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Actividad de las Aminoacil ARNt Sintetasas (AARS) y composición de clases lipídicas como potenciales biomarcadores de crecimiento y calidad de huevos y paralarvas de pulpo común (*Octopus vulgaris* Cuvier, 1797).

Aminoacyl t-RNA Synthetases (AARS) activity and lipid classes composition as potential biomarkers of growth and quality of eggs and paralarvae of common octopus (*Octopus vulgaris* Cuvier, 1797).

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DECLARAN:

Que la memoria presentada por la Licenciada en Biología Marina, **Dña. Alin González Delgadillo** titulada **"**Actividad de las Aminoacil ARNt Sintetasas (AARS) y composición de clases lipídicas como potenciales biomarcadores de crecimiento y calidad de huevos y paralarvas de pulpo común (*Octopus vulgaris* Cuvier, 1797), Aminoacyl t-RNA Synthetases (AARS) activity and lipid classes composition as potential biomarkers of growth and quality of eggs and paralarvae of common octopus (*Octopus vulgaris* Cuvier, 1797)**",** ha sido realizada bajo su dirección y consideran que reúne todas las condiciones de calidad y rigor científico requeridas para optar a su presentación como Trabajo de Fin de Máster, en el Máster Oficial de Postgrado de Biología Marina: Biodiversidad y Conservación de la Universidad de La Laguna, curso 2017-2018.

Y para que así conste y surta los efectos oportunos, firman el presente informe favorable en San Cristóbal de La Laguna a 8 de enero de 2018.

Fdo. Dra. Covadonga Rodríguez González Fdo. Dr. Eduardo Almansa Berro

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Resumen

El pulpo común (*Octopus vulgaris*. Cuvier, 1797) es una especie cuyas características biológicas y gran valor comercial la vuelven una candidata importante para la acuacultura marina. Aunque esta especie es la más estudiada entre los cefalópodos y se ha intentado su crianza en cautividad desde la década de los 60, aún existen innumerables complicaciones para lograr su acuicultura a nivel comercial. Entre los cuellos de botella existentes destacan la dificultad que representa el poder llevar a las paralarvas hasta su etapa bentónica juvenil; lo que ha llevado a buscar biomarcadores que indiquen el estado de los huevos y paralarvas para intentar mejorar su desarrollo. En el presente trabajo se presentan los resultados del primer acercamiento al uso de la actividad aminoacil ARNt sintetasa (AARS) y la composición de clases lipídicas en paralarvas y huevos como posibles biomarcadores de tasa de crecimiento y bienestar de los organismos. Aun siendo estos resultados preliminares se encontró cierta relación entre el crecimiento y la actividad AARS en las paralarvas de pulpo común y a su vez con la composición lipídica de estos, abriendo las puertas a experimentos más específicos destinados a afinar la utilización de estos elementos como biomarcadores útiles de la calidad de huevos y paralarvas del pulpo común.

Palabras clave: Acuicultura, huevos, paralarvas, biomarcadores, AARS, clases lipídicas, *Octopus vulgaris*.

Abstract

The common octopus (*Octopus vulgaris*. Cuvier, 1797) is a species which biologic characteristics and high commercial value make it an important candidate for marine aquaculture. Even when this species is the most studied of cephalopods and its captivity rearing has been tried since the sixties, there are still numberless complications to achieve their aquaculture to a commercial level. Among the existing bottlenecks one that stands out is the difficulty that represents the rearing of the paralarvae until their juvenile benthonic stage; which have gotten to the search of biomarkers that could indicate the condition of eggs and paralarvae and to take these actions to improve their development. In this work the results of the first approach to the use of aminoacyl tRNA synthetase (AARS) and the composition of lipid classes in paralarvae and eggs as possible biomarkers of growth rate and welfare of the organisms was achieved. Even when these results are preliminary some relationship between growth rate and the common octopus paralarvae AARS and lipid composition was found, opening the gates to experiments more specific about this topic to develop a proper use of these elements as useful biomarkers of octopus eggs and paralarvae quality.

Key words: Aquaculture, eggs, paralarvae, biomarkers, AARS, lipid classes, *Octopus vulgaris*.

1. INTRODUCTION

1.1 Cephalopods importance

Cephalopods have approximately 800 living species, they have high growth rates and short life spans, all of them are active foraging predators with separate sexes and direct development, they have a single period of sexual maturity during which they have multiple spawning. At the ecological level, the cephalopods are considered keystones species, as active predators and important sources of prey for organisms on different food chain levels (Boletzky & Villanueva, 2014).

Cephalopods are very important for human direct consumption, but also, they are really demanded because of their by-products such as marine oil, ink, chitin and chitosan, collagen, calcium and hydroxyapatite, functional peptides, peptones, enzymes, digestive gland proteinases that can be used to prepare fish sauces, and organic fertilizers among others. Their importance as research models for a good number of sciences, such as medical and biochemical research, physiology, neuroscience, nutritional biochemistry, ageing, molecular biology and immunology (Oestmann *et al.*, 1997), is due to some unusual characteristics including their nervous system and sense organs (Lee, 1994). All these characteristics have generated a fishery industry with cephalopods as first target and, in the past 50 years the development of a cephalopod aquaculture that has advanced at various rates depending of each species characteristics and needs (Boletzky & Villanueva, 2014).

1.2 Octopus importance

Octopus vulgaris is the most studied species from the genus *Octopus*, they have a pear-shaped body, can reach up to 1.3m of size and a top weight of 15.2kg, with a mean of 3kg. They are camouflage masters as they can change their skin color and texture easily thanks to their chromatophores; they are usually solitary and defend their territory, with seasonal migrations from shallow parts to deeper in winter and the opposite in summer with the objective to mate. This octopus can be found most of the time hidden between cracks or holes, but can also live on sandy bottoms and algae fields up to 200m deep in temperate seas, with a temperature between 10 to 30ºC distributed in four principal regions, oriental center Atlantic (Mediterranean and Atlantic zone), Brazil and south Africa (Hernández, 2017). For a long time, the *Octopus sinensis* was known as the Japan population of *O. vulgaris* but very recent genetic studies discovered it was a different species and even nowadays, investigators as Amor *et al.* (2016) support the existence of multiple *O. vulgaris*- like species that are incorrectly treated as one single species.

O. vulgaris is a species that support industrial and artisanal fisheries, with local fishermen catching them with hooks, lures and pots, and industrial fishermen getting large quantities of organisms in the oceanic sub-littoral areas using trawls operated from large fishing boats (Iglesias & Fuentes, 2014). According to data from the food and agriculture organization (FAO, 2017) the declared annual world catches attributed to *O. vulgaris* declined from more than 100,000 tons in

the late 1970s to 34,000 tons in the 2015. Spain is the sixth exporter at global level of the species and has the biggest captures in the nor east Atlantic where the decline is also notable, going from 38,000 tons in 1998 to 4,000 tons in 2015; which proves that the octopus fishery has been exploited in the past decades (Fernández-Gago, 2017).

In order to prevent this overexploitation of octopus wild stocks, some options have been analyzed. These including the protection of spawning areas, the regulation of fishery to allow animals to reach market size, the control of fishing effort in coastal areas, the use of selective gears to minimize by-catch problems, to protect high seas stocks by international agreements and the development of cephalopod fishery certification schemes. But on top of that, the option of making *O. vulgaris* culture completely functional and able of supply part of the production stock desired, is one of the best and the more studied choices nowadays (Iglesias & Fuentes, 2014).

1.3 Aquaculture of octopus

The common octopus, *Octopus vulgaris*, is an important candidate for marine aquaculture, due to characteristics as a high growth rate, easy adaptation to captivity and different food sources, and being a species of high market value; nevertheless, the culture to develop the whole cycle of this species has only been possible at a laboratory scale (Iglesias & Fuentes, 2014).

One of the principal characteristics that makes *O. vulgaris* an excellent candidate for marine aquaculture, is its high growth rate, that goes between 3 and 13% of weight per day (Villanueva & Norman, 2008). This is possible thanks to the high rates of protein synthesis and retention of synthesized protein, while their protein degradation rates are very little (Houlihan *et al.*, 1990); these capacities depend above all on the adequate feeding of organisms, when fed properly they are extremely efficient in all these processes (Carter *et al.*, 2009). Also, at paralarvae and juvenile states they can reach food conversion rates between 15-43%, increasing their weight up to 10.5% per day (Mangold, 1983).

The octopus culture, is of relatively recent development and started with Coates *et al.* (1965), who developed an automatic food dispenser for *O. vulgaris* useful to maintain these animals in captivity and take observations on the amount, time and frequency of octopus feeding. A decade later, Guerra (1978) described a seawater setup to maintain *O. vulgaris* in the laboratory and tried a wide variety of live and dead prey as food, concluding that octopuses prefer crustaceans to fish. Next, Hanlon and Hixon (1983) wrote about methods for laboratory maintenance and culture of octopuses and squids that could be applied to *O. vulgaris* paralarvae. With all the knowledge generated, at 1989 *O. vulgaris* was identified as the most probable species to be mass cultured in Europe (Boucaud-Camou, 1989).

Years later, Villanueva (1994) studied the suitability of decapod crab zoeae as first food for *O. vulgaris* paralarvae and just a year later Villanueva (1995) was able to rear *O. vulgaris* paralarvae until settlement. From the mid-1990s onwards the culture of common octopus has been of particular interest for Mediterranean countries and in Spain a lot of research centers are dedicated to this task, while there are some companies dedicated to the fattening of *O. vulgaris* subadults (García *et al.*, 2004). The complete culture cycle was first achieved in 2001 by Iglesias *et al.* (2004) just at experimental level, using *Artemia sp.* and spider crab zoeae (*Maja squinado*) as

live prey. A year later, Carrasco *et al.* (2003) got similar results using the same prey; these zoeae has gotten the best growth rates and survival of paralarval phase but the method is not transferable to a commercial state as there is limited availability of live zoeae.

Nowadays, the available technology for the culture of the whole life cycle of *O. vulgaris* is scarce and has only been achieved at laboratory scale, mostly because there is no commercial diet available. As a result, the production system is based on the capture of young subadults that are kept in captivity and fatted until they reach the commercial weight. An option that seems to be more economical is the use of sea cages, but considering that this means direct effect over a larger ecosystem and uncontrolled conditions for the octopuses, is a field that needs more investigation before being put in action. The proper development of the octopus culture to be a sustainable economic activity and a good industry requires solving two limiting issues: mass production of subadults and a suitable commercial diet (Iglesias & Fuentes, 2014).

