Digestive enzyme ratios are good indicators of hatchling yolk reserve and digestive gland maturation in early life stages of cuttlefish Sepia officinalis L.: application of these new tools in ecology and aquaculture

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Digestive enzyme ratios are good indicators of hatchling yolk reserve and digestive gland maturation in early-life stages of cuttlefish *Sepia officinalis* L.: application of these new tools in ecology and aquaculture.

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Abstract

In *Sepia officinalis* (Linnaeus, 1758), the digestive gland matures during the first month post-hatching while a shift from intracellular acid to extracellular alkaline digestion occurs. The purpose of this study was to investigate the possibility of using enzymatic ratios for the description of digestive system maturation in early life stages of *S. officinalis.* Secondly, it is intended to apply these new tools as eco-physiological indicators for understanding the impact of cuttlefish eggs' life history from different spawning sites of the English Channel on digestive performance of juveniles. An experimental rearing was performed over 35 days after hatching (DAH) on juveniles from wild collected eggs in 2010 and 2011. Four digestive enzyme activities and their ratios [*i.e.* trypsin, cathepsin, acid (ACP) and alkaline (ALP) phosphatase, ALP/ACP and trypsin/cathepsin] were studied along with histological features [*e.g.* internal yolk surface and digestive gland development]. The two enzyme ratios were good indicators of digestive system maturation allowing the study of the digestive gland's development. They were highly correlated to juveniles' weight increase and histological features of the gland in early DAH. These ratios described more accurately the shift occurring between the intracellular acid and the extracellular alkaline modes of digestion in *S. officinalis* and were more specific than separated enzyme activities. Their application as eco-physiological tools revealed that enzyme ratios reflected yolk content and digestive gland development in new hatching juveniles. Finally, ALP/ACP ratio was shown to be a powerful tool to describe growth performance of *S. officinalis* which is useful for aquaculture optimization.

Keywords: *Sepia officinalis*; early life stages; enzyme ratio; indicator; digestive gland; cathepsin; phosphatase; trypsin

1. Introduction

Cephalopods are a highly developed group of marine mollusks with digestive physiology that shares several similarities with that of fish. Indeed, the extracellular digestion occurs in the stomach, while keeping some characteristic features of their molluscan ancestry, with the intracellular digestion (Boucaud-Camou and Yim 1980; O'Dor and Webber 1986). The digestion of proteins by intracellular enzymes in fish larvae is thought to aid in digestion to compensate for the lack of a functional stomach (Georgopoulou et al. 1985; Govoni et al. 1986; Cahu and Zambonino Infante 1995; Lazo et al. 2007). However, in cephalopods, this "ancestral" intracellular digestion is described at all life stages in parallel with the "advanced" extracellular digestion therefore both leading to a rapid growth of these animals due to the efficient assimilation of nutrients (Boucaud-Camou and Roper 1995; Swift et al. 2005). The combination of intracellular and extracellular digestion makes the cephalopods digestive system particularly performant. The efficiency of this digestive system is mostly attributed to the digestive gland and its multiple roles in digestion, namely enzyme secretion, absorption of molecules, intracellular digestion, nutrient and lipid storage as well as excretion of waste products, to

mention only the main ones (Boucaud-Camou and Yim 1980; Boucaud-Camou and Boucher-Rodoni 1983; Boucher-Rodoni et al. 1987; Budelman et al. 1997; Semmens 2002; Martínez et al. 2011; Costa et al. 2014; Lopez-Peraza et al. 2014).

Given the carnivorous diet of cephalopods (Boucaud-Camou and Boucher-Rodoni 1983), the dominant enzymes are expected to be a suite of proteases. Cephalopod enzymatic activities, which are localized in the digestive system, were determined as non-specific proteolytic, α-amylasic, alkaline and acid phosphatasic activities (Boucaud-Camou 1973; Boucher-Rodoni 1981; Perrin et al. 2004). The non-specific proteolytic activities include trypsin and cathepsin enzymes. Trypsin (EC 3.4.21.4) is a member of a large family of serine proteases, which specifically hydrolyses proteins and peptides at the carboxyl group of arginine and lysine residues and plays major roles in biological processes such as digestion and activation of zymogens of chymotrypsin and other enzymes (Kolodziejska and Sikorski 1996; Jellouli et al. 2009). Cathepsins are intracellular enzymes, mainly aspartic and cysteine endopeptidases, active at acid conditions (Morishita 1972; Gildberg 1988; Balti et al. 2010). Acid phosphatases (EC 3.1.3.2, ACP) are characteristic of lysosomes (Boucaud-Camou 1974), and alkaline phosphatases (EC 3.1.3.1, ALP) are membrane-bound enzymes that can be found in cell membrane in which the active transport takes place (Boucaud-Camou and Roper 1995). ACP and ALP catalyze the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at acid and alkaline pHs, respectively (Mazorra et al. 2002; Lacoue-Labarthe 2010). These enzyme activities were observed in the digestive system of several cephalopod species such as *Octopus maya* (Aguila et al. 2007; Rosas et al. 2011; Martínez et al. 2011), *Dosidicus gigas* (Gárdenas-López and Haard 2009), *Robsonella fontaniana* (Pereda et al. 2009), *Sepioteuthis lessoniana* (Semmens 2002) and *Sepia officinalis* (Perrin et al. 2004; Balti et al. 2010; Lacoue-Labarthe et al. 2010) and are considered as key elements in the digestive process.

In cephalopods, enzyme activities are generally used by researchers in relation to diet and growth (Perrin et al. 2004; Le Bihan et al. 2006a; Pereda et al. 2009; Rosas et al. 2011), contaminants (Lacoue-labarthe et al. 2010; Le Pabic et al. 2015) or are localized to describe their function in the digestive system (Boucaud-Camou 1982; Boucaud-Camou and Roper 1995). Although some enzyme activities were used as environmental descriptors in relation with marine population dynamics (Bergeron et al. 2013), such approaches are still rare in cephalopods. In an ecological context, enzyme activities are expected to be highly variable; this could be one of the main reasons for this lack of studies. Howbeit, it is common for studies involving humans to use ratios as health indicators in physiological studies (*e.g.* lipoprotein or enzyme ratios). Lipoprotein ratios are used in cases of heart diseases (Fuduka et al. 2011; Soska et al. 2012) whereas enzyme ratios can be markers of liver fibrosis (Fabris et al. 2006; Shin et al. 2008) or age-related oxidative stress (Ozturk et al. 2012). In cephalopods, the only tools applied right now for trophic dynamics and

ecological description are stable isotope ratios, lipids and fatty acids signature as well as trace metal accumulation (*e.g.* Jackson et al. 2007; Chouvelon et al. 2011; Lourenço et al. 2014). Therefore, enzymatic tools, and more particularly enzyme ratios, still need to be developed in ecological studies that have not been used for such purposes yet. The potential use of enzyme ratios as health indicators in cephalopods (e.g. describing growth performance or contaminants' impact on digestive system) is also important to be investigated.

The European common cuttlefish, *Sepia officinalis* (Linnaeus, 1758) lives in the Mediterranean and in the waters of the Eastern Atlantic from southern Norway to the north-western coast of Africa reaching the border limit between Mauritania and Senegal (Jereb et al. 2015). In the English Channel, the population of *S. officinalis* performs large migrations offshore in winter and inshore in spring for reproduction (Boucaud-Camou and Boismery 1991) and become mainly, but not exclusively, sexually mature at 2 years old (Gras et al. 2016). The littoral zones of the English Channel are thus important spawning locations for *Sepia*. Once mating occurs, cuttlefish lay their eggs on benthic structures in coastal waters, essentially between April and June and die shortly afterwards (Boucaud-Camou and Boismery 1991). The eggs then undergo local environmental conditions that influence their development (Bloor 2016). After hatching, juveniles stay in coastal waters until autumn migration offshore. These early-life stages affect life- history characteristics, distribution and abundance (Bouchaud and Daguzan 1989; Pierce et al. 2008; Bloor et al. 2013). Hence, early-life history is thus assumed to be one of the most critical phase in *Sepia* life cycle and is a key factor for recruitment success (Bloor et al. 2013). Finally, the digestive system of cuttlefish goes through a critical maturing phase during the first month of life. A transition from a predominant acid intracellular digestion to extracellular alkaline digestion occurs (Boucaud-Camou et al. 1985). Digestive gland cells, that are immature at hatching, are progressively filled with various cytoplasmic inclusions, such as vacuoles, lipid droplets and "balls" (digestive vesicles) while maturing (Boucaud-Camou and Yim 1980).

The present study investigates, for the first time, the possibility of using enzymatic ratios (as new indicators) for the description of the digestive system maturation in early life stages of *S. officinalis.* For that purpose, the activities of four enzymes, involved in intra- (*i.e.* cathepsin and ACP) and extracellular (*i.e.* trypsin, and ALP) digestion and their ratios (*i.e.* trypsin/cathepsin, ALP/ACP) were studied along with digestive gland histological observations. These selected enzymes were identified and commonly used in early-life stages of *S. officinalis* and are crucial during the digestive gland maturation (Boucaud-Camou 1982; Boucaud-Camou et al. 1985; Boucaud-Camou and Roper 1995; Perrin et al. 2004; Lacoue-labarthe et al. 2010; Le Pabic et al. 2015).

The aim of this work was to (1) update and complete the description of the main transitional process during the digestive gland maturation of *S. officinalis* (first month of life), (2) test the relevance of enzymatic ratios (*i.e.* ALP/ACP

and trypsin/cathepsin) in digestive gland maturation and (3) use these enzymatic ratios as eco-physiological indicators and as indicators of growth performance. This work would allow developing and testing new eco-physiological indicators while refining the description of the main digestive gland maturation process of *S. officinalis.*

2. Material and methods

2.1 Egg sampling and experimental juvenile growth survey

Mature eggs (*i.e.* very swollen eggs indicating advanced embryonic development) of wild *Sepia officinalis* were collected from four sites among the main spawning grounds of cuttlefish in the English Channel (Boucaud-Camou et Boismery 1991; Dunn 1999). Two of them are located on the French coast [Agon Coutainville (AC; 49°02‘35”N, 1°34’32’’W) and Bay of Seine (BS; 49°18‘53’’N, 0°21’0’’W)] and two others on the UK coast [Torbay (TB; 50°27‘08’’N, 3°33’25’’W) and Selsey (SE; 50°44‘06’’N, 0°47’23’’W)] (**Fig. 1**). Between 1000 and 2400 eggs were sampled per site in July 2010 and 2011 and were transferred to the marine research center of the University of Caen Normandy (CREC, Normandy, France). Eggs were conditioned in boxes half filled with seawater and algae for stabilization during the transport. When the eggs came to the marine research center, they were placed on sieves (0.36 x 0.28 m, 1-mm mesh size) distributed in large tanks containing circulating seawater at a temperature of 18.5 ± 0.5°C (Semi-closed system previously described by Koueta and Boucaud-Camou 1999).

In order to avoid the use of premature juveniles resulting from transport stress, one can generally see it from their remnant external yolk, eggs were acclimatized for 3 days prior to juveniles’ collection among experimental rearing (*i.e.* All juveniles hatched during the 3 days of eggs acclimatization were automatically removed from the tanks). Mature eggs were specifically chosen so that the major incubation period would be achieved in the original natural spawning site. After hatching, juveniles were reared for 35 days between July and September 2010 and 2011. In order to synchronize the launch of all rearing groups (*i.e.* BS, AC, TB and SE), a large number of mature eggs were collected from spawning sites (*i.e.* between 1000 and 2400 eggs/ site). Throughout the hatching period, hatching peaks were observed on specific days. Juveniles used for the growth survey were collected during these peaks so that to have 150 cuttlefish per site which hatched on the same day (*i.e.* being the same age). Even though we managed to have 150 juveniles/site having the same age to be launched for rearing, it was difficult to have juveniles from the four sites (*i.e.* BS, AC, TB and SE) launched for rearing on the same date. It is worth noting that in order to avoid any bias due to the delay in launching the rearing process between sites (*i.e.* a maximum of two weeks delay), a strict control of the abiotic conditions was constantly applied; moreover, the food (*i.e. Crangon crangon*) was prepared in June and frozen at -80˚C in order to have the same food source and quality for all.

