*, 2006b). *S. officinalis* has short life cycles (Warnke, 1994), which is influenced by the temperature. This species is poikilothermic (Boletzky, 1974b; Domingues*, et al.*, 2003). Temperature regulates the feeding and growth rates (Bloor*, et al.*, 2013). It produces large eggs that allow its easy handle and transportation (Sykes*, et al.*, 2006b). The hatchlings have similar appearance to adults, their feeding behaviour is identical through lifespan, i.e. benthic feeding and it has short life cycles (Domingues*, et al.*, 2003).

The cultivation of *S. officinalis* carried so far has been used mainly for research purposes, however, it can be commercially appreciated. For instance, non-edible parts of the cuttlefish, about a third of all body, may be used in the fish meal industry or used as bait (Sykes*, et al.*, 2006b). Also, the species production may promote the reduction of illegal catches of undersized cuttlefish, which are considered a delicacy in many countries, namely Portugal, and display higher monetary value (Sykes*, et al.*, 2006b). Cuttlefish is also easily digested and a healthy meal (Boucaud-Camou, 1990; Sykes*, et al.*, 2006b). Although cuttlefish is mainly composed by water [81% (Boucaud-Camou 1990)], it is a good source of protein (16.1%), essential lipids (EPA/DHA), mineral salt and vitamins (Sykes*, et al.*, 2006b).

For cuttlefish, both wild and laboratory studies were carried to better understand its stock dynamics and culture technology. Experimental culture of cephalopods started in the 60's by Asian countries (Korea and Japan). Since then, the reports regarding culture management and influence of both biotic and abiotic parameters have been published (Boletzky, 1979; Sykes*, et al.*, 2006b). An important progress in cuttlefish cultivation was observed with the reports of successful rearing of more than 3 consecutive generations (Boletzky, 1979; Boletzky*, et al.*, 1971; Forsythe*, et al.*, 1994). Other studies on cuttlefish feeding, digestive system and biochemical processes, as well as growth, were also performed which added information to improve cultivation (Boucaud-Camou, Pequignat, 1973; Sykes*, et al.*, 2006b). Moreover, through the 90's it was reported studies on sexual and social behaviour, crowding, diseases and pathologies (Boletzky, Hanlon, 1983; Sykes*, et al.*, 2006b). Despite the successful results bottlenecks were also observed. For instance, this species has low fertility and fecundity. Forsythe*, et al.* (1994) Obtained results of about 3000 eggs per female of *S. officinalis* within English Channel populations in captivity, yet the viability of the eggs was about 6%. It was observed at this study that females seem to mature later than males, which made them to respond with constant jetting and inking. Such stress caused by the time lapse between males and females maturation may also be a cause for the non-viability of the eggs. Recently, it was suggested several parameters that might affect reproduction in culture conditions (Sykes*, et al.*, 2012b). It was shown that tank density could influence the fertility and fecundity (Correia*, et al.*, 2005). Correia*, et al.* (2005) suggested that, in tanks with greater density, females had high fertility but lower fecundity (834 eggs/female). Furthermore, the bottom size/volume may also influence the fertility in *S. officinalis*. However, according to Sykes*, et al.* (2012b) it might be strongly related to the daily temperature variations in the tank. Another issue being faced is that a new group of breeders is always required in consequence of the species semelparous life history, i.e. female cuttlefish spawn once and then die (Laptikhovsky*, et al.*, 2003).

The nutritional aspect is probably the greatest challenge that cuttlefish culture faces and it is creating bottlenecks effects (Domingues*, et al.*, 2008; Lee, 1994; Sykes*, et al.*, 2009a). As any other culture, an appropriate artificial diet is crucial for cuttlefish production. The use of a commercial diet may reduce feed costs up to 80%, therefore reinforcing cuttlefish large-scale production (Domingues*, et al.*, 2009). Lee*, et al.* (1991) studied the use of pellet diets and surimi in octopus and cuttlefish. Although these were accepted by the animals, low growth (<0.5% body weight per day) and survival were obtained. Similar studies (Castro, 1991; Castro, Lee, 1994; Castro*, et al.*, 1993) also reported pellet acceptance by cuttlefish but low growth and survival rates were also registered. Albeit the advances in cuttlefish culture, there's still no prepared diet that promotes similar growth and survival as verified with natural foods (Domingues*, et al.*, 2008; Sykes*, et al.*, 2006a; Sykes*, et al.*, 2009a). The lack of an artificial diet remains a bottleneck in the development of *S. officinalis* culture (Sykes*, et al.*, 2012a). The effects of diverse type of food - live or death (frozen) - have been intensified during the past 13 years (Domingues*, et al.*, 2001b). Both adults and hatchlings rather feed on live preys, showing better growth and survival. However, feeding with live food is costly and can be difficult to produce or capture (Sykes*, et al.*, 2006b). So far, it has been problematic to define only one type of food for the entire production. During the species life cycle, live food is used during the hatchling stage and frozen (fish, shrimp and crab) are used to feed juveniles and adults (Domingues*, et al.*, 2003). The use of live food is impracticable for large-scale production due to its costs and also to its availability, which may create bottlenecks (Baeza-Rojano*, et al.*, 2010). Normally, live mysid shrimp is the first food given at initial stages allowing acceptable growth rates and low mortality (Domingues*, et al.*, 2001a; Sykes*, et al.*, 2006a). However, this food is not commercially viable because the production of mysids can be difficult due to its costs and its low fecundity. *Artemia* sp. had been also used but the low lipid content does not allow cuttlefish to growth and survive (Sykes*, et al.*, 2006a). Nevertheless, the use of only one type of food is cheaper and will facilitate its culture in terms of labour (Sykes*, et al.*, 2006a). The use of live grass shrimp (*P. varians*) has proven successful as first diet in the post-hatchling culture of *S. officinalis* for an entire cycle. The growth rate was higher than 5% $BW.d^{-1}$ (Domingues*, et al.*, 2006). In order to better formulate an artificial diet information regarding the development of the digestive tract and the species metabolic requirements are needed. A distinction of the digestive tract can be observed during the early stages of development but the metabolic requirements are still uncertain (Boletzky, 1989; Sykes*,* *et al.*, 2009a). Nevertheless, cuttlefish metabolism is mainly based on protein and amino acids, therefore it is an important point to address when formulating an artificial diet. Protein is probably the main energy supply that will contribute for growth (Lee, 1994). In order to obtain a high growth rate, which should be between 3 and 15% BW.d^{-1}, the cuttlefish will need great quantities of amino acids for protein synthesis. Thus the protein/energy ratio should be 50g protein/MJ (Lee, 1994; Valverde*, et al.*, 2012). Additionally, Sykes*, et al.* (2009a) suggested the existence of a carbohydrate metabolism that may affect the nutritional content of the eggs and metabolism of *S. officinalis*. This species eggs have water soluble glycol-lipoprotein, which has 20% lipid and 12.6% carbohydrates, in its composition (Navarro*, et al.*, 2014). On the other hand, according to these same authors, eggs collected in Faro (Portugal) present more carbohydrates than lipids. Different temperatures at different specific geographical locations may influence the metabolism of *S. officinalis* and the nutritional content of its eggs (Navarro*, et al.*, 2014). Lipids are considered to be less important in the energy metabolism as it represents less than 2% (Boucaud-Camou, 1990; Sykes*, et al.*, 2009a). However, these have been shown to be very important during early development (Domingues*, et al.*, 2005; Sykes*, et al.*, 2009a). It is important to supply a prey rich in polyunsaturated fatty acids (PUFA), phospholipids and cholesterol to hatchlings and juveniles (Navarro*, et al.*, 2014). Similarly to other marine animals, PUFAs, such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA) are vital for development, growth, and reproduction of cuttlefish (Navarro*, et al.*, 2014). These fatty acids must be included in the diet as they cannot be synthesised from the precursors (linolenic acid - 18:3n-3 for EPA and DHA or linoleic acid - 18:2n-6 for ARA), (Sykes*, et al.*, 2006b). It is necessary to know the appropriate nutritional requirements avoiding the creation of the bottlenecks previously described. These requirements can be estimating by determining the yolk reserve nutrients during the embryonic development (Sykes*, et al.*, 2009a). In addition, embryological studies can also be used to better understand the biology of cuttlefish and to improve its culture (Sykes*, et al.*, 2009a).

1.3. Embryological studies and their importance for cuttlefish aquaculture development

In contrast to fish, cuttlefish hatchlings closely resemble miniatures of juveniles and exhibit similar behavioural traits and habits (Boucaud-Camou, Boucher-Rodoni, 1983; Nixon, Mangold, 1998). Such characteristics might facilitate hatchlings nutritional studies, which have been considerate in the last few years (Sykes*, et al.*, 2009a). Moreover, the large size of the *S. officinalis* eggs and its simple observation with a microscope makes it easy for embryonic development studies. The egg size allows to follow the different initial stages, from fertilization to hatching (Boletzky*, et al.*, 2006).

Embryological studies have shown to be a helpful tool to determine a suitable type of food in terms of nutritional content. From embryonic to post embryonic, studies have recognized the need for knowledge regarding digestive physiology and the way in which the nutrients are consumed, catabolized, absorbed and excreted (Martínez*, et al.*, 2011).

During embryonic development, the embryo will use a certain quality and quantity of nutrients, which have been previously deposited by the female in yolk. A deficiency in the nutrients present at the yolk may originate deformations and unfit cuttlefish (Boyle*, et al.*, 2001). At the initial stages of development, cuttlefish embryos display an outer and an inner yolk sac (Boletzky, 1989; Sykes*, et al.*, 2009a). The outer yolk sac plays respiratory and circulatory functions (before gills and heart are fully developed and functional) and also induces continuous movement of the perivitelline fluid (Boletzky, 1989). The outer yolk epithelium becomes progressively active with the completion of an ectodermic cover (Boletzky, 1974b; 1989). According to Boletzky (1975), the circumoral musculature contracts driving the outer yolk to the inner yolk and an increase in size of the second can be observed. Towards hatching, the inner yolk sac becomes more active and the outer yolk sac reduces or disappears. A premature hatching with large external yolk sac corresponds to death (Boletzky, 1974b). On the other hand the inner yolk sac increases in size rapidly working as a food reserve. Its size varies greatly among cephalopods (Boletzky, 1974b; Boucaud-Camou*, et al.*, 1985). The food that is present in the inner yolk sac will be transferred to the digestive gland through sanguineous flow, at post-embryonic stages (Boletzky, 1974b). The Martínez*, et al.* (2011) study on *Octopus maya* showed that, although the inner yolk sac and the digestive gland are independent organs (separated by connective tissues), the nutrients flow from one to another through blood. Those nutrients will be later absorbed and distributed by the formed circulatory system at later embryonic stages. Furthermore, the yolk-derived material would be incorporated into the endolysosomal system during the process. Such nutrient movement and incorporation was also observed in *S. officinalis* (Boletzky, 1975; Martínez*, et al.*, 2011; Safi, 2013). Boletzky (1974b) observed that newly hatched animals may die if not fed, as their reserves are entirely absorbed. However, if prey are available they will start feeding before the depletion of reserves (Safi, 2013). Until then there is yolk digestion and digestive gland development, i.e., cell multiplication, with no digestive function can be observed (Boucaud-Camou*, et al.*, 1985). The embryonic development ends at first meal (Boletzky, 1989). The digestive gland is the most visible organ at early stages of cephalopod development (Martínez*, et al.*, 2011). In cuttlefish, the digestive gland has a colourless appearance in embryos and it is formed by two masses of glandular tubules that are located against the internal yolk sac (Safi, 2013). It is responsible for the production of a variety of digestive enzymes and also for the storage of nutrients, mainly lipids (Martínez*, et al.*, 2011; Safi, 2013).

During cuttlefish embryological stages, yolk provides the nutrients that allow the embryo to growth and survive. Histological techniques can help to add information on the primary function of the outer and inner yolk sac, as well as, the reserves movement from one to another, respectively.