The common octopus is included in the European Union's directive on animal welfare for the protection of animals used for scientific purposes since 2010 (Directive 2010/63/EU). Years later, Fiorito *et al.* (2015) proposed some "Guidelines for the care and welfare of cephalopods" that are followed nowadays when working with these organisms. The principal projects, investigation centers and books dedicated to octopus development and culture in Spain are:

- Instituto Oceanográfico de Canarias: responsible of some of the projects developed in the last years for octopus culture (OCTOPHYS, OCTOWELF, CephsInAction, …) with Dr. D. Eduardo Almansa, José Iglesias, and Dra. Virginia Martín as main investigators, their principal objective is to discover the optimal conditions and diet for the paralarvae to be able to reach the juvenile state.
- OCTOPHYS project: (from 2013), with a strong input of D. Juan Carlos Navarro from IATS (CSIC, Castellón) and D. Covadonga Rodríguez from Universidad de la Laguna, contributed with essential information about lipid requirements of common octopuses and generated the design of an enrichment protocol that helped in the paralarvae growth.
- OCTOWELF project studies the welfare and health of *O. vulgaris* at the first stages of life and has been run from 2014 to 2017 with Dr. D. Eduardo Almansa and Dra. Virginia Martín as main investigators.
- CEPHCOST – Cephs*In*Action: is an European network that count with the contribution of Dr. D. Eduardo Almansa Berro and promotes the exchange of tools and knowledge to improve the maintenance and take care of the cephalopods used in science or education.
- Instituto Oceanográfico de Vigo: this is where Dr. Jose Iglesias was able to complete the culture cycle of *O. vulgaris* on an experimental level using *Artemia sp.* and crustacean zoeae as food.
- IEO - Cephalopod Culture: a compilation book done at 2014 by 50 cephalopods specialists with the more recent and significant results about the culture of different species of cephalopods.
- JACUMAR (Junta Nacional Asesora de Cultivos Marinos): this proyect coordinated investigations such "El cultivo del pulpo *Octopus vulgaris*" from 2001 to 2003. An international working group where experts from different regions debated about culture problems and possible solutions, and their "Nutrición y alimentación de paralarvas del pulpo de roca (*Octopus vulgaris*)" in 2005 which objectives were to select the best culture method for the octopus paralarvae and to create a commercial food for the subadults.

1.4 Main bottlenecks in octopus aquaculture

Some of the problems the octopus culture must deal with are that they are solitary organisms, a lack of sociality which makes it difficult to have high densities of organisms all together in small tanks and which is believed to contribute to another problem, the cannibalism, which must be avoided by keeping animals of the same size together with a good supply of preferred food or building complex environments where organisms can escape and hide. Another problem is that octopuses do not like to stay in restricted areas more than some days or weeks, making them attempt to leave, with *O. vulgaris* as the most likely species to escape (Sánchez *et al.*, 2014). It also has been proven that the captive cephalopods have a different physiology than those from the wild and this generates changes at tissue level (Pecl & Moltschaniwkyj, 1999).

About the octopus likely to get ill, even when the cephalopods have an immune system effective to attach the pathogens they can be exposed to, and virus-like particles have been found associated with tumors in *O. vulgaris* (Hanlon & Forsythe, 1990), while bacterial infections are often developed on the organisms mantle after injuries and the infections can be spread to other individuals in the tank (Hanlon *et al.*, 1988). Reports on fungal activity are scarce and mostly related to eggs and embryonic development (Iglesias & Fuentes, 2014).

Still with all the problems before mentioned being part of the problematic to have a functional *O. vulgaris* culture, they are just secondary, and the main trouble is based on the massive mortality rates (near 100%) observed during the first two months of paralarval rearing, just before they change their pelagic behavior to a benthic life, which is known as settlement. There have been a lot of studies aiming to know the causes of these high mortalities, but even when there is a consensus about the nutritional factor being important, the reason remains unclear and there are many inconsistencies on results in studies of survival and growth (Garrido *et al.*, 2017).

Some of the possible causes of the high mortality of paralarvae that have been studied and tested in laboratories very recently are the effect of the environment on early life stages organisms for its future metabolism and health due to their plasticity (Díaz-Freije *et al.*, 2017), driving to changes in the epigenetic of the octopuses, such as the DNA methylation process, which is controlled by age and diet (García-Fernández *et al.*, 2017). This also seems to be part of problems that the paralarvae could carry from the embryonic state, such as a smaller inner yolk, when the eggs are maintained in high temperatures after the XV stage of development, causing paralarvae to lose weight easier and to have less time to effectively capture its prey, accelerating and increasing the mortality (Nande *et al.*, 2016).

Talking about the effect of the diet in the paralarvae, the proteins are the most abundant macronutrients in cephalopods and a large protein content in their diet is required for growth and energy demands (Lee, 1994). For *O. vulgaris* hatchlings the protein content represent 73% of their dry weight (Yebra *et al.*, 2017) with glutamate and aspartate as the most abundant nonessential amino acids; and lysine, leucine and arginine as very important essential amino acids (Villanueva *et al.*, 2004). Also, the vitamins that have been studied in octopus, and proved to be particularly high in paralarval cephalopods are vitamin A and E, as in other marine molluscs and fish larvae, but the importance of other vitamins have not been investigated. In the topic of

minerals, the common octopus paralarvae most abundant ones are copper, for which there could be a specific nutritional requirement, and cobalt, that seems to be also important in the development of adenochrome, the red-violet pigment present on the octopus branchial hearts and involved in excretion processes (Villanueva & Norman, 2008). Also, the low growth rates that the cultured organisms register in comparison with the wild octopuses is a problem that could have an origin in what the cephalopods use as energy substrate, that can be proteins (Lee, 1994) or carbohydrates (Storey & Storey, 1983). Other effect of the diet could be the reduction of bacterial diversity on the gut of paralarvae reared in captivity; about this Roura *et al.* (2017) recently found just 5 bacteria species in captivity paralarvae against the 50 species found in the wild organisms, which can affect the nutrition and health of the host, and make them easier to get ill.

When all the nutritional requirements of the paralarvae are not fulfilled, a new problem and possible cause of mortality is developed, the so-called nutritional stress, which causes changes in the antioxidant defenses and lipid peroxidation, just as Varó *et al.* (2013) found in *O. vulgaris* paralarvae, showing differences in total peroxidase activity depending on diet. There is also stress that can be generated by handling, producing an increment in dopamine or corticosterone (Tur *et al.*, 2017a). Some ambient parameters that have been also proved to affect the paralarvae development and welfare, are the light, which intensity and color can change the octopuses growth and survival (Tur *et al.*, 2017b); and, water circulation, temperature and chemical parameters which can cause gas supersaturation on the paralarvae, and which could also lead to extensive mortalities (Hargreaves & Tucker, 1999).

In order to overcome this bottleneck in the common octopus culture and considering the studies that have been done in the last few years, there are some possible solutions that have been raised or tested by diverse scientists, mostly to overcome the diet as possible cause to the massive mortality. In first place, a reduced number of paralarvae have been reared successfully to juvenile state when being fed with crustacean zoeae in co-feeding with *Artemia sp.* (Moxica *et al.*, 2002; Carrasco *et al.*, 2003; Iglesias *et al.*, 2004; Villanueva, 1994). In this sense and in order to overcome the problem of zoeae poor availability and high economic value Moxica *et al.* (2002) developed the option of a diet based on *Artemia sp.*, phytoplankton and crab zoeae, which advantage is the needing of little zoeae amounts and which have reached survivals of 0.2% after two months with a good growth rate. Other diet option is the use of copepods of the genus *Centropages* and *Temora*, with which Nande *et al.* (2017) got similar results at the ones with crustacean zoeae, and with higher growth rates than when the paralarvae are only feed with *Artemia sp.*. There is also a recent idea of using not living food but inert diets, but some aspects must be considered for this, especially if flours are used, since these must get processed at temperatures under 60ºC, so they will not lower the resultant growth rates. But still the digestivity of these flours need to be proved (Hamdan *et al.*, 2014). In the same topic, there is evidence about the paralarval acceptance of food without movement, as crab eggs, through resuspension and bubbling, which could help to get a diet with better nutritional characteristics (Hernández, 2017). Other possibility is to exploit the capacity of the paralarvae to absorb small organic molecules that are in the water, such as essential amino acids and nutrients through the skin, as another path to fulfill their necessities (Villanueva *et al.*, 2004).

Also, a lot of physical and chemical parameters need to be take into account at *O. vulgaris* culture, such as water circulation, salinity, which is recommended to be between 33 to 35

practical salinity units (psu); and also dissolved oxygen, pH, nitrite and ammonia levels should be monitored (Sánchez *et al.*, 2014). In the case of temperature, the recommendation of Nande *et al.* (2016) is to rear the eggs at high temperatures for a rapid development, and after the XV stage to reduce the temperature gradually to enhance inner yolk accumulation and allow the paralarvae to have more time to become effective hunters or in case required, maintain their weight longer time due to lower energy requirements. The light is also an important variable, and low intensity seems to reduce the growth rates, while semi-covered tanks and the light in an oblique angle present better survival rates (Tur *et al.*, 2017b).

The characteristics of the space where the paralarvae live are significant as well, an example is the effect of the tank volume. About this De Wolf *et al.* (2011) studied the relationship between survival and tank volume, finding higher survival of paralarvae when large volumes and low paralarvae density were tested. Tank color seems to affect too, getting better survivals in black than in white tanks (Estefanell *et al.*, 2015). The rearing of paralarvae seems to require a better simulation of natural conditions, in relation with water column, water quality and turbulence (Villanueva & Norman, 2008); after all the fact that *O. vulgaris* paralarvae have an oceanic strategy, living far from the continental shelf and an affinity for surface waters at night do not have to be forgotten (Roura *et al.*, 2016).

Finally, the possibility that the high mortalities of paralarvae could be a consequence of problems carried from the egg phase or even as problems transmitted from mother to child exist should be also considered. Therefore an specialized treatment and care must be given to the reproductive adults, to ensure that the paralarvae obtained from them will be healthy for their rearing and use for experimental studies (Villanueva & Norman, 2008).