Cuttlefish from each site were placed into six rectangular sieves (0.36 x 0.28 m; 1mm mesh size; 25 juvenile /sieve). Sieves were randomly distributed across three tanks thus mixing the sieves with juveniles from different origins in order to avoid any bias related to the parameters of the tank (e.g. controlled temperature). Cuttlefish were fed *ad libitum* with frozen shrimps *C. crangon*. Mean water temperature was 19.4 ± 0.1 in 2010 and 19.3 ± 0.2 in 2011; the rearing system allowed 80% renewal of seawater per day so as to avoid changes in salinity content, pH as well as accumulation of nitrogen compounds.

Juvenile were sampled 0, 7, 14, 21, 28 and 35 days after hatching (DAH). Weight measurements were conducted on 24 juvenile cuttlefish/site/DAH with a Denver Instrument balance ((Digital blanc, Washington, precision of 0.001 g). For enzymatic assays, 5 juvenile cuttlefish/site/DAH were anesthetized in a 2 % ethanol solution in seawater, which is widely used as an anaesthetic agent in cephalopods (Fiorito et al. 2015). Once anaesthetized, *i.e.* observable decrease of locomotor activity and loss of normal posture, the animals were frozen in liquid nitrogen and kept at -80°C until biochemical analysis. This quick euthanasia method is adequate to avoid animal suffering with minimum pain and distress (Fiorito et al. 2015). For histological experiments, 6 and 10 juveniles (in 2010 and 2011 respectively) were anesthetized in a 2 % ethanol solution in seawater, fixed in a Davidson's solution (10% glycerol, 20% formaldehyde, 30% ethanol, 40% filtered seawater) for 24h at 4°C and then transferred in a 70% ethanol storage solution.

2.2 Enzymes extraction and assays

2.2.1 Enzyme extraction

Sampled juveniles (*i.e.* 5 juvenile cuttlefish/site/DAH) were individually weighed before being separately grounded in liquid nitrogen. The digestive enzymes are highly concentrated in the digestive system with the digestive gland playing a major role in their secretion (Boucaud-Camou et al. 1985). Several authors have investigated the digestive enzymes' evolution in early life stage of *S. officinalis*, using the entire animal to reflect the digestive system maturation in relation to the animal's growth performance (Koueta et al. 2000, Perrin et al. 2004, Le Bihan et al. 2006a, Le Pabic et al. 2015). The same approach is applied here for enzymatic assays.

Powder obtained after grinding was homogenized in a known amount (0.1 g to 10 ml) of Tris buffer pH 8 (10 mM Tris-HCl and 150 mM NaCl) and stored at 4°C for 1 hour. The mixture was then centrifuged for 10 min at 15000 g and 4°C. The supernatant was used for digestive enzyme assays and for determination of total protein concentration.

2.2.2 Total protein concentration

The total protein content was assayed according to the Bradford method (1976) using Bovine Serum Albumin (Sigma-Aldrich, France) as standard.

2.2.3 Enzymatic assays

Trypsin activity was measured according to Tsunematsu et al. (1985) using 1 mM Nα-benzoyl-Arg-p-nitroanilide as substrate in a 0.1 M Tris buffer at pH 9. Twenty µl of supernatant were added to 100 µl of substrate in triplicates in sterile 96-well flat bottom plates (BD, USA) and samples were incubated for 1 hour at 25°C. The final absorbance was recorded at 410 nm using Mithras LB940 luminometer (Berthold, Thoiry, France) and the enzyme activity was expressed as trypsin specific activity (U.mg $prot^{-1}$) where one enzymatic activity corresponds to 1 µmol of pNa $formed.min^{-1}$.

Cathepsin activity was measured according to Bonete et al. (1984) using 100 µl of supernatant, 50 µl of 0.4 M acetate buffer at pH 4 and 50 µl of 2% (w/v) haemoglobin solution. In parallel, intrinsic proteolytic end products were measured by replacing 50 µl of 2% haemoglobin by Milli-Q water. Samples were then incubated at 37 °C for 1 hour. The reaction was stopped by adding 1 ml of 3% (w/v) trichloroacetic acid. After 10 min, the assays were centrifuged at 800 g for 10 min at 4°C. Fifty µl aliquots were used to estimate the released proteolytic end products by using the Bradford (1976) method with Bovine Serum Albumin (Sigma-Aldrich, France) as standard. The activity was expressed as cathepsin specific activity (U.mg $prot^{-1}$).

Total acid and alkaline phosphatase (respectively ACP and ALP) activities were respectively determined according to Moyano et al. (1996) and Principato et al. (1982) using p-nitrophenyl-phosphate 2% as substrate in a 1 M Tris buffer at pH 3 for ACP and pH 10 for ALP. For both activities, 10 µl of supernatant were added to 10 µl of substrate in 96-well flat bottom plates (BD, USA). After 30 min of incubation at 25 °C, 100 µl of NaOH 1 M were added to stop the reaction and reveal the color. The absorbance was measured at 405 nm using Mithras LB940 luminometer. Total acid and alkaline phosphatase activities were expressed as specific activity (U.mg $prot^{-1}$) where one enzymatic unit corresponds to 1 µmol of p-nitrophenol $formed.min^{-1}$.

2.2.4 Enzymatic ratios

During the first month post-hatching, a shift from predominant acid intracellular digestion to extracellular alkaline digestion was observable in *S. officinalis* juveniles (Boucaud-Camou et al. 1985). Enzymatic ratios were calculated in order to describe this shift between intracellular digestion carried out by enzymes acting in acidic medium and extracellular digestion carried out by alkaline enzymes. Two types of ratios were calculated, one between the two proteolytic enzyme activities (trypsin/cathepsin) and one between the two phosphatase activities (ALP/ACP).

2.3 Histology

A histological study of the maturation of the digestive gland was undertaken in parallel to enzymatic assays. For this purpose, the samples stored in 70% ethanol solution were washed, dehydrated and embedded in paraffin. Serial sections of 5 µm were cut with a manual rotary microtome Leica RM2135 (Leica, Nanterre, France), processed and stained with

Prenant-Gabe's trichrome according to a classical protocol (Gabe 1968). Digital pictures and cell measurements of the digestive gland were achieved with the Nikon C system combining Eclipse 80i microscope / DXM1200-C digital camera and NIS-elements D 3.0 software.

The maturation of the digestive gland was assessed from 0 to 35 DAH with special emphasis on the hatching day (*i.e.* D0) reflecting the incubation conditions in the different spawning sites with no experimental interference. In 0 DAH juveniles, internal yolk and digestive gland surfaces (respectively IYS and DGS, mm^2) were measured (**Fig. 2**). The digestive gland development (DGD, %) was estimated according to the following equation:

DGD (%) = [DGS/(DGS + IYS] x 100

From 0 to 35 DAH, cytological features, that are specific to maturing digestive cells such as the mean number of "balls" (proteinaceous inclusions characteristic of most cephalopods)/cell (NBC) (**Fig. 2**) and the mean digestive cell length (CL, µm), were estimated from observations of 50 digestive cells/individual using 6 juveniles/DAH/site that had been sampled in 2010.

2.4 Statistical analysis

All results are given as mean ± standard error. For statistical analysis, preliminary tests of normality (Shapiro test) and homoscedasticity (Bartlett test) allowed the use of parametric methods. Biological responses (weight, enzymatic activities, enzymatic ratios and histological features) were compared across samples (see **Table S1** which synthetizes the number of samples/analysis). Two-ways ANOVA (for factors site and age) were used for samples of the same year. The statistical significance threshold was set at $p < 0.05$. When significant differences were observed, then a *post hoc* Tukey test was used to look for homogenous groups of batches. An ANCOVA analysis was conducted for inter-annual comparisons (2010-2011) of growth evolution, enzymatic activities and enzyme ratios.

At hatching day (0 DAH), differences between juveniles were sought in relation to the origin of the eggs (i.e. spawning sites: BS, AC, TB and SE). Differences in enzymatic variables were analyzed in two steps. In the first step, the matrix describing enzyme activities and ratios at 0 DAH was analyzed using a MANOVA analysis to test significant differences between origin sites. In a second step, a Linear Discriminatory Analysis (LDA) was performed to display juveniles in the plane of the first discriminant axes and to visualize site differences.

During the first month post-hatching (i.e. 0 – 35 DAH) the correlations between average juvenile growth (i.e. weight and age), digestive enzyme activities and ratios, and histological features (i.e. NBC and CL) were analyzed using a Principal Component Analysis (PCA) in order to determine which variables contributed most to the description of changes in physiology .

Spearman rank correlation tests were also performed at 0 DAH and during the first month post-hatching (i.e. 0 – 35 DAH) to test the relationships between variables used in the LDA and those used in the PCA.

R software and packages were used for statistics and graphics (Fox and Weisberg 2011; Hervé 2012; R Core Team 2012).

3. Results

3.1 Growth survey

Cuttlefish growth was monitored on reared juveniles hatched from eggs collected at four different spawning sites of the English Channel in 2010 and 2011 (**Fig. 3a, b**). The general profile of juveniles' growth showed a "no net growth" phase between 0 and 7 DAH. Then a significant weight increase was observed between 14 and 35 DAH (**Table 1**). Slopes fitted to the weight natural logarithm were significantly lower in 2011 (mean weight of 1.5 g at 35 DAH) than in 2010 (mean weight of 2 g at 35 DAH), thus revealing a significantly lower growth rate in 2011 (ANCOVA, $F(1,6)= 62.86$, $p_{value}= 2.14e^{-04}$).

The main spatial difference in juvenile's weight was observed between BS and AC in 2010 (**Fig. 3a**) and in 2011 (**Fig. 3b**). This difference was not observed at 7 DAH but the weight distribution reappeared afterwards from 14 until 35 DAH with marked significant differences at 28 and 35 DAH (**Table 1**). The UK juveniles (*i.e.* TB and SE) showed no significant difference in weight (**Table 1**). When compared to French sites (*i.e.* BS and AC), The UK juvenile's weight distribution appeared to be closer to BS juveniles.

3.2 Digestive enzyme activities and ratios

3.2.1 Enzymatic activities: general profile description

Trypsin mean activity varied between 0.5 and 1.6 IU.mg $prot^{-1}$ during both monitoring years (**Fig. S1a, b**). In 2010 (**Fig. S1a**), an increase of this activity was observed between 0 and 7 DAH then this activity was stabilized until 35 DAH. In 2011 (**Fig. S1b**), a significant increase was noticed between 0 and 14 DAH (**Table S2**) followed by an important variability in activities between 14 and 35 DAH. Cathepsin mean activity varied between 5 and 25 IU.mg $prot^{-1}$ (**Fig. S1c, d**) during the two monitoring years of the study. In both years, a significant increase of cathepsin activity was described during the first week (**Table S2**) followed by a decrease in activity and a stabilization afterwards between 21 and 35 DAH. The trypsin/cathepsin ratio (**Fig. 4a, b**) had a general profile that varied between the two years. However, a significant peak of this ratio was observed at 28 DAH for both years (**Table 2**).

Mean alkaline (ALP) and acid (ACP) phosphatase activities measured from 0 to 35 DAH varied respectively between 1.7 and 4 IU.mg $prot^{-1}$ for ALP and between 6 and 13 IU.mg $prot^{-1}$ for ACP (**Fig. S1e to S1h**). ALP activity profiles (**Fig. S1e, f**) showed a significant increase in early DAH then a stabilization until 35 DAH (**Table S2**). ACP activity profiles (**Fig. S1g, h**) showed a significant increase of activity, between 0 and 7 DAH, then a significant decrease up to 35 DAH (**Table S2**) with a stabilization phase from 21 DAH observed only in 2010 (**Fig. S1g**). ALP/ACP ratios (**Fig. 4c, d**) revealed a significant increase of this ratio up to 35 DAH in both years of study (**Table 2**).

