Sexual maturation and embryonic development in octopus: use of energy and antioxidant defense mechanisms using Octopus mimus as a model.

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Abstract

Sexual maturation and reproduction influence the status of a number of physiological processes and consequently the ecology and behaviour of cephalopods. Using *Octopus mimus* as study model, the present study was focused in the changes in biochemical composition that take place during gonadal maturation of octopus females and its consequences in embryo and hatchlings characteristics, putting special attention to energetic metabolites, digestive enzymes and antioxidant defence mechanisms. To do that, a total of 32 adult females of *Octopus mimus* were sampled during ovarian maturation and the biochemical composition (metabolites and digestive enzymes) of digestive gland (DG) and ovaries (only metabolites) were followed during physiological and functional maturation. Levels of protein (Prot), triacyl glycerol (TG), cholesterol (Chol), glucose (Glu) and glycogen (Gly) were evaluated. In DG also the activity of alkaline and acidic enzymes was measured. Simultaneously, groups of eggs coming form mature females were also sampled along development, and metabolites (Prot, TG, Glu, Gly, TG, Chol), digestive enzymes activity (Lipases, alkaline and acidic), antioxidant defence mechanisms and radical oxygen species (ROS) were evaluated. Results obtained showed that ovarium is a site for reserve of some nutrients for reproduction. Presumably, TG where stored at the beginning of the maturation processes followed by Chol, both at the same time were energetically supported by Glu, derived from Gly following gluconeogenic pathways. Also, was observed that embryos during organogenesis nutrients and enzymes (metabolic, digestive and REDOX system) where maintained without significant changes and in a low activity. Results obtained in the present study shows that that activity could be not energetically costly. In contrast, was observed that during the embryo growth there were mobilization of nutrients and activation of the metabolic and digestive enzymes, joint with increments in consumption of yolk and glycogen, and reduction in molecules associated with oxidative stress, allowing paralarvae hatch with the antioxidant defence mechanisms ready to support the ROS production.

Keywords: Metabolites, Cephalopods, *Octopus mimus*, Reproduction, Embryo development

Introduction

Sexual maturation and reproduction influence the status of a number of physiological processes and consequently the animal's ecology and behaviour (Zamora & Olivares 2004). Studies made in *O. vulgaris*, *O. defilippi* (Rosa et al. 2004) and in the Ommastrephid Squids *Illex coindetii* and *Todaropsis eblanae* (Rosa et al. 2005) indicated that those species take the energy for egg production directly from food rather than from stored products in digestive glands or muscle, putting in evidence the importance of trophic ecology for reproduction of those species. In this sense, laboratory studies have confirmed that type of food have a strong influence in the biochemical characteristics of octopus eggs, embryos and hatchlings. Studies made in *O. maya* demonstrated that the type of diet (fresh or formulated) during female reproduction have influence on biochemical and morphological characteristics of embryos and hatchlings (Caamal-Monsreal et al. 2015; Tercero-Iglesias et al. 2015). In those studies, was concluded that octopus females feed mixed diets provide a higher quality yolk, allowing hatchlings a better performance during the first days of culture, in comparison with hatchlings from females feed with a mono-diet. Also was observed that the health status of the *O. maya* females, derived of their nutritional condition determined, between others, the capacity of the animals to maintain their physiological integrity during post-spawning period, when female require enough energy to protect the spawn during the embryo development (Roumbedakis et al. 2017). In *O. vulgaris* also was showed that a mixed maternal diet provoked more hatchlings from females than those fed mono-diet (only crabs) (Márquez et al. 2013), suggesting that the relationship between the nutrients of the diet (AAs and FAs), metabolic pathways and embryos characteristics previously observed in *O. maya* (Caamal-Monsreal et al., 2017) could be operating in the same form in other octopus species. Although there are enough evidences that indicate that the type of food has an important role on embryos and hatchlings characteristics, at the date there are no information related with the biochemical dynamics involved in the form in which nutrients are processed in females during maturation processes, stored in eggs and after used by embryos for their development. Recent studies have demonstrated that during octopus embryo development there are physiological changes that are

related with the form in which nutrients stored in yolk are used to synthesize organs and tissues. Caamal-Monsreal et al., (2016) and Sánchez-García et al., (2017) showed that from stage XV on wards the yolk consumption of *O. maya* embryos was significantly higher than observed in previous stages indicating that is in the stage XV when embryo metabolism is accelerated to stimulate the growth of embryos after the organogenesis. There are evidences that indicate that from stage XV joint with energetic metabolism, there is an increment in catabolic enzymes activity that transform yolk in molecules physiologically useful for embryos (Caamal-Monsreal et al. 2016). That reserves mobilization provoke on the one hand, an increment in oxygen consumption with consequences in energy production, and on the other the production of radical oxygen species (ROS) as a result of the increment of metabolic rate (Repolho et al. 2014; Sánchez-García et al. 2017). Although at the date there are evidences that indicate that octopus embryos are not capable to eliminate ROS in an efficient form when are exposed at thermal changes (Repolho et al. 2014; Sánchez-García et al. 2017), there are no data bases that allow see how is the relationship between the use of yolk reserves, the antioxidant defence mechanisms and ROS production during octopus embryos development.

Octopus mimus is one of the most important species of octopus in the Pacific Ocean at the South of Ecuador, inhabiting from north of Peru to San Vicente bay in Chile where sustains important artisanal benthonic fisheries grounds in both countries (Cardoso et al. 2004; Cortez et al. 1999; Olivares et al. 1996). O. mimus reproduces throughout the year, with one or two seasonal peaks in mature females that are specific for each locality (Cardoso et al., 2004; Castro-Fuentes et al., 2002; Olivares et al., 1996). Egg laying by an individual can extend over 20 d, due to asynchrony in oocyte development and the loss of ovarian function due to their semelparous reproductive strategy (Zamora and Olivares, 2004). The length of time required for embryonic development changes with environmental temperature; in winter at 16 °C it is 67 to 68 d, whereas in summer at 20 °C it is between 38 and 43 d (Castro-Fuentes et al., 2002; Warnke, 1999). Results obtained in laboratory conditions indicated that the optimum temperature for *O. mimus* embryos is in the range of 15 to 18°C (Uriarte et al. 2012), while paralarvae of one to three d old have a thermal

preference of 23 to 26°C (Zuñiga et al. 2013). At the date there are also information about the environmental conditions to obtain spawns in laboratory, including type of tanks, type of light and feeding (Cortez et al. 1995; Olivares et al. 1996; Zuñiga et al. 1995), histology, biochemistry and reproduction (Cortez et al. 1995; Olivares et al. 2017; Olivares et al. 2003; Olivares et al. 2001; Zamora & Olivares 2004), embryo development (Castro-Fuentes et al. 2002; Uriarte et al. 2012; Warnke 1999), thermal tolerance of paralarvae (Zuñiga et al. 2013), growth and nutrition (Baltazar et al. 2000; Carrasco & Guisado 2010; Gallardo et al. 2017), taxonomy and genetics (Perez-Losada et al. 2002; Söller et al. 2000). All those studies were stimulated both, on the potential interest to use this species in Chilean aquaculture and to know the potential effects of environmental changes in the natural populations.

At the date there are fragmented and disperse information about the relationship between physiological characteristics of cephalopod females, and the physiology of embryos and hatchlings that allow establish a reference for comparison of physiological process that occurs from brood stock to the next generation. In this sense the present study was focused in the changes in biochemical composition that take place during gonadal maturation of octopus females and its consequences in embryo and hatchlings characteristics, putting special attention to energetic metabolites, digestive enzymes and antioxidant defence mechanisms of *O. mimus*.

2. Material and methods

2.1. Animals

A total of 32 females *Octopus mimus* 1179 $\pm$ 651 g BW were collected by scuba diving and using the gear hook (the local method) from a depth of 1 to 5 m and off coast of Antofagasta, Chile (23°38'39 S, 70°24'39 W). All captured females above these sizes were anatomically ready with a developed reproductive system (Zuñiga *et al*. 2014).

2.2 Reproductive condition

Once in laboratory 23 females were immersion in cold sea water (6-8°C) as was recommended for sub-tropical cephalopod species (Roper and Sweeney 1983)

to induce loss of sensation and to enable humane killing (Andrews *et al*. 2013). Soon after dormancy, the organisms were dissected and four weights (±0.001 g) were recorded per octopus: BW, g–total body weight; Ovw, g – ovarium weight; RSW–reproductive system weight, identified as Ovarium with oviducts and oviducal glands; and DG, g - digestive gland weight (g). Once dissected, eviscerated wet weight was also obtained and DG and ovarium samples were placed in Eppendorf tubes and freeze at -80ºC. With those information several indices were calculated:

Reproductive system weight (RSWI,%) = (RSW/BW – RSW) x 100
Gonadosomatic index (GI,%) = (Ovw/BW – Ovw) x 100
Digestive gland index (DGI, %) = (DG/BW - DG) x 100

Octopus mimus females were classified according with maturation status, being 8 immatures and 15 sampled during gonadal maturation. Maturating females were classified as physiological maturation (Phys Mat; n = 4), early functional maturation (Ea Func Mat; n = 6) and late functional maturation (La Func Mat; n = 5), being Phys Mat animals at initial vitelogenesis (stage III of ovocytes), Ea Func Mat as females with eggs in reproductive coeloma and La Func Mat as females with eggs at the end of maturation process (Avila-Poveda et al. 2016; Olivares et al. 2017). To do that, preserved ovarium samples (one per each sampled female, n= 23) were cut through its middle level into transverse and longitudinal sections and washed in 70% ethanol and dehydrated in ethanol series, cleared in benzene, and infiltrated and embedded in paraplast ®. Serial sections were cut at a thickness of 5 µm using a Leitz 1512 manual rotary microtome, and were mounted on glass slides and stained using the routine Harris hematoxylin-eosin regressive method (HHE_2; Luna 1968, Howard and Smith 1983). Alcian blue at pH 1.0 was used to contrast acidic mucopolysaccharides (Humason 1962).

2.3 Embryo development

The rest of females (n = 8) were individually placed in 108 L tanks with open and aerated seawater flow at optimum temperature of 16 to 20ºC (Uriarte et al. 2012) in a semi-dark environment to stimulate the spawn, which occurred 8 to

20 d after. A rope of eggs of each female were samples every 4 to 7 d and embryos classified, separated and freeze dried for biochemical analysis. Stages of development of *Octopus mimus* embryos were classified according to Naef (1928). *O. mimus* parlarvae 1 to 2 d old were also sampled and stored at -80ºC. Before the analysis, samples of females, embryos and paralarvae were freeze dried and transported to Unidad Multidisciplinaria de Docencia e Investigación of Faculty of Science, of Universidad Nacional Autónoma de México located in Sisal, México. There, biochemical analysis were done.