1.4. Histological techniques applied in invertebrates

For many years, histology has been used in a variety of studies in both vertebrates and invertebrates. However, invertebrate histology and histochemistry for structure identification, characterization and nomenclature studies are less advanced in comparison with vertebrate research (Costa*, et al.*, 2014). Histological techniques allow a better understanding of internal animal tissues, organs and its different components. The microanatomy information of cephalopods is scarce due to technical difficulties, i.e., organs decays when preparing samples. Knowledge on complex organs and its microstructural details is lacking, which results on a gap in cephalopod biology in general (Costa*, et al.*, 2014). Albeit cephalopods histological studies were initially avoided due to their lack of skeleton and heavy musculature (Roper, Sweeney, 1983), many different studies were performed. Cephalopods are very visual active predators due to their evolved nervous system (Bozzano*, et al.*, 2009), which is bigger among invertebrates (Shigeno*, et al.*, 2001b). The development of brain lobes of cephalopods using histological examinations has been studied and have identified the variety of brain structure, i.e., optic lobes, eye development and photomechanical responses (Bozzano*, et al.*, 2009). Consequently different life styles (e.g. feeding behaviour), within the class were observed, as well as phylogenetic relationships. For example Shigeno*, et al.* (2001a) studies have explained the morphological process of the brain development on post-hatching of oegopsid squid *Todarodes pacificus.* Using conventional histological and Cajal's silver impregnation methods their study suggested that in contrast to some cephalopods, early paralarvae of *T. pacificus* are suspension feeders (Shigeno*, et al.*, 2001a). Processes such as digestion and energy intake allow us to understand the lifestyle of an animal, i.e., their growth, life-span and reproductive cycle (Swift*, et al.*, 2005). The digestive system efficiency is mostly attributed to the digestive gland (Yim, Boucaud-Camou, 1980). Physiological and morphological maturation of the digestive gland and its digestive cells complexity have been shown, through histological studies. The Lemaire*, et al.* (1976) study of embryonic development of *S. officinalis* showed that, at hatching, the cells of the digestive gland have simple structures and little differentiation. The nutrients needed for the animal to grow are transferred through blood and the digestive activity is mainly localized at the yolk syncitium that digests the yolk (Boletzky, 1975). However, as soon as feeding begins at post-embryonic stages, the digestive system functional activity begins (Boucaud-Camou 1982). The cell differentiation evolves and a complexity of cell types with different function can be observed within histological sections (Yim, Boucaud-Camou, 1980). Yim, Boucaud-Camou (1980) showed that the digestive cell presented different progressive stages that correspond to the different *S. officinalis* development stages. At the digestive gland epithelium, "boules" or digestive cells were observed histologically, replacing cells and vacuole cells (Yim, 1978; Yim, Boucaud-Camou, 1980). The "boules" cells are sizable inclusions of proteic nature (Yim, 1978), that appear in great quantities in the digestive diverticula. These cells are responsible for the synthesis of digestive enzymes, absorption and excretion during digestion (Yim, Boucaud-Camou, 1980). Moreover, lipid reserves (different triglyceride forms) in the form of droplets can be observed at the cytoplasm of the "boules" cells (Boucaud-Camou, 1971; Costa*, et al.*, 2014). The replacing cells are triangular shaped cells with little differentiation and are responsible for the regeneration of the epithelium (Yim, 1978). The vacuoles cells are less numerous, composed by granules (Yim, 1978) and its function is unclear (Costa*, et al.*, 2014). Furthermore, the digestive gland is the main source of digestive enzymes in *S. officinalis* (Boucaud-Camou 1982). Those enzymes can be also identified through histology (Swift*, et al.*, 2005). The digestive enzymes are mainly of proteolytic nature that allows to metabolize proteins and amino acids (Safi, 2013). The presence of those type of enzymes is strongly related to cephalopods being carnivorous (Boucaud-Camou 1982).

1.5. Objective

This study aimed to characterize the development of digestive tract during embryogenesis of European cuttlefish *Sepia officinalis.* The external and internal yolk sacs were mainly focused, as well as their nutritional content (using specific stains) and function. Analysis at macroscopical scale (weight and measurements) and microscopical scale (photographs, histology and image analysis) was performed.

2. Material and Methods

2.1. Samples collection

A total of 364 cuttlefish eggs were collected after being spawned by a F2 broodstock at the Centre of Marine Sciences (CCMar) Ramalhete research station (37° 0' 30.21" N 7° 57' 54.89" W). All eggs were from the same generation (F3), but the batches were from different females. After being removed from the spawning tank and sorted according to Sykes*, et al.* (2014), eggs were placed in a hatching basket, inside a 1500L tank, containing 2 air stones to allow egg movement and optimized oxygenation (Sykes*, et al.*, 2014). Salinity (34.5 ± 0.63 ‰), temperature (19.4 ± 0.95 ºC) and dissolved oxygen (93.7 ± 3.58 %) were verified daily with a VWR EC300 salinity meter (VWR International, LLC, Radnor, PA, USA) and an OxyGuard Handy Polaris (OxiGuard, Birkerod, Denmark), respectively.

2.2. Data collection and analysis

2.2.1. Embryonic development at macroscopical scale

Every day, during 31 days, 5 eggs were randomly collected from the hatching basket. These were weighed in a Kern EG 300-3M (KERN & SOHN GmbH, Balingen, Germany), and measured (length and width up to 1mm) using a digital Powerfix caliper (OWIM GmbH & Co. KG, Neckarsulm, Germany). Then, a correlation test was performed to check if there was any relationship between egg measures (weight, width and length). A comparison between mean wet weight of eggs throughout embryogenesis from the present study and that of Sykes*, et al.* (2009a) was performed to assure proper development.

2.2.2. Embryonic development at microscopical scale

Each egg was cut open and, firstly, the embryo was removed from the embryonic sac and, secondly, from the chorion (Figure 2.2.2.1). Only then, each embryo was photographed using a Dino-eye AM-4023X digital camera (ANMO, Taiwan), which was attached to a stereo microscope Zeiss STEMI 2000-C (Carl Zeiss AG, Oberkochen, Germany). Afterwards, embryos were fixed in 4% formaldehyde at 20:1 (volume by sample).

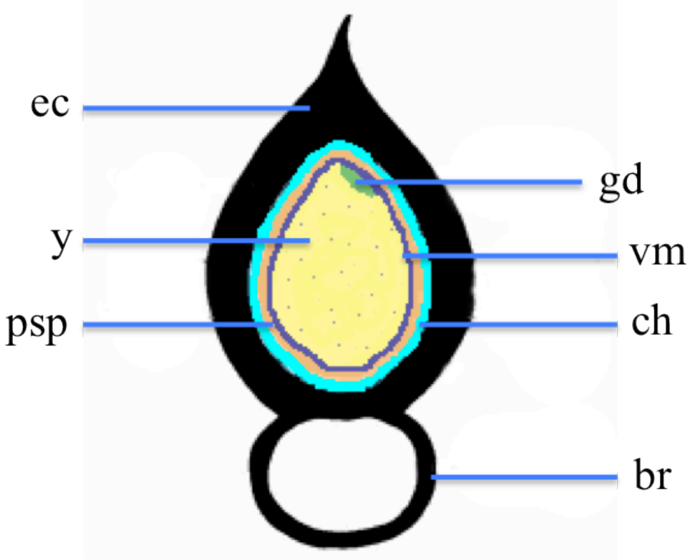

Figure 2.2.2.1. Schematic view of the egg of *S.officinalis*. (br) basal ring; (Ch) chorion; (gd) germinal disc; (psp) perivitelline space; (ec) external capsule; (vm) vitelline membrane; (y) yolk. Adapted from Wolf*, et al.* (1985).

Image J 1.46r (Fiji, http://imagej.nih.gov/ij) was used to measure an estimate area of the embryo and also of the external yolk sac, as shown in figure 2.2.2.2. Data was then used to check the correlation between both areas throughout embryonic development by linear regression (Dytham, 2003; Ennos, 2007). The area of both embryo body area and that of the external yolk sac where used to calculate the total embryo area (TEA). In addition, a possible correlation between egg weight and TEA was verified (Dytham, 2003; Ennos, 2007).

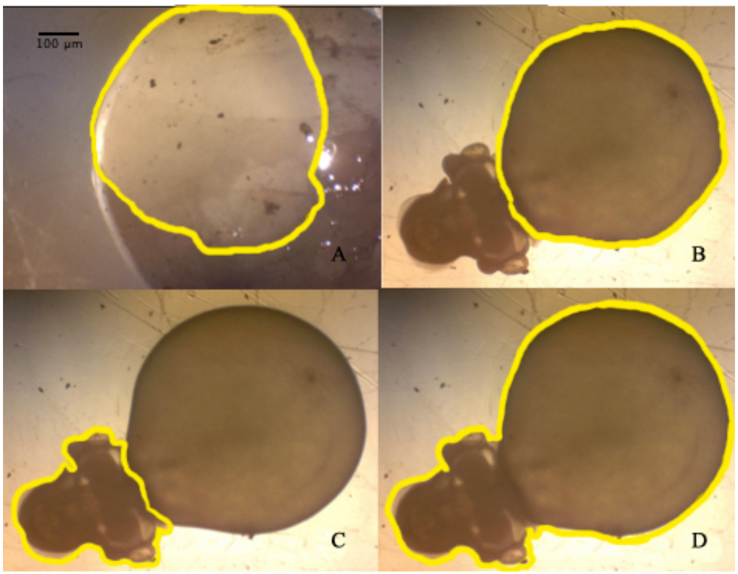

Figure 2.2.2.2 Measurement of an estimate area of a cuttlefish embryo: (A) internal egg mass (B) embryo external yolk sac (C) embryo body (D) embryo. Nomenclature used according to Wolf*, et al.* (1985).

Embryonic development stages were then determined through the identification of given structures, according to Naef's (1928) description. Images were pre-treated prior analysis (removal of image background and, when needed, images were adjusted to invert function) with Adobe© Photoshop© CS5 (Adobe Systems Incorporated, USA).

2.2.2.1. Histology characterization of yolk sac and digestive gland

Samples collected were first dehydrated through washing with increasing percentages of ethanol (70%, 90%, and 95%) for established periods of time (table 2.2.2.1.1).

Table 2.2.2.1.1 Percentages of ethanol used to wash the samples for the correspondent amount of time

Ethanol percentage	Washing numbers	Time of washes (hours)
70	4	3 to 4
90	3	3 to 4
95	2	12 3 to 4

Pre-infiltration, infiltration and polymerisation was carried with Heraeus Technovit 7100 (Heraeus Kulzer GmbH, Germany). Samples were placed in 200ml of resin (Technovit 7100) containing 200ml of ethanol (95%) "intermediate preparation solution" for 24h, to initiate resin inclusion. Then, a preparation solution was prepared by mixing 1g of the activator (hardener I) to 100ml of the Technovit 7100 resin. Afterwards, 30ml of the preparation solution was mixed with 1.5ml of hardener II (at a 1:15 proportion) for one minute. After, 1 to 3 ml of that solution was placed in small moulds (ice trays) and samples were carefully placed within, avoiding the formation of any bubbles. Samples where then left drying for 48h before being removed from the moulds with the help of a tweezers.

After, 3µm longitudinal sections cuts were obtained using a Microm HM 340 E microtome (Microm International GmbH, Germany). These cuts were placed in warm water (40ºC-50ºC) into a Micron SB80 (Germany) in order to expand. Next, cuts were placed into microscope slides (3 to 5 cuts per slide) and stained with both toluidine blue O and periodic acidSchiff (PAS). Staining solutions were acquired from Carl Roth GmbH + Co. KG (Germany).

After drying, slides were checked under a stereo microscope Zeiss STEMI 2000-C (Germany) and a microscope Zeiss Axioskop 50 (Germany). Digital photographs were acquired with a VWR Visicam 10.0 (Leuven, Germany) attached to either. For amplifications, a Leica camera DFC480 attached to a Leica DM200 was used.