1.5 Biotechnology tools and useful biomarkers

In search of a sustainable octopus aquaculture the use of tools to predict and estimate growth, to know physiological and nutritional conditions and to quantify the possible stress the organisms can be exposed to, has driven to the recent discover of useful biomarkers, which are based in the different mechanisms that respond to environmental and intracellular stimuli (Garrido *et al.*, 2017).

Some used biomarkers are the heat shock proteins (HSP), within which the HSP70 is consider one of the major HSP families in molluscs and have been proved as a successful biomarker for stress and health status in cephalopods. Also, RNA/DNA and protein levels have being positively correlated to growth rates in *O. vulgaris* but just partially, due to the great variability these biomarkers can show, meaning they could become good growth indicators for octopus paralarvae with some limitations depending on the diet and geographical region of the organisms (Garrido *et al.*, 2017).

Growth is a dynamic process linked with environmental conditions that the organisms are exposed to. This is why growth rate is considered a good way to calculate production during culture of certain organisms, but the direct estimation of growth requires repeated sampling, incubations and is difficult for small animals, such as the zooplankton and the octopus paralarvae (Yebra *et al.*, 2017). As a result to such problems, a biochemical approach to predict and estimate

growth for small aquatic organisms have been developed; such as the estimation of bromodeoxyuridine (BrdU) into the DNA (Gómez *et al.*, 2001). The quantification of nucleic acid, that has a relationship with growth or reproduction; and methods that measure enzymes activities involved in synthesis pathways, with the advantage that these do not require incubation of organisms, have simple procedures and quick measurement with high precision that are easy to repeat (Yebra *et al.*, 2017).

Focusing on the enzyme methods to calculate growth rate of organisms, a strong relationship is present between growth and DNA polymerase in *Artemia salina* (Yebra *et al.*, 2017), also the activity of aspartate transcarbamylase (ATC) has been proposed as an index of growth for molluscs, after showing good relationship in fishes (Bergeron, 1981) but not with planktonic crustaceans (Alayse-Danet, 1980). Other method, that was assayed by Berges *et al.* (1990) is based on the nucleoside diphosphate kinase (NDPK) but the results just showed a scaling factor between individuals biomass and the enzymatic activity, years later Berges and Harrison (1993) proved the NDPK as proxy of growth in a marine diatom but obtained a poor correlation.

Some problems that the use of enzymes as growth estimators have are that the relationship between enzymatic activity and metabolic rates are not straightforward, and that depending of the method used, this has different effects. For example, ATC and NDPK do not measure the protein building, giving low precision. Also when measuring ATC, the technique can be affected by methodological constrains; on the other hand, these methods can depend on the well feed of the organisms and some protocols have to be changed or completely eliminated as options because of the need of radioactive isotopes to run the essay (Yebra *et al.*, 2017).

1.6 AARS as biomarker

The protein synthesis is a very complex process where proteins get assembled by ribosomes, at the same time occurs the generation of messenger RNA, and the aminoacylation of transfer RNA, during which the Aminoacyl t-RNA synthetases (AARS) fulfil the function of attach the different amino acids with their specific t-RNAs, in a reaction that release pyrophosphate (PPi) (Lehninger, 1988). So, the AARS activity is directly related to the protein synthesis, which is related to somatic growth, being the reason to consider AARS a good candidate as proxy of octopus paralarvae growth.

There are two non radiochemical methods to measure AARS activity, both based on the release of PPi during the amino acylation. Upson *et al.* (1996) developed a sensitive method, which is not useful for cell homogenates, but Chang *et al.* (1984) established a very simple method which use a spectrophotometer and a commercial PPi-reagent kit to register the maximal potential activity of the enzymes. This method was changed years later by Yebra and Hernández-León (2004) stopping the addition of amino acids as substrate to measure the capability to synthesize proteins the organisms have at the moment of capture, revealing their previous food and development history.

The good relationship between AARS activity and somatic growth rates has been proven on different organisms, as yeast (Johnson *et al.*, 1977) and fishes (Bolliet *et al.*, 2000). After these first approaches, the AARS activity has been successfully calibrated as index of growth for

marine calanoid and cyclopoid copepods (Herrera *et al.*, 2012; Yebra *et al.*, 2005), and euphausiid larvae (Guerra, 2006), and fish larvae (Herrera, 2014). This AARS method has been used as proxy of growth rate on different geographical areas, seasons and diets with positive results in *Calanus finmarchicus* (Yebra *et al.*, 2006), and in a bulk of zooplankton (Herrera *et al.*, 2017) in the Canary Islands waters. Also, the AARS method was compared to theoretical models of growth, which allow to know the degree of accuracy that the method can obtain with different organisms (Yebra *et al.*, 2017).

Some advantages of the AARS method are that it is simple, can be performed with a quick non-radioactive assay, it gives an *in situ* approach to the growth rate assessment, is applicable to different taxonomic groups and can work altogether with other biochemical measurements (Yebra *et al.*, 2017). On the other hand, it has some limitations, as the necessity of calibration, and the existence of different sources of PPi that can interfere with the method, such as the β-oxidation of fatty acids that occurs in starved organisms (Hawkins, 1985) and during the biosynthetic conversion of glucose into glycogen where PPi is also released (Lehninger, 1988).

1.7 Lipids as biomarkers

The octopus paralarvae have some dietary requirements that must be fulfilled to ensure their good development. One of the few factors that have been determinate as extremely important in the diet over the *O. vulgaris,* are lipids. The octopus lipid-rich nervous system represents a quarter of the animal fresh weight, suggesting their importance (Packard & Albergoni, 1970). Different studies have proven that *O. vulgaris* requires preys rich in polar lipids (PL) including phospholipids, long-chain polyunsaturated fatty acids (LC-PUFA) and possibly cholesterol (Navarro & Villanueva, 2000; 2003), which resembles a natural diet based on crustacean larvae and planktonic organisms, but is far from any kind of enriched *Artemia sp.* composition (Iglesias & Fuentes, 2014).

In the case of fatty acids, the paralarvae have high levels of docosahexaenoic acid (22:6n-3, DHA), eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA), being the first one essential for normal neural development and the second one particularly important for normal growth of the paralarvae (Garrido *et al.*, 2017). Recent studies about the fatty acyl desaturase and elongase capacity of *O. vulgaris* have shown that ARA, EPA and DHA are essential fatty acids for them. At the same time, the octopus present low levels of triacylglycerol (TAG) along their whole life (Navarro & Villanueva, 2000; Reis *et al.*, 2015). This composition, with low TAG and high phospholipids rich in LC-PUFA, could mean that in this organism the lipids may have a predominant structural function as happens with the cuttlefish (*Sepia officinalis*) (Sykes *et al.*, 2009). This probably defer depending the area the population live in, because the energy may be taken from carbohydrates at subtropical temperatures, while the lipids and proteins could be the main energy source at lower temperatures (Navarro *et al.*, 2014).

Artemia sp. is the most common organism used as live prey for the paralarvae, but this prey has an inadequate lipid composition, with low polar lipid levels, and poor contents of DHA, which is of great importance for octopus paralarvae development (Navarro & Villanueva, 2000; 2003). To aggravate it, a study from Reis *et al.* (2015) revealed that even when paralarvae can incorporate

fatty acids from the sea water, if these are supplied through *Artemia* sp. the assimilation is reduced, especially for DHA, from which just an approximately 5% get into the octopus.

Even when the octopus metabolism of lipids is not fully understood, it is known that they display a poor capacity for mitochondrial lipid oxidation (O'Dor *et al.*, 1984), and lipids as cholesterol and phospholipids has been suggested as critical dietary components for the early stages of these species. Even when in adult tissue the activity of a Δ5 Fad has been registered (Monroig *et al.*, 2012), in the case of paralarvae the activity of this enzyme has not been detected in vivo, making it impossible for them to biosynthesize ARA from 18:2n-6 or EPA from 18:3n-3 (Reis, 2016) as happens in the *S. officinalis* (Sykes *et al.*, 2009). Being the lipids molecules so important for structure and biological functions and consider as essential nutrients, their composition may serve as a useful biomarker of the *O. vulgaris* hatchlings and eggs status of development and quality.

Taking into account all these considerations exposed in the introduction, the objective of the present work was to test the suitability of AARS activity and lipid class composition as possible biomarkers of the growth and quality of eggs and paralarvae of *O. vulgaris* to predict and improve the rearing of this organism.

2. OBJECTIVES

General objective

The main objective of this study was to investigate the possible use of AARS activity and lipid class composition of *O. vulgaris* paralarvae and eggs as useful biomarkers of welfare, growth and quality of the organisms.

In order to achieve this goal, the following specific objectives were followed:

1. Study the AARS activity and compare its changes in relation with the growth or development of the different organisms sampled.

2. To characterize the lipid class composition of the organisms and look for changes determined by their growth or development status.

3. Look for a correlation between the AARS activity and lipid class composition to know if there is dependence between them and the growth or development rate.

4. In the case of the eggs, to search for correlations between some mother characteristics and the lipid class composition found.

3. MATERIALS AND METHODS

All experimental works were performed according to Spanish law (RD 53/2013) based on the European Union's directive on animal welfare for the protection of animals used for scientific purposes (Directive 2010/63/EU). Guidelines for the care and welfare of cephalopods proposed by Fiorito *et al.* (2015) were followed in this study. The present study was also approved (register document CEIBA2014-0108) by the Ethics Committee for Animal Research and Welfare (Comité de Ética de la Investigación y Bienestar Animal, CEIBA) from the University of La Laguna (Spain).

The experiments were done at two centers belonging to the Spanish Institute of Oceanography (IEO), namely the Oceanographic Centre of the Canary Islands in Tenerife (TF) and the Oceanographic Centre of Vigo (VG). The analyses of lipids were performed at laboratory facilities of Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna. Three experiments were carried out; two of them were done to test different diets for the paralarvae, in order to know how they affect their development, growth, AARS activity and lipid composition. The third one was done with eggs from the ordinary reproduction that takes place at the Oceanographic Centre of the Canary Islands, to know the AARS activity and lipid classes composition of *O. vulgaris* eggs at different stages of development.