2.4 Metabolites, digestive enzymes, antioxidant mechanisms and AChE during maturation process, embryo development and hatchlings

Metabolites. Samples were homogenized in cold buffer Tris pH 7.4 at 100 mg tissue/mL using a Potter-Elvehjem homogenizer. After, samples were centrifuged at 10,000 × g for 5 min at 4°C and the supernatant was separated and stored at -80°C until analysis. Metabolites were analyzed with commercial kits acylglycerols (AG) with Ellitech TGML5415 kit; cholesterol with Ellitech CHSL5505 kit and glucose with Ellitech GPSL0507 kit) following manufacturer's instructions. Sampler were later diluted 1:300 for soluble protein determination using a commercial kit (Bio-Rad; Cat. 500-0006) (Bradford 1976). Determinations were adapted to a microplate using 20 µL of supernatant and 200 µL of enzyme chromogen reagent. Absorbance was recorded using a microplate reader (Benchmark Plus, Bio-Rad) and concentrations were calculated from a standard substrate solution and expressed an mg/ml.

Enzyme activity assays. Acid proteases activity at pH 4 was evaluated according to Anson (1938) with adjustments, using a solution of 1 % (w/v) hemoglobin (BD Bioxom- USB Products – 217500 hammersten quality) in universal buffer (Stauffer, 1989). Alkaline proteases activity of the extracts at pH 8 was measured using the method of Kunitz (1947) modified by Walter (1984), using 1% (w/v) casein (Affymetrix, 1082-C) as substrate in 100 mM universal buffer. In both assays, 0.5 ml of the substrate solution was mixed in a reaction tube with 0.5 ml of universal buffer and 20 µl of enzyme preparation (1:100 dilution) and incubated for 10 min at 35 ºC and 40 °C for alkaline proteases and acid proteases, respectly. Then, the reaction was stopped by adding 0.5 ml of 20% (w/v) trichloroacetic acid (TCA) and cooling on ice for 15 min. The

undigested substrate precipitated was separated by centrifugation for 15 min at 13,170 g. The absorbance of the supernatants was measured spectrophotometrically at 280 nm. Control assays (blanks) received a TCA solution before the substrate was added.

For trypsin, the adjusted methods of Charney and Tomarelli (1947) was used. The trypsin activity of the extracts was measured using 1 mM BAPNA (Benzoyl-$_L$-Arg-*p*-nitroanilide) as substrate TRIS 0.1 M at pH 7. This assay was made in microplate: 250 µl of the substrate solution was mixed with 5 µl of enzyme preparation (1:2). The absorbance was measured spectrophotometrically at 405 nm every minute, during 2 minutes. A mix of 5 µl distilled water with 250 µl of substrate solution was used as control assay (blank).

Lipases activity was measured in microplate using 200 µl substrate solution (TRIS 0.5 M, pH 7.4, sodium taurocholate 5 mM, sodim chloride 100 mM, 4 nitrophenyl octanoate 0.35 mM) and 5 µl homogenate diluited (1:2) in TRIS 0.5 M, pH 7.4 (Gjellesvik, et al. 1992). Absorbance was read at 415 nm, every minute, during 10 minutes

For all enzymes, units of activity were expressed as change in absorbance per minute per milligram of protein ($U=Abs_{nm}\ min^{-1}mg^{-1}$ protein).

Antioxidant defence system. Samples were snap-frozen in liquid nitrogen, lyophilized and stored at -20°C until homogenization. Samples were homogenized in cold buffer Tris 0.05 M pH 7.4 at 10 mg tissue/mL using a Potter-Elvejhem homogenizer. For enzyme activity assays, homogenates were centrifuged at 10,000 × g for 5 min at 4°C and the supernatant was separated for analysis. All samples were stored at -80°C until analysis.

Redox potential was measured with a probe (ArrowDox Measurement System, ORP-146CXS, Los Angeles, USA) in each homogenate (in mV), after homogenization. Posteriorly, homogenate was divided for triplicate assays of activities of acetylcholinesterase (AChE), carboxylesterase (CbE), catalase (CAT), glutathione S-transferase (GST), and for levels of lipid peroxidation (LPO) and total glutathione (GSH). All spectrophotometric measurements were realized in a micro-plate reader. AChE activity, was measured using a modification of the method of Ellman et al. (1961) adapted to a microplate

reader (Rodríguez- Fuentes et al. 2008). Each well contained 10 µL of the enzyme supernatant and 180 µL of 5, 5′ -dithiobis (2 nitrobenzoic acid) (DTNB) 0.5 M in 0.05 mM Tris buffer pH 7.4. The reaction started by adding 10 µL of acetylthiocholine iodide (final concentration 1 mM) and the rate of change in the absorbance at 405 nm was measured for 120s. CbE activity, a detox enzyme, was measured using ρ-nitrophenyl-α- arabinofuranoside (ρNPA) substrate, as indicated by Hosokawa and Satoh (2001) with some modifications (25 µL of the supernatant and 200 µL of ρNPA were mixed and the reaction was recorded for 5 min at 405 nm).

CAT activity was measured using Goth (1991) method with the modifications made by Hadwan and Abed, (2016). In this method, the undecomposed H_2O_2 after 3 minutes is measured with ammonium molybdate to produce a yellowish color that has a maximun absorbance at 374 nm. GST activity was determined from the reaction between reduced glutathione and 1-chloro-2.4-dinitrobenzene at 340 nm (Habig and Jakoby 1981). AChE, CbE, CAT, and GST activities were reported in nmol min^{-1} mg $protein^{-1}$. Proteins were analyzed in the supernatant according to Bradford (1976) and was used to normalize enzyme activities. Total glutathione (GSH) was measured with a Sigma-Aldrich Glutathione Assay Kit (CS0260). This kit utilizes an enzymatic recycling method with glutathione reductase (Baker *et al*., 1990). The sulfhydryl group of GSH reacts with Ellman´s reagent and produces a yellow colored compound that is read at 405 nm. LPO was evaluated using PeroxiDetect Kit (PD1, Sigma-Aldrich, USA) following manufacturer's instructions and was reported in nmol mL^{-1}. The procedure is based on peroxides oxide iron (Fe^{3+}) that forms a coloring component with xylenol orange at acidic pH measured at 560 nm.

2.5. Statistical analysis

General data were expressed as mean (± standard deviation, SD). Differences of the mean values of each measurement variables, female tissue, embryo and paralarvae (i.e., BW, Ovw, RSW, and DG weights), were tested each one using one-way ANOVA followed by Fisher LSD (least significant difference) test (Sokal and Rohlf 1995). Before ANOVA, assumption tests were carried out to determine the homogeneity of variances for each measurement and those that

did not fit the premises for ANOVA were transformed using more appropriate measurement scales (McCune *et al*. 2002). Statistical analyses were carried out using STATISTICA® 7. The significance of the statistical difference was accepted at $p < 0.05$.

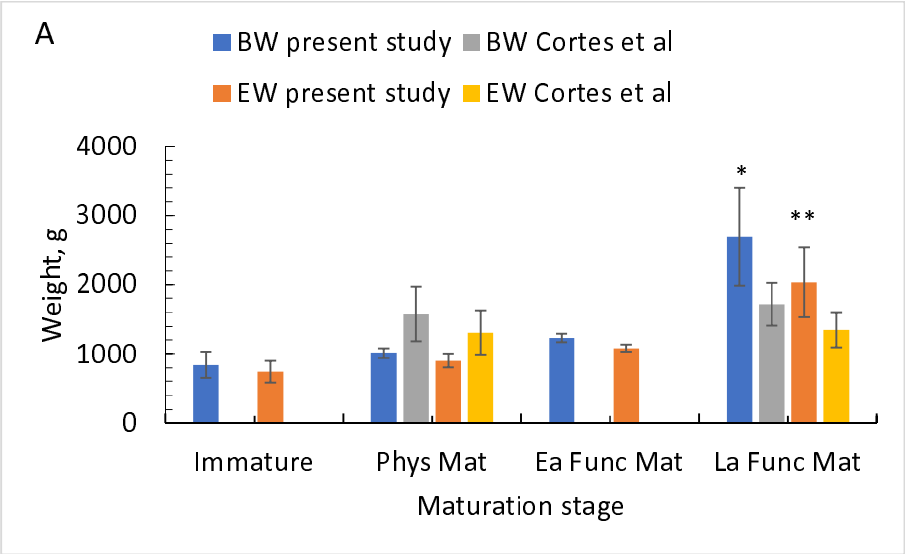
Once made ANOVAs, we also evaluate the synergistic way in which the physiological processes are carried out in the females of *O. mimus* during the maturation of the gonads and in the embryos during development. To do that, a multivariate sets of descriptors were analysed: 1) reproductive condition: BW, OVW, DG, EBW and RSW (mg); 2) metabolite concentration in ovaries: Glycogen, Glucose, Chol, Triacyl and Prot (mg/ml); 3) metabolite concentration and enzyme activity in the digestive gland: Glycogen, Glucose, Chol, Triacyl and Prot, AcidProt and AlkaProt (UI/mg protein). Data on *O. mimus* embryos and paralarvae were analysed using three multivariate sets of descriptors and considering that there are four well recognized phases during embryo development: i) organogenesis (namely Pre); ii) the end of organogenesis around stages XIV and XV (namely Organ); iii) Growth (post organogenesis, namely Post) and iv) paralarvae with one d old (1st) and two d old (2nd): The descriptors used were 1) metabolite concentration in body as a whole: Glycogen, Glucose, Chol, Triacyl and Prot (mg/ml); 2) enzyme activity: acid and alkaline proteases (UI/mg protein) and 3) Antioxidant defence mechanisms: Ache, CbE, GsT, SOD, CAT (nMol/min/mg protein), ORP (mV), GsH (nMol/ml), LPO (nMol peroxido/ml). Principal Coordinate Analysis (PCoA) was applied on Euclidean distance matrices of samples in each data set (Legendre and Legendre, 1998). Data were square root (female data) or log-transformed (embryo and paralarvae data) and normalised by centring and dividing between the standard deviation of each variable prior to analysis (Legendre and Legendre, 1998).

A permutational multiple ANOVA was applied on the distance matrices to detect differences amongst female octopuses in four different stages of gonadic maturation (fixed factor: Imm, PhyMat, EarFunMat, LatFunMat), and amongst embryos and paralarvae in different stage of development (Pre, Organ, Post, 1st and 2nd paralarvae). Permutational multiple paired t-tests were used to compare the centroids of the different stages in each data set; 9999 unrestricted

permutations of raw data were used to generate the empirical *F* and *t*-distributions (Anderson, 2001; McArdle and Anderson, 2001).