3. Results

3.1. Embryonic development at macroscopical scale

Changes in egg weight, width and length throughout embryonic development are shown in figure 3.1.1 (A to C, respectively). All three variables showed a similar pattern during embryogenesis. This was characterized by a reduction in weight, length and width during the first 10 days, followed by a rapid increase of all 3 from the 10th day until the end of development. Although the great majority of the eggs had hatched at day 30, egg measurements were carried until day 32. The external cases of those eggs were highly thin and many ruptured to touch, hence its lower values.

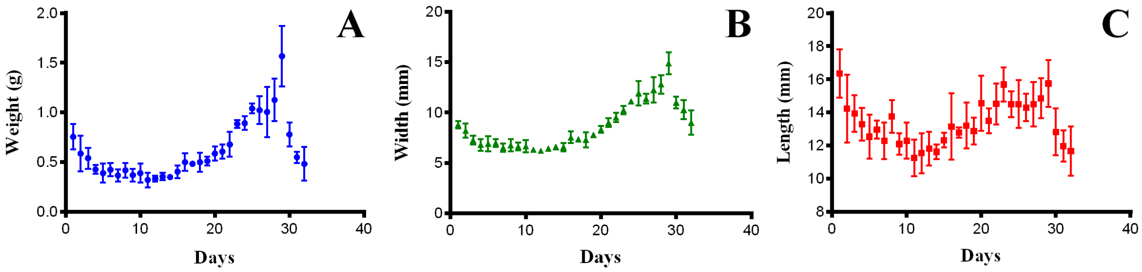

Figure 3.1.1 Changes in the mean eggs weight (A), width (B) and length (C) during embryonic development ($p < 0.001$)

A significant positive correlation ($p < 0.001$) between weight and width (figure 3.1.2 A), weight and length (figure 3.1.2 B), width and length (figure 3.1.2 C) was observed.

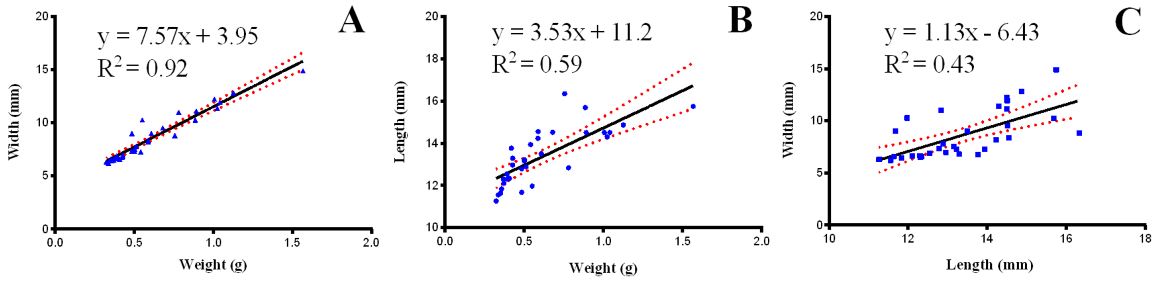

Figure 3.1.2 The correlation between each set of measurements: (A) weight *versus* width (B) weight *versus* length (C) width *versus* length ($p < 0.001$). Red lines represent the threshold for the 95% confidence interval.

However, the correlation between the weight and width was stronger than the correlation between weight and length and width and length (table 3.1.1).

Table 3.1.1 Correlation within the set of measurements

	Pearson Correlation	p	N
Weight *versus* width	0.937	0.001	160
Weight *versus* length	0.700	0.001	160
Width *versus* length	0.558	0.001	160

The mean wet weight of the eggs from the present study followed a similar pattern than those described in the Sykes, *et al.* (2009a) study (figure 3.1.3). Nonetheless, eggs of the present study always displayed higher weight than those of Sykes, *et al.* ($p < 0.05$) throughout embryogenesis.

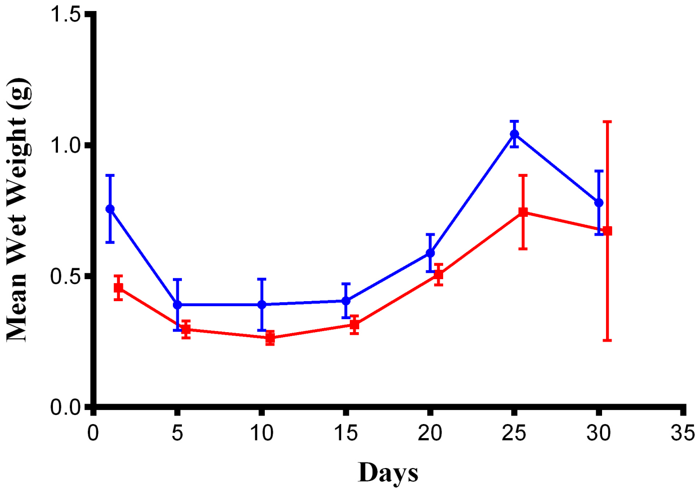

Figure 3.1.3 Mean wet weight variation of *S. officinalis* eggs throughout embryogenesis. Blue line -●- corresponds to present data and red line -■- to data from Sykes, *et al.* (2009a).

3.2. Embryonic development at microscopical scale

The *S. officinalis* yolk sac area decreased as the body of the embryo grew in the egg (figure 3.2.1) throughout embryonic development (observed from 12 days post posture, dpp).

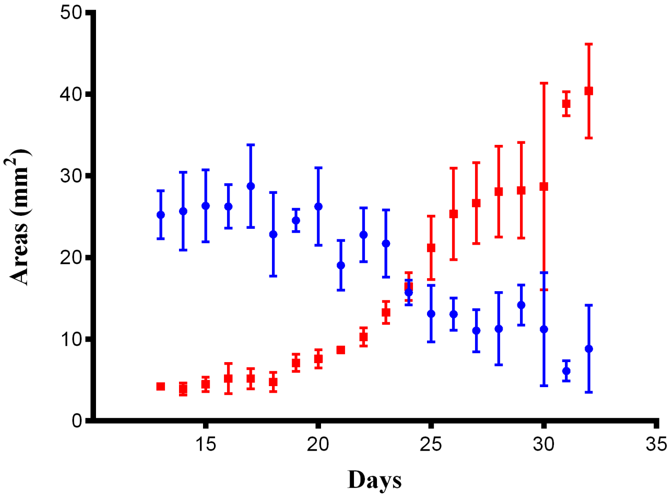

Figure 3.2.1 Area (mm^2) of the external yolk sac (•) and of the embryo body (■) during embryonic development of *S. officinalis*.

In fact, there was a high correlation between the decrease of the area of the yolk sac and the increase of the body of the embryo area (p <0.001; figure 3.2.2).

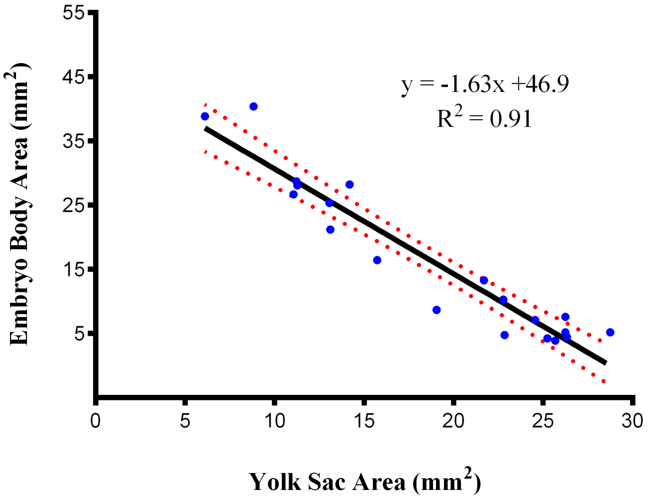

Figure 3.2.2 Linear correlation between the external yolk sac area and the embryo body area of *S. officinalis* during embryonic development (p <0.001). Red lines represent the threshold for the 95% confidence interval.

The total embryo area (TEA) presented a linear growth ($R^2 = 0.52$) and the wet weight of the eggs had exponential growth from 12 dpp until the end of embryogenesis (figure 3.2.3). The eggs weight, measured each day, increased more rapidly than those of TEA during that period (figure 3.2.3).

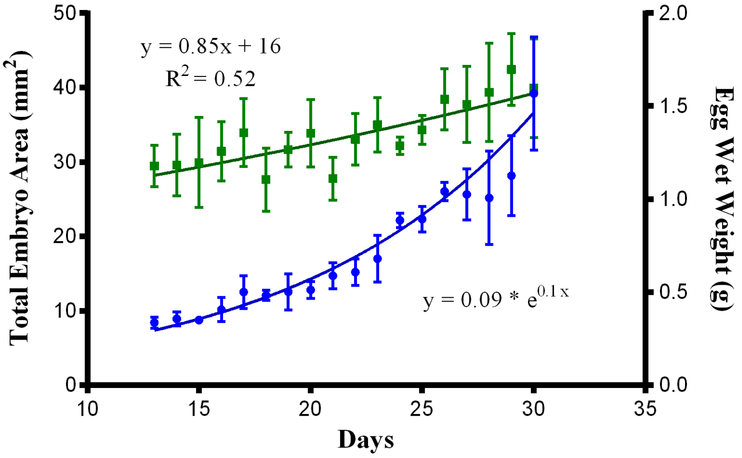

Figure 3.2.3 Total embryo area (■), (external yolk sac with the embryo body; mm^2) and wet weight (g) of the eggs (•) from 12 dpp until the end of embryonic development of *S. officinalis*.

A positive significant correlation ($R^2 = 0.69$) between the total embryo area and the wet weight of the eggs ($p < 0.001$) was established (figure 3.2.4).

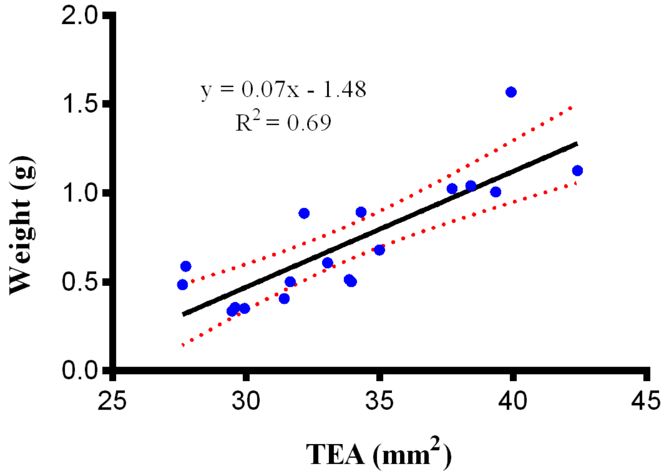

Figure 3.2.4 Correlation between the TEA and the egg weight from 12 dpp until the end of embryonic development of *S. officinalis* ($p < 0.001$). Red lines represent the threshold for the 95% confidence interval.