The ANCOVA analysis applied on these enzyme activities revealed (i) a higher decrease in cathepsin and ACP activities after 7 DAH in 2010 (**Fig. S1c, g**) compared to 2011 (**Fig. S1d, h**) [ANCOVA, cathepsin: $F(1,6)= 7.08$, $p_{value}=0.037$; ACP: $F(1,6)= 18.31$, $p_{value}=0.005$] and (ii) a higher slope in ALP/ACP ratio in 2010 (**Fig. 4c**) compared to 2011 (**Fig. 4d**) (ANCOVA, $F(1,6)= 5.41$, $p_{value}=0.049$).

3.2.2 Comparing enzymatic activity between sites

Trypsin mean activity did not reveal any significant differences between sites on the same DAH (**Fig. S1a, b and Table S2**). In contrast, cathepsin activity exhibited significant differences between sites at 0 and 7 DAH (**Fig. S1c, d and Table S2**). Two groups were observed in the two years of study, the first including AC and SE and the second including BS and TB. Indeed, at hatching day (0 DAH), SE and AC had significantly higher cathepsin activity than TB and BS and this distribution was maintained at day 7. These two groups were also observed with trypsin/cathepsin ratios (**Fig. 4a, b**) at 0 and 7 DAH.

ALP and ACP activities did not give a clear pattern related to spawning sites (**Fig. S1e, f, g, h**). However, when ALP/ACP ratio was applied (**Fig. 4c, d**), the same two groups were formed as observed for trypsin/cathepsin ratios, the first including AC and SE showing lower ALP/ACP ratio at 0 and 7 DAH; the second including BS and TB with a higher ALP/ACP ratio. These differences tended to disappear afterwards until 35 DAH.

3.3 Histological features of the maturing digestive gland

3.3.1 Histological features at hatching (0 DAH)

Internal yolk surface (IYS) (**Fig. 5a, b**) and digestive gland development (DGD) (**Fig. 5c, d**) distribution were similar in the two years. Two juvenile groups appeared at 0 DAH, one including BS and TB juveniles with lower IYS but higher DGD as compared to the second group with AC and SE.

3.3.2 Histological features during the rearing period (0 to 35 DAH)

Mean number of "balls" per cell (NBC) (**Fig. 6a**) and digestive cell length (CL) (**Fig. 6b**) were only studied in 2010, due to the absence of clear patterns in relation to the origin of juveniles (*i.e.* no significant differences were observed in CL and NBC when comparing different sites at the same DAH at the exception of AC at 14 DAH). CL significantly increased up to 28 DAH then decreased from 28 to 35 DAH (**Table 3**). "Balls" were only observed from 7 DAH. From this stage, NBC started to increase up to 21 DAH and then stabilized between 21 and 35 DAH, as observed for enzyme activities.

3.4 Multivariate analysis of enzyme and histological descriptors

3.4.1 Site related differences at hatching day

The MANOVA performed on juvenile enzyme activities and ratios showed significant differences ($p_{value} = 1.12.10^{-6}$) between spawning sites. The descriptive LDA (**Fig. 7**) highlighted on the first axis the differences between the origin sites (almost 80 % of the variance were represented on the first axis – **Fig. 7b**). Juveniles from BS and TB appeared clearly separated from AC and SE juveniles (**Fig. 7a**). The correlation circle (**Fig. 7c**) provided additional information explaining the underlying differences of this separation between sites. The first axis was explained by enzyme ratios and cathepsin activity. Higher enzyme ratios in BS and TB set them apart from AC and SE which had significantly lower enzyme ratios and higher cathepsin activities. The results obtained here are in agreement with the previously made observations of the histological features (**Fig. 5**) and enzymatic ratios at 0 DAH (**Fig. 4**). Spearman rank correlation test revealed that the enzyme ratios were highly correlated to DGD and inversely correlated to IYS at hatching (**Table S3**). The shift between IYS and DGD was shown by the inverse correlation between each other (**Table S3**). The digestive gland maturation at hatching (*i.e.* higher DGD) was better described using enzyme ratios (i.e. ALP/ACP and trypsin/cathepsin) rather than with each enzymatic activity either alkaline (*i.e.* ALP and trypsin) or acid (*i.e.* ACP and cathepsin). Ratios and histological features showed higher R-squared and lower p_{values} in the correlation matrix between variables observed at 0 DAH (**Table S3**). Finally enzyme ratios provided a measure of the maturation stage of the digestive gland at 0 DAH similar to what can be achieved with histological observation.

3.4.2 Principal Component Analysis (PCA)

The relationship between juveniles' growth, enzymatic activities, enzyme ratios and histological features (*i.e.* NBC and CL) was investigated with a PCA (**Fig. 8**). With these variables, the first two dimensions of the PCA accounted for almost 68% of the variability in the dataset. The first dimension, which accounted for 49.08% of the variability, represented mainly changes in the enzyme ratios, in weight and in histological features (*i.e.* NBC and CL). The second

dimension, which accounted for 18.85% of the variability, explained mainly enzyme activities. Projections in the first plane of the PCA revealed four groups of variables in the correlation circle (**Fig. 8b**). The first two groups included enzyme activities with a high correlation between the intracellular acid enzymes from one side (*i.e.* ACP and cathepsin) and a high correlation between the extracellular alkaline enzymes from the other side (*i.e.* ALP and trypsin) (see also Spearman rank correlations in **Table S4**). The third group showed that the enzyme ratios were highly correlated with the juvenile's growth (*i.e.* weight) and with histological features (*i.e.* NBC and CL). Considering the importance of the balance between alkaline (*i.e.* ALP and trypsin) and acid (*i.e.* ACP and cathepsin) enzyme activities in *S. officinalis'* early life stages, the use of enzymatic ratios (*i.e.* ALP/ACP and trypsin/cathepsin) has increased the significance of correlations between the enzyme ratios and the histological features that describe the digestive gland maturation (see Spearman rank correlations in **Table S4**). The fourth group was formed by the juveniles' survival which did not reveal significant correlations with the variables included in the PCA (see Spearman rank correlations in **Table S4**). It is worth noting that samples were reared in the same condition during the first month and that the projection of the individual samples on the plane formed by the first two principal axes (**Fig. 8a**) showed no pattern related to juveniles' origin site, at the exception of 0 DAH juveniles' differences already revealed with the Linear Discriminatory Analysis (**Fig 7**). The correspondence between plot a and plot b in the **Fig. 8** allows for further analysis of temporal changes. The colored clusters in **Fig. 8a** were mainly related to cuttlefish age evolution (i.e. from 0 to 35 DAH), evolving clockwise from the bottom left quadrant to the bottom right quadrant. The red and green clusters (**Fig. 8a**), which were respectively juveniles' samples at 7 and 14 DAH, showed a high correlation with the ACP and cathepsin enzyme activities (**Fig. 8b**), underlying their importance during the cuttlefish first days after hatching. The alkaline enzymes (i.e. trypsin and ALP), the histological features of the maturing digestive gland (i.e. NBC and CL) and the enzyme ratios showed higher correlation with the older cuttlefish, aged 21 to 35 DAH and respectively represented by blue, light-blue and purple clusters in the right panel of the PCA (**Fig. 8a**). These observations represented the changes that were occurring during the first month post hatching in cuttlefish juveniles, transitioning from an important role of intracellular digestive enzymes during yolk digestion to an increasing role of extracellular digestive enzymes with the maturing digestive gland.

4. Discussion

In the course of this work, a complete update of the main transitional process knowledge, occurring with the maturation of digestive gland in *Sepia officinalis,* was conducted. This information was used to validate the suitability of developed indicators (*i.e.* enzymatic ratios) and was then used to understand the impact of life history of eggs (*i.e.* maternal and

ecological effects) on digestive performance of *S. officinalis* early-life stages. The structure of the discussion thus follows the three aspects of this study with (i) knowledge update of the main transitional processes during digestive gland maturation, (ii) the test of indicators (*i.e.* enzymatic ratios) to describe digestive gland maturation and (iii) the application of these indicators to an ecological case study and for aquaculture optimization.

4.1 Main transitional process during digestive gland maturation

The digestive gland of cuttlefish develops in the first month after hatching until it reaches its definitive adult physiology (Yim and Boucaud-Camou 1980; Boucaud-Camou and Boucher-Rodoni 1983). In this process, the digestive gland goes from a state of reserve and yolk distribution organ to a state of an organ responsible for the processing of ingested food (Boletzky 1975). Boucaud-Camou et al. (1985) defined three periods in the early-life stages of cuttlefish *S. officinalis*. The embryonic phase, which starts with the egg development and lasts until juvenile's first meal. In this first phase, food is only provided by the yolk which is digested by the intracellular enzymatic activities of the yolk syncytium. The post-embryonic phase, which begins with the first meal, shows a coexistence between the embryonic mode of nutrition (yolk digestion) and the post-embryonic mode (capture of prey and extracellular digestion in the digestive tract). The third phase (*i.e.* juvenile-adult phase) is characterized by the acquisition of an adult pigmentation and physiology of the digestive gland. In this study, the transition from embryonic to post-embryonic digestion was observed at 7 DAH whereas transition from post-embryonic to the juvenile-adult phase was observed at 21 DAH.

4.1.1 Embryonic to post-embryonic digestion transition

The transition between embryonic and post-embryonic digestion occurred during the first week of juveniles' life when internal yolk reserve is being digested and juveniles start exogenous feeding by catching preys (*i.e.* around day 4). During this transition period, a "no net growth" phase has been observed with a decrease in juveniles' survival (**Fig. 9**). A low growth in early post-hatching days has been described in several cephalopod species such as *Loligo opalescens* (Vidal et al. 2002), *O. maya* (Moguel et al. 2010) and *S. officinalis* (Boucaud-Camou and Boucher-Rodoni 1983). Boucaud-Camou and Boucher-Rodoni (1983) attribute this delay in growth to the fact that the extracellular digestion has not started because of yolk absorption. However, from a physiological point of view, several changes take place. A significant increase in enzyme activities (*i.e.* trypsin, cathepsin and phosphatases) was observed between 0 and 7 DAH. In the digestive gland cells, digestive vesicles known as "balls" emerged at 7 DAH (**Fig. 6a**). These are densely staining spheres containing digestive enzymes that are released in the stomach for primary digestion (Boucaud-Camou and Yim

1980; Boucaud-Camou and Boucher-Rodoni 1983). Their appearance is a sign of the establishment of extracellular digestion which induced the increase in extracellular enzyme activities (i.e ALP and trypsin) between 0 and 7 DAH. An increase in cathepsin and acid phosphatase activities was also registered during the embryonic phase, from 0 to 7 DAH, and mainly marked by cathepsin activity. Umezawa (1982) presented evidence that the most abundant yolk protein is primarily and proteolytically processed by the cathepsin-B-like enzyme which was identified in *S. officinalis* digestive gland (Le Bihan et al. 2006b). The optimal pH for this enzyme activity is low to ensure its stability (Le Bihan et al. 2006b) while pH appears to be a key regulator of yolk degradation (Fagotto 1990, Martínez et al. 2011). The yolk platelets are initially neutral but become acidic on a later phase during development, causing pro-enzyme maturation and yolk degradation (Fagotto 1991). These mechanisms were described in cephalopods which probably shared the same regulatory enzymatic mechanism for yolk degradation (e.g. *O. maya;* Martínez et al. 2011). Peaks in digestive enzyme activity in *O. maya* juveniles in early DAH (Moguel et al. 2010) coincide well with the decrease in density of yolk platelets (Martínez et al. 2011). Thus, in *S. officinalis*, the increase in cathepsin and in ACP activities in the first week post-hatching could be a result of yolk acidification thus inducing cathepsin pro-enzyme activation. Moreover, the increase of these enzyme activities could also be supplemented by the synthesis of *de novo* enzymes (Boucaud-Camou and Yim 1980; Lacoue-Labarthe et al. 2010; Costa et al. 2014). Lacoue-Labarthe et al. (2010) suggested this hypothesis after studying the cathepsin and ACP activities during egg development of *S. officinalis* and after having observed an important increase of these activities in the final days before hatching. The increase in cathepsin and ACP activities revealed in this study after hatching seems to be a continuum of increasing activity of these enzymes in the post hatching phase, with a peak observed at 7 DAH (**Fig. 9**).