3.1 Experimental trials

3.1.1 Paralarval nutrition; Experiment 1. *Artemia* – *Maja brachydactyla* zoeae (Vigo).

These samples were taken from a specific experiment performed to improve the octopus culture conditions and to search for alternative preys. The preys used after the paralarvae reached 30 days old and also some of the culture parameters assayed are under patent process. In consequence, the results exposed in the present memory correspond to paralarvae aging between 0 and 30 days-old which were fed with either 7 days-old *Artemia* (Sep-Art EG, INVE, Aquaculture, Belgium) grown with phytoplankton (T-Iso) as the control treatment or a co-feeding treatment which combined *Artemia* and live *Maja brachydactyla* zoeae as the experimental treatment. In general terms, culture conditions were based on Garrido *et al.* (2017) using 1,000L fiberglass cylindroconical black tanks with a density of 5 paralarvae per L, fluorescent light, 18ºC of water temperature and oxygen close to saturation. Other data including water renovation and light intensity changes (among others) differed from those of Garrido *et al.* (2017) but are under patent process.

10 paralarvae from both the control and experimental treatments were taken at days 0, 20, and 30; collected, frozen in liquid nitrogen (-196°C) and kept in individual marked vials at -80°C and still frozen sent to the TF center for their analysis.

3.1.2 Paralarval nutrition; Experiment 2. *Artemia*- Inert Diet (Tenerife).

The next experiment tested an inert diet as the experimental treatment, which consisted in inert blue crab *Callinectes sapidus* tissue homogenized and encapsulated through direct spherification

with alginate (1.3%). It was performed between the months of July and August 2017. In this case, 3,000 paralarvae (500 paralarvae per tank; 5 paralarvae/L) were reared over 15 days in 100L black tanks, having a total of 3 tanks (n=3), with a temperature of 23.5°C, oxygen close to saturation, and a daily water renovation of 400%. Light of 4.5W was applied in an oblique position from the tank edge with an intensity of 600 lux during day and at 15 lux at nigh-time. Another 3 tanks worked as the control group with the paralarvae being fed with 0.5 one-day old *Artemia/*mL (Sep-Art BF, INVE, Aquaculture, Belgium) and per day. The 3 tanks serving as the experimental group, were fed with a mix of 0.5 one-day old *Artemia/*mL and 0.75g of the Inert diet.

20 paralarvae were taken from each tank at days 0, 6, 11 and 14 and kept in the same conditions as was described before, until their use for analysis.

3.1.3 Experiment 3. Eggs development

The broodstocks whose eggs were taken as samples were captured by local fishermen using artisanal octopus traps in Tenerife coastal waters (Canary Islands, Spain) and maintained in the facilities of the Oceanographic Centre of the Canary Islands (Spanish Institute of Oceanography). Adult specimens were kept in 4,000 L tanks (1 male: 2 females) with water renovation (5 L/min), under oxygen saturation conditions and low light intensity. The eggs spawnings took place between May and June of 2016, and the mother chip was used to identify the origin of each one of the spawns. Diverse variables changed between them and they were also considered; such as the mother's captivity time before the spawning, the mothers weight when they arrived at the institute (Table 1) and the temperature that the eggs developed in. Taking in count that the Tenerife's IEO use an open system for pumping water from the ocean and so, the natural changes also affect the temperature of the water the organisms received, varying between 19.95°C and 22.13°C depending on the specific days of the spawning, as can be seen in Figure 1.

Table 1. Mother's characteristics of the spawnings sampled

Chip	**9517**	**2506**	**0043**	**0481**	**9707**
Captivity time (days)	30	52	62	45	45
Initial weight (kg)	1.2	2	1.4	3.3	2

Lastly, 25 eggs were taken from each different spawning at three different egg stages, which corresponded in general terms to VIII-X, XIV and XVII-XIX; these samples were also collected in the same way, frozen in liquid nitrogen and kept in individual marked vials at -80 °C until their use for analysis.

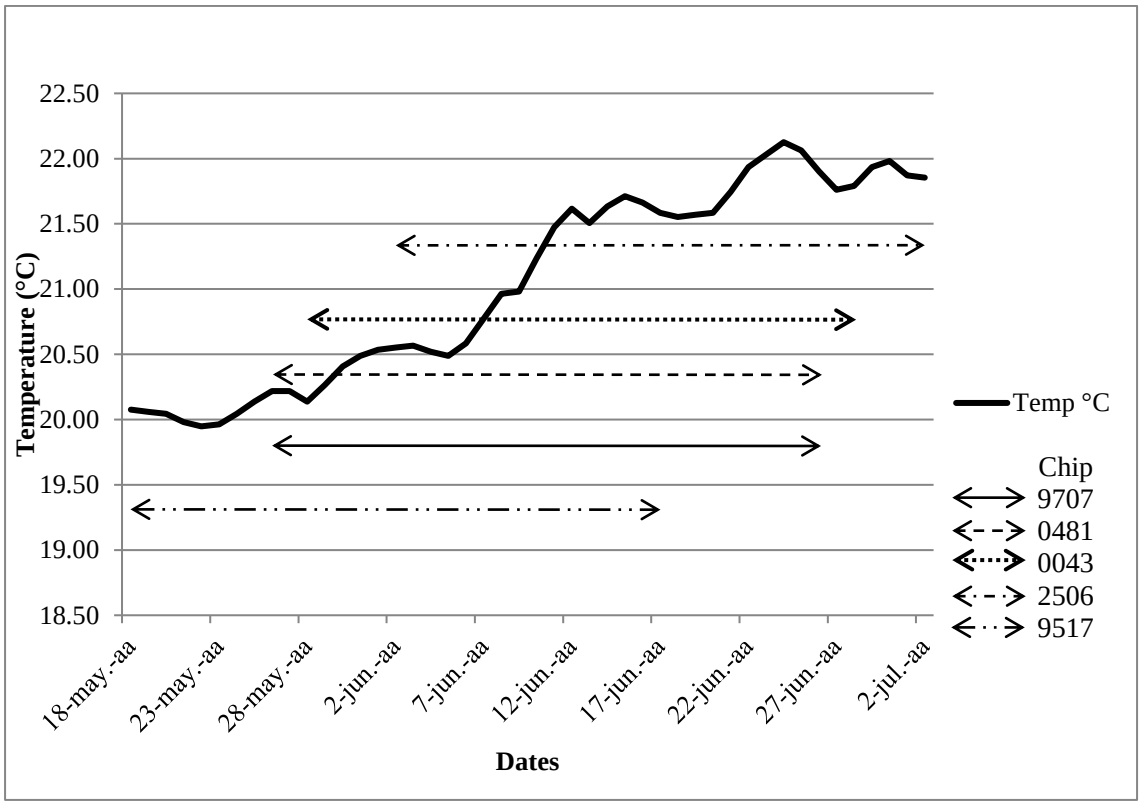

Figure 1 Temperature variation with time for each egg spawning.

In the case of the eggs (Experiment 3), 10 eggs were taken from each, kept in seawater, and analyzed under microscope to know the egg stage and development conditions.

Growth/ development assessment

In the first experiment, at VG, the dry weight of 10 paralarvae per tank was determined every five days (day 0, 5, 10, …, 30) Paralarvae were euthanized in chilled water (-2°C), then washed three times with distilled water and put in aluminum pre-weighted bases to be dried in an oven (100°C, 24 hours) and weighted with a Mettler Toledo AT201 scale (Barcelona, España), to then calculate the actual paralarvae dry weight (DW) taking off the aluminum base weight. Having the DW of the organisms the Instantaneous relative growth (IGR, % DW/day) was calculated for each tank as (Ln DWf-Ln DWi) 100/(tf-ti), where DWf and DWi are the dry weight at final time (tf) and initial time (ti).

At the TF experiment, the dry weight (DW) of 10 paralarvae specimens per tank was also obtained at day 0, 6, 11 and 14 using the same methodology as in Vigo and obtaining the IGR afterwards. The wet weight (WW) of all the samples was calculated considering that the DW is equivalent to approximately 15% of the WW of the paralarvae.

3.2 Analysis

Each sample was homogenized in Tris HCl buffer with a proportion of 4:1 (4µl of buffer per mg of WW of paralarvae/or egg); then 10µl of the sample was separated in a new vial and 20µl of buffer was added, these new vials were kept in -80°C to be used for the lipid extractions. The remaining original samples were then centrifuged with a Hettich Universal 320 R centrifugate (Tuttlingen, Germany) at 30,000 g (5°C) for 10 min and the supernatant was used for the AARS activity assay and protein content.

3.2.1 Aminoacyl t-RNA synthetases (AARS) activity

This procedure was based in the method of Yebra and Hernandez-Leon (2004), modified by Yebra *et al.* (2011) to use microplate readings (Herrera, 2014), as is described here: a mixture of 40 µL of pyrophosphate (PPi) reagent (Sigma, P-7275), 60 µL of Milli-Q water and 50 µL of the supernatant obtained after the centrifugation of each sample was added in each well. The reaction absorbance was monitored at 340 nm for 10 min on a SAFAS flx-xenius BioTek Synergy HT spectrofluorometer (Vermont, USA) with microwell plates. With this, the NADH oxidation rate (dAbs·min-1) produced by the release of PPi during the aminoacylation of the tRNA was registered as a decrease in absorbance (dAbs) and converted to PPi release rate (AARS activity, nmol PPi·h-1) using the following corrected equation by Herrera *et al.* (2017).

$$\text{nmol PPi} \cdot \text{h}^{-1} = \left(\frac{\frac{\text{dAbs}}{\text{min}} \cdot 10^3 \cdot 60 \cdot V_{rm}}{V_s \cdot 6.22 \cdot 2 \cdot 0.46} \right) \cdot V_{hom}$$

where dAbs·min-1 is the rate of decay in absorbance per minute, 10^3 is the conversion of µmol to nmol, 60 is the conversion from minutes to hours, Vrm is the volume of the reaction mixture (mL), Vs is the volume of sample (mL), 6.22 is the millimolar absorptivity ($L \cdot mmol^{-1} \cdot cm^{-1}$) of NADH at 340 nm, 2 is the number of moles of β-NADH oxidized per mole of PPi consumed, 0.46 is the path length correction (cm) for micro well plate and Vhom is the volume of homogenate (mL). Finally, the AARS activity was corrected for the in-situ temperature of each experiment, using 8.57 kcal·mol-1 as activation energy (Yebra *et al.*, 2005) to obtain the AARS in situ activity. This activity was then divided by the number of individuals per sample to obtain the individual AARS activity (AARSs·ind^{-1}) expressed in nmol PPi·$ind^{-1} \cdot h^{-1}$.