3.2.1. Stages identification and histological characterization

Different stages of embryonic development of *S. officinalis* were identified based on the growth of the area of the embryo body (Figure 3.2.1.1). Furthermore, throughout the identified stages, both the external and internal yolk sac were characterized. Eleven of the twenty Naef's stages were identified:

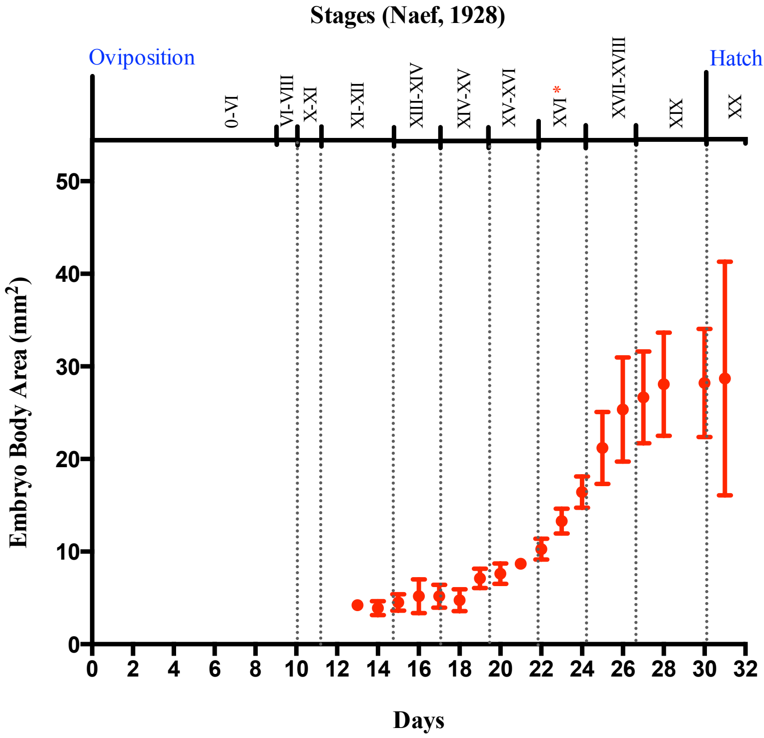

Figure 3.2.1.1 Stages identified according to Naef (1928) and the growth of the embryo body area throughout *S. officinalis* embryonic development. (*) Stage repeated due to presence of transitional embryonic changes.

a) *Stages 0 - VI*

This period corresponded to the first 12 days. Until 12 dpp it was only observed the basic structure of the *S. officinalis* egg (as shown in figure 2.2.2.1).

b) *Stages VI - VIII*

Eggs from the 13 dpp (figure 3.2.1.2) presented an opaque white mass with a circular, slightly darker ring shape (R), and the arms crown (ac). The ring may correspond to the primordial mantle (mp) or primordial shell gland (psg).

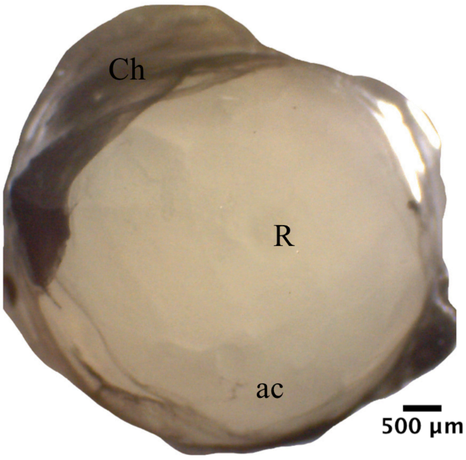

Figure 3.2.1.2 Egg of *S. officinalis* at stages VI-VIII. Chorion (Ch), ring (R), arm crown (ac).

c) *Stages X-XI*

By 14 dpp, the animal becomes more distinct, being the "embryonic body" and some of its organs better observed (Figure 3.2.1.3 A, B). Rudimentary organs, such as the eye vesicles (e) that are closed and the arm rudiments (a) that are closer to the funnel tube (FT) were observed (Figure 3.2.1.3 A, B). Another characteristic at this stage is the distinct full enclosure of the external yolk sac envelope (figure 3.2.1.3 B), which will be visible until hatching.

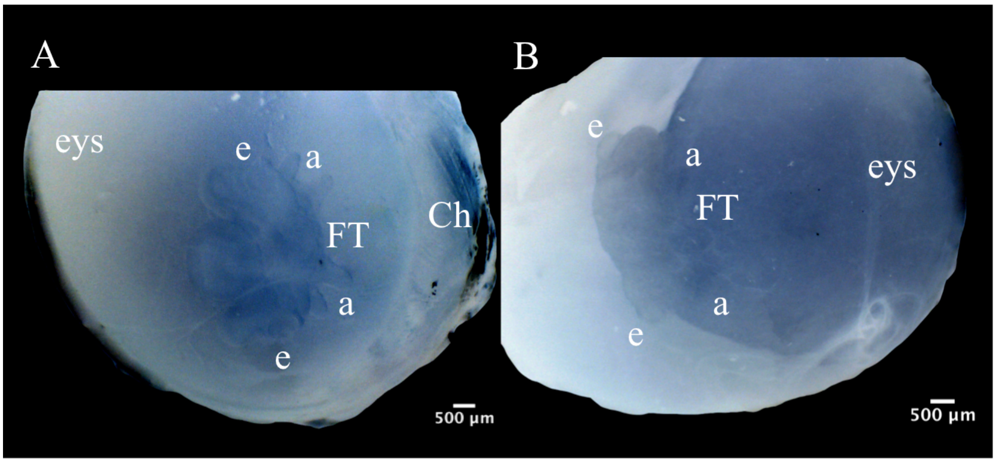

Figure 3.2.1.3 Eggs of *S. officinalis* at stage X-XI. Arm (a), chorion (Ch), eye (e), external yolk sac (eys), funnel tunnel (FT). Photoshop© inverted function used.

d) *Stages XI-XII*

Similar rudimentary organs to stages X-XI (figure 3.2.1.4) were observed. However, at this stage, the body of the embryo had slightly grown. The posterior ends of the head, or the "cheek humps" (chu) were much salient in relation to the body. The arm (a) rudiments have now elongated outwards and they are laid slightly further from the funnel tube (FT).

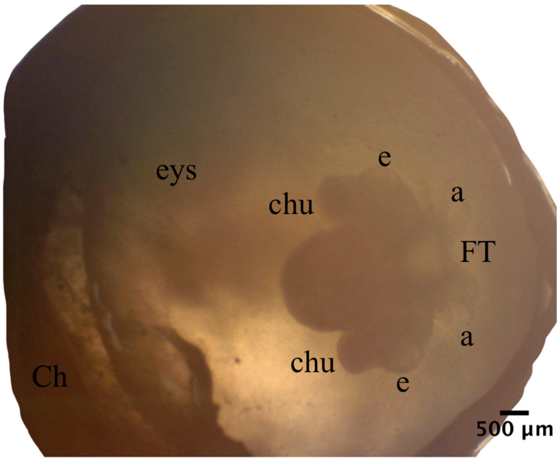

Figure 3.2.1.4 Egg example at stage XI-XII of *S. officinalis*. Arm (a), chorion (Ch), eye (e), external yolk sac (eys), funnel tunnel (FT), cheek hump (chu).

e) *Stages XIII-XIV*

From the 17 dpp onwards it was possible to remove the egg chorion without damaging the embryo, which allowed for proper histological procedure. Close to the end of stage XII, beginning of stage XIII, the arms of the embryos are pulled under the head or close to the mouth. The yolk sac, now presents a pear-shape form (figure 3.2.1.5. A). At this stage, the posterior limit of the "cheek hump" (chu) is less prominent than at stage XI and the collar (c) or funnel pouch, which is the posterior edge of the funnel apparatus, is now drawn into the mantle cavity (figure 3.2.1.5. A). The eyes have a circular shape with a rudimentary iris (figure 3.2.1.5. A, B). The embryo crouched position (figure 3.2.1.5 B, C) is typical at the transitional stage (in-between stages XIII to XIV). The tentacle (T) displayed a strong hook-shaped curvature (figure 3.2.1.5 A and C) and a clear differentiation of the arms (a), (figure 3.2.1.5 A to C).

The use of both colorations allowed to differentiate the external yolk sac (eys) and its outer membrane (arrow in figure 3.2.1.5 C), continuously connected to the internal yolk sac (iys) (figure 3.2.1.5 B and C). Both, eys and iys, were PAS positive, while the different rudiments and yolk membrane were Toluidine O positive (figure 3.2.1.5 B to F). The content of the internal and external yolk sacs were characterized by a high concentration of yolk “platelets” (yp; figure 3.2.1.5 C to F). No digestive cell differentiation within the iys was observed. However, packed diverticula was noticed (figure 3.2.1.5 F). The build-up of cellular differentiation was visible (“yolk epithelium” (ye), (figure 3.2.1.5 F) separating the yp and the connective tissues (ct).

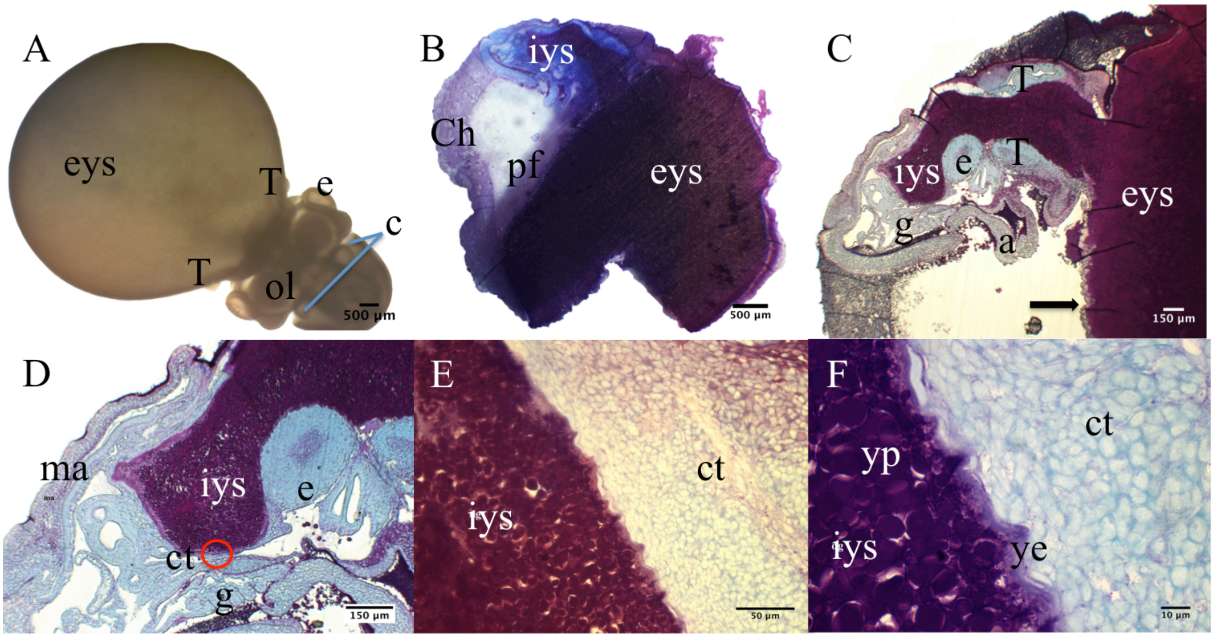

Figure 3.2.1.5 A Photograph of living *S. officinalis* embryos using a Zeiss STEMI 2000-C stereo microscope, and histological images (**B** to **F**) for identification of stages XIII-XIV. **C** and **D** longitudinal cut of the embryo. (Cut allowed the observation of external and internal yolk connection). **E** and **F** closing up of internal yolk. Arm (a), Collar (c), connective tissue (ct), epithelium (ep), eye (e), external yolk sac (eys), gill (g), internal yolk sac (iys), mantle (ma), perivitelline fluid (pf), tentacle (T), optic lobe (ol), yolk “platelet” (yp). Black arrow shows the eys membrane. Red circle shows the focus area of image E.

f) *Stages XIV-XV*

At 19 dpp, the dorsal arms have covered the mouth completely, a nearly undetected oval shell lamella started to appear into the shell (sh) sac and the corneal fold is completely closed (figure 3.2.1.6 A, B). The arm suckers (sk) rudiments are arranged in rows (not visible in figures) and the fins (f) are now visible (figure 3.2.1.6 A). The gills (g) attachment were distinctly elongated anteriorly (figure 3.2.1.6 B).