4.1.2 Transition from post-embryonic to juvenile-adult digestion

The transition from post-embryonic to juvenile-adult digestion was observed at 21 DAH and corresponded to the establishment of extracellular digestion with exogenous feeding. The transition at 21 DAH indicates also a reversal in juveniles' survival trend. Survival decrease from embryonic phase up to the end of the post-embryonic phase reaching a rate of less than 95% of survival at 21 DAH. After 21 DAH, the juveniles' survival rate starts to increase (**Fig. 9**). The beginning of exogenous feeding is a major characteristic of the post-embryonic phase and induces the start of exponential growth (Boucaud-Camou et al. 1985) as observed after 7 DAH (**Fig. 9**). The first meal triggers the secretory activity of the digestive gland (ensured by the "balls" vesicles), while yolk is still being digested by the yolk syncitium (Boucaud-Camou and Boucher-Rodoni 1983). In this study, "balls" could be seen starting 7 DAH and their number increased rapidly before stabilizing from 21 DAH, similarly to digestive cell length (**Fig. 9**). As for enzymes, a decrease

411 in intracellular acid enzymes (*i.e.* ACP and Cathepsin) is detected during the post-embryonic phase while extracellular
412 alkaline enzymes (*i.e.* ALP and trypsin) starts to stabilize. After 21 DAH, extracellular and intracellular enzymes
413 showed stabilized trends during the juvenile-adult phase, at the exception of the acid phosphatase that decreased up to
414 35 DAH. A stabilization phase is observed in enzyme activities of cephalopods when the digestive system matures
415 (Solorzano et al. 2009). The appearance of well-developed digestive gland cells and plenty of "balls" between 21 and
416 35 DAH demonstrated that *S. officinalis* reached its digestive maturity when the enzyme activity was stable. However,
417 the balance between extracellular and intracellular enzyme activities, mainly described with ALP/ACP ratio, did not
418 seem to be fully mature at 21 DAH as this ratio continued to increase up to 28 DAH. ALP/ACP revealed a constant
419 increase between hatching day (0 DAH) and 28 DAH (**Fig. 9**). This ratio permitted to describe the fine adjustments that
420 occur during the first month post-hatching in *S. officinalis* between the two complementary modes of digestion (*i.e.*
421 intracellular and extracellular digestion).
422
423 *4.2 Enzymatic ratios as indicators of digestive gland maturation*
424 The parallel between enzyme activities and digestive system maturation has been made in cephalopods such as *S.*
425 *officinalis* (Boucaud-Camou 1982; Boucaud-Camou and Roper 1995; Perrin et al. 2004). However, to our knowledge,
426 up to now no study has used enzyme ratio to characterize digestive system maturation.
427
428 *4.2.1 Indicators applied to new hatching juveniles (0 DAH)*
429 The transition from dependence on maternally derived yolk reserves to independent active feeding represents a critical
430 period in the early-life history of cephalopods (O'Dor and Wells 1975; Vecchione 1987; Boletzky 1989). At hatching
431 time, cuttlefish juveniles have important yolk reserves that enable them to survive until exogenous feeding is
432 established (Boucher-Rodoni et al. 1987). Estimating these yolk reserves is thus important to get an idea of the survival
433 capacities of juveniles. In this study, histological observations were achieved in parallel with enzymatic assays in order
434 to look into the correlations between histological features and enzyme ratios. ALP/ACP and trypsin/cathepsin ratios
435 were found to be inversely correlated with internal yolk surface (IYS) and highly correlated with digestive gland
436 development (DGD) at 0 DAH (**Table S3**). At hatching, the digestive gland of *S. officinalis* is being formed and
437 replaces the IYS during maturation of the digestive system (Boucaud-Camou et al. 1985; Boucher-Rodoni et al. 1987).
438 Thus, the ALP/ACP and trypsin/cathepsin ratios appeared to be very good indicators of the development of the
439 digestive system in new hatching juveniles. The higher these ratios are, the more developed is the digestive gland and
440 inversely, lower ratios are correlated with higher yolk reserves.

441

442 *4.2.2 Indicators used to describe digestive gland maturation during the first month post-hatching (0 to 35 DAH)*

443 The adjustment in enzymatic activities that occurs during the first days post-hatching is a signal of juvenile digestion
444 changing to adult digestion (Yim and Boucaud-Camou 1980; Vecchione and Hand 1989; Perrin et al. 2004). In this
445 study, digestive gland maturation was well described by the ALP/ACP and trypsin/cathepsin ratios (**Fig. 8**). These ratios
446 were highly correlated with juvenile age and weight increase up to 35 DAH (**Fig. 8**) even if the correlation was less
447 noticeable with Trypsin/cathepsin ratio (**Table S4**). These enzyme ratios were also highly correlated with digestive
448 gland maturing features (*i.e.* CL and NBC). Enzyme ratios are thus good indicators to describe the digestive system
449 maturation in early life stages of cuttlefish. These ratios were found to be more specific than separated alkaline or acid
450 activities for the description of digestive system maturation (**Fig. 8**). By integrating the acid activities into the
451 establishment of the alkaline digestion with the digestive system maturing, the enzymatic ratios reflected the balance
452 made between the two digestion modes (intracellular and extracellular digestion) during the first month post-hatching.
453 The switch between the initial intracellular acid digestion into extracellular alkaline digestion in cuttlefish is best
454 represented with these ratios and especially with the ALP/ACP ratio. This approach is very promising as it can give
455 additional information on the digestive system maturation using simple tools.

456

457 *4.3 Use of enzyme ratio indicators in ecology and aquaculture optimization*

458 4.3.1 Indicators applied to an ecological case study

459 The development of tools such as enzyme ratio indicators to investigate the spatial digestive performance capacity of
460 cuttlefish juveniles is much needed. This approach could lead in the future to a greater understanding of the relationship
461 between stock abundance and early life history of *S. officinalis* in coastal habitats. Understanding the contribution of
462 spawning sites to the recruited stock is still not well explained and the physiological performance of juveniles in those
463 sites could be a key information to elucidate that.

464 The transition from the embryonic to post-embryonic stage was highly dependent on the juveniles' internal yolk
465 content. This seemed to be influenced by the eggs' origin (*i.e.* spawning site) and thus, by the mother yolk deposit in
466 them and afterwards, by the conditions of incubation of the eggs in the various sites. If a mother is able to predict the
467 quality of her offspring's environment, she may increase their survival chances by adjusting their phenotype to the
468 expected conditions (Segers and Taborsky 2010). Several examples of such maternal effects have been reported,
469 demonstrating a wide range of mechanisms by which females can alter offspring phenotype by adapting to the
470 environment (De Fraipont et al. 2000; Eising et al. 2001; Uller et al. 2007) and thus influencing the quality of egg

content as described in *O. vulgaris* (Lourenço et al. 2014). Such hypotheses suggest that the female cuttlefish would adjust the yolk content in eggs depending on the environmental parameters where eggs are laid (such as the availability and access to prey for juvenile cuttlefish). Higher yolk content would enable better chances of survival of offsprings in an environment that is not rich in preys. It is good to know that, at hatching, the digestive gland of *S. officinalis* is still developing and digestive cells are still undifferentiated as most of them being immature (Yim and Boucaud-Camou 1980). At hatching time, the nutrients are exclusively obtained from the yolk by the digestive activity of the yolk syncitium (Boucaud-camou et al. 1985). In this study, the internal yolk content of juveniles at hatching was observed to be stable in space (*i.e.* AC and SE always had a significant higher yolk content than BS and TB) and time (*i.e.* differences were observed during the two years of study). These differences were also revealed by the enzyme ratios (**Fig. 7**) due to the high correlation between enzyme ratios and histological features at hatching (*i.e.* DGD and IYS) (**Table S3**). Despite the mother effect on eggs yolk content, many physico-chemical factors may have influenced the observed spatio-temporal distribution of internal yolk content in juveniles. Physico-chemical factors play an important role in the early life stages of *Sepia* (Bloor 2016) and may locally influence the incubation time of the eggs. Embryonic development of many cephalopods has been shown to be highly temperature-dependent as eggs develop faster in warmer waters (Semmens et al. 2007); this influences the amount of remaining yolk at hatching (Bouchaud 1991). The observed spatio-temporal distribution of internal yolk content in juveniles is hence a result of a combination between the amount of yolk deposited by the mother when laying eggs and the local environmental parameters during incubation period.

During the first phase of transition from an embryonic to a post-embryonic stage, the internal yolk reserve (IYS) and the digestive gland development (DGD) helped understanding the variability of enzyme ratios and juveniles' growth. Higher IYS advantages AC and SE in the first transitional period (*i.e.* from 0 to 7 DAH). This was mirrored by the absence of significant differences in juveniles' weight at 7 DAH even though significant weight differences were registered at 0 DAH (**Fig. 3**). But this advantage was temporary as the extracellular alkaline digestion was taking place more rapidly in BS and TB during the same period. The DGD was already different at hatching and the ALP/ACP and trypsin/cathepsin ratios highlighted a faster maturation of the digestive system in BS and TB juveniles since ratios were greater at 0 and 7 DAH compared to those of AC and SE.

During the post-embryonic phase, weight differences between juveniles from different spawning sites, which were already described at 0 DAH reappeared at 14 DAH (**Fig. 3**). However, in 2010, the difference at 0 DAH was significant between BS juveniles and the other three sites. At 14 DAH, SE and TB showed a greater growth compared to AC juveniles as their weights became closer to BS juveniles. The advantage gained by SE and TB can be explained in two

ways. SE juveniles had significantly more IYS than the other three sites, which induced higher intracellular acidic activities in the first phase of growth post-hatching. At 14 DAH, SE presented a high ALP/ACP ratio, similar to those found in BS and TB juveniles, whereas AC had a significantly lower ratio. Thus having the advantage in the first phase in intracellular digestion, juveniles of SE have had a DGD maturation equivalent to BS juveniles at the beginning of the post-embryonic phase. This maturation was reached only after 21 days in AC juveniles, which is later than those of the other sites. Intracellular acidic (with higher IYS) and extracellular alkaline (with higher ALP/ACP ratio) digestion helped understanding the higher growth of SE juveniles between 7 and 14 DAH. Similarly, TB juveniles had a higher DGD at 0 DAH when compared to AC juveniles and higher ALP/ACP which gave them the advantage in the second transitional phase.

Differences between cuttlefish batches were particularly noticeable between 0 and 14 DAH for both enzyme ratios and histological features (*i.e.* IYS and DGD). The differences in enzymatic activities and ratios disappeared during the post-embryonic and the juvenile-adult phases (*i.e.* between 14 and 35 DAH) in experimental standardized rearing due to the fact that all batches received the same food and due to the acquisition of similar digestive performances (*i.e.* a stabilization phase was observed in enzyme activities, in CL and NBC of digestive cells). When integrating the biological and physiological information (i.e. age, weight, enzyme activities, enzyme ratios and histological features) with the multivariate analysis, only the Linear Discriminatory Analysis applied on new hatched juveniles (i.e. 0 DAH) allowed the observation of clusters by origin sites. The Principal Component Analysis applied on the whole rearing period (i.e. 0 to 35 DAH) showed no site related clustering and this is due to the standardization of rearing conditions during the experiment. The origin site influence (i.e. the maternal yolk deposit and the incubation conditions) was mainly observable on new hatched juveniles and soon diminished after experimental interference.