The protein content of the samples was measured following the Smith *et al.* (1985) method, adapted with a Pierce BCA Protein Assay Kit (23225), which includes the use of Albumin as standard; the solution absorbance was measured at 562 nm using a SAFAS flx-xenius spectrofluorometer. Obtaining these measurements, the AARS in situ activity was divided by the protein content, to obtain the protein-specific AARS activity (spAARSs) in nmol PPi·mg $prot^{-1} \cdot h^{-1}$.

3.2.2 Lipid extraction and lipid class analysis

The lipid extraction was done with chloroform methanol (2:1) following the Folch method as described by Christie (2003) with some modifications, taking in count the little amount of sample available and using Chloroform: Methanol (2:1) and butylated hydroxytoluene (BHT) as antioxidant. The organic solvent was then evaporated under a stream of nitrogen and the lipid content was gravimetrically determined. To analyze the lipid classes 30 µg of each lipid extract was applied to perform a double high performance thin layer chromatography (HPTLC) (The plate was developed to one-half distance with methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (5 : 5 : 5 : 2 : 1.8, by volume), to separate polar lipid classes, and then fully developed with isohexane/diethyl ether/acetic acid (22.5 : 2.5 : 0.25, by volume), for the neutral lipid separation. Lipid classes were visualized by charring at 160 ºC for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid) following the Olsen & Henderson (1989) method, and the resultant lipid classes bands were quantified by calibrated densitometry using a Camag TLC visualizer and Camag VideoScan TLC/HPTLC evaluation software (Version 1.02.00), to obtain the percentage of each lipid class per sample.

3.3 Statistical analysis

Results are presented as means ± SD. Data were checked for normal distribution with the one-sample Shapiro-Wilk test, as well as for homogeneity with the Levene test (Zar, 1999). Arcsine square root transformation was applied for all data expressed as percentage (Fowler *et al.*, 1998). Correlation matrices were done with the data of the different experiments using the Pearson's correlation, and the p-value was adjusted with the Bonferroni's correction. Differences between octopus paralarvae variables under different treatments in the same experiment and between eggs of different spawnings were tested using Student´s *t*-test (Zar, 1999). Differences between paralarvae or characteristics along the time on the same treatment or spawning were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (Zar, 1999). All statistical analysis was performed using the IBM SPSS statistics 22.0 (IBM Co., USA).

4. RESULTS AND DISCUSSION

4.1 Experiment 1

In the case of the Vigo zoeae experiment (Fig 2A), the biggest difference of DW was found at day 30, when the organisms with the zoeae diet reached 1.80mg, while the control group weighted just 1.16mg. The t-student done proved there is a difference in the DW between treatments at day 20 ($p<0.001$) and 30($p<0.001$). When the Individual Growth Rate (IGR) was compared (Fig 2B), the values range was from 5.08 to 11.48%, and the organisms fed with zoeae got the higher values in the first days post hatching, never getting below the control group, while this last one never registered values over 7.14% and got the lowest values of all the experiment at the last 10 days, of around 5.8%. The survival achieved in this experiment at day 40 was of 65% for the *Artemia* treatment and 90% for the zoeae diet group which is consider really high in both cases (Iglesias & Fuentes 2014).

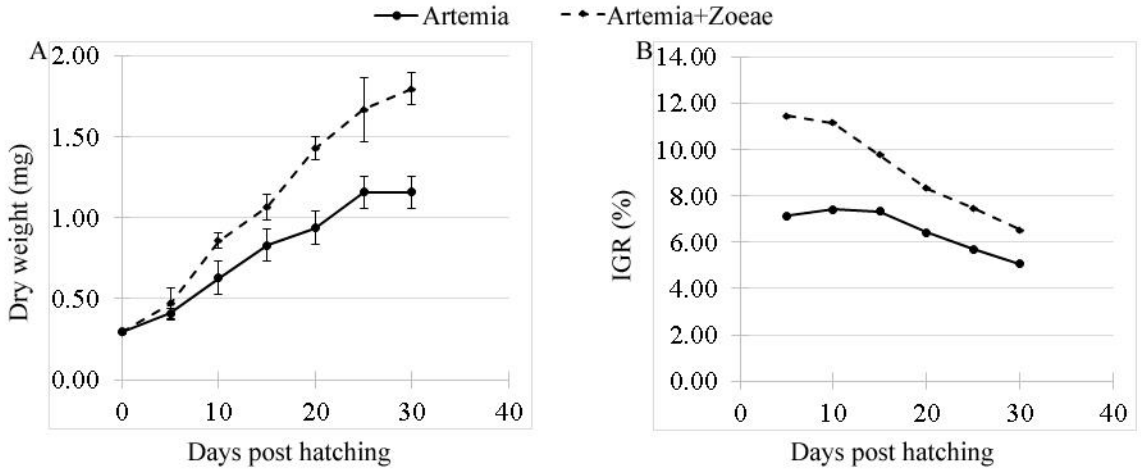

Figure 2. Relationship between Days post hatching and A) Dry weight (mg) and B) IGR (%) at *Artemia*-Zoeae experiment.

At the same zoeae experiment the individual AARSs activity (nmol PPi·ind^{-1}· h^{-1}) (Fig 3A) had values between 4.02 and 31.46nmol PPi·ind^{-1}·h^{-1}, with the activity tending to increase with the increase of growth in the time and with the *Artemia* treatment always having the lowest values. On the other hand, the spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) (Fig 3B) registered increasing values until a maximum of 100.08nmol PPi·mg prot^{-1}·h^{-1} in the specimens fed with the zoeae diet at day 20, but after that, it decreased to 69.10 nmol PPi·mg prot^{-1}·h^{-1} for day 30. Besides this, the *Artemia* group values remained stabilize within the development, with values between 77.59 and 81.68 nmol PPi·mg prot^{-1}·h^{-1} in all essays.

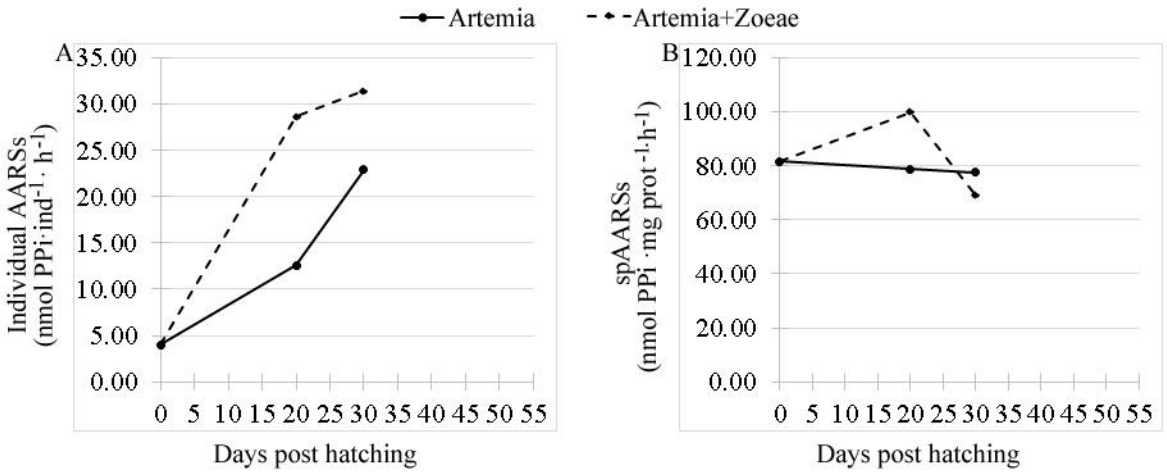

Figure 3. Relationship between days post hatching and A) Individual AARSs (nmol PPi·ind^{-1}· h^{-1}) and B) spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) at *Artemia*-Zoeae experiment.

When the correlation of the variables was analized the data from both treatments had to be taken into account. The dry weight (mg) presented a very good correlation with the individual AARSs activity (nmol PPi·ind^{-1}· h^{-1}) (Fig. 4A), with values of r^2=0.97 and p<0.01, but the correlation of dry weight (mg) and spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) (Fig. 4B) was not significant at all, r^2=-0.40 and p=0.50.

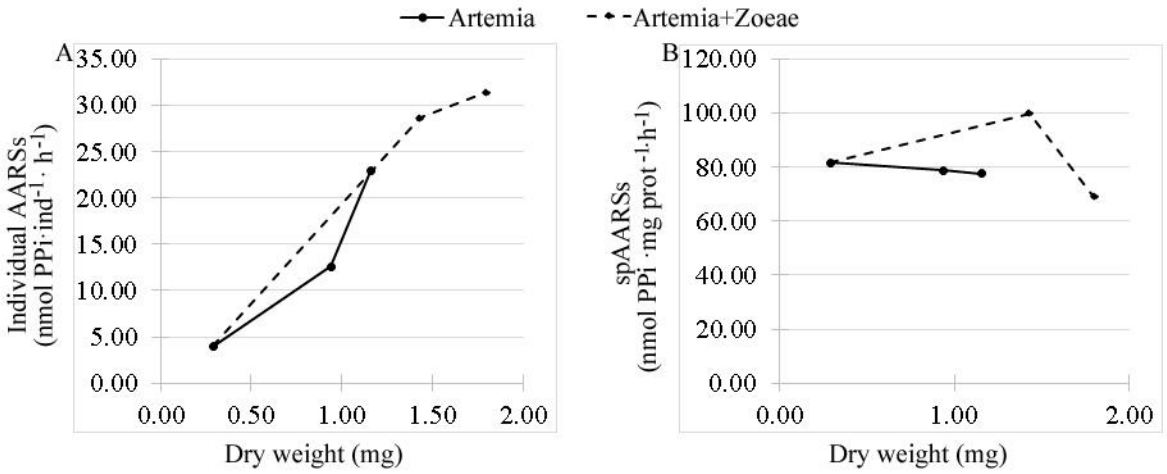

Figure 4. Relationship between Dry weight (mg) and A) Individual AARSs (nmol PPi·ind^{-1}· h^{-1}) and B) spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) at *Artemia*-Zoeae experiment.