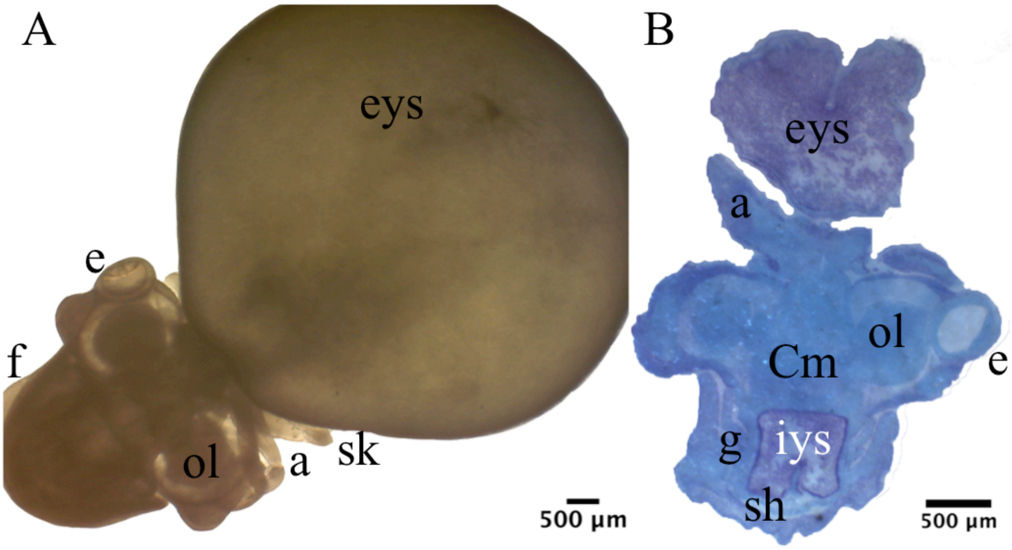

Figure 3.2.1.6 A Photograph of living *S. officinalis* embryos using a Zeiss STEMI 2000-C stereo microscope and **B** histological images for identification of stages XIV-XV. Arm (a), cephalic mass (Cm), fin (f), eye (e), external yolk sac (eys), gill (g), internal yolk sac (iys), optic lobe (ol), shell (sh) suckers (sk)

At this stage, the yolk platelet (yp) concentration seemed reduced in both eys and iys, leaving some emptiness within the structures (figure 3.2.1.7). At the eys (figure 3.2.1.7 A to C), it was observed an extra-embryonic ectoderm (exe) followed the perivitelline sinus (ps). Closely next to the ps there was a darker layer, the yolk syncitium (ys), (figure 3.2.1.7 C). Some nuclear cells were identified at the ys (arrows at figures 3.2.1.7 B and C) that seemed to be engulfed (figure 3.2.1.7 C). At the iys, it was closer observed the initial shell formation and the Hoyle's organ (figure 3.2.1.7 D). Along the lateral and posterior lobe of the iys (figure 3.2.1.7 D to F) it was observed a darker purple coloration (arrow at figure 3.2.1.7 D and F).

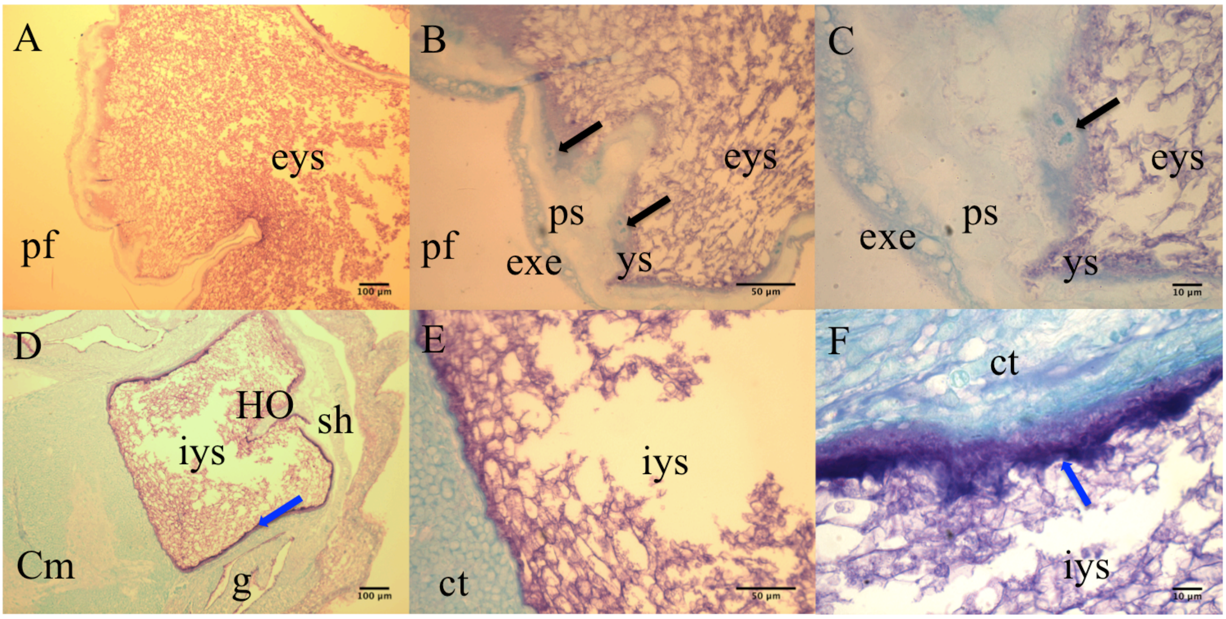

Figure 3.2.1.7 Histological images of stage XIV-XV obtained with a Leica DM200. **A** to **C** close up of the external yolk sac. Black arrows shown cells pinocytosis; **D** to **F** close up of the internal yolk sac. Blue arrows show possible iys epithelium. Cephalic mass (Cm), connective tissue (ct) external yolk sac (eys), extra-embryonic ectoderm (exe), gill (g), Hoyle's organ (HO), internal yolk sac (iys), perivitelline fluid (pf), shell (sh), yolk syncytium (ys).

g) *Stages XV-XVI*

Slightly differences of the animal organs were observed by 20 and 21 dpp (figure 3.2.1.8 A and B). The fins (f) had grown larger and start more anteriorly. The eyes (e) presented an orange coloration with distinct lens (l) formed. Also, the initial formation of the ink sac was observed, as well as, the chromatophores first appearance (both not visble at the seleted image). In both images of Figure 3.2.1.8, the cuttlebone, or initial shell (sh) formation, is more evident than in the previous stages. At this stage, the mouth reached a definitive position. The observation of distinct buccal mass (Bm) from this stage forward, represent a evidence of that (figure 3.2.1.8 B).

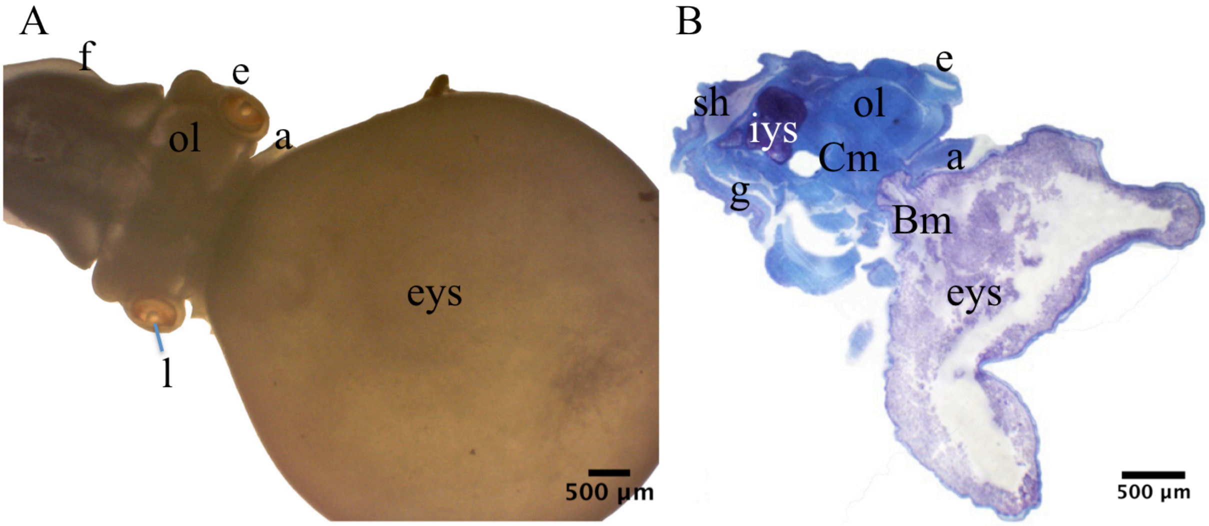

Figure 3.2.1.8 A Photograph of living *S. officinalis* embryos using a Zeiss STEMI 2000-C stereo microscope and **B** histological images for identification of stages XV-XVI. Arm (a), buccal mass (Bm), cephalic mass (Cm), fin (f), eye (e), external yolk sac (eys), gill (g), internal yolk sac (iys), optic lobe (ol), shell (sh) suckers (sk).

The empty-like structures (possible lipid droplets) at both, eys and iys, were still observed (figure 3.2.1.9). Nonetheless, the yolk platelets (yp) concentration seemed to decrease at the eys (figure 3.2.1.9 A to C), while in the iys the agglomerate seemed to be much concentrated (figure 3.2.1.9 D to F). At the eys (figure 3.2.1.9 A to C) it was, again, observed an extra-embryonic ectoderm (exe) followed by the perivitelline sinus (ps). And, it was also detected the yolk syncytium (ys), with more cells exchanging (arrows at figures figure 3.2.1.9 B and C). Some material accumulation at the outer side of the exe was noticed, which leads to a possible cilia activity (arrows at figures 3.2.1.9. B and C). Cells exchanging were greater than in the previous histological figures. From the exe to the ys, two different types of cells were observed, either being engulfed (red circle at figure 3.2.1.9. C) or being absorbed (green circle at figure 3.2.1.9. C). Furthermore, at ps an unknown mucus-like differentiation was detected (white arrow 3.2.1.9. C). At the iys (figure 3.2.1.9 D to F) it was closer observed the shell (sh) and the Hoyle's organ (HO), (figure 3.2.1.9 D). At these stages, the shell has greater cuttlebone formation than in previous. Similar darker purple coloration, with a vein-like appearance was observed along the lateral and posterior side of the iys "yolk epithelium" (ye; arrow at figure 3.2.1.9 D and F). This could be the primordial state of the digestive gland wall. Within the ye it was noticed a large cell with similar shape to hemocytes (blue arrows at figure 3.2.1.9 E and F). The left side of the ye seemed to display some cellular activity (orange circle at figure

3.2.1.9 F) but further magnification would be needed to confirm that. Within the iys, it was observed a packed diverticula. Nonetheless, the magnification used did not allow further characterization of the granules-like cells. Even so, there is a possibility of initial digestive cells differentiation (green arrows at figure 3.2.1.9 E and F). But then again, further magnification should have been used.