4.3.2 Indicators applied to aquaculture optimization

The introduction of *S. officinalis* as a new species for aquaculture is a challenging question that researchers have been working on for several years now (Koueta and Boucaud-Camou 1999; Koueta et al. 2000; Perrin et al. 2004; Domingues and Márquez 2010; Sykes et al. 2013). The establishment of the best rearing conditions and the setting up of a more efficient and inexpensive diet for industrial production of this species are still being explored. However, recent studies have underlined the need to develop physiological tools to allow accurate description of growth performance when testing different diets or different rearing conditions (Sykes et al. 2013). Several indices have been used to describe growth performance in cephalopods, such as biochemical indices for somatic growth (*e.g.* ARN/ADN and protein ratio) or enzyme activities (*e.g.* Aspartate transcarbamylase, total proteolytic activity, proteases) in relation to

instantaneous growth rate (Clarke et al. 1989; Pierce et al. 1999; Koueta et al. 2000; Moltschaniwskyj and Jackson 2000; Villanueva et al. 2002; Roark et al. 2009; Rosas et al. 2011). Among these indices, the influence of digestive enzymes activities have been suggested as determinant for growth performance description (Sykes et al. 2013).

A delay in the digestive system maturation is noticeable between 2010 and 2011. The growth rate was lower in 2011, with mean juvenile weights at 35 DAH around 1.5 g while it reached 2 g in 2010 (**Fig. 3**). The discrepancy between the two years resulted from a delay in the physiological maturation of the digestive system. The decrease in intracellular acid activities (*i.e.* ACP and cathepsin activities) was lower in 2011 during the post-embryonic to juvenile-adult digestion transition compared to 2010 (**Fig. S1**). In 2011, the stabilization phase for cathepsin activity was observed starting from 28 DAH and the ACP decrease was seen up to 35 DAH whereas in 2010 ACP and cathepsin stabilized as soon as 21 DAH. Perrin et al. (2004) also noticed that a faster decrease of ACP activity was a sign of a faster maturation of the digestive system. The evolution of ACP and cathepsin activities was consistent with these author's description when comparing juveniles' growth between 2010 and 2011. Trypsin and ALP activities were stabilized from 14 DAH. However, in 2011 the trypsin activity was very variable compared to 2010 (**Fig. S1**). This last observation could be an indicator of a lower maturation of the digestive system inducing a lower growth rate. Lemieux et al. (1999) worked on a large number of digestive enzymes including trypsin in cod *Gadus morhua* and this enzyme was described as the only measured one that could be suspected to potentially limit growth rate. In carnivorous species such as cuttlefish, trypsin activity is expected to play a major role in protein digestion (Vonk and Western 1984). High variability in its activity could thus indicate a lower efficiency of extracellular digestion of proteins, which limits the growth rate.

Enzyme activities (*i.e.* Cathepsin, ACP, ALP and trypsin) were shown to be good markers of juvenile growth performance during early DAH. Nevertheless, when investigating enzyme ratios, integrated, stronger and simplified information is being captured efficiently describing the changes in growth performance. This is particularly noticeable with ALP/ACP ratio. ALP/ACP ratio showed a decrease in the slope of its linear correlation with juvenile growth in 2011 compared to 2010 (*i.e.* the increase in ALP/ACP ratio between 0 and 35 DAH was faster in 2010 compared to 2011). The ALP/ACP slope decrease reflected the decrease in juveniles' growth performance between the two years. Thus, this ratio is highly relevant to compare growth performance. As for the trypsin/cathepsin ratio, the differences in ratio profiles between 2010 and 2011 were highly influenced by the changes in trypsin activity profiles from one year to the other.

5. Conclusion

This study has led to the development of new indicators (*i.e.* ALP/ACP and trypsin/cathepsin ratios) for the description of digestive gland maturation in *Sepia officinalis* L. during early-life stages. The use of enzyme ratios allowed a more accurate description of the shift occurring between acid and alkaline digestive enzyme activities during the first month post hatching in cuttlefish juveniles. These indicators were highly correlated with juvenile weight increase and were also correlated with digestive gland maturing features (*i.e.* mean number of digestive 'balls'/ cell and digestive cell length). This approach is very promising as it gives information on the digestive system maturation with simple tools. The work undertaken in this study allowed updating knowledge of the main transitional process during digestive gland maturation in *S. officinalis* L. early-life stages. Cathepsin and ACP activities (*i.e.* intracellular acidic enzymes) revealed an increasing profile up to 7 DAH before a new decrease before finally stabilizing. While trypsin and ALP activities (*i.e.* extracellular alkaline enzymes) increased from hatching until 14DAH and then stabilized until 35 DAH. The appearance of well-developed digestive gland cells and plenty of "balls" between 21 and 35 DAH demonstrated that *S. officinalis* reached its digestive maturity when the enzyme activity was stable. However, ALP/ACP ratio has permitted to describe that fine adjustments between intracellular and extracellular digestion still occur at least up to 28 DAH. The relevance of the enzyme ratios as ecological indicators was also demonstrated. The enzyme ratios were inversely correlated to yolk content (IYS) in new hatching juveniles with low values reflecting a high yolk content. This gave an advantage in growth performance for juveniles during the first transition period (*i.e.* from embryonic to post-embryonic transition). Inversely, higher enzyme ratio values were correlated to a faster digestive gland development (DGD) thus giving it the advantage in the second transitional period (*i.e.* from post-embryonic to juvenile-adult transition). The enzyme ratios allowed the distinction of two groups of juveniles by reflecting their IYS content and DGD. Combining enzymatic ratio with other tools, such as isotope analysis, should be used in future work to relate the cuttlefish recruitment from coastal spawning sites to juveniles' physiological performance. Finally, a comparison between the two years of study (*i.e.* 2010 and 2011) revealed the possibility to use enzyme ratios, with mainly the ALP/ACP ratio, as good markers to describe juvenile growth performance.

583 **Compliance with Ethical Standards**

584 **Conflict of interest** The authors declare that they have no conflict of interest.

585 **Ethical approval** All procedures performed in this study involving animals were in accordance with the ethical
586 standards of the institution or practice at which the study was conducted. The present study does not contain any
587 experiments carried out by its authors on human participants.

588

589 6. References

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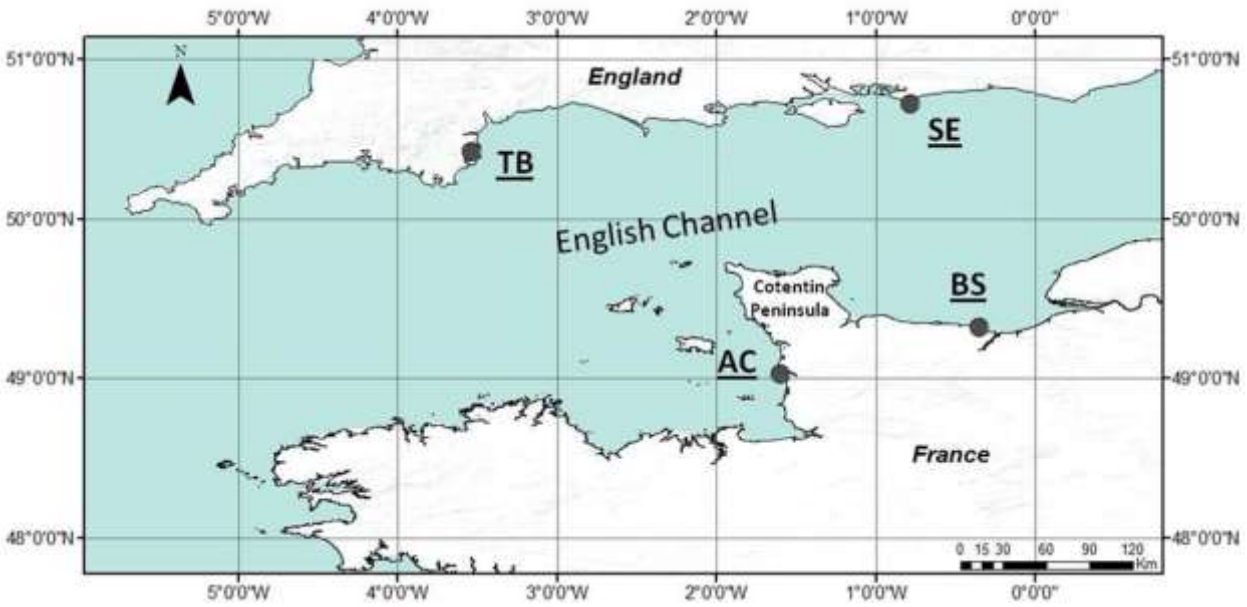
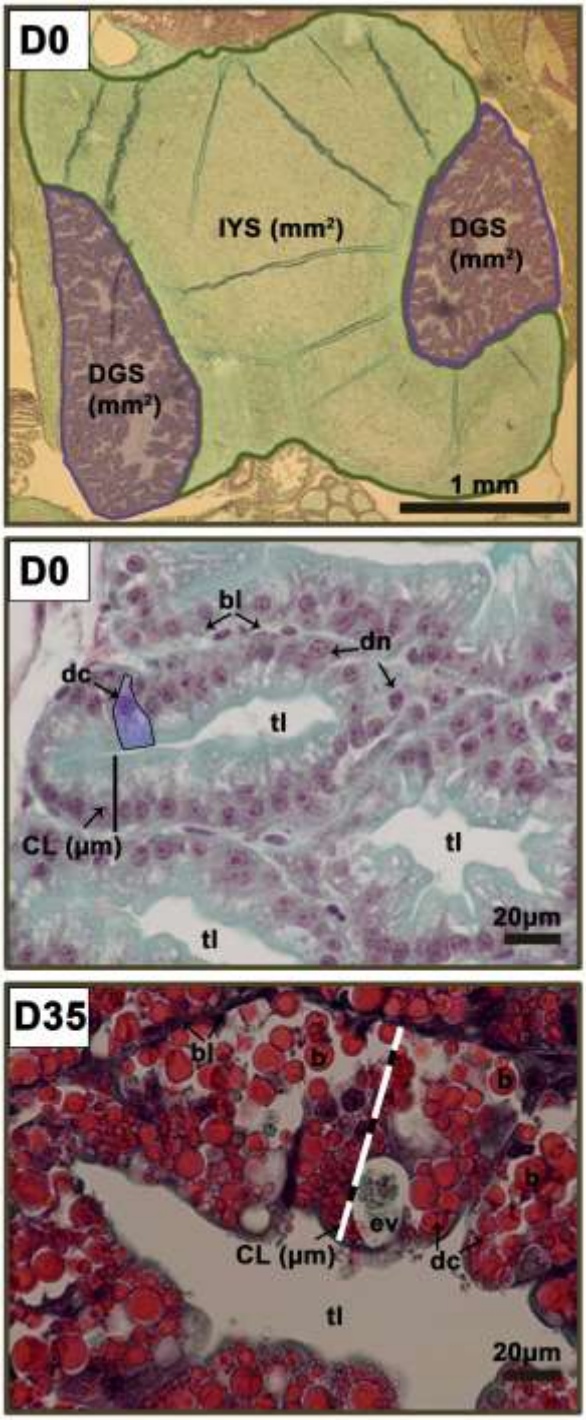


805

806 **Fig. 1** Spawning sites distribution of *Sepia officinalis* in the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB

807 (Torbay-UK) and SE (Selsey-UK)



808

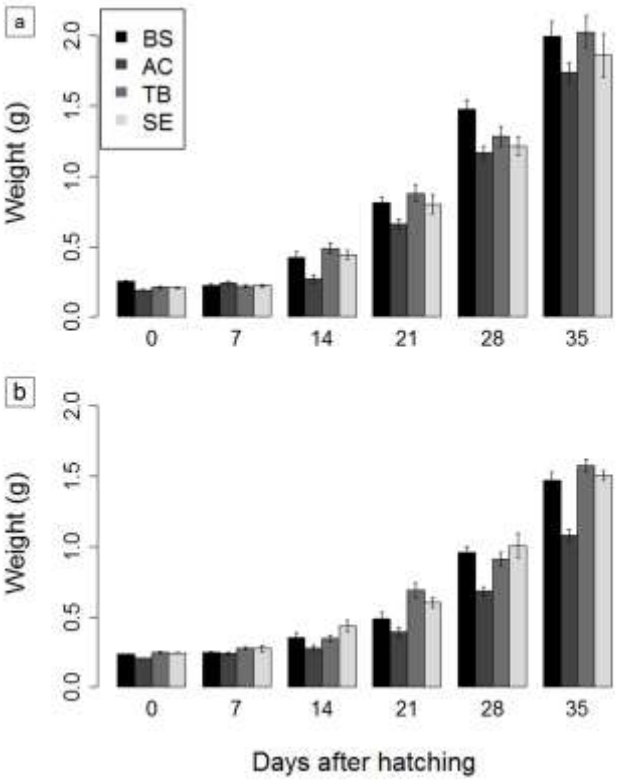
809 **Fig. 2** Structural analysis of *Sepia officinalis* digestive gland at hatching (D0) and 35 days after hatching (D35).