3. Results

3.1. *Octopus mimus* females reproductive condition



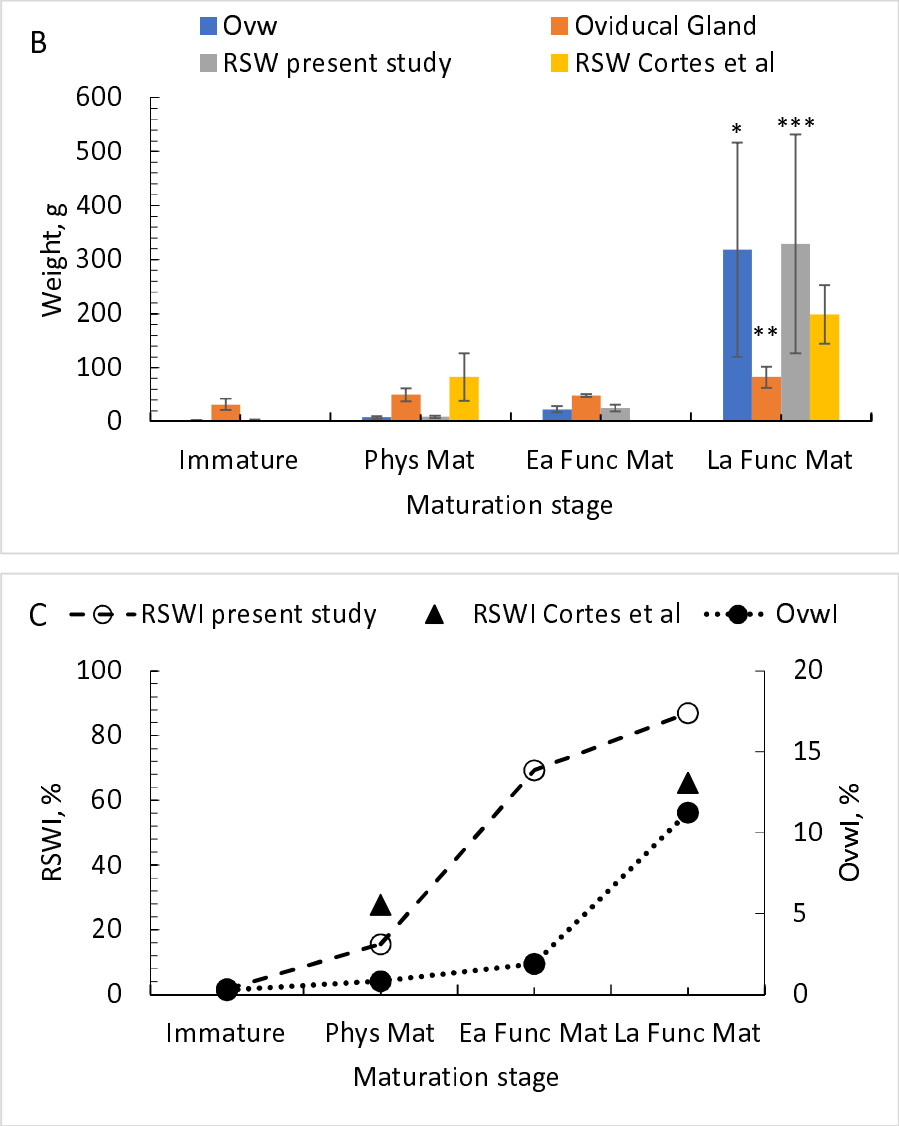


Fig. 1. Total, eviscerated (A), reproductive tract (B), and gonadosomatic and reproductive system indices (C) of *O. mimus* females along the maturation stages obtained in the present study and by Cortes et al. (1995). Physiological maturity (Phys Mat) was obtained from females with a Ovwl < 2 and in stage III of oocytes development. Early functional maturation stage (Ea Fun Mat), was recognized from females with eggs in the reproductive coelom and with Ovwl > 2. Late functional maturation stage (La Func Mat), was obtained from females close to spawn (Arkhipkin, 1992; Avila-Poveda *et al.*, 2016; Olivares *et al.*, 2017). RSWI = reproductive system index; Ovwl = ovarium Index.

Total and eviscerated wet weight changed with maturation of females with low values in females at immature stage and high in females in late functional maturity stage (Fig. 1A). At the end of maturation total and eviscerated weight resulted 3.2 and 2.7 higher than observed in immature females ($p < 0.0001$; Fig.

1A). In a similar form, an increment of reproductive complex system (RCS), ovarium and oviductal wet weight were registered along the maturity stages; RSW, Ovw and oviductal gland wet weight values at the end of maturity stages were 108, 135 and 2.55 times higher than in immature females, respectively (Fig 1B). As a consequence, increments in RSWI and OvwI were observed along the maturity stages (Fig. 1C).

Digestive gland wet weight changed along the maturity stages with low values in immature females and high at the end of maturation process ($p < 0.001$; Fig. 2). In contrast, hepatosomatic index (HI, %) increased with maturation stages to reach a peak at the beginning of functional maturation, when oocytes were end to reproductive coelom (Fig. 2). At the end of maturation stage, HI was reduced.

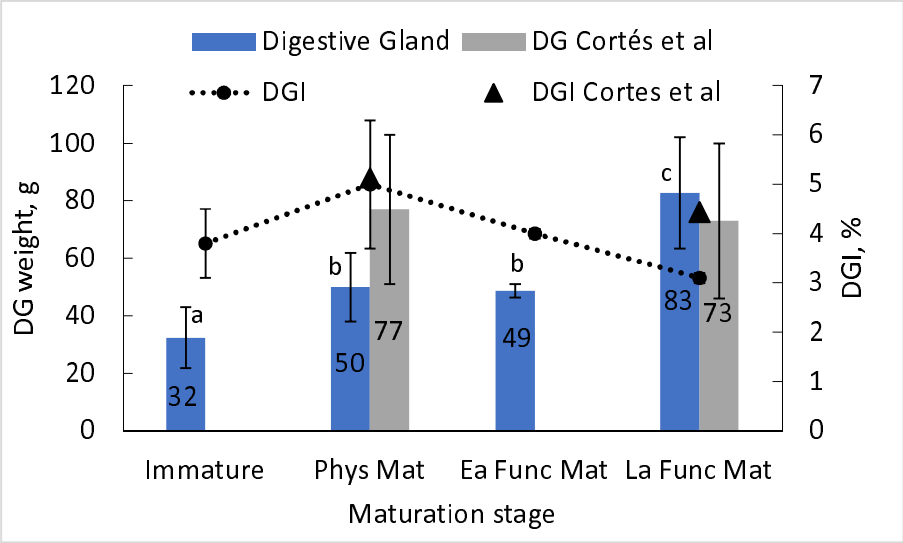
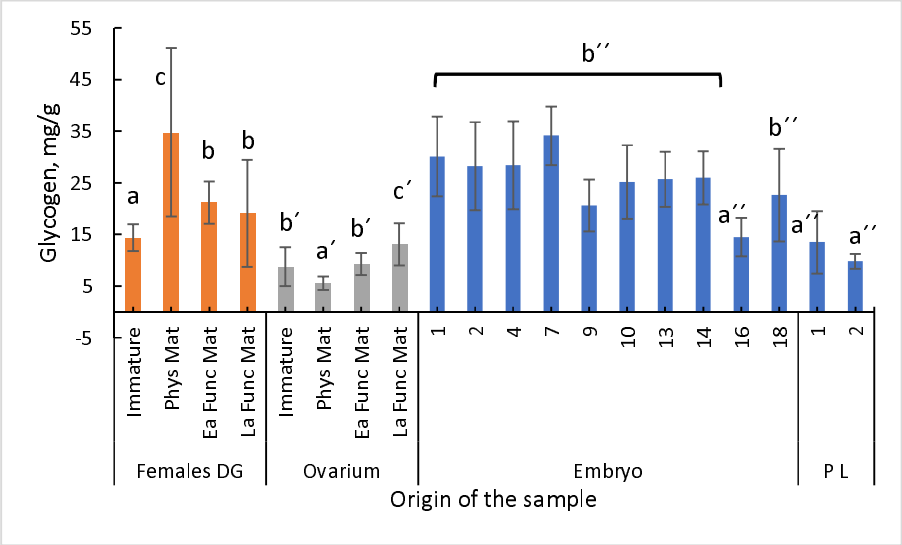


Fig. 2. Digestive gland weight (DG, g) and digestive gland index (DGI, %) of *O. mimus* females along the maturation stages obtained in the present study and by Cortes et al. (1995). Physiological maturity (Phys Mat) was obtained from females with a IGS < 2 and in stage III of oocytes development. Early functional maturation stage (Ea Fun Mat), was recognized from females with eggs in the reproductive coelom and with OvwI > 2. Late functional maturation stage (La Func Mat), was obtained from females close to spawn (Arkhipkin, 1992; Avila-Poveda*, et al.*, 2016; Olivares*, et al.*, 2017)

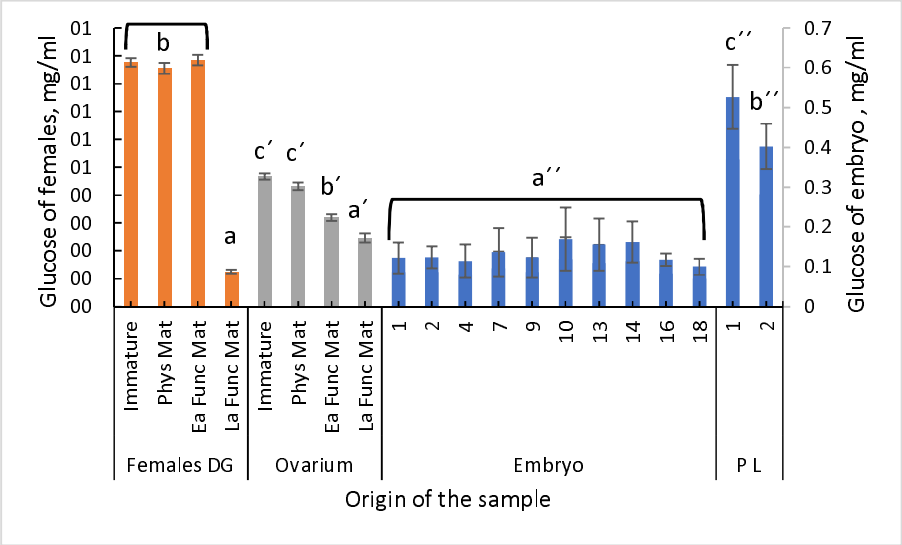
3.1. Biochemical composition

a. Glycogen.