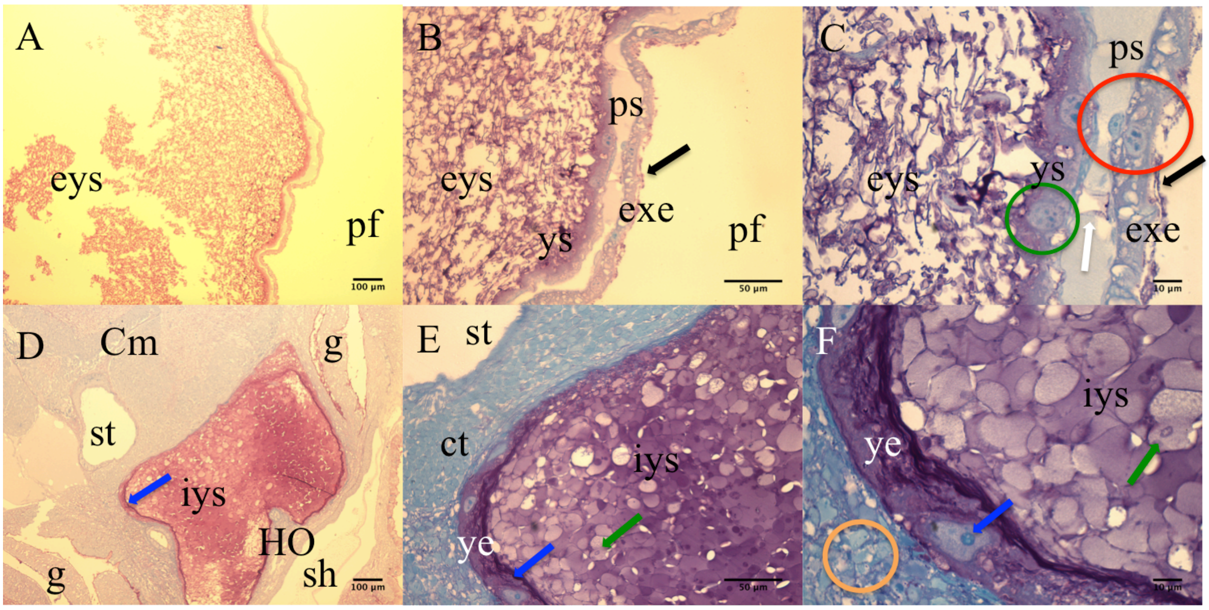

Figure 3.2.1.9 Histological images of stage XV-XVI. **A** to **C** close up of internal yolk sac. Black arrow shows exe cilia. Red circle shows absorption at the exe and green circle pinocytosis; **D** to **F** close up of external yolk sac. Blue arrows show possible hemocyte while green arrows show possible digestive cells formation. Cephalic mass (Cm), connective tissue (ct) external yolk sac (eys), extra-embryonic ectoderm (exe), gill (g), Hoyle's organ (HO), internal yolk sac (iys), perivitelline fluid (pf), shell (sh), yolk syncytium (ys).

h) *Stage XVI*

At this stage the embryo undergoes slightly important transitional development differences (figure 3.2.1.10 A and B). The head cover has increased its surface and the posterior limit or "cheek hump" (chu; figure 3.2.1.10 A) have reduced significantly (compared to previous stages). Chromatophores started to be more frequent, being abundantly visible in the mantle (not visible in the selected image). Both, cuttlebone and ink sac (is; ventrally; arrow at figure 3.2.1.10 A) were visible under mantle skin. By the end of 25 dpp, the embryo had the eys volume roughly equal to its body volume (figure 3.2.1.10 A). Such was also confirmed at the correlation curve between the external yolk sac and the embryo body area (figure 3.2.1).

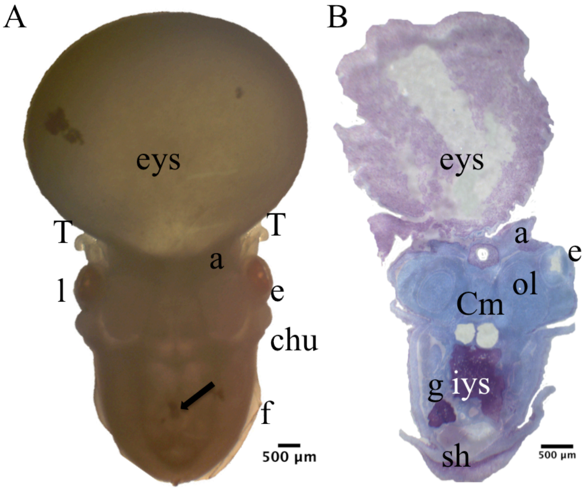

Figure 3.2.1.10 A Photograph of living *S. officinalis* embryos using a Zeiss STEMI 2000-C stereo microscope and **B** histological images for identification of stages XVI. Black arrow shows ink sac (ventrally). Arm (a), cephalic mass (Cm), cheek hump (chu), fin (f), eye (e), external yolk sac (eys), gill (g), internal yolk sac (iys), lens (l) optic lobe (ol), tentacles (T), shell (sh), suckers (sk)

At the eys (figure 3.2.1.11 A to C) it seemed to have much cells exchanging (black arrow at figure 3.2.1.11 C) from the perivitelline fluid (pf) to the eys. At the iys, besides previous features observed (from previous stages) it was also identified the digestive duct appendages (dda; figure 3.2.1.11 D). Also, the posterior end of the iys, seemed to have a much stronger pink saturation (blue arrows at figure 3.2.1.11 E and F).

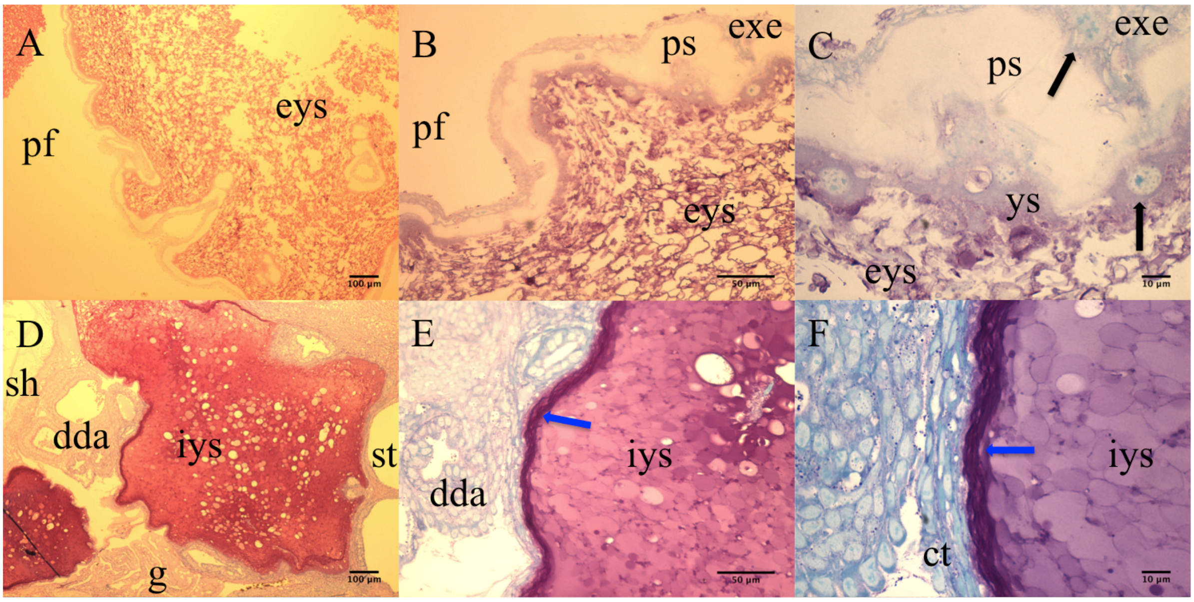

Figure 3.2.1.11 Histological images at stage XVI**. A** to **C** close up of external yolk sac and **D** to **F** close up of the internal yolk sac. Black arrows at C show cells pinocytosis while blue arrows at E and F show possible iys epithelium. Cephalic mass (Cm), connective tissue (ct), digestive duct appendages (dda), external yolk sac (eys), gill (g), internal yolk sac (iys), shell (sh).

i) *Stages XVII-XVIII*

The tentacles (T) were into the tentacle pouch (ventral view). Here, they were not observed, suggesting the retraction to the pouch, which grows deeper with the embryo growth (figure 3.2.1.12. A). The form of the suckers (sk) in the tentacles (T) and arms (a), were closer to its final form but will still grow in the remaining stages (not visible at image selected). The ink sac was observed to lie closer to the ventral integument (not visible at selected image) and chromatophores were now much frequent than at previous stages (3.2.1.12. A). The shell (sh) was better visible under dorsal mantle and it displayed greater volume (3.2.1.12. A and B). At the mouth of the embryo it was observed the annular muscle (amm) that drives yolk to the proper embryo (figure 3.2.1.12 B). At this stage, the eys and iys seemed to have roughly equal volumes (figure 3.2.1.12 B).

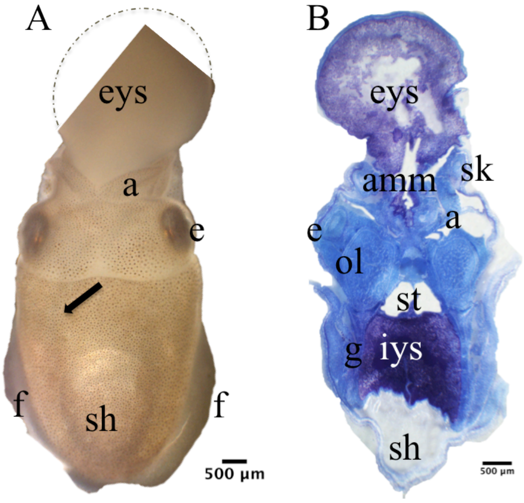

Figure 3.2.1.12 A Photograph of living *S. officinalis* embryos using a Zeiss STEMI 2000-C stereo microscope and **B** histological images for identification of stages XVII-XVIII. Black arrow shows, at A, mantle with chromatophores. Annular mouth muscle (amm), Arm (a), fin (f), eye (e), external yolk sac (eys), gill (g), internal yolk sac (iys), optic lobe (ol), shell (sh), statocysts (st), suckers (sk).

Empty-like structures were frequent at both eys and iys, which could be lipid droplets (figure 3.2.1.13 A to F). The overall structuration of the eys outer layer seemed to be much defined (figure 3.2.2.13 A to C). It was noticed a fourth layer outside the exe (black arrow at figure 3.2.1.13 B), or simply a greater amount of cilia. Cells exchanging activity was still observed. Besides those cells, it was also observed smaller nucleus within the eys (green arrow at figure 3.2.1.13 C). At the iys, it was observed the pair of statocysts (st) as well as the Hoyle's organ (HO; figure 3.2.1.13 D). Similarly to previous figures, iys "yolk epithelium" (ye) that have the vein-like appearance, was still observed along the lateral and posterior side (blue arrow at figure 3.2.1.13 D).

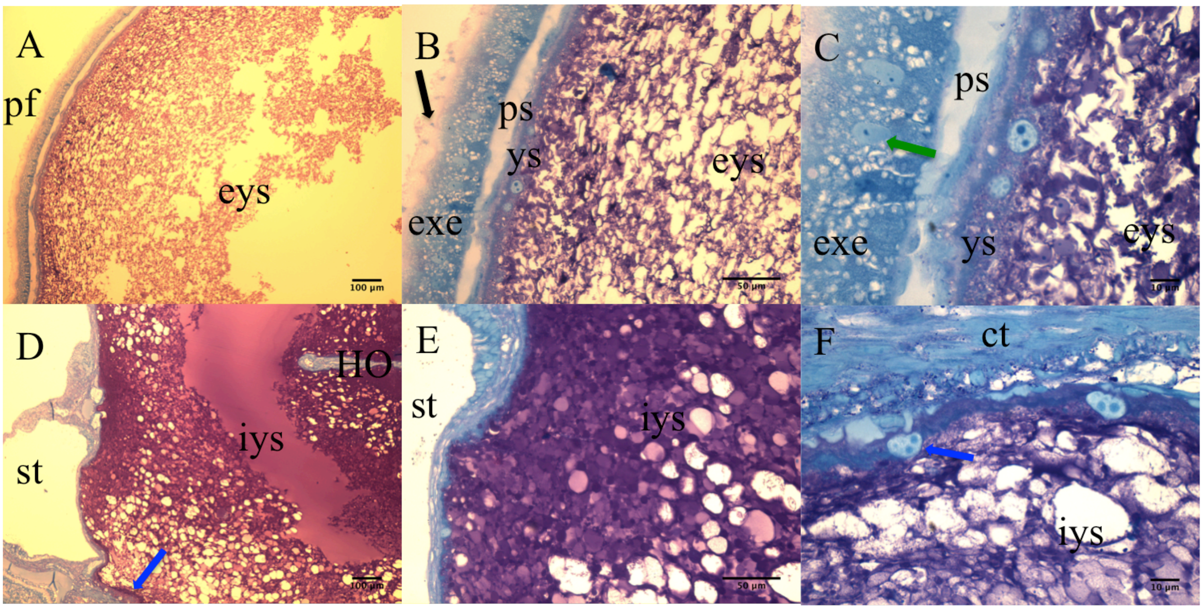

Figure 3.2.1.13 Histological images of stage XVII-XVIII**. A** to **C** close up of internal yolk. Black arrow at B shows exe cilia; **D** to **F** close up of the external yolk sac. Blue arrows show cells at iys epithelium. Cephalic mass (Cm), connective tissue (ct) external yolk sac (eys), gill (g), Hoyle's organ (HO), internal yolk sac (iys), shell (sh).