The *O. vulgaris* paralarvae lipid classes composition at the *Artemia*-zoeae experiment (Table 2) got cholesterol (CHO) as the main lipid component in paralarvae followed by the phosphatidylethanolamine (PE). Even when statistics could not be done in this *Artemia*-zoeae experiment, cause of having just an n=1 some tendencies can be seen, mostly due to the PE which seems to change a lot between treatments, increasing from 18.58 to 27.12% at the *Artemia* group and decreasing from 18.58 to 12.38% in the zoeae group at day 30. Interestingly the PE increment with age in the *Artemia*-control treatment is accompanied by a decrement of PC and PS

whereas the decrement of PE in the *Artemia*-zoeae experimental treatment is not followed by these evident changes in PC and PS. Among main phospholipids PI tended to increase with age in both treatments. An inverse tendency to that described for PE seems to be followed by the Free Fatty Acids (FFA) which decreases with age in the *Artemia*-control group, reaching just 10.92% at day 30, while the zoeae group displayed the opposite trend and reached 19.40% at the same age. In the other hand, the Triacylglycerols (TG) tended to increase in both treatments with time but at a different rate, with the Artemia group reaching 8.51% at day 30, while the zoeae group got just 3.75 at the same day.

Table 2 Lipid classes composition of paralarvae (% of total lipids) from *Artemia*- Zoeae experiment.

		Artemia		*Artemia* + Zoeae	
Days post hatching	0	20	30	20	30
∑ Polar lipids	**45.40**	**47.05**	**49.76**	**42.70**	**44.53**
Lysophosphatidylcholine	1.87	1.82	1.99	3.81	4.09
Phosphatidylcholine	8.37	7.26	5.86	9.37	8.68
Phosphatidylserine	6.58	4.63	4.81	5.32	6.01
Phosphatidylinositol	2.65	4.39	6.29	5.66	5.75
Phosphatidylglycerol	7.35	5.86	3.68	6.77	7.62
Phosphatidylethanolamine	18.58	23.09	27.12	11.78	12.38
∑ Neutral lipids	**54.60**	**52.95**	**50.24**	**57.30**	**55.47**
Cholesterol	30.08	27.38	24.51	24.46	25.76
Free fatty acids	16.84	16.29	10.92	19.18	19.40
Triacylglycerols	2.48	4.49	8.51	3.42	3.75
Sterol esters	5.19	4.79	6.30	10.23	6.56

A table of correlations was also done to look for any important relationship between protein contents ($mg \cdot ind^{-1}$), and both the AARS activity and DW, and also the lipid classes studied (Table 3). As shown in this table a few significant correlations were found, such as those corresponding to PI, cholesterol or DW with Ind AARSs. However, subsequent Bonferroni's correction let us stablished $p<0.012$ as the significant value, and accordingly none significant correlation was found.

Table 3. Pearson's correlation values for each variable of *Artemia*- zoeae experiment.

	Proteins ($mg \cdot ind^{-1}$)	Ind.AARSs ($nmol\ PPi \cdot ind^{-1} \cdot h^{-1}$)	spAARSs ($nmol\ PPi \cdot mg\ prot^{-1} \cdot h^{-1}$)	Dry Weight (mg)
Polar lipids	-0.207 0.738	-0.263 0.669	-0.489 0.403	-0.256 0.677
Lysophosphatidylcholine	0.795 0.108	0.829 0.082	0.153 0.806	0.817 0.091
Phosphatidylcholine	0.187 0.763	0.203 0.743	0.441 0.458	0.200 0.746
Phosphatidylserine	0.041 0.948	-0.235 0.703	-0.116 0.853	-0.264 0.667
Phosphatidylnositol	0.712 0.177	.904 **0.035**	-0.019 0.976	0.859 0.062

Phosphatidylglycerol	0.159 0.798	-0.043 0.946	0.066 0.916	0.026 0.967
Phosphatidylethanolamine	-0.456 0.440	-0.448 0.449	-0.310 0.611	-0.447 0.450
Neutral lipids	0.202 0.744	0.264 0.668	0.500 0.391	0.255 0.679
Cholesterol	-0.607 0.278	-.892 **0.042**	-0.198 0.750	-0.823 0.087
Free fatty acids	0.298 0.627	0.229 0.711	0.234 0.705	0.301 0.623
Triacylglycerols	0.129 0.836	0.246 0.690	-0.264 0.667	0.190 0.759
Sterol esters	0.308 0.615	0.675 0.211	0.741 0.152	0.536 0.352
Dry Weight (mg)	0.915 **0.029**	0.971 **0.029**	-0.103 0.870	1

The p-value in bold indicates a significance below 0.05

The dry weight was compared with some lipids classes considered particularly important at marine initial development (Monroig *et al.*, 2012; Reis *et al.*, 2015; Sykes *et al.*, 2009), but statistically talking none of them showed a significant correlation. For instance, phosphatidylcholine (PC) got an r^2= 0.20, p=0.74, PE r^2= -0.44, p=0.45 and triacylglycerols (TAG) obtained an r^2= 0.19, p=0.75 as shown at table 3. Despite this, and at a graphical level (Fig. 5), we can see the different behavior of these lipids depending of the diet the organisms are fed with, and the complete opposite happening at the *Artemia* diet, except for TAG, which get a lot higher with the *Artemia* treatment and just a little higher with zoeae.

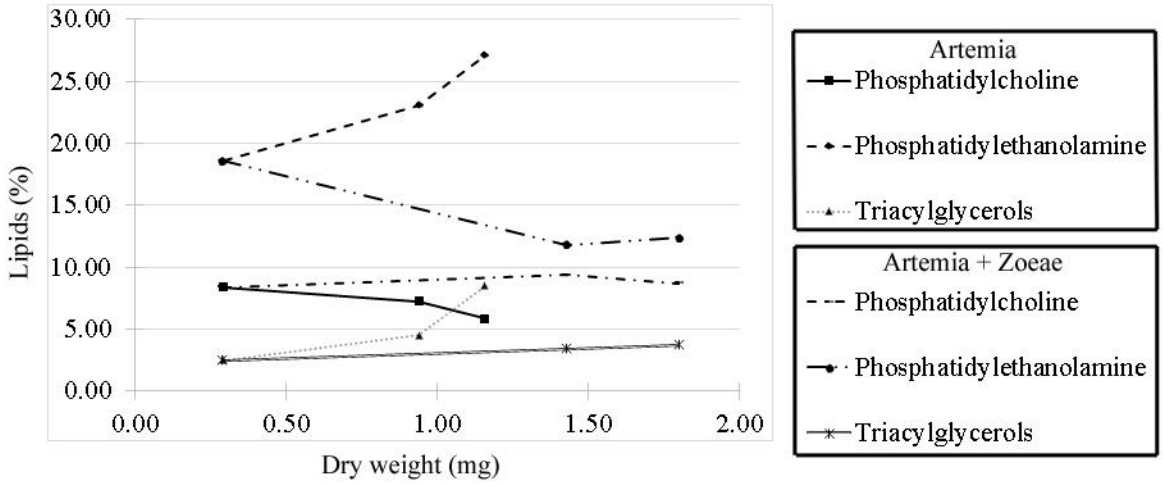

Figure 2. Relationship between dry weight (mg) and lipid classes (%) in the paralarvae from *Artemia*- Zoeae experiment.

Even when Bonferroni was not accomplished by any correlation performed with lipid classes, the individual AARSs activity (nmol PPi·ind^{-1}· h^{-1}) got the most significant correlations with 2 of the lipid classes studied. The first was phosphatidylinositol (PI) (Fig. 6A) with which it reached a correlation of r^2=0.904 and a p=0.03, showing a positive mutual increasing; and the second one was CHO (Fig. 6B), where the correlation was of r^2= -.892 and p= 0.042, showing a tendency to decrease while the individual AARSs (nmol PPi·ind^{-1}· h^{-1}) increase.

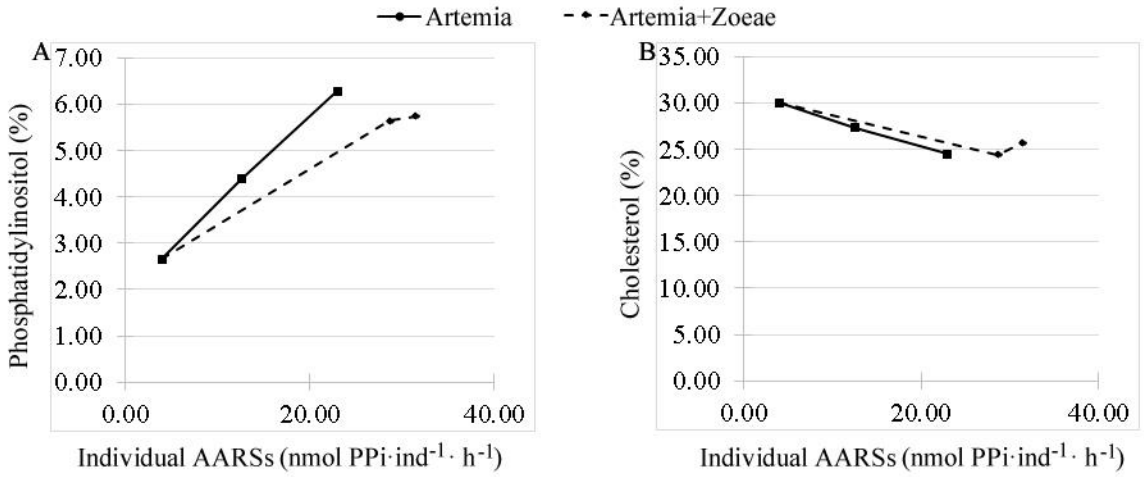

Figure 6. Relationship between Individual AARS (nmol PPi·ind^{-1}· h^{-1}) and A) Phosphatidylinositol (%) and B) Cholesterol (%) at *Artemia*-Zoeae experiment.

The little growth achieved by the paralarvae fed with *Artemia* could be explained taking in count that *Artemia* is already known as an inadequate diet for the paralarvae. This because it generally has low polar lipid levels and a poor incorporation of DHA, tending to accumulate this essential fatty acid into TAG and not into phospholipids (Navarro & Villanueva, 2000; 2003; Reis, 2016) which is in contrast with the known little capacity of the octopus to assimilate fatty acids coming from the *Artemia* TAG (Reis *et al.*, 2015). In spite of the *Artemia* used in the present experiment seems to help maintaining paralarvae total polar lipid contents, the poor incorporation of DHA in the *Artemia* is probably an important cause for the poor growth achieved by specimens fed this control diet. At the same time, the high growth in paralarvae fed with the zoeae diet is alike the results of a lot of experiments done before (Carrasco *et al.*, 2003; Iglesias *et al.*, 2004; Villanueva, 1994) where paralarvae fed partially or totally with zoeae could even be reared until the juvenile state. A good balance of reach DHA phospholipids has also been pointed out as good reasons to explain this better growth.