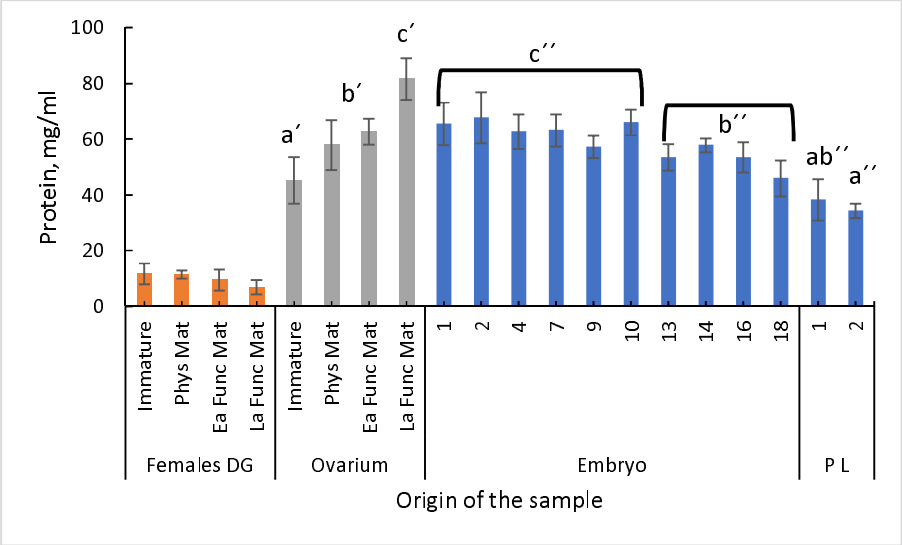
Immature *O. mimus* females showed digestive gland glycogen levels 59% lower than females at the beginning of maturation process (Fig. 1A; $p < 0.001$). After, a significant increment of DG glycogen was registered in females in physiological maturation, reaching values 2.4 times higher than observed in immature females ($p < 0.001$; Fig. 3A). A reduction in 38% of digestive gland glycogen values was registered ($p < 0.01$; Fig. 3A). Increments of ovarium glycogen concentration were during maturation with high glycogen levels obtained in ovarium of females at the end of the maturation process (Fig. 3A; $p < 0.01$). In embryos of *O. mimus,* glycogen levels of stages I to XIV and XVIII did not show statistical differences with a mean value of 26.8 $\pm$ 9.0 mg/g ($p > 0.05$; Fig. 3A). Glycogen levels of embryos of stages XVI and paralarvae 1 and 2 were statistically lower than the rest of the embryos (Fig. 3A; $p < 0.01$).


Glycogen, mg/g
55
45
35
25
15
5
-5
a
c
b
b
b´
a´
b´
c´
b´´
a´´
b´´
a´´
a´´
Immature
Phys Mat
Ea Func Mat
La Func Mat
Immature
Phys Mat
Ea Func Mat
La Func Mat
1
2
4
7
9
10
13
14
16
18
1
2
Females DG
Ovarium
Embryo
P L
Origin of the sample

391


Glucose of females, mg/ml
Glucose of embryo , mg/ml
b
a
c´
c´
b´
a´
a´´
c´´
b´´
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Immature
Phys Mat
Ea Func Mat
La Func Mat
Immature
Phys Mat
Ea Func Mat
La Func Mat
1
2
4
7
9
10
13
14
16
18
1
2
Females DG
Ovarium
Embryo
P L
Origin of the sample

392


Protein, mg/ml
100
80
60
40
20
0
a´
b´
c´
c´´
b´´
ab´´
a´´
Immature
Phys Mat
Ea Func Mat
La Func Mat
Immature
Phys Mat
Ea Func Mat
La Func Mat
1
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Females DG
Ovarium
Embryo
P L
Origin of the sample

393

Fig. 3. Glycogen, glucose and protein of *O. mimus* females (digestive gland: DG) and ovarium), embryos and paralarvae (PL) maintained in laboratory conditions at 16°C. Values as mean ± SD. Different letters indicate statistical differences at P < 0. 05 level.

b. Glucose

Digestive gland glucose levels were similar in immature and during almost all maturation process ($p > 0.5$; Fig 3B). Only in females in late functional maturation process glucose levels resulted significantly lower than in the rest of the maturation conditions (Fig. 3B; $p < 0.001$). A reduction in glucose levels were recorded in ovarium according with maturation process, with higher levels in immature females (0.5 mg/ml) than in late functional maturation condition (Fig 3B; $p < 0.001$). In embryos, glucose levels were similar along the development. In consequence a mean value of 0.13 ±0.02 mg/ml was calculated for all embryo stages (Fig. 3B; $p > 0.05$). Glucose levels in paralarvae 1 d old was 4 times higher than in embryos and 1.3 times higher than observed in paralarvae 2 d old (Fig. 3B; $p < 0.002$).

c. Protein

Digestive gland protein did not change during the female maturation process (Fig. 3C; $p > 0.05$). In contrast, an increment of soluble protein was recorded in ovarium in relation with maturation process, with low values in immature females and high at the end of maturation process (Fig. 3C; $p < 0.001$). No statistical differences were recorded in protein levels of embryos of stages I to X, with a mean value of 63.8 ± 3.7 mg/ml (Fig. 3C; $p > 0.05$). After those stages, a reduction of soluble protein levels was registered in embryos and paralarvae, to reach the lower value in 2 d paralarvae of *O. mimus* (Fig. 3B; $p > 0.05$).

d. Triacyl glycerol (TG)

Digestive gland TG values did not change with female maturation stages; for that reason, a mean value of 0.35 ± 0.08 mg/ml was calculated (Fig. 4A; $p > 0.05$). In contrast, in females, ovarium there were strong changes in TG values with low values in immature females (1.05 mg/ml) and at the end of the maturation stage (1.6 mg/ml) ($p > 0.05$) and high values in females both at the end of physiological maturation and during early maturation process (Fig. 4A; p

< 0.001). In embryos, levels of TG were highly variables with high levels in stages I, IV, VII, and X to XVIII and low in stages II and IX (Fig. 4A, P < 0.01). Values of TG recorded in paralarvae of 1 and 2 d old resulted 1.5 times higher than maximum value recorded in embryos of stage X (Fig. 4A, P < 0.01).

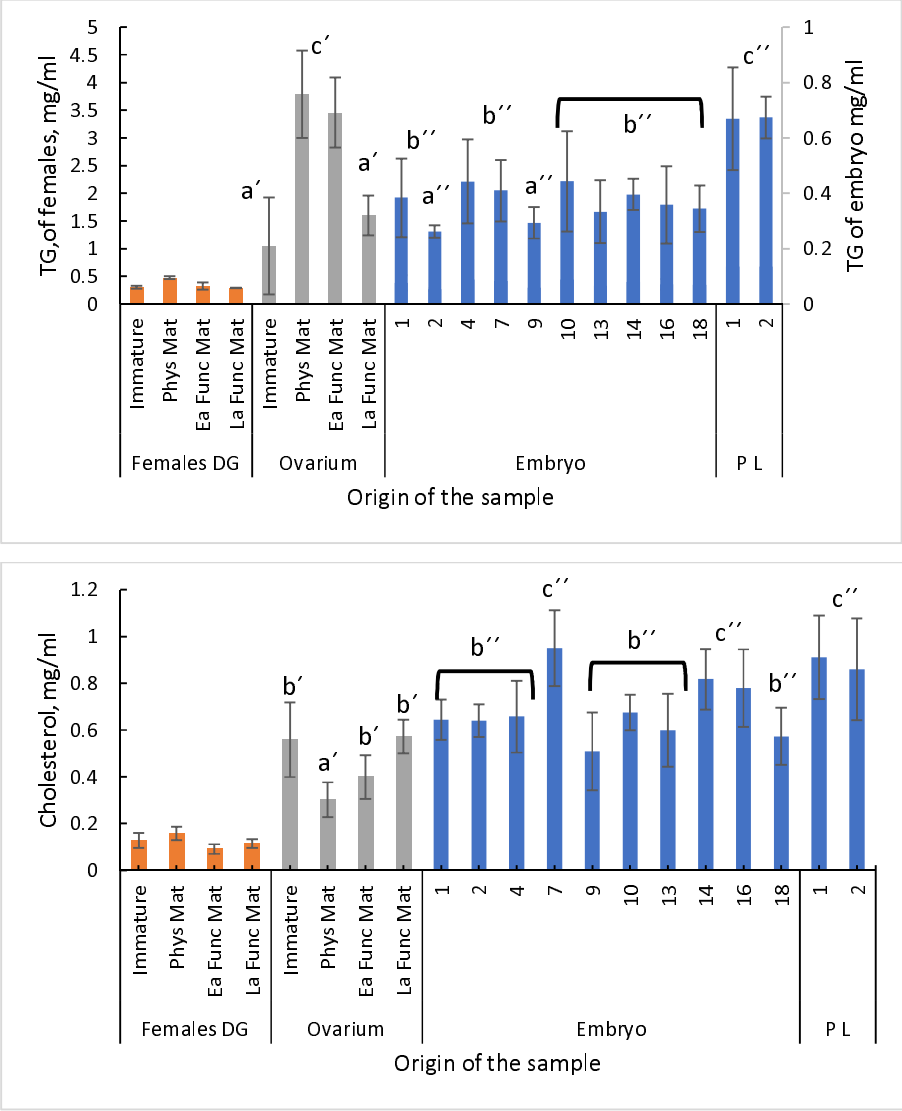

Fig. 4. Triacyl glycerol and cholesterol of *O. mimus* females (digestive gland: DG) and ovarium), embryos and paralarvae (PL) maintained in laboratory conditions at 16°C. Values as mean ± SD. Different letters indicate statistical differences at P < 0. 05 level.

e. Cholesterol

Digestive gland cholesterol levels remained constant along the maturation stages of *O. mimus* females (Fig. 4B; p > 0.05). As a difference, cholesterol

levels in ovarium changed with maturation stages of females with high levels in immature animals, followed by a reduction at the beginning of maturation process and an increment in the next maturation stages, to reach the maximum concentration level at the end of maturation process (Fig. 4B; $p < 0.002$).