j) *Stage XIX*

Nearly hatch embryos with closed resemblance to adults were noticed from 27 to 29 dpp. Dorsal (figure 3.2.1.14 A and C) and ventral views (figure 3.2.1.14 B and D) were observed. The eys was of sizable volume, i.e., if embryo prematurely hatch it might had survived. The chromatophores, which were of brown-orange colours, were visible over all body mantle, except in certain areas, such as in fins (f). It also was observed the shell (sh) under the pigmented mantle (figure 3.2.1.14 A). The arms (a) were fully differentiated and tentacles had larger suckers (sk) than previous stages (suckers were better observed histologically). Moreover, the tentacles were totally hidden in pouch (green arrow at figure 3.2.1.14 B). The eyes (e) were enlarged, shifted laterally and its lids were completely formed. The ink sac (is), with silvery colour under the mantle, was observed at ventral view (figure 3.2.1.14 B and D). At the buccal cavity it was observed greater amount of yolk (figure 3.2.1.14 C and D).

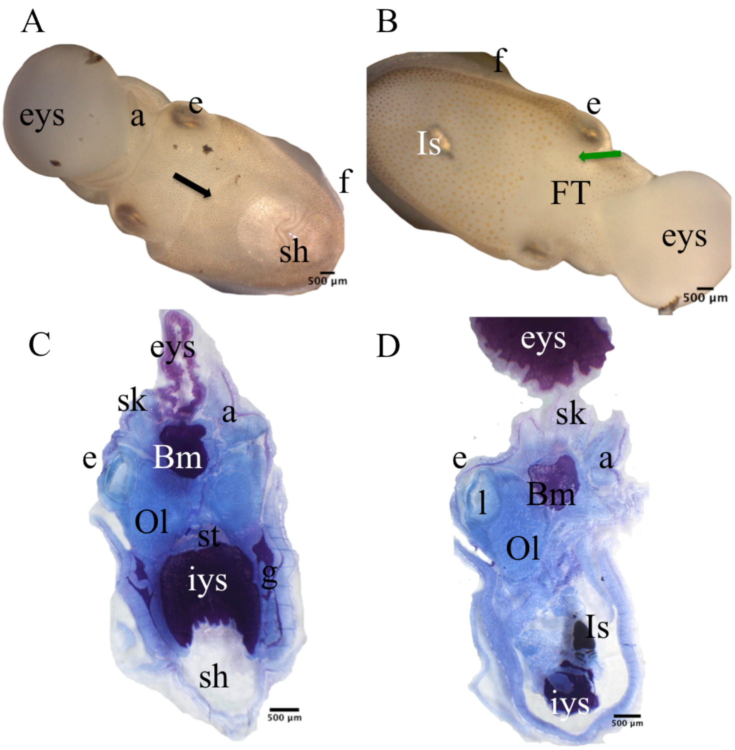

Figure 3.2.1.14 A and **B** Photograph of living *S. officinalis* embryo using the STEMI 2000-C stereo microscope Zeiss. Black arrow shows shell pouch (sp), green arrow shows tentacle pouch (Tp); **C** and **D** histological image for identification of the stages XIX. Arm (a), buccal mass (Bm), fin (f), funnel tunnel (FT), eye (e), external yolk sac (eys), gill (g), internal yolk sac (iys), ink sac (Is), lens (l), optic lobe (ol), tentacles (T), shell (sh) suckers (sk)

At the eys, the cell exchange had continued, as previously (figure 3.2.1.15 A to F). Also, at both ventral and dorsal views, of the eys, the cilia activity was still noticed (black arrows at figure 3.2.1.15 C and F). However, such is better observed at ventral view of the selected image (figure 3.2.1.15 F).

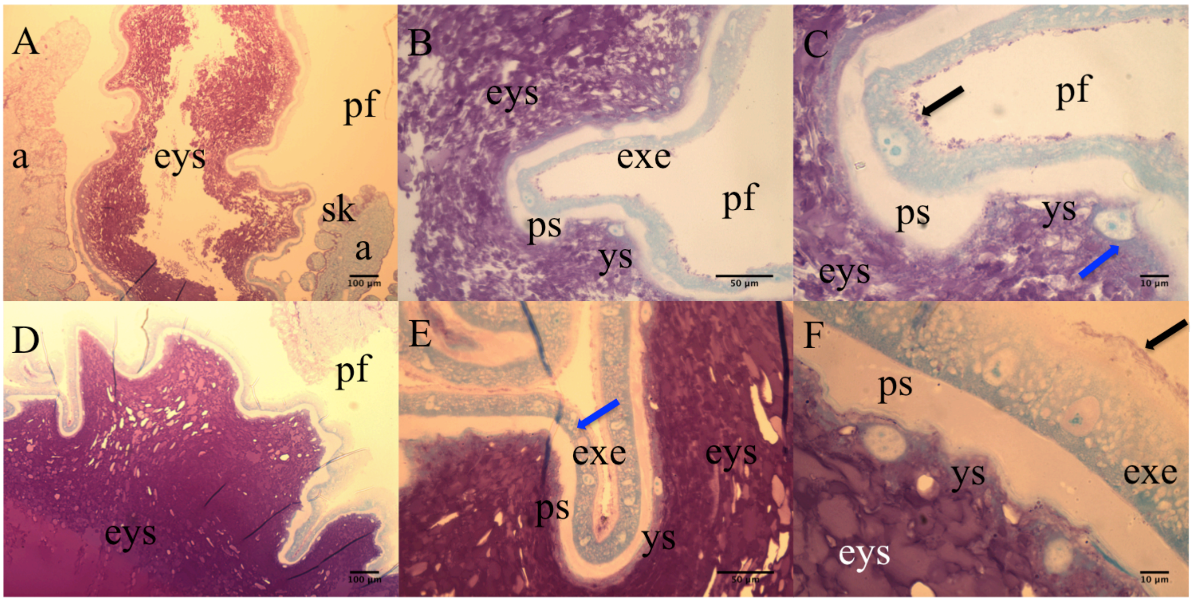

Figure 3.2.2.15 A to **C** dorsal and **D** to **F** ventral view of the external yolk sac at stage XIX. Black arrows show exe cilia and blue arrows show cells pinocytosis. Arm (a), external yolk sac (eys), shell (sh), suckers (sk), tentacles (T).

At the iys dorsal view, it can still be observed the agglomerated yolk platelets (yp) or packed diverticula (figure 3.2.1.16 A to C). However the magnification used have not allowed further characterization of the granules-like cells found within the iys (figure 3.2.1.16 B). The darker purple coloration, with a vein-like appearance was still observed along the lateral and posterior side of the iys “yolk epithelium” (ye; black arrows at figure 3.2.1.16 B and C). Similar to previous stages, within the ye it was noticed cells, as well as, a considerable amount of empty-like structures. Ventrally, a well-developed ink sac was observed (figure 3.2.1.16 D). The iys content had more plane appearance, with little or none “platelets”. Some empty-like structures were also observed (figure 3.2.1.16 E and F).

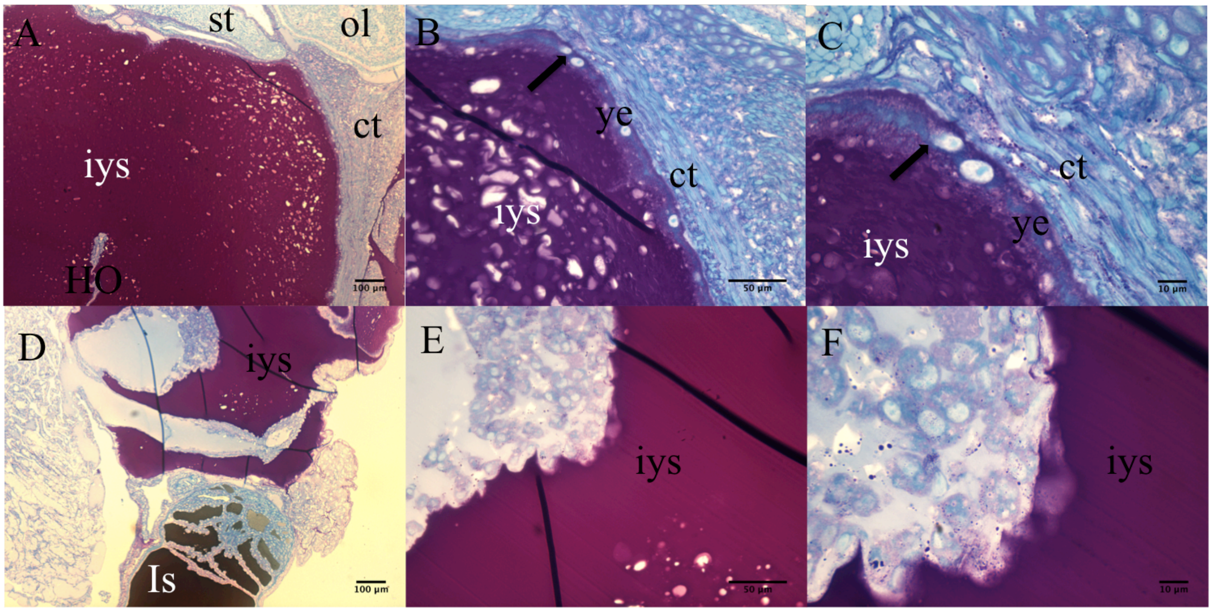

Figure 3.2.1.16 A to **C** dorsal and **D** to **F** ventral view of the internal yolk sac at stage XIX. Black arrows show cells at iys epithelium. Connective tissue (ct), internal yolk sac (iys), ink sac (Is), optic lobe (ol), shell (sh), statocysts (st) suckers (sk), yolk epithelium (ye).

k) *Stage XX*

Most hatching occurred.

4. Discussion

4.1. Embryonic development at macroscopical scale

S. officinalis eggs are an attractive material for embryological studies (Boletzky*, et al.*, 2006). Firstly, the eggs selected were visually viable. They were black or dark brown colour, with and oval shape, which increased the probability to select fertilised eggs (Boletzky*, et al.*, 2006; Sykes*, et al.*, 2006b). During this study, the eggs were at constant temperature of 19.4 ± 0.95 ºC, therefore the rate of embryonic development was fast (30 days), as expected based on literature (Boletzky, 1974a; 1975; 1982; Boletzky*, et al.*, 2006; Naef, 1928). Water temperature has a direct effect on the embryonic development of cephalopods. It influences the cuttlefish egg size and length of development (Boletzky, 1974b; Sykes*, et al.*, 2009a).

In the present study, the changes of eggs weight, width and length were analysed during the 30 days of embryogenesis (figure 3.1.1).