A high correlation was also found between the dry weight and individual AARS activity (nmol PPi·ind^{-1}· h^{-1}) with values of r^2=0.97 and p<0.01, even with the little amount of data available. This could be due to the bigger size the paralarvae presented at Vigo facilities from hatching and to the good development they achieved thanks to the diet given. These results can mean that the individual AARS activity (nmol PPi·ind^{-1}· h^{-1}) could be related with the size of the organisms and open the possibility to use this variable as an alternative to know the size of the organisms instead of the DW but more studies must be done to ensure this assertment.

The lipid class composition found in the paralarvae from this experiment was generally similar to that previously reported for *O. vulgaris* paralarvae (Reis, 2016; Navarro and Villanueva, 2000, 2003) and confirms the importance of preys rich in phospholipids and cholesterol, and with moderate content of neutral lipids (NL) as suggested by Navarro & Villanueva (2000, 2003) and Navarro *et al.* (2014). Even when statistically analysis could not be done, the zoeae diet seems to allow the octopus to have a more balanced polar lipids composition, where the percentage of phosphatidylethalonamine decreases but the other polar lipids are able to increase or to be maintained stable. Meanwhile, the neutral lipid fraction and particularly TAG poorly increases resembling the low contents of TAG in wild paralarvae (Navarro & Villanueva, 2003). The free fatty acids are always higher with the zoeae diet, which could be part of the reasons this diet is so good for the paralarvae. This fact denoting a more active catabolism of lipids which seems to match well with the decrement of PE and the increments of PI or the maintenance of other important phospholipids such as PC or PS with growth, but the implications of these changes in the lipid classes composition should be studied further.

The correlation found between individual AARSs activity (nmol $PPi \cdot ind^{-1} \cdot h^{-1}$) and phosphatidylinositol (PI) and cholesterol (CHO), even when was not statistically significant after applying the Bonferroni's correction, may denote their importance of these lipid classes for a good growth and a good physiological status of the organisms. The relation of AARS with PI could be due to the importance of this phospholipid for its implication in different metabolic processes of transduction and in osmoregulation processes (Tocher, 2003) also being a reservoir of important fatty acids such as arachidonic acid (20:4n-6) (Rodriguez *et al.*, 2009). The PI is also a precursor of two biology important messengers, that control important cellular processes as the insulin secretion (Lodish *et al.*, 2002; Bell y Sargent, 2003) and in fish, higher survivals and less deformity rates have been achieved when PI is added to their diet (Geurden *et al.*, 1997, 1998; Cahu *et al.*, 2003).

4.2 Experiment 2

The experiment performed to test the Inert diet registered little differences in terms of dry weight and IGR between the control and the inert diet groups. As occurred in the zoeae experiment, the experimental group (*Artemia*+ inert diet) was able to grow better, reaching 0.38mg DW (Fig 7A) while the control group weighted just 0.22mg DW at day 14. According to the t-student done this difference was significant, p=0.014. On the other hand, the IGR (Fig 7B) of all the organisms was between 1.11 and 4.29%, but all the values of those ones fed with Inert diet were higher, always above 3.10%, while the control group highest value was 2.84%. The survival reached at day 14 was a of 30.04%±9.09 for the group with the *Artemia* diet and 31.21%±17.61 for the inert diet group, being this consider normal because the massive mortalities detected in most of the studies started as early as 15-20 days post hatching (Iglesias & Fuentes, 2014).

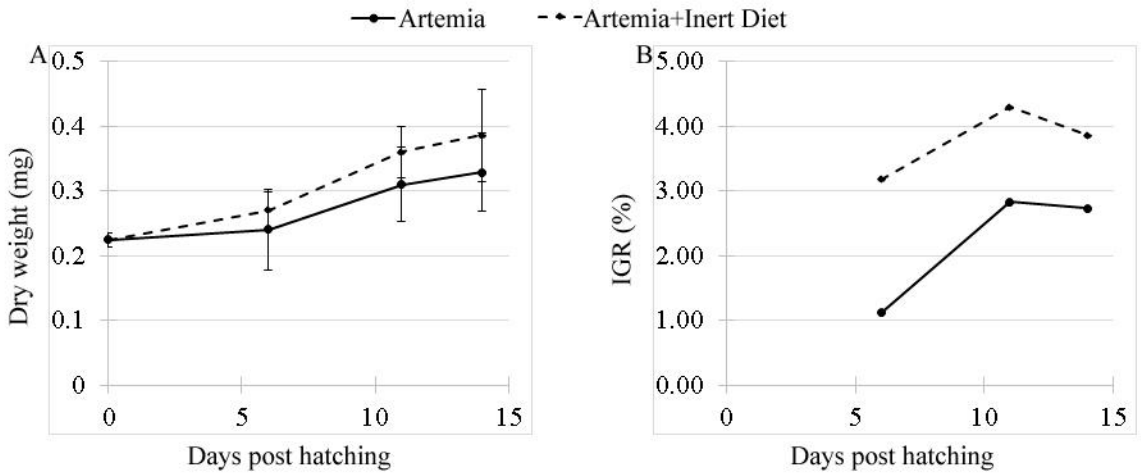

Figure 7. Relationship between Days post hatching and A) Dry Weight (mg) and B) IGR (%) at *Artemia*-Inert diet experiment.

In this experiment, the values of individual AARSs activity (nmol PPi·ind^{-1}· h^{-1}) (Fig 8A) were between 5.59 and 13.45nmol PPi·ind^{-1}· h^{-1}, with little differences between treatments. Still the individuals fed with Inert diet had higher values at day 6 and 11 but without a significant difference from the control group. When comparing the spAARSs activity (nmol PPi·mg prot^{-1}·h^{-1}) (Fig 8B), the values were between 69.06 and 133.44 nmol PPi·mg prot^{-1}·h^{-1}; and although the differences were not significant, the individuals fed just with *Artemia* got higher values that the ones with Inert diet at days 11 and 14. Anyway, the paralarvae with the Inert diet reached the highest value of spAARSs at the 6th day post hatching, with 133.44nmol PPi·mg prot^{-1}·h^{-1}.

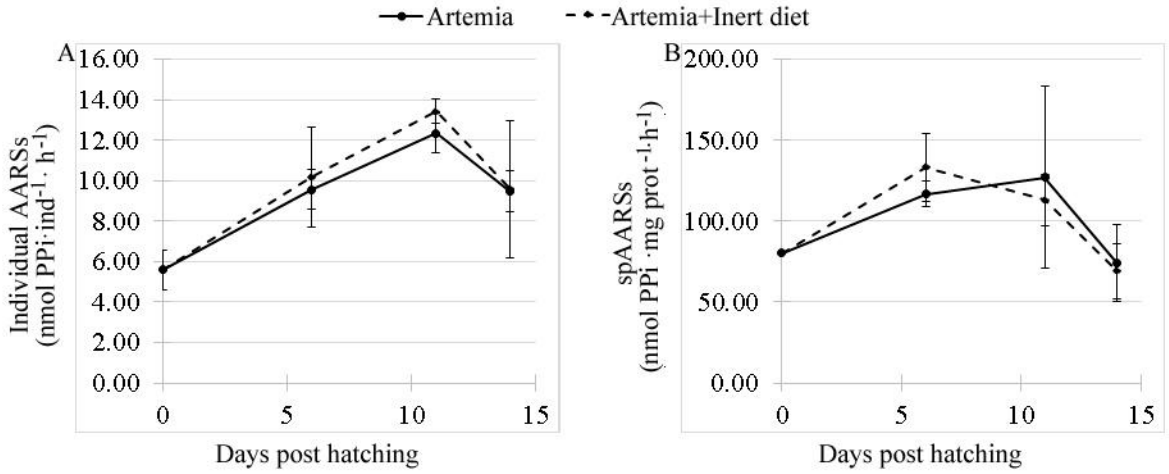

Figure 8. Relationship between days post hatching and A) Individual AARSs (nmol PPi·ind^{-1}· h^{-1}) and B) spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) at *Artemia*-Inert diet experiment.

The dry weight (mg) correlation with AARS activity was not significant in any case; using all the data together, individual AARSs (nmol PPi·ind^{-1}· h^{-1}) got r^2=0.255 and p=0.29, while the spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) correlation was r^2= -0.422 and p=0.07. When the data was divided per treatment, the individual AARSs (nmol PPi·ind^{-1}· h^{-1}) (Fig. 9A) of *Artemia* group got

an r^2= 0.26 and p=0.46 and the organisms fed with the Inert diet got r^2= 0.40 and p=0.25. In the case of spAARSs (nmol PPi·mg $prot^{-1}·h^{-1}$) (Fig. 9B) the *Artemia* group obtained an r^2= -0.332 and p=0.34, while the Inert diet group got r^2=-0.40 and p=0.24.

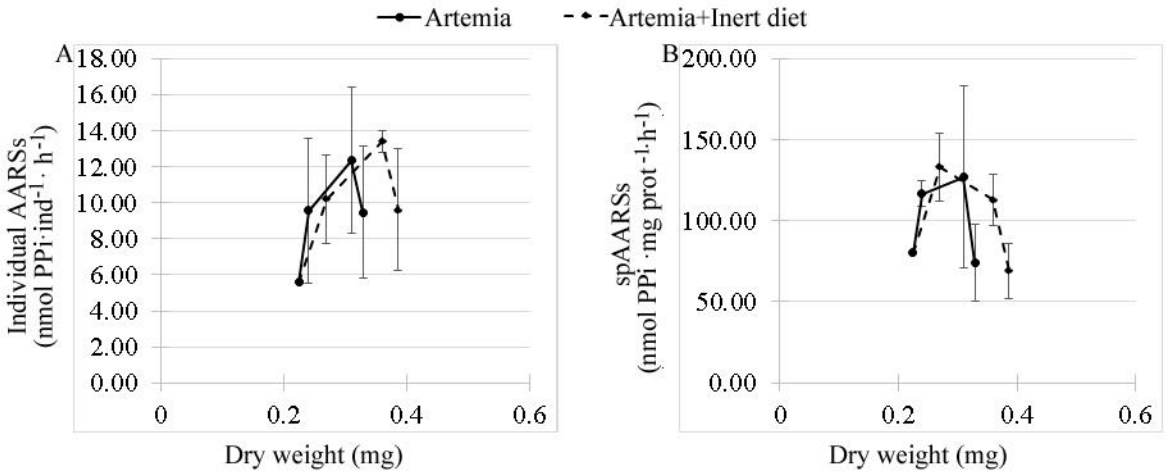

Figure 3. Relationship between Dry weight (mg) and A) Individual AARSs (nmol PPi·ind^{-1}· h^{-1}) and B) spAARSs (nmol PPi·mg $prot^{-1}·h^{-1}$) at *Artemia*-Inert diet experiment.