3.2. Digestive enzymes

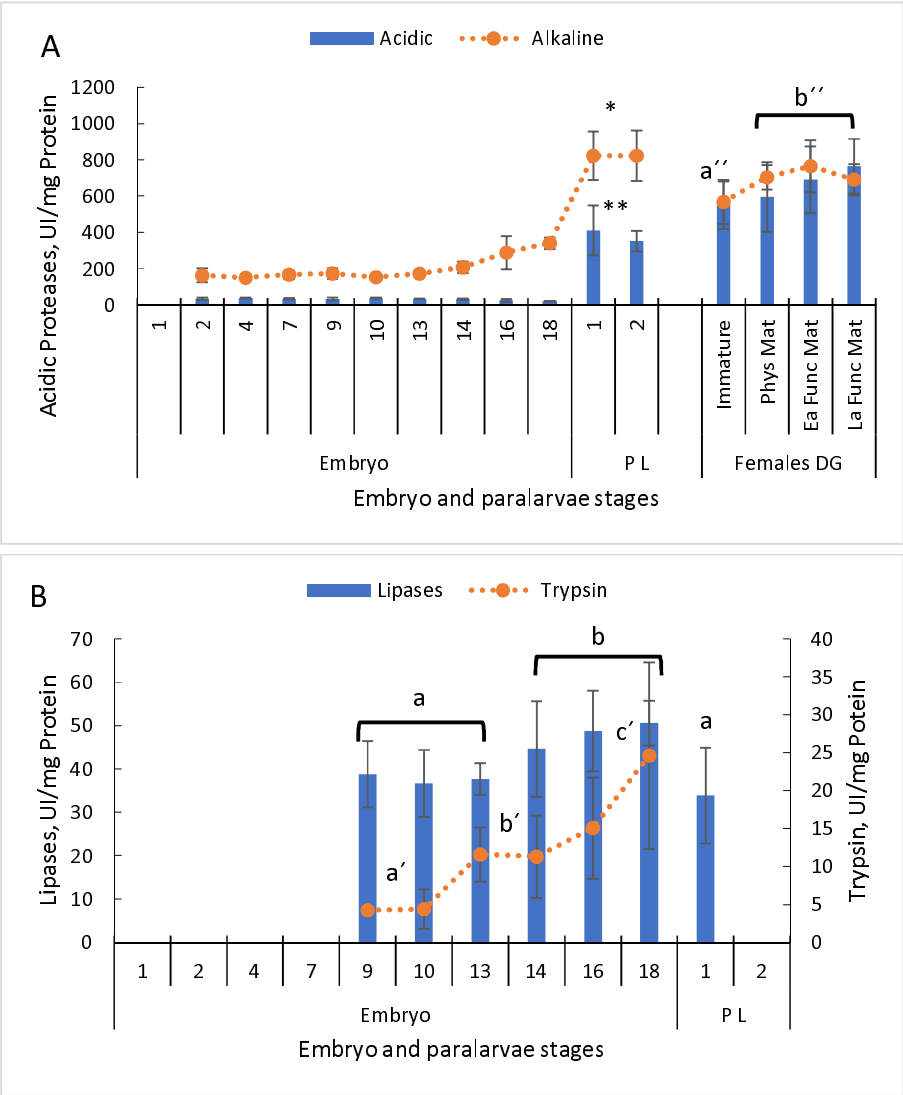

Fig. 5. Digestive enzymes activity in entire embryos, paralarvae (PL) and in the digestive gland of *O. mimus* females obtained in animals maintained in laboratory conditions at 16°C. Values as mean ± SD. Different letters or asterisk indicate statistical differences at $P < 0.05$ level.

Acidic and alkaline proteases activity was constant along the embryo development until stage XIV by alkaline and stage XVIII by acidic proteases (Fig. 5). After stage XIV, alkaline proteases activity increased to reach its maximum and significant activity after hatch ($p < 0.001$; Fig 5A). In a similar form, a maximum acidic proteases activity was recorded in *O. mimus* after hatch. In adult females, there were no statistical differences in activity of acidic proteases along the maturity stages ($p > 0.05$; Fig 5A). in contrast, a lower activity of alkaline proteases was registered in immature females in comparison with maturating and mature *O. mimus* females (Fig. 5A). Lipases and trypsin activities changed with embryo development, with low values in stages lower than XIII, and higher in stages XIV to XVIII ($p < 0.001$; Fig. 5B). A reduction on lipases activity was recorded in paralarvae 1 d old ($p < 0.001$; Fig. 5B).

3.6. Acetyl cholinesterase (AChE) and antioxidant defence mechanisms (ADM)

AChE increased along the embryo development with low values at the beginning of embryo development and high after hatch when animals emerged as paralarvae ($p < 0.01$; Fig 6A). Antioxidant defence mechanisms (GST, SOD and CAT) showed the same tendency; low values at the beginning of embryo development and high after hatch ($p < 0.01$; Fig. 6 B to D). In consequence a reduction on oxidative damage (CbE; ORP and LPO) and GSH were observed with high values in embryos at the beginning of the development and low after hatch. It is interesting to note that LPO and GSH levels start to be reduced around stage XV to maintain a low level until hatch ($p < 0.001$; Fig 6).

Ordination by PCoA of the reproductive condition of female *O. mimus* showed that the PCo1 explained 94.9 % of total variation in the data, with EBW, BW, OVW and RSW greatly contributing to ordination in the horizontal axis (Figure 7A). The PCo2 explained only 4.2% of total variation, with differences in DG contributing to ordination in the vertical axis. Values of all the reproductive condition descriptors increased as females advanced from immature to physiological and functional stages of gonadic maturation (Figure 7A), and females in late functional maturity were distinctly separated and associated to the highest weight values.

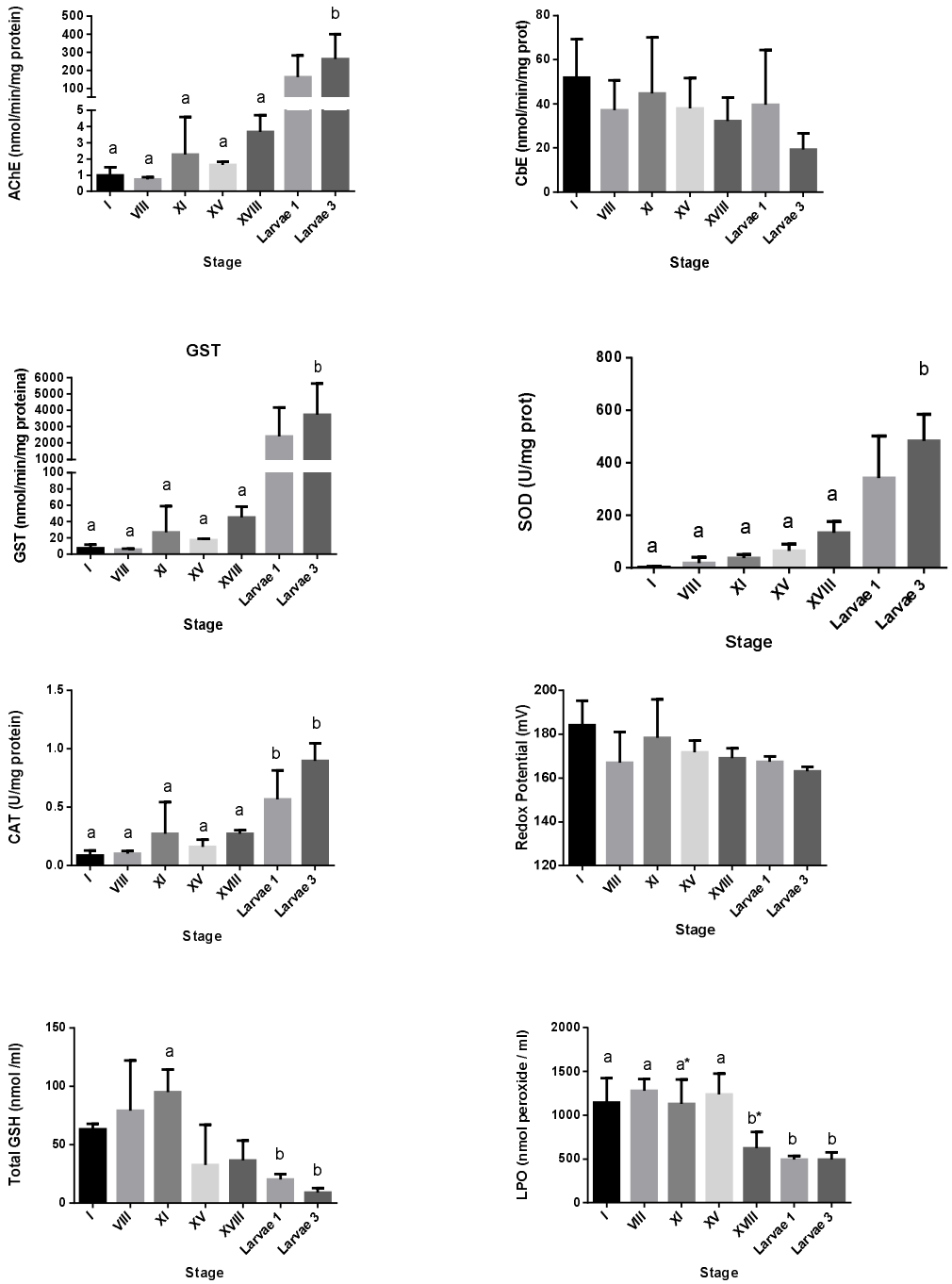

Fig. 6. Antioxidant defence mechanisms and acetyl cholinesterase activity of embryos and paralarvae of *O. mimus* obtained in laboratory conditions at 16°C. Values as mean ± SD. Different letters means statistical differences between treatments at $p < 0.05$ level.

The multiple ANOVA showed overall significant differences between stages of gonadic maturations (Table 1), but paired comparisons amongst centroids revealed that only immature females and those in either early or late functional maturity differed significantly in the reproductive condition descriptors measured (Table 2).

Ordination of the concentration of metabolites in female ovaries showed that the PCo1 and PCo2 explained 74% of total variation in the data (54.8% and 19.2%, respectively; Figure 7B). Concentration of Glycogen, Chol and Prot contributed most to ordination on the horizontal axis, whereas Glucose and Triacyl did so on the vertical axis. The multiple ANOVA showed overall significant differences between stages of gonadic maturations (Table 1), and paired comparison between centroids allowed for three distinct groups to be formed based on ovary metabolites: immature; physiologically and early functionally mature; and late functionally mature females (Table 2).

Ovary samples from immature females were high in glucose and cholesterol, but low in triaclyglicerides and proteins when compared to samples from late functionally mature females; physiologically and early functional mature females presented intermediate ovary concentrations of these metabolites (Figure 7B).