The eggs capsule was large at the beginning of the study with a very thick gelatinous external coat. However, this coat is reduced until 10 dpp, as well as the eggs weight (figure 3.1.1 A), width (figure 3.1.1 B) and length (figure 3.1.1 C). The water within the capsulated egg is released, which makes eggs to shrink and harder (Boletzky, 2003; Sykes*, et al.*, 2009a). Different cephalopods studies have also shown similar egg size evolution (Gomi*, et al.*, 1986; Lacoue-Labarthe*, et al.*, 2009; Sykes*, et al.*, 2009a). Encapsulation in cephalopods works both as predator and microbiological defence of the developing animal and as a "trap" of the nutrient needed to the animal (Bloor*, et al.*, 2013; Boletzky, 2003). The egg shrinking may work as a nutrient filtration and pathological microorganisms discharge. Therefore, the egg hardening may be related to egg fertilization and embryo development. From 10 dpp and until hatching, a rapid increase of the eggs in weight (figure 3.1.1 A), width (figure 3.1.1 B) and length (figure 3.1.1 C) was observed. The reabsorption of the seawater to the egg is linked to the swelling of the egg that, together with chemical processes, will facilitate the hatching by softening the jelly layers and the chorion (Boletzky, 2003; Sykes*, et al.*, 2009a). In the present study, measurements were carried until 32 dpp, corresponding to nearly hatching. At this stage, the external cases of the eggs were highly thin and many ruptured to touch, hence its lower values.

As the weight of the eggs increase it also increases the length and width (figure 3.1.2 A, B and C). A significant positive correlation between the weight, length and width of the eggs was observed ($p < 0.0001$). However, the correlation between the weight and width was greater ($R^2 = 0.91$). Many different studies have suggested that it is accurate to use weight to define the growth of cephalopods (Domingues*, et al.*, 2002). Likewise, the use of weight or width of the eggs seemed to be appropriate for eggs variations in the present study. On the other hand, the length of the eggs may be an unpredictable measurement. This is due to the amount of nidamental gland that females produce to make the gelatinous capsule of the eggs varies (Boletzky, Boletzky, 1973). Moreover, the egg positioning at the substrate (normally a plastic net) and its removal process will also influence egg length.

The dehydration and re-hydration of eggs during embryogenesis was also observed by Sykes*, et al.* (2009a) study. This seems to be a common feature of *S. officinalis* eggs. Hence the comparison with that and the present study (figure 3.1.3). Although the mean wet weight of the eggs at this study were greater than those observed by Sykes*, et al.* (2009a) study, the egg weights were significantly different between both studies ($p < 0.05$) at each day. In the present study, females were bigger than those that laid eggs at Sykes*, et al.* (2009a) study. It is known that bigger females lay bigger eggs (Boletzky, 1983). Also, there is a maternal effect on the cuttlefish eggs, which can be affected by the surrounding conditions. For instance the stress caused by males sexual behaviour may contribute to egg posture (Bloor*, et al.*, 2013). Furthermore, there may have been different maturation phases of the different females and different reproduction times (Bloor*, et al.*, 2013), thus the variation on the eggs laid between both studies. Additionally, each female lay each egg at different times, hence it may influence the embryonic development (Sykes*, et al.*, 2009a).

4.2. Embryonic development at microscopical scale

The duration of the embryonic development is shorter at higher temperatures (Boletzky, 1974b; Sykes*, et al.*, 2009a). Embryos show low utilisation of the yolk and catabolic processes, such as respiration and excretion that accelerates the metabolism linked to it (Bouchaud, 1991a; b). Therefore, there is an inverse correlation between the volume of the yolk and the hatching (Bouchaud, 1991b). Similarly, at the present study, a high inverse correlation was observed between both areas measured of the external yolk sac and the embryos ($P < 0.001$). There was a significant effect of the outer yolk sac size on the embryo size. As expected, the outer yolk sac was consumed and the animal increased its size within the egg capsule. There was a direct utilization of the nutritional external yolk sac to embryonic development. In the present study, it was not possible to estimate the area of the internal yolk sac nor through live pictures neither histologically (further section). However, it is known that the proper development of the embryo is dependent on the outer yolk sac formed (Boletzky, 2003). Therefore, it was carried the sum of the external yolk area measured with the embryo area measured (or TEA), which increased from 12 dpp until the end of embryogenesis. Likewise, the weight of the egg increased and there was a correlation between TEA and egg weight. However, the correlation only happened 68% of the times, suggesting the water intake may be an important element on the development of the egg and embryo, as seen in many cuttlefish species (Gomi*, et al.*, 1986).

4.2.1. Stages identification and histological characterization

Independently of the development staging system used it is possible to standardize development based on individual variation (Boletzky*, et al.*, 2006; Naef, 1928). The system of development stages presented by Naef (1928) is the most used. Therefore, this was the one selected and used at the present study, throughout which, the organs development were observed essentially for the stages identification. Organs, such as: eyes, arms, tentacles, funnel tube, head (cover and shape) and yolk sac; allowed to determine in which stage the individuals were in. However, within the same day, two or three different stages were possible to be identified. Hence the grouping of stages at this study. Additionally, in Naef (1928) there is a description of transitional stages that were commonly observed during the present study. Although it was constant, the length of the embryonic development is greatly influenced by temperature. Within a range of 16ºC to 20ºC the development tends to be faster than at lower temperature ranges, such as 10ºC to 16ºC (Boletzky, 1974b). In the present study, egg sampling was performed every 24 hours, and the higher temperature verified will have surely influenced the existence of 2-3 stages.

From stage I to stage X blastulation and gastrulation occurred (Boletzky*, et al.*, 2006) hence it was not possible to gather histological cuts. At this stages, a series of folding events and invaginations (figure 3.2.1.2) of the egg occurred (Boletzky*, et al.*, 2006; Naef, 1928). These events originated, in later stages, the different rudimentary organs (Boletzky, 1989; Boletzky*, et al.*, 2006).

Throughout embryogenesis, the embryos were enclosed in the chorion, inside the external capsule. According to Boletzky (2003), the chorion is responsible for retaining the spermatozoon inhibiting polyspermy and confines the perivitelline fluid that have a tranquillizing effect on the embryo, preventing early hatching. Moreover, water and molecules exchange occurs between the perivitelline fluid and the sea water (Boletzky, 2003; Dorey*, et al.*, 2012). For instance, the hypoxic hypercapnic condition created within the perivitelline fluid allows the regulation of the oxygen and carbon dioxide for the developing embryo (Hu*, et al.*, 2011).

The first egg internal differentiation was observed at stage VI - VIII (figure 3.2.1.2). The circular darker ring (R) observed can be the primordial shell gland (psg) or as shown at later phase of that stages by Naef (1928), the mantle primordium (mp).

At stages X to XII (figure 3.2.1.3 and figure 3.2.1.4), a distinct full enclosure of the external yolk sac was observed. The external yolk sac soft globules (or yolk platelets) surrounded by a membrane with water soluble glycol-protein composition was observed (Sykes*, et al.*, 2009a). Those yolk platelet were coloured pink, which is PAS positive for polysaccharides, with large empty spaces in between. That emptiness might suggest lipid content (Costa*, et al.*, 2014), which were damaged during sample collection and preservation (Roper, Sweeney, 1983). The external yolk sac has nutritional, respiratory and circulatory functions (Boletzky, 2003; Sykes*, et al.*, 2009a). Those functions may be carried by the structures observed on the external yolk sac (figures 3.2.1.7, 3.2.1.9, 3.2.1.11, 3.2.1.13, 3.2.1.15). Before any definitive organ is formed, the external yolk sac works as a transient "gill and heart" that encloses a nutrient reservoir (Boletzky, 2003). It maintains an intensive muscular activity to which all embryonic development is dependent. The entire yolk sac is covered by a dense cilliature (greatly visible at figure 3.2.1.13 B, arrow) that enhances the gill temporary function surface. The epidermal ionocytes covering the yolk epithelium appears to be responsible for the ionic regulation (Hu*, et al.*, 2011). Furthermore, the perivitelline fluid circulation is done by the continuous activity of these kinocilia that allow the diffusion of oxygen and embryo' uptake (figure 3.2.1.9 C), (Boletzky, 2003). Peristaltic waves allow contractions that will run over the external yolk moving the blood from the lacuna (ps, white arrow at figure 3.2.1.9 C) of the external yolk sac into the blood sinuses of the proper embryo - primary circulatory system (Boletzky*, et al.*, 2006). Moreover, that same peristaltic movement will be responsible for the circulation of the hemolymph, which is responsible for the immune system (Hu*, et al.*, 2011).

The internal yolk sac shape and storage capacity varies among cephalopods, it is an organ subdivided into lobular niches (Boletzky, 2003). In the present study, it was not possible to estimate the exact size of the internal organ, nor to compare its area to the measures from the external yolk sac. However, the yolk transfer from the external to the internal sac occurs and facilitates the nutrients absorption (Lacoue-Labarthe*, et al.*, 2010). Throughout the embryogenesis, the internal yolk sac content was also PAS-positive, which also suggested a great presence of polysaccharides. Besides its coloration, its content presented an agglomerate of granules substances (figures 3.2.1.5, 3.2.1.9, 3.2.1.11, 3.2.1.13, 3.2.1.16) and empty-like characteristic (figure 3.2.1.7). Due to the lack of microanatomy information of cephalopods early stages it was difficult to determinate the exact microstructural details (i.e. general structure, cells and cell types). Nevertheless, the agglomerates of these structure might be related to the primordial formation of the digestive gland vacuoles and "boules" that were recently described from the digestive gland of subadults *S. officinalis* (Costa*, et al.*, 2014). Similarly to the external yolk sac, the emptiness in the yolk droplets may also be related to the presence of lipid droplets, which were damaged during the histological process. The presence of those in both external and internal yolk sac may emphasise the idea that to the yolk is transferred from one to another (Boletzky, 2003).

During the embryonic development, the internal yolk sac had a denser coloration on its lateral and posterior side, which may be, as observed in later stages, the yolk digestion by the syncytial internal yolk epithelium that passes directly or by the circulatory system to the developing organs of the embryo (Boletzky, 1974b). However, when that denser coloration was magnified it was visible some unknown triangular shaped cells. Those could be the initial stage of cuttlefish digestive cells. However, basal cells were identified by Yim (1978) at stage 22 after hatching. Those cells are responsible for the cell replacement and detoxification, they have similar shape to those at present study. Likewise the excretory cells that are responsible for the solubilisation of salts could also be developing, as they also have similar shape and at these initial stages ionic regulation is high (Costa*, et al.*, 2014; Hu*, et al.*, 2011). Moreover, those cells may be hemocytes, responsible for the immune system and for the elimination of bacteria from the hemolymph (Beuerlein*, et al.*, 2002). Those types of cells were found in the immune system response of other cephalopods such as the *Octopus vulgaris* (Novoa*, et al.*, 2002) and *Euprymna scolopes* (McFall-Ngai*, et al.*, 2010).

At later embryonic stages (figures 3.2.1.12 to 3.2.1.16) of the present study, the overall external aspect of the embryos started to resemble morphologically that of juvenile and adult cuttlefish (Boletzky 1989). Remarkable organs changes were observed, such as: the disappearance of prominent eye stalks combined by the formation of a second head cover; the formation of primary lid that closed together as the cornea of the eye lens are formed; the intensification of the eye pigmentation; the cuttlebone perfectly observed in closed shell sac (closed during organogenesis); several differentiated chromatophores, in contrast to other cephalopods species (Boletzky, 1989; Boletzky*, et al.*, 2006); And finally, one of the most obvious changes was the reduction of the outer yolk sac due to the transfer and absorption to inner yolk sac (Boletzky 1974). Some of the animals at the end of this study were already hatched and some were swimming with their external yolk sac attached. However the “hatching stage” cannot be defined morphologically or physiologically (Boletzky*, et al.*, 2006). Exogenous factors may influence on earlier hatching than at stage XX of Naef’s. The representative amount of inner yolk sac together with the relative viability (i.e., the influence of the yolk system at tissue and organs differentiation) will determinate the competence for hatching (Boletzky*, et al.*, 2006). The initial feeding condition of the hatching will be influenced by the volume of the yolk remaining at the inner sac of late embryonic stages (Boletzky, 2003).

6. References

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