810 b: "balls" (proteinaceous inclusions characteristic of most cephalopods), bl: basal lamina, CL: digestive cell

811 length (µm), dc: digestive cell, DGS: digestive gland surface (μm^2), dn: digestive cell nucleus, ev: excretory

812 vacuole, IYS: internal yolk surface (μm^2), tl: tubule's lumen. Histological sections were stained with Prenant-

813 Gabe's trichrome



814

815 **Fig. 3** Growth of cuttlefish juveniles *Sepia officinalis* during their early days after hatching (DAH) in two
816 different years (a: 2010; b: 2011) (see Table S1 for N juveniles/site/DAH values: mean ± standard error).
817 Cuttlefish hatched from eggs collected from different spawning locations in the English Channel, namely BS:
818 Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

819

820 **Table 1** Statistical results of a two-ways ANOVA (factors site and age) applied to growth of *Sepia officinalis*
821 juveniles, as presented in Fig. 3. Cuttlefish hatched from eggs collected from different spawning locations in the
822 English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

year	site	Age (days)					
		0	**7**	**14**	**21**	**28**	**35**
2010	BS	hi	i	ghi	efg	bc	a
	AC	i	hi	hi	fgh	cde	ab
	TB	i	i	fghi	def	cd	a
	SE	i	i	ghi	efg	cde	a
2011	BS	fg	fg	efg	def	b	a
	AC	g	fg	fg	efg	cd	b
	TB	fg	fg	efg	cd	bc	a
	SE	fg	fg	defg	de	b	a

823

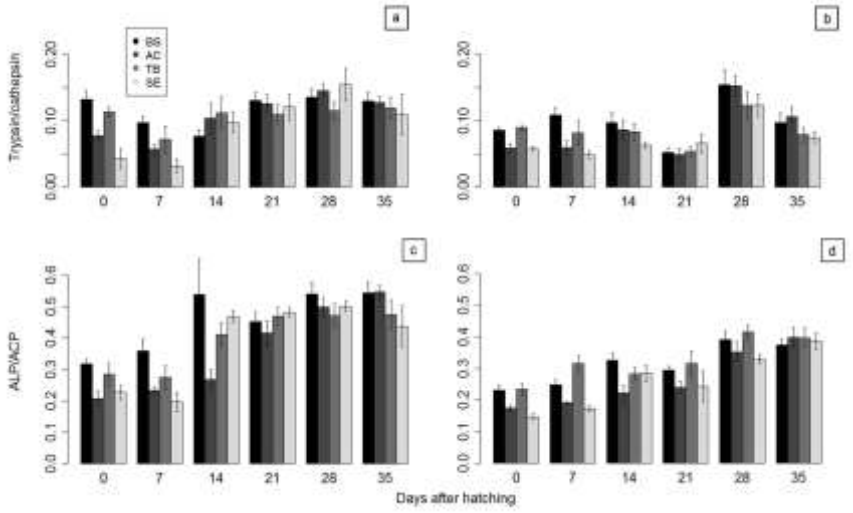
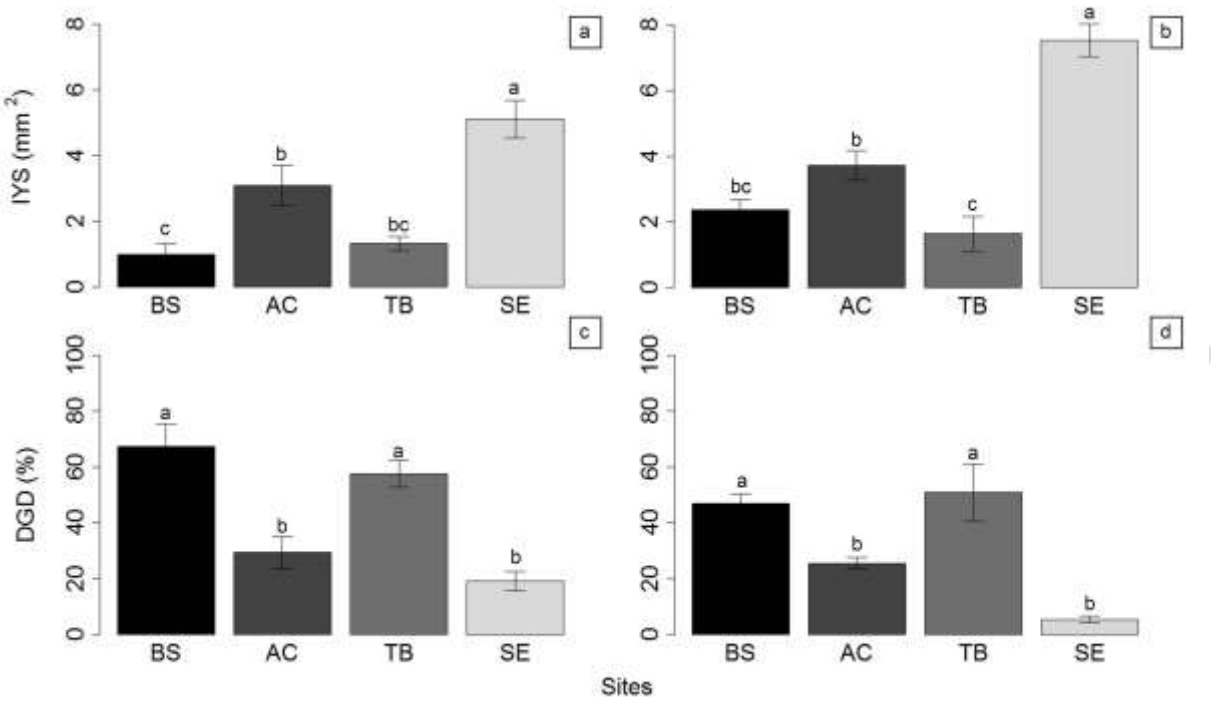


Fig. 4 Enzyme ratios' evolution (*i.e.* trypsin/ cathepsin and alkalin and acid phosphatases, ALP/ACP) in *Sepia officinalis* juveniles during their early days after hatching (DAH) in 2010 (Barplots a and c) and 2011 (Barplots b and d). N=5 juveniles/site/DAH, values: mean ± standard error. Cuttlefish hatched from eggs collected from different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

Table 2 Statistical results of a two-ways ANOVA (factors site and age) applied for enzyme ratios'evolution in *Sepia officinalis* juveniles presented in Fig. 4. Cuttlefish hatched from eggs collected from different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

Enzyme ratio	year	site	Age (days)					
			0	**7**	**14**	**21**	**28**	**35**
Trypsin/ cathepsin	2010	BS	abc	abcd	abcd	abc	ab	abc
		AC	abcd	bcd	abcd	abc	ab	abc
		TB	abcd	abcd	abcd	abcd	abcd	abcd
		SE	cd	d	abcd	abcd	a	abcd
	2011	BS	ab	ab	ab	b	a	ab
		AC	b	b	ab	b	a	ab
		TB	ab	ab	ab	b	ab	ab
		SE	b	b	b	b	ab	ab
ALP/ACP	2010	BS	bcdefg	abcdefg	a	abcde	ab	ab
		AC	fg	efg	defg	abcd	abc	ab
		TB	cdefg	cdefg	abcdefg	abcd	abcd	abcd
		SE	efg	g	abcd	abcd	abc	abcdef
	2011	BS	cdef	bcdef	abc	abcde	a	ab
		AC	ef	def	cdef	bcdef	abc	a
		TB	cdef	abcd	abcde	abcd	a	a
		SE	f	ef	abcde	bcdef	abc	a



835

836 **Fig. 5** *Sepia officinalis* digestive gland characteristics at hatching (0 DAH) in 2010 (Barplots: a and c; n=6) and
837 2011 (Barplots: b and d; n=10, values: mean ± standard error). IYS: Internal Yolk Surface (mm^2), DGD=
838 Digestive Gland Development (%).Cuttlefish hatched from eggs collected from different spawning locations in
839 the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey. Barplots not
840 bearing the same subscript letter are significantly different ($p < 0.05$, one way ANOVA for factor site)

841

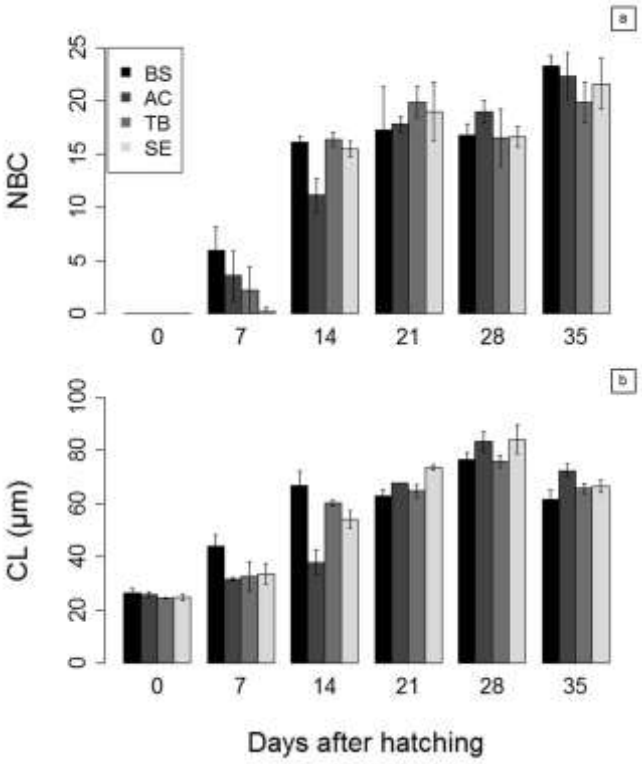
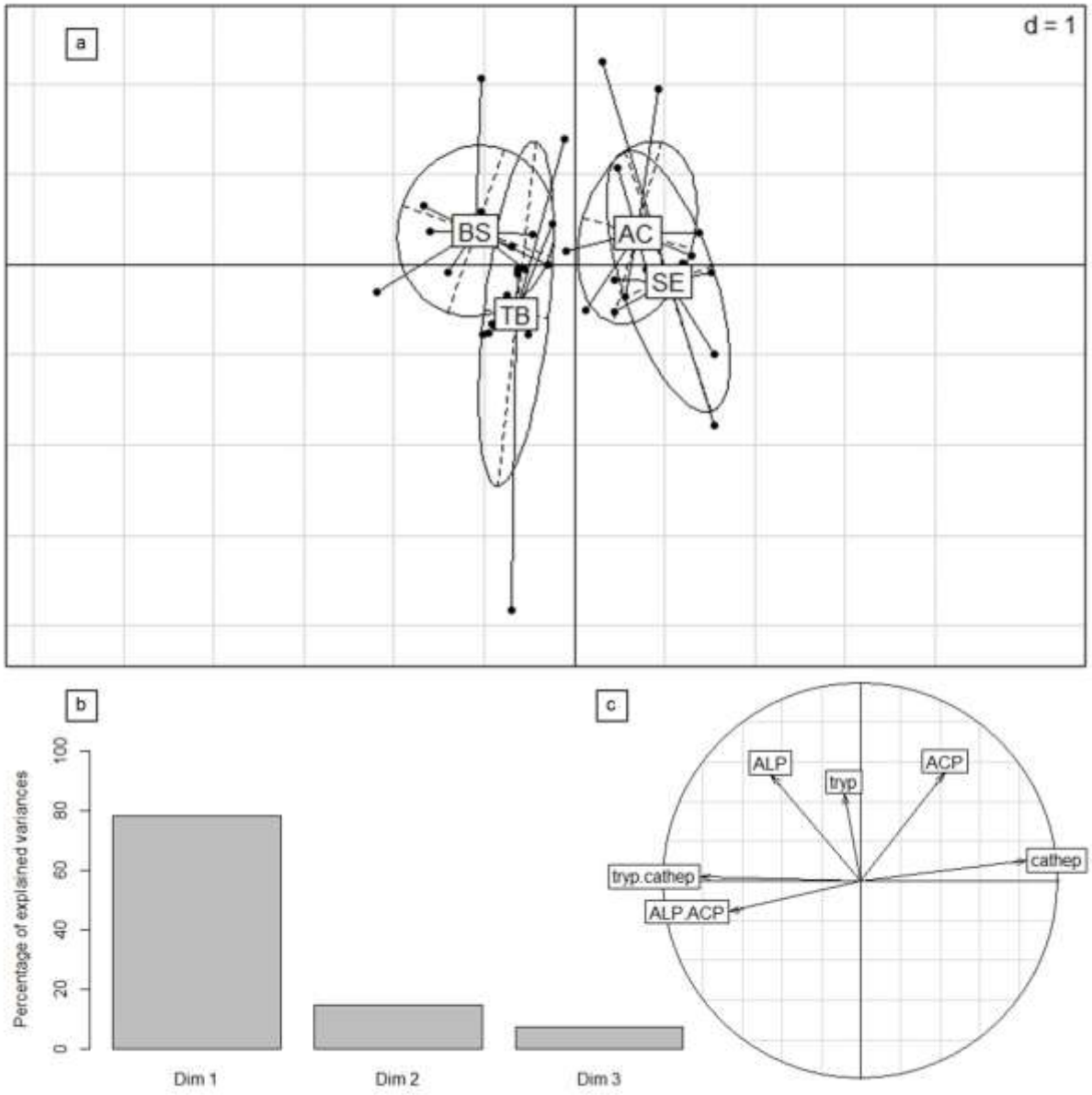