The first and second PCo together only explained 63.8% of the total variation in metabolite concentration and enzyme activity of the digestive gland in female *O. mimus* (39.5% and 24.3 %, respectively; Figure 7C), the percentage of total variation increasing to 79% when the third PCo is considered. Glucose was inversely correlated with glycogen, triacylglicerides and cholesterol, whilst protease activity was so with protein concentration in all three principal coordinates. In this case, significant differences between stages of gonadic maturation were also detected by the general multiple MANOVA (Table 1), but paired comparisons between centroids only revealed significant differences immature females and those in late functional maturity (Table 2).

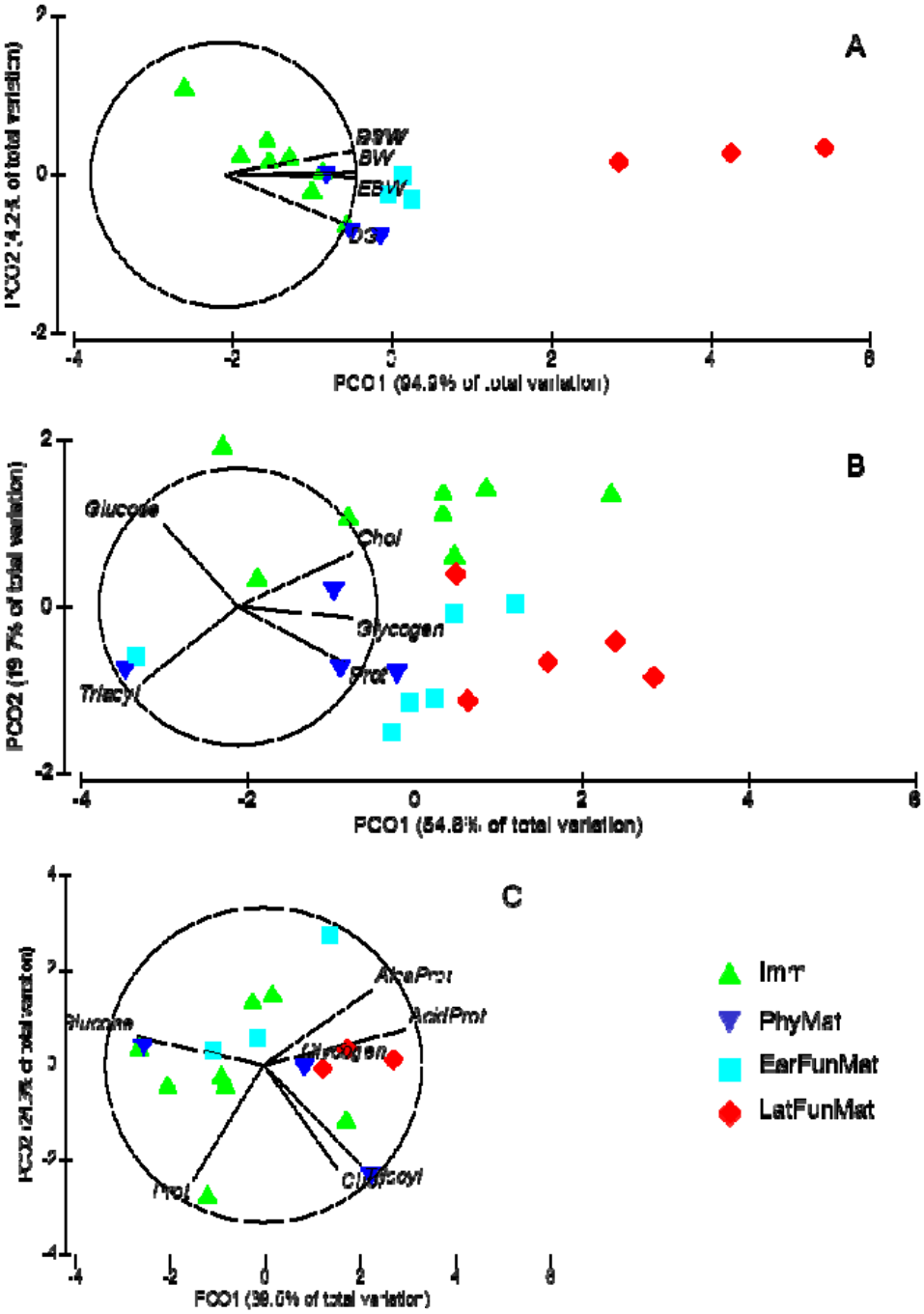

Fig. 7. Principal coordinate analysis (PCO) of females of *O. mimus* during ovarian maturation. A. Body weight characteristics (Total body weight: BW; Eviscerated weight: EBW; reproductive tract weight: RTW; Digestive gland weight: DG). B. ovarian metabolites (Chol: Cholesterol; Prot: protein; Triacyl: triacyl glycerol). C. Digestive gland metabolites and digestive enzymes (Chol: Cholesterol; Prot: protein; Triacyl: triacyl glycerol; Alcal prot: Alkaline proteases; Acid Prot: Acidic Proteases). Measures made in immature (imm), physiological maturity (PhyMat), early functional maturity (EarFunMat) and late functional maturity (LatFunMat).

In the digestive gland immature females had high concentrations of glucose and both acid and alkaline protease activity, whereas fully functionally mature females had relatively higher concentration of glycogen and lower protease activity.

Regarding the metabolite concentration in embryos and paralarvae, the first and second PCo explained 56.7 and 20% of total data variation (Figure 8A). Glucose and triacylglicerides were inversely correlated with glycogen and proteins and contributed largely to sample ordination on the horizontal axis. Cholesterol concentration contributed to order samples on the vertical axis (Figure 8A). The multiple MANOVA detected overall significant differences amongst stages of embryo and paralarvae (Table 1), and significant differences in paired comparisons allowed to distinguish three groups: embryos in stages prior to organogenesis (Pre); those in stages characterised by organogenesis and immediately after (Organ and Post); and the first and second paralarvae. Embryos prior to enter organogenesis had high glycogen and protein concentration, but low glucose and triacylgliceride concentration; embryos at stages Organ and Post had high cholesterol concentrations and intermediate values in all other metabolites; 1st and 2nd stage paralarvae had high glucose and triacylgliceride and low glycogen and protein concentrations (Figure 8A).

Ordination of protease activity in *O. mimus* embryos and paralarvae showed that the PCo1 and PCo2 explained 92.6 and 7.4% of total variation, respectively (Figure 8B). Enzyme activity in general increased as embryos advanced from stages before organogenesis towards the 2nd paralarvae, paralarvae having higher alkaline protease, but lower acid protease activity, than embryos. The multiple ANOVA showed significant differences between stages of development (Table 1), and significant differences were detected between all pairs of centroids except those representing the 1st and 2nd paralarvae (Table 2). These results show four distinct groups of samples regarding enzyme activity: embryos before, during and after organogenesis, and paralarvae (Figure 8B).

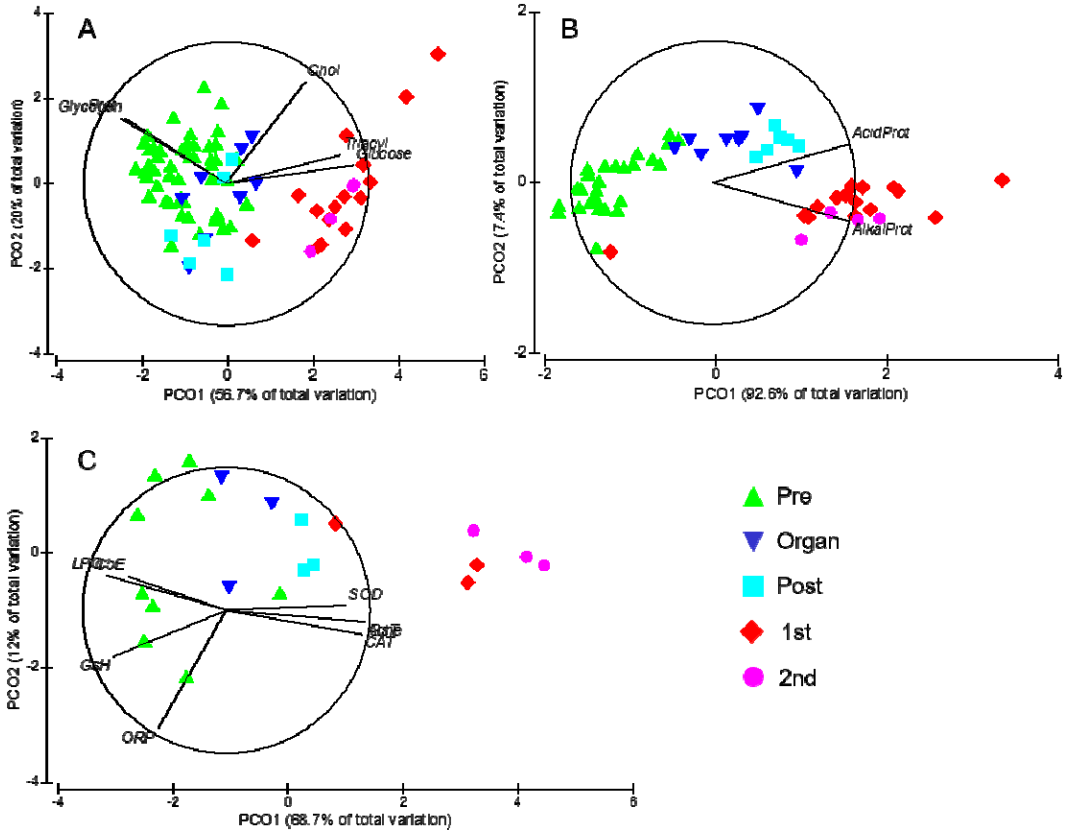

Fig. 8. Principal coordinate analysis (PCO) of *O. mimus* embryo metabolites (A), digestive enzymes (B) and antioxidant defence mechanisms (C) measured along development. Pre = Organogenesis: stages I to XII; Organ = The end of organogenesis: stages XIV to XVI; Post = Post organogenesis: stages XVII to XIX; 1 d old Paralarvae: 1st; 2 d old paralarvae: 2nd.

Analysis of the antioxidant defence mechanisms in *O. mimus* embryos and larvae showed that the first and second PCo explained 68.7 and 12% of total data variation (Figure 8C).

Table 1. Results of one-way permutational multiple MANOVAs applied on six multivariate sets of data obtained from *O. mimus*: 1) female reproductive condition; 2) metabolite concentration in female ovaries; 3) metabolite concentration and enzyme activity in the digestive gland of female; 4) metabolite concentration; 5) enzyme activity; and 6) antioxidant defence mechanisms in embryos and paralarvae. The degrees of freedom (df), multivariate sum of squares (SS), mean square (MS), pseudo-*F* and p-values, and the number of unique permutations is given for each test.