The *O. vulgaris* paralarvae lipid classes composition at Inert diet experiment (Table 4) resulted in CHO being the main lipid component followed by PE and PC. When the lipid classes composition was compared between the *Artemia* and the Inert diet group, some differences were found between treatments. At day 6 the unique difference found was a lower content of free fatty acids (FFA) p<0.01 in the Inert diet fed paralarvae whereas at day 14 the differences were found in the higher contents of PC (p<0.01) and PI (p=0.01) in paralarvae from this treatment. About the existing differences within the same treatment, in the *Artemia* group the only one was with the observed increment of sterol esters (SE) with time while the *Artemia* + Inert diet showed more differences between lipids in the different days. This is the case of the increasing trends of PC, and the opposite trends observed for phosphatidylglycerol (PG), and the total neutral lipid (NL) fraction as a whole.

Table 3. Lipid classes composition of paralarvae (% of total lipids) from *Artemia*- Inert diet experiment.

	Artemia			*Artemia* + Inert diet		
Days post hatching	0	6	11	14	6	11	14
∑ Polar lipids	**43.11**	**39.95 ± 1.50**	**38.18 ± 2.31**	**39.66 ± 2.47**	**39.68 ± 1.28a**	**41.16 ± 2.46ab**	**44.59 ± 0.51b**
Lysophosphatidylcholine	0.66	2.14 ± 1.50	1.683 ± 1.15	1.32 ± 0.49	1.48 ± 0.92	0.62 ± 0.25	0.87 ± 0.36
Phosphatidylcholine	11.09	10.01 ± 2.05	9.67 ± 1.86	10.50 ± 1.05	9.95 ± 0.74^{a}	10.93 ± 1.44^{a}	14.65 ± 0.93^{b}*
Phosphatidylserine	4.64	5.04 ± 0.68	4.83 ± 0.65	4.45 ± 0.54	4.44 ± 0.59	4.30 ± 0.53	4.56 ± 0.48
Phosphatidylinositol	7.37	5.37 ± 2.57	6.03 ± 0.37	5.67 ± 0.72	6.54 ± 1.02	6.47 ± 1.52	7.64 ± 0.41*
Phosphatidylglycerol	1.2	3.48 ± 1.47	2.76 ± 0.37	2.73 ± 0.80	2.77 ± 0.39^{b}	2.64 ± 0.32^{b}	1.74 ± 0.30^{a}
Phosphatidylethanolamine	18.15	13.91 ± 2.78	13.21 ± 3.63	14.99 ± 2.87	14.51 ± 0.71	16.19 ± 0.23	15.13 ± 1.41
∑ Neutral lipids	**19.15**	**60.05 ± 1.50**	**61.82 ± 2.31**	**60.34 ± 2.47**	**60.32 ± 1.28^{b}**	**58.84 ± 2.46^{ab}**	**55.41 ± 0.51^{a}**
Cholesterol	38.56	33.80 ± 4.27	34.68 ± 1.42	31.97 ± 2.02	35.66 ±2.15	35.78 ± 5.14	33.07 ± 0.51
Free fatty acids	8.61	9.73 ± 0.79^{c}	8.00 ± 0.42^{b}	6.14 ± 0.83^{a}	7.22 ± 0.49*	5.17 ± 2.93	5.21 ± 1.39
Triacylglycerols	5.1	6.49 ± 3.51	5.93 ± 2.15	6.25 ± 3.06	5.82 ± 2.20	6.05 ± 0.50	5.29 ± 1.72
Sterol esters	4.62	10.04 ± 1.66^{a}	13.20 ± 1.82^{ab}	15.98 ± 2.50^{b}	11.62 ± 1.80	11.84 ± 1.91	11.84 ± 1.39

Data are represented as means ± SD (n=3). * Represents differences between *Artemia* and Inert diet groups at the same days. Different letters in superscript within the same row represent significant differences within different ages at the same treatment ($p<0.05$).

A table of correlations was done to look for any important relationship between proteins (mg·ind-1), both measures of AARS activity and DW, and all the lipid classes studies (Table 5). As mentioned for Experiment 1, the Bonferroni's correction let us stablished $p<0.012$ as the significant value; and therefore, the only significant one was found between the dry weight (mg) and the protein contents ($mg \cdot ind^{-1}$).

Table 4. Pearson's correlation values for variables of Inert diet experiment.

	Proteins ($mg \cdot ind^{-1}$)	Ind.AARSs ($nmol\ PPi \cdot ind^{-1} \cdot h^{-1}$)	spAARSs ($nmol\ PPi \cdot mg\ prot^{-1} \cdot h^{-1}$)	Dry Weight (%)
Polar lipids	-0.332	-0.359	-0.169	-0.359
	0.164	0.131	0.488	0.131
Lysophosphatidylcholine	-0.343	-0.355	-0.160	-0.374
	0.150	0.135	0.514	0.114
Phosphatidylcholine	-0.331	-0.361	-0.172	-0.357
	0.167	0.129	0.482	0.133
Phosphatidylserine	-0.335	-0.361	-0.169	-0.361
	0.161	0.128	0.489	0.128
Phosphatidylnositol	-0.333	-0.361	-0.170	-0.359
	0.164	0.129	0.486	0.131
Phosphatidylglycerol	-0.345	-0.355	-0.156	-0.373
	0.148	0.136	0.524	0.115
Phosphatidylethanolamine	-0.331	-0.357	-0.168	-0.358
	0.166	0.133	0.491	0.132
Neutral lipids	-0.338	-0.358	-0.165	-0.364
	0.157	0.132	0.500	0.126
Cholesterol	-0.335	-0.359	-0.167	-0.360
	0.161	0.131	0.494	0.130
Free fatty acids	-0.341	-0.362	-0.166	-0.367
	0.154	0.127	0.498	0.123
Triacylglycerols	-0.332	-0.354	-0.166	-0.361
	0.165	0.137	0.496	0.129
Sterol esters	-0.327	-0.354	-0.170	-0.358
	0.172	0.137	0.486	0.133
Dry Weight (mg)	.859	0.255	-0.422	1
	0.000	0.292	0.072	

The p-value in bold indicates a significance below 0.05 and the ones in bold and underlined indicate significance after Bonferroni correction.

The dry weight (mg) did not show any correlation with PC, PE or TAG (Fig. 10) using the data corresponding to both treatments (values given in Table 5).

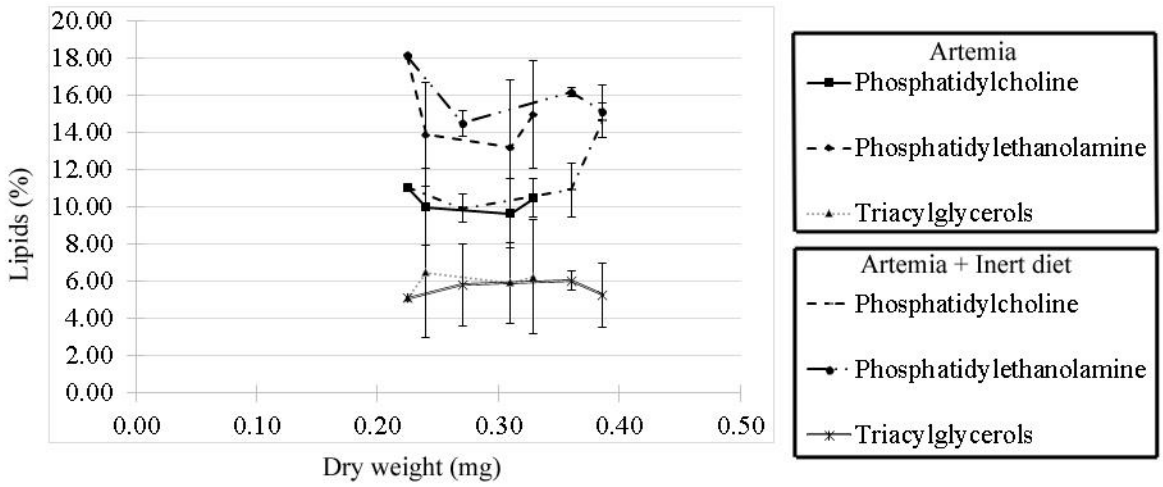

Figure 4. Relationship between Dry Weight (mg) and Lipids (%) at *Artemia*- Inert diet experiment.

Just as in the zoeae experiment, the lowest growth found was in the organisms with the *Artemia* diet, this possibly partly due to the low polar lipid levels and poor contents of DHA of this prey (Navarro & Villanueva, 2000; 2003). In this experiment, even when it was the first time that spherized particles of crustacea were used as inert diet for the octopus paralarvae it helped the organisms to get a better growth, although in a smaller scale than the zoeae. To know the actual suitability of this inert diet more experiments are needed.

Even when it was not statistically significant some correlation can be seen between the dry weight and individual AARS activity *(*nmol PPi·ind^{-1}· h^{-1}), in fact we think that the correlation is not so clear because of the dry weight difference being so little in comparison with the zoeae experiment. Still this tendency of the individual AARS activity to increase along with the dry weight may support the possibility of using this activity as a way to measure the size of the organisms, although further experiments focused on this must be done.

The decrease of individual AARS activity at 14 days-old in both treatments as well as the decrease in the IGR values could be caused by an impairment in the metabolic process prior to a massive mortality event. Morales *et al.* (2017) observed an imbalance in the paralarval metabolism (related with the redox status and other enzymatic activities such as Glicerol Kinase) prior to massive mortalities even at 12 days-old. The low survival (30%) at 14 days-old obtained in these experiments and the