Fig. 6 *Sepia officinalis* mean number of « balls » per cell (NBC, barplot a) and digestive cell length (CL, barplot b) during early post hatching days (35 DAH) (n= 6/site/DAH, values: mean ± standard error). Cuttlefish hatched from eggs collected in 2010 from different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

Table 3 Statistical results of a two-ways ANOVA (factors site and age) used to characterize the histological features of *Sepia officinalis* juveniles, as presented in Fig. 6. NBC: mean number of « balls » per cell; CL: cell length. Cuttlefish hatched from eggs collected from different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

Histological feature	site	Age (days)					
		0	7	14	21	28	35
NBC	BS	d	bcd	abc	ab	abc	a
	AC	d	cd	abcd	ab	ab	a
	TB	d	d	abc	a	abc	a
	SE	d	d	abc	ab	abc	a
CL	BS	fg	bcdefg	abc	abcde	ab	abcde
	AC	g	efg	cdefg	abc	a	ab
	TB	g	defg	abcdef	abcde	ab	abcde
	SE	g	cdefg	abcdefg	ab	a	abcd



852

853 **Fig. 7** Linear discriminant analysis performed on juveniles cuttlefish at hatching day (0 DAH), sampled in four
854 spawning sites from the English Channel (i.e. BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey) in
855 2010 and 2011. (a) Plane formed by the first two principal dimensions with the projection of the individual samples; (b)
856 Percentage of explained variances on the first three dimensions; (c) Correlation circle. ACP: acid phosphatase enzyme
857 activity; ALP: alkaline phosphatase enzyme activity; cathep: cathepsin enzyme activity; tryp: trypsin enzyme activity;
858 tryp.cathep and ALP.ACP: enzyme ratios.

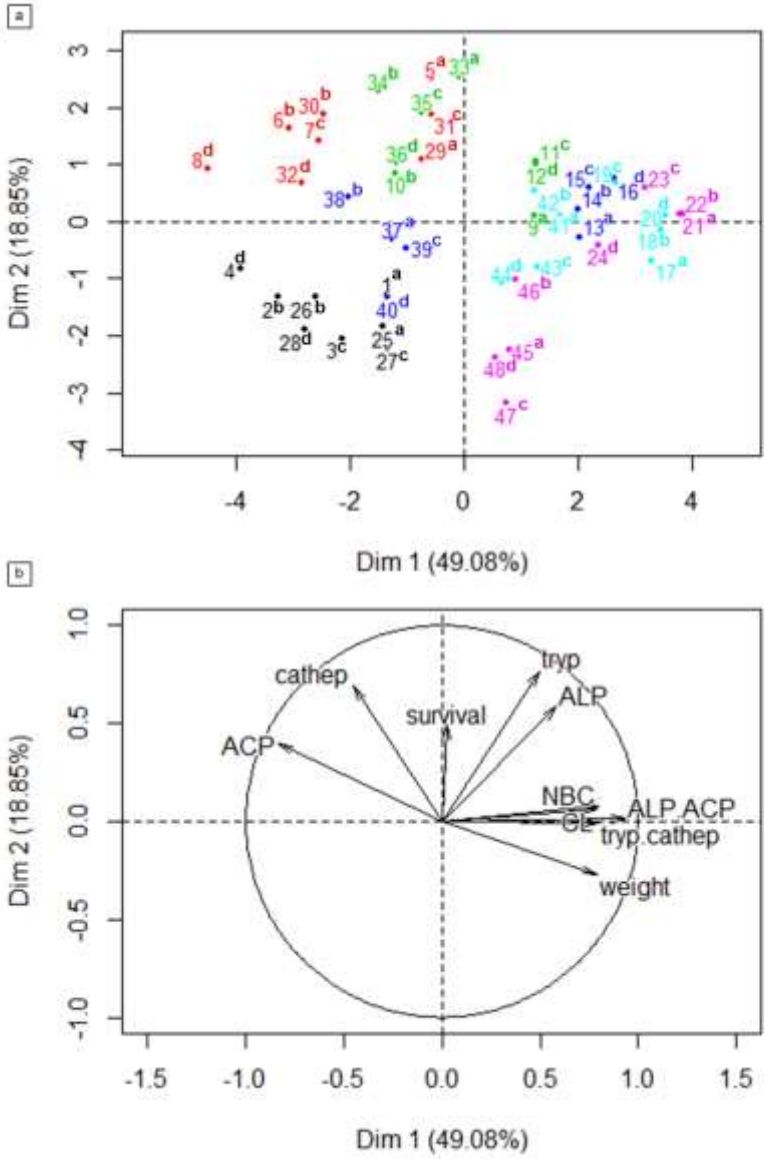
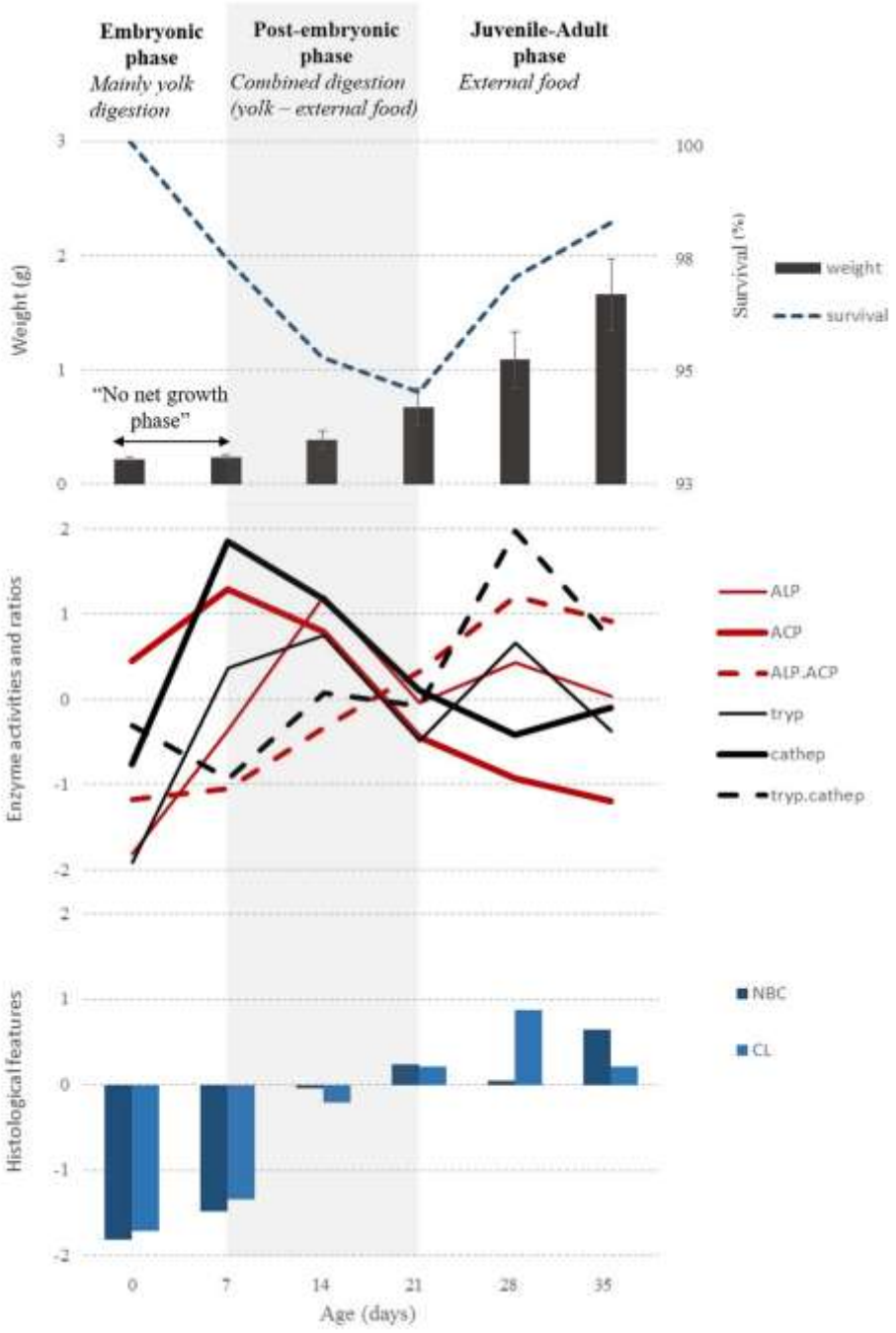


Fig. 8 Principal Component Analysis showing the projection on the plane formed by the first two principal dimensions of individual samples (categorized according to cuttlefish age) (a) and the related variables in the correlation circle (b). In plot a, the color code correspond to the cuttlefish age, being respectively 0 DAH (black), 7 DAH (red), 14 DAH (green), 21 DAH (blue), 28 DAH (light-blue) and 35 DAH (purple). Numbers 1 to 24 are for samples of 2010 and numbers 25 to 48 for samples of 2011. The four studied sites are identified by the superscript letters a (BS), b (AC), c (TB) and d (SE).ACP: acid phosphatase enzyme activity; ALP: alkaline phosphatase enzyme activity; cathep: cathepsin enzyme activity; CL: cell length; NBC: mean number of digestive 'balls'/cell; tryp: trypsin enzyme activity; tryp.cathep and ALP.ACP: enzyme ratios.



868

869 **Fig. 9** Outline of the main physiological and digestive processes occurring in early post hatching days of *Sepia*
870 *officinalis*. ACP: acid phosphatase enzyme activity; ALP: alkaline phosphatase enzyme activity; cathep: cathepsin
871 enzyme activity; CL: cell length; NBC: mean number of digestive 'balls'/cell; tryp: trypsin enzyme activity; tryp.cathep
872 and ALP.ACP: enzyme ratios. Results shown in this figure are mean values of all results obtained in this study for
873 juveniles' weight, survival, enzyme activities, enzyme ratios and histological features. Enzyme activities, enzyme ratios
874 and histological features were centered-reduced for comparison.