Source of variation	Df	SS	MS	pseudo-*F*	*p*	Unique permutations
1. Female reproductive condition						
Stage	3	70.60	23.53	32.54	< 0.001	9949
Residuals	13	9.40	0.72			
2. Metabolites in female ovaries						
Stage	3	39.66	13.22	3.57	< 0.01	9945
Residuals	19	70.34	3.70			
3. Metabolites and enzyme activity in female digestive gland						
Stage	3	35.52	11.84	2.01	< 0.05	9931
Residuals	13	76.48	5.88			
4. Metabolites in embryos and paralarvae						
Stage	4	202.95	50.74	19.81	< 0.001	9927
Residuals	75	192.05	2.56			
5. Enzyme activity in embryos and paralarvae						
Stage	4	96.88	24.22	58.66	< 0.001	9944
Residuals	56	23.12	0.41			
6. Antioxidant defence mechanisms in embryos and paralarvae						
Stage	4	110.8	27.7	9.01	< 0.001	9944
Residuals	16	49.20	3.08			

Lipid peroxidation and carboxylesterase were high amongst embryos in stages before organogenesis, and were inversely correlated to acetylcholinesterase, catalase, glutathione S-transferase and superoxide dismutase, which had the highest values amongst 1st and 2nd day paralarvae (Figure 8C).

Table 2. Results of permutational paired t-tests that compared centroids representing data in six multivariate sets of data obtained from female *O. mimus* in different stages of gonadic maturation (Imm: immature, PhyMat: physiological maturity, EarFuncMat: early functional maturity, and LatFuncMat: late functional

maturity), and from embryos and paralarvae in different stage of development (Pre, Organ and Post: stages before, during, and after organogenesis, respectively, and 1st and 2nd paralarvae). Values are permutational *p*-values for each test.

1. Female reproductive condition			
	Imm	PhyMat	EarFuncMat
PhyMat	0.053	-	-
EarFuncMat	< 0.05	0.102	-
LatFuncMat	< 0.01	0.100	0.100

2. Metabolites in female ovaries			
	Imm	PhyMat	EarFuncMat
PhyMat	< 0.05	-	-
EarFuncMat	< 0.05	0.351	-
LatFuncMat	< 0.01	< 0.05	< 0.05

3. Metabolites in female digestive gland			
	Imm	PhyMat	EarFuncMat
PhyMat	0.225	-	-
EarFuncMat	0.271	0.498	-
LatFuncMat	< 0.01	0.204	0.104

4. Metabolites in embryos and paralarvae				
	Pre	Organ	Post	1st
Organ	< 0.01	-	-	-
Post	< 0.001	0.161	-	-
1st	< 0.001	< 0.001	< 0.001	-
2nd	< 0.001	< 0.01	< 0.01	0.567

5. Enzyme activity in embryos and paralarvae				
Organ	< 0.001	-	-	-
Post	< 0.001	< 0.05	-	-
1st	< 0.001	< 0.001	< 0.01	-
2nd	< 0.001	< 0.01	< 0.01	0.617

6. Antioxidant mechanisms in embryos and paralarvae				
Organ	0.125	-	-	-
Post	< 0.01	0.098	-	-
1st	< 0.01	0.097	0.098	-
2nd	< 0.01	0.099	0.096	0.299

Whilst he multiple ANOVA showed overall significant differences throughout development (Table 1), paired tests amongst centroids revealed significant differences only between extreme stages (Table 2).

Discussion

Previous studies of *O. mimus* from North of Chile have investigated size at maturity and, as a result it is well attested, as was observed in the present study, that females heavier than 2000 g are mature (Cortez et al. 1995). In the present study body, reproductive tract, and digestive gland weights and their indices followed the same behaviour as was previously observed when reproductive condition of 991 *O. mimus* females were analysed (Cortez et al. 1995). As was expected, increments on RSW and reproductive indices were observed along the reproductive maturity stages, indicating that during ovarian development there were mobilization of energy and nutrients to reproductive organs. Previous studies made in *O. vulgaris*, *O. defilippi* (Rosa et al. 2004) and in the Squids *Illex coindetii* and *Todaropsis eblanae* (Rosa et al. 2005) suggested that those species take the energy for egg production directly from food rather than from stored products in a specific tissue. That conclusion was based in the observation that, while mature females experience gonad increments on protein, lipid and glycogen contents, digestive gland and muscle where without apparent changes between both maturation stages, indicating that there were no evidences that storage reserves were transferred from tissue to tissue during ovaria maturation. Although the present study corroborates that conclusion, now we showed that, during maturation process there were nutrients mobilization both DG and ovarium that where not observed early. As can be expected in the DG of *O. mimus* females, free glucose resulted in an important source of metabolic energy, being highly concentrated along the maturation process until early functional maturation, when oogonia are growing in the ovarium. In *O. maya* was observed that precisely during early functional maturation there were an elevated level of progesterone, hormone involved in oocytes vitellogenesis (Avila-Poveda et al. 2015), suggesting that due to its complexity these processes required high levels of metabolizable energy. In this sense, previous results obtained in *O. mimus* and *O. maya* showed that glycogen and glucose in digestive gland, joint with soluble proteins were used as a source of metabolic energy during digestive processes (Gallardo et al. 2017), and during the growth of juveniles and pre-adults of *O. maya* (Aguila et al. 2007; Rosas et al. 2011) putting in evidence the importance of those nutrients in physiology of cephalopods. According to Martínez et al., (2014) this

energy is the result of gluconeogenic pathway that are supported by protein metabolism, that results of the carnivorous eating habits of cephalopods. For that reason we hypothesise that, as in DG (Martinez et al., 2014) in the ovarium part of the glycogen and glucose registered followed the glycogenic pathways (Hochachka & Fields 1982) (Fig. 9), following the metabolic processes previously described in muscle of different cephalopod species (Gallardo et al. 2017; Hochachka & Fields 1982; Morales et al. 2017).

The dynamics of glycogen and glucose observed in the *O. mimus* ovarium suggest that there are biochemical regulatory mechanisms involved in storing and mobilization of nutrients. In the present study high glucose levels were observed in ovarium in immature females and during physiological maturation suggesting that, at the beginning of the process, glucose could be used as a source of energy during the complex processes involved in oocytes synthesis. However, after a reduction on glucose levels were recorded during functional maturation with the lowest levels at the end of the process. If glucose is used only as a source of energy why a reduction on glucose was recorded when the vitellogenesis process are in its maximum level? To answer that question there are two hypotheses: i) or in the last part of vitellogenesis ovarium required less energy than previous maturation stages, using facilitating transport mechanisms to vitellogenesis and to store yolk into the eggs, or ii) glucose was reduced to avoid its inhibitory effects. Taking into consideration that in insects has been reported that excess of glucose inhibits vitellogenin uptake (Kunkel et al., 1987), is possible to think that in octopus a reduction of ovarium glucose levels could be related with mechanisms to avoid physiological problems during the last part of the reproductive process, maintaining so only glucose levels to be used as a source of metabolic energy, allowing in this form the adequate vitellogenin uptake. If some control mechanisms exist, then it also is involved in glycogen synthesis and storing. During late functional maturation, an increment on glycogen was registered in ovarium, suggesting that those molecules were directly stored into the eggs to be used as a source of energy during embryo development and/or to maintain the physiological integrity of females during parental care that occurs after the spawn (Roumbedakis et al. 2017).

As was previously observed in *O. vulgaris* (Rosa et al., 2004), in *O. mimus* there were no an apparent mobilization of lipids from DG to ovarium, supporting the hypothesis that suggests that in cephalopods the nutrients channelled to the ovarium are obtained from the food and not from reserves tissues, as was also observed in laboratory studies (Caamal-Monsreal et al. 2015; Rosa et al. 2005; Tercero-Iglesias et al. 2015).

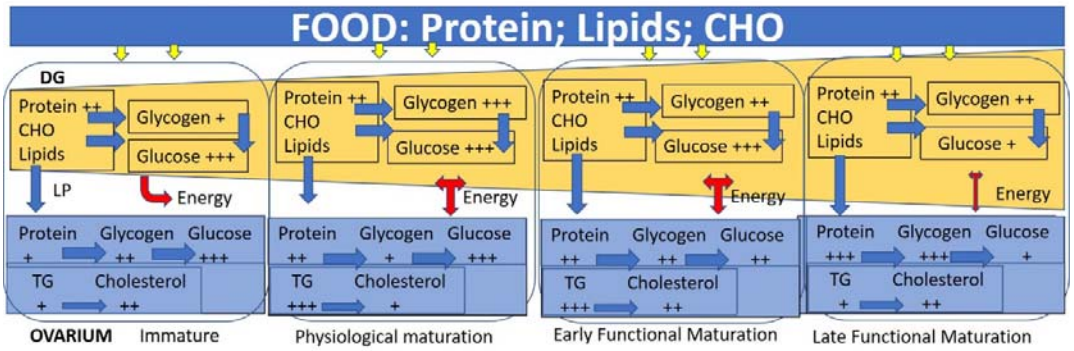

Fig. 9. Physiological processes that occurs in digestive gland and ovarium during *O. mimus* female maturation. Nutrients, coming from the ingested food are processed into the digestive gland (DG) where proteins are used along maturation both as a source of carbohydrates via gluconeogenic pathway and to be transported as peptides and amino acids to internal tissues. Ingested carbohydrates (CHO) and glucose liberated from glycogen are used mainly as a source of energy into the DG and other tissues at the beginning of maturation and until the vitellogenesis start (early functional maturation) (Gallardo el al., 2017). In ovarium, protein and glycogen follow the same process than DG, supporting the glucose production until early functional maturation stage. Lipids (measured as triacyl glycerides: TG) and free cholesterol are absorbed in DG and transported by lipoproteins (LP) to ovarium, between other tissues (Heras & Pollero, 1990; Heras & Pollero, 1992). TG are accumulated during physiological maturation and early functional maturation and presumably converted at least in part, as cholesterol at the end of the maturation process (Estefanell *et al.*, 2017).

In the present study, high levels of TG and cholesterol were registered in immature females, indicating that even before maturation, the females used the ovary as a lipid reserve site. It means that, although it is true that the nutrients come directly from the food and that these are not stored in muscle or digestive gland (Rosa et al., 2005), the results obtained now show that the ovary itself is a reserve site for the nutrients that will be used during maturation, at least at the beginning of the process (Fig. 9). That nutrients probably be reflects

crustaceans ingested during the stage of somatic growth (Cardoso et al. 2004; Carrasco & Guisado 2010).

Once the maturation processes started, an increment of TG was observed followed by a reduction on cholesterol indicating that, during physiological maturation (oocytes growth) cholesterol was requested, probably to be used as structural component into the oocytes membranes. This process is characterized, at the beginning, by formation of oocytes which after growth, will be transformed in secondary oocytes surrounded by the follicle cells without yolk (Avila-Poveda et al. 2016). During this process is highly probable that the oocytes and follicle cells synthesis will require cholesterol, because it is an essential constituent of the biological membranes (Zubay 1983). Once the vitellogenesis started (early functional maturation process) high levels of TG were registered, indicating that fatty acids are also stored in the ovarium, probably to be used in the yolk synthesis. Proof of that, is that at the end of the maturation process (late functional maturation) low TG levels were registered in the ovarium of the *O. mimus* females, suggesting that fatty acids accumulated as TG, were transformed and stored into the eggs as yolk. At the end of maturation process moderated cholesterol levels were recorded indicating that they were probably stored also in the yolk to be used by the embryos throughout their development (Estefanell et al. 2017).

As can be expected, the biochemical and physiological processes in embryos are highly dynamics following two well identified developmental phases: organogenesis and growth (Boletzky 1987; Naef 1928). In many species the first phase occurs between stage I to XII-XIII where the first embryo inversion allows to the embryo growth in the proximal side of the egg (Boletzky, 1987). During this phase, nervous system is developed and the retina pigmentation is evident around stage X, to reach the complete organogenesis in *O. mimus* embryos at stages XII-XIII (Castro-Fuentes et al. 2002).

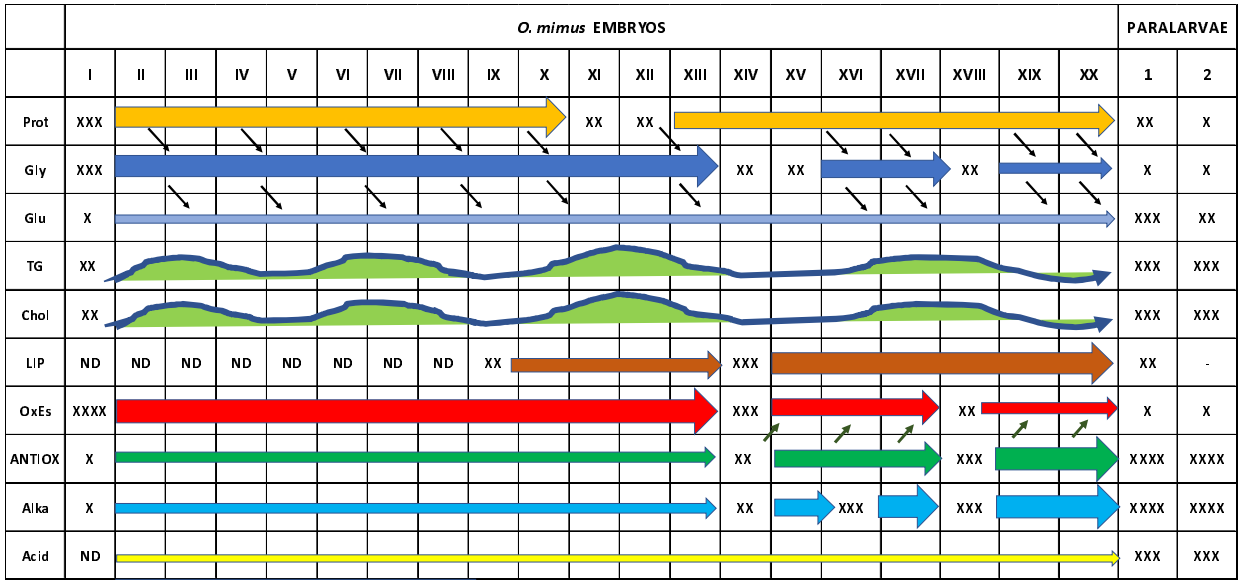

Fig. 10. *O. mimus* biochemical and physiological dynamics through embryo development and after hatch. Gly: glycogen; Glu: glucose; Prot: soluble protein; TG: triacyl glycerides; Chol: Cholesterol; Alka: Alkaline enzymes; Acid: Acidic Enzymes; LIP: Lipases; OxEs: Oxidative stress; ANTIOX: Antioxidant defence mechanisms. Arrows indicate the possible metabolic pathway that are carried out into the embryos. Soluble proteins support the glycogen production via gluconeogenic pathways, while glycogen liberate glucose in a stable and permanent form through embryo development to maintain a stable and efficient metabolizable energy supply. Coming from the yolk, TG and Cholesterol are used in pulses while lipases activity was evident from the stage X onwards, when the organogenesis starts. At the beginning of the embryo development high levels of oxidative stress were observed, probably from residual ROS produced in the female ovarium. *O. mimus* embryos were able to start the elimination of ROS when they reached the stage XIV, when the activity of the antioxidant defence mechanisms started activities. Activities of alkaline and acidic digestive enzymes were evident at the end of embryo development and in hatchlings, when presumably the digestive gland is in preparation to ingest food.

Present results indicate that soluble proteins and its amino acids could be used as a source of glycogen allowing maintain a stable and permanent supply of glucose along embryo development, even during the growth phase. These results suggest on the one hand, that glucose is not the most important source of energy for embryos and, for the other hand, regulation of gluconeogenic pathway works as mechanism of the glucose supply. That regulation is coupled with the phases of embryo development being without control until stage XI and control from stage XII onwards (Fig. 10). Also, results obtained in the present study putted in evidence that energetic demands of the embryo in the first phase of development were relatively low, without significantly mobilization of energetic substrates and its associated enzymes (Fig. 10). Also, was observed that molecules identified with redox stress were maintained in the eggs without changes, indicating that the antioxidant defence mechanisms were inactive until the stage XIV.

Joint with a reduction on soluble protein and glycogen an increment on lipases activity was recorded on embryos in stage XIV onward, indicating that at that stage the mobilization of yolk started (Fig. 10). Studies made in *O. maya* and *O. mimus* embryos also find significant mobilization of yolk from stage XIV-XV indicating after that stages mobilization of nutrients from yolk mark the start of the embryonic growth (Caamal-Monsreal et al. 2016; Sánchez-García et al. 2017). In *O. mimus* was observed that hearth beats increase with the embryo development reaching a relatively stability from stage XV onward, when the growth of embryos was evident (Warnke 1999). Also in *Enteroctopus megalocyathus* an power growth rate of embryos was obtained from stage XII onwards (Uriarte et al. 2016), suggesting that this phase of growth after organogenesis is a common characteristic between octopus species. The metabolic demand of cephalopod embryos rises during development as was observed in *Sepia officinalis* and *Loligo vulgaris* (Pimentel et al. 2012), *E. megalocyathus* (Uriarte et al. 2016), *O. vulgaris* (Parra et al. 2000) *O. mimus* (Uriarte et al. 2012) and *O. maya* (Caamal-Monsreal et al. 2016; Sánchez-García et al. 2017). During this process reactive oxygen species (ROS) are produced, which consequently leads to oxidative stress (Regoli et al. 2011). To

prevent oxidative stress and keep the balance of the cellular, aerobic organisms have evolved an efficient antioxidant defence system that consists of both non-enzymatic small antioxidant molecules (e.g. reduced glutathione (GSH), ascorbic acid (AA), carotenoids, etc.) and a cascade of enzymes (e.g. superoxide dis-mutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Regoli & Giuliani 2014). A recent study on the role of the enzymatic antioxidant system in the thermal adaptation of *O. vulgaris* and *O. maya* embryos suggests that early developmental stages of cephalopods have temperature-regulated mechanisms to avoid oxidative stress (Repolho et al. 2014; Sánchez-García et al. 2017). In the present study was observed that ROS in the eggs of *O. mimus* embryos were almost totally eliminated during the growth phase of the embryo development, thanks to the activation of the antioxidant defence mechanisms in the stage XIV, indicating the coupling between metabolic demands and the functioning of the antioxidant defense system against oxidant stress.

Studies made in *O. vulgaris* hatchlings revealed that during the burst of anaerobic swimming of paralarvae the energy is obtained from glucose and from Arginine phosphate system mediated by lactate dehydrogenase (LDH) and octopine dehydrogenase (ODH) respectively (Morales et al. 2017). Both systems require pyruvate, either by the gluconeogenic route or via the degradation of amino acids by transamination (Zubay 1983). Although we don't have data to demonstrate what kind of mechanisms could be operating in *O. mimus* embryos, it is possible to hypothesize that those mechanism should be related with amino acids and lipid catalysis, because both substrates (mainly arginine and glycerol) are involved in pyruvate production in cephalopods (Morales et al. 2017). In this sense we think that the no detected activity of lipases until stage X, and the moderated activity of alkaline and acidic enzymes registered along *O. mimus* embryo development could be involved in physiological regulation of the energy supply in those organisms. Studies made in *O. maya* embryos demonstrated that the yolk consumption starts after the stage XIV, when the organogenesis ends (Caamal-Monsreal et al. 2015; Sánchez-García et al. 2017), just when the antioxidant defense mechanisms start its activity.

Conclusions

Results obtained in the present study demonstrate that ovarium is a site for reserve of some nutrients for reproduction. Presumably, acyl glycerides where stored at the beginning of the maturation processes followed by cholesterol, both at the same time were energetically supported by glucose, derived from glycogen following gluconeogenic pathways. It means that probably, a control mechanism of protein-glycogen-glucose could be operating in *O. mimus* ovarian, were we planted the hypothesis that suggest that glucose, while is the energetic support for ovarian maturation at the beginning, have a potential role as inhibitor of vitellogenin uptake at the end of the processes. Also, was observed that embryos during organogenesis, characterized by gastrula stages when pairs of arm rudiments are evident joint with first visible organs at the disc-shaped animal pole (early organogenesis) (Boletzky 2003), nutrients and enzymes (metabolic, digestive and REDOX system) where maintained without significant changes and in a low activity. During this phase yolk was maintained almost constant indicating that, as was previously observed, blastulation and gastrulation appear uninfluenced by the size of yolk mass (Boletzky 2003). In this phase, the outer yolk sac envelope joint with its role of reserve of nutrients have the provisional function of gill and heart. Its activity is supported by a dense ciliature covering the entire yolk sac surface that maintain perivitelline fluids in circulation and oxygen uptake by the embryo (Boletzky 1989). Although there are no evaluations of energy cost of this activity, results obtained in the present study shows that that activity could be not energetically costly. In contrast, was observe that during the embryo growth and when the activity of hearth was evident (Castro-Fuentes et al. 2002), there were mobilization of nutrients and activation of the metabolic and digestive enzymes, joint with increments in consumption of yolk and glycogen, and reduction in molecules associated with oxidative stress, allowing paralarvae hatch with the antioxidant defence mechanisms ready to support the ROS production.

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