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"Digestive enzyme ratios are good indicators of hatchling yolk reserve and digestive gland maturation in early life stages of cuttlefish *Sepia officinalis* L.: application of these new tools in ecology and aquaculture"

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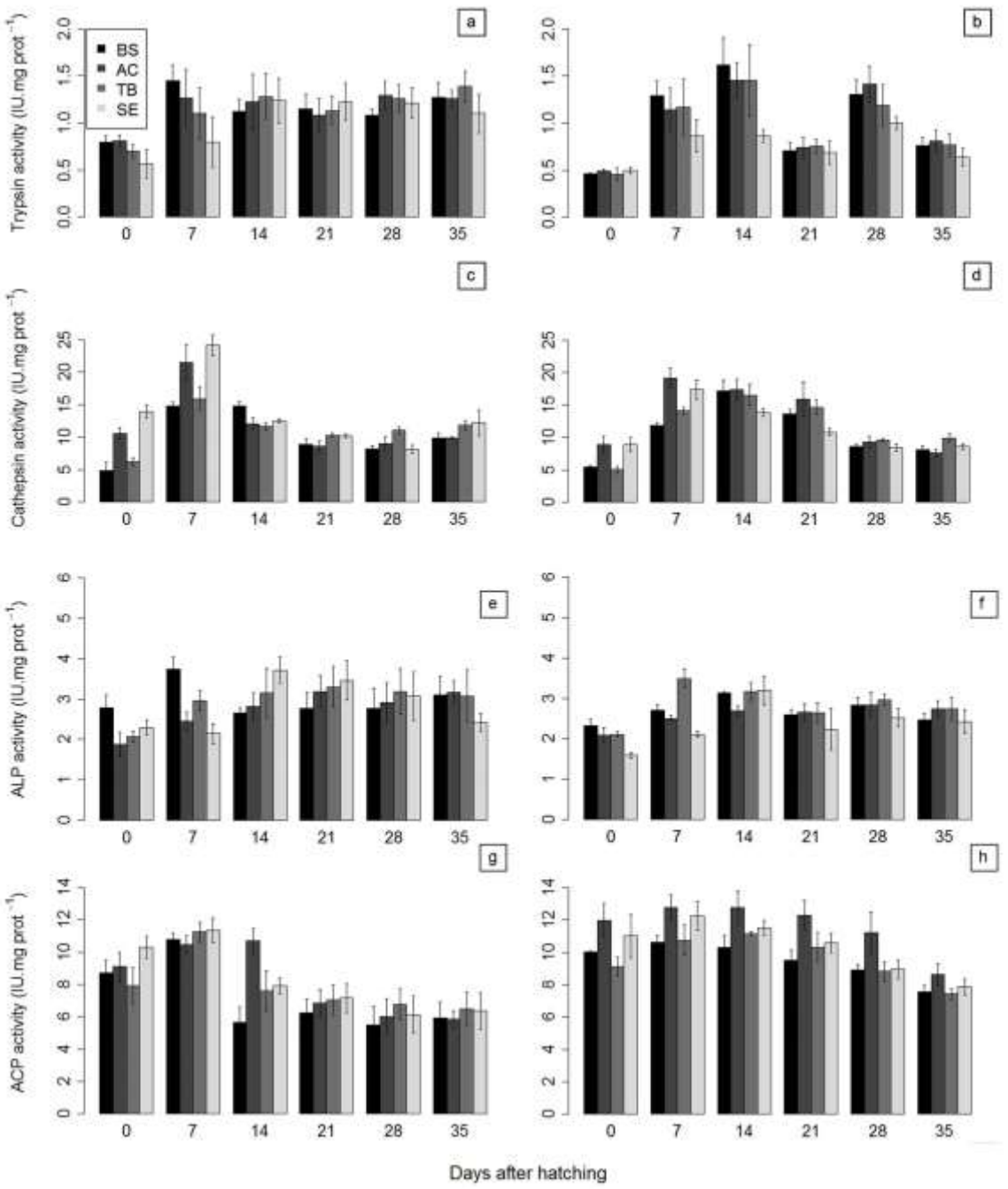
Table S1 Number of samples (N) used to describe biological responses (*i.e.* weigh, enzyme activities and histological features) of juvenile cuttlefish *Sepia officinalis* during their early days after hatching in 2010 and 2011. Cuttlefish hatched from eggs collected from different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

Biological responses (N)	year	site	Age (days)					
			0	7	14	21	28	35
Weight	2010	BS	24	21	19	24	23	30
		AC	24	21	21	23	20	28
		TB	24	22	20	22	23	29
		SE	24	15	13	18	23	30
	2011	BS	24	21	21	20	23	24
		AC	24	23	22	20	22	30
		TB	24	21	9	14	10	13
		SE	24	23	23	15	18	25
Enzyme activities	2010	BS	5	5	5	5	5	5
		AC	5	5	5	5	5	5
		TB	5	5	5	5	5	5
		SE	5	5	5	5	5	5
	2011	BS	5	5	5	5	5	5
		AC	5	5	5	5	5	5
		TB	5	5	5	5	5	5
		SE	5	5	5	5	5	5
Histological observations	2010	BS	6	6	6	6	6	6
		AC	6	6	6	6	6	6
		TB	6	6	4	6	5	6
		SE	6	6	6	6	6	6
	2011	BS	10	-	-	-	-	-
		AC	10	-	-	-	-	-
		TB	10	-	-	-	-	-
		SE	10	-	-	-	-	-



893

894 **Fig. S1** Trypsin, cathepsin, alkaline and acid phosphatases **(**respectively ALP and ACP**)** enzymes' activity in *Sepia*
895 *officinalis* juveniles during their early days after hatching (DAH) in 2010 (Barplots a, c, e and g) and 2011 (Barplots b, d,
896 f and h). N=5 juveniles/site/DAH/year, values: mean ± standard error. Cuttlefish hatched from eggs collected from
897 different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE:
898 Selsey.

899

Table S2 Statistical results of a two-ways ANOVA (factors site and age) applied for enzymatic activities (*i.e.* trypsin, cathepsin, alkaline and acid phosphatases) in *Sepia officinalis* during their early days after hatching. Enzyme activities barplots are presented in Fig. S1. N=5 juveniles/site/DAH/year. Cuttlefish hatched from eggs collected from different spawning locations in the English Channel, namely BS: Bay of Seine, AC: Agon Coutainville, TB: Torbay, SE: Selsey.

Enzyme ratio	year	site	Age (days)					
			0	7	14	21	28	35
Trypsin	2010	BS	a	a	a	a	a	a
		AC	a	a	a	a	a	a
		TB	a	a	a	a	a	a
		SE	a	a	a	a	a	a
	2011	BS	b	ab	a	ab	ab	ab
		AC	ab	ab	ab	ab	ab	ab
		TB	b	ab	ab	ab	ab	ab
		SE	ab	ab	ab	ab	ab	ab
Cathepsin	2010	BS	d	abcd	abcd	cd	cd	cd
		AC	cd	ab	bcd	cd	cd	cd
		TB	cd	abc	bcd	cd	cd	bcd
		SE	abcd	a	bcd	cd	cd	bcd
	2011	BS	cd	abcd	ab	abcd	bcd	bcd
		AC	abcd	a	ab	abc	abcd	bcd
		TB	d	abcd	ab	abcd	abcd	abcd
		SE	abcd	ab	abcd	abcd	bcd	bcd
ALP	2010	BS	a	a	a	a	a	a
		AC	a	a	a	a	a	a
		TB	a	a	a	a	a	a
		SE	a	a	a	a	a	a
	2011	BS	abc	abc	ab		abc	abc
		AC	bc	abc	abc	abc	abc	abc
		TB	bc	a	ab	abc	ab	abc
		SE	bc	bc	ab	abc	abc	abc
ACP	2010	BS	abcd	abc	cd	abcd	cd	cd
		AC	abcd	abcd	abc	abcd	cd	cd
		TB	abcd	ab	abcd	abcd	abcd	abcd
		SE	abcd	a	abcd	abcd	bcd	abcd
	2011	BS	abcdefg	abcdef	abcdefg	cdefgh	gh	gh
		AC	abcd	a	ab	abcde	efgh	gh
		TB	bcdefgh	abcde	abcde	defgh	gh	gh
		SE	abcdef	abc	abcde	cdefgh	fgh	h

Table S3 Spearman rank correlation test applied to investigate relationships between enzyme activities (*i.e.* trypsin, cathepsin, alkalin "ALP" and acid "ACP" phosphatases), enzyme ratios (*i.e.* trypsin/cathepsin and ALP/ACP) and histological observations (Digestive Gland Development "DGD" and Internal Yolk Surface "IYS") at hatching day (*i.e.* 0 day after hatching) in *Sepia officinalis* (N=8). Correlation coefficients are above the diagonal and pvalues below. Significant correlations (pvalue < 0.05) and their corresponding correlation coefficient are in bold.

		Trypsin	Cathepsin	Tryp.cathep	ACP	ALP	ALP.ACP	DGD	IYS
		Correlation coefficient							
Trypsin		-	0.29	0.14	-0.52	-0.17	0.14	0.17	-0.05
Cathepsin		0.493	-	**-0.86**	0.43	-0.55	**-0.71**	**-0.81**	**0.86**
Tryp.cathep		0.736	**0.007**	-	**-0.81**	0.38	**0.86**	**0.98**	**-0.95**
ACP	**pvalues**	0.183	0.289	**0.015**	-	-0.17	**-0.81**	**-0.83**	**0.76**
ALP		0.693	0.160	0.352	0.693	-	0.64	0.50	-0.55
ALP.ACP		0.736	**0.047**	**0.007**	**0.015**	0.086	-	**0.93**	**-0.90**
DGD		0.693	**0.015**	**0.000**	**0.010**	0.207	**0.001**	-	**-0.98**
IYS		0.911	**0.007**	**0.000**	**0.028**	0.160	**0.002**	**0.000**	-

Table S4 Spearman rank correlation test applied to investigate the relationships between weight, age, survival, enzyme activities (*i.e.* trypsin, cathepsin, alkalin "ALP" and acid "ACP" phosphatases), enzyme ratios (*i.e.* trypsin/cathepsin and

913 ALP/ACP) and histological features (mean Number of Balls/Cell "NBC" and Cell Length "CL") during early days after
914 hatching "DAH" (*i.e.* 0 – 35 DAH) of juvenile cuttlefish *Sepia officinalis* (N= 24 for NBC and CL; N= 48 for the rest of
915 the variables). Correlation coefficients are above the diagonal and pvalues below. Significant correlations (pvalue <
916 0.05) and their corresponding correlation coefficient are in bold.

		Weight	ALP	ACP	ALP.ACP	Trypsin	Cathepsin	Tryp.cathep	NBC	CL	Survival	Age
		Correlation coefficient										
Weight		-	**0.37**	**-0.80**	**0.80**	0.25	**-0.32**	**0.55**	**0.91**	**0.79**	0.08	**0.96**
ALP		**0.010**	-	-0.25	**0.63**	**0.68**	0.12	**0.49**	**0.49**	**0.45**	0.19	**0.34**
ACP		**0.000**	0.082	-	**-0.89**	-0.12	**0.54**	**-0.66**	**-0.73**	**-0.77**	-0.02	**-0.72**
ALP.ACP		**0.000**	**0.000**	**0.000**	-	**0.41**	-0.32	**0.72**	**0.78**	**0.82**	0.13	**0.73**
Trypsin		0.089	**0.000**	0.398	**0.003**	-	**0.34**	**0.50**	**0.48**	0.38	0.22	0.27
Cathepsin	pvalues	**0.024**	0.421	**0.000**	0.025	**0.019**	-	**-0.58**	-0.26	-0.34	0.02	-0.23
Tryp.cathep		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	-	**0.54**	**0.63**	0.24	**0.47**
NBC		**0.000**	**0.016**	**0.000**	**0.000**	**0.018**	0.219	**0.007**	-	**0.75**	0.38	**0.93**
CL		**0.000**	**0.027**	**0.000**	**0.000**	0.071	0.104	**0.001**	**0.000**	-	0.18	**0.82**
Survival		0.632	0.234	0.909	0.419	0.177	0.881	0.140	0.098	0.447	-	0.00
Age		**0.000**	**0.019**	**0.000**	**0.000**	0.064	0.110	**0.001**	**0.000**	**0.000**	0.981	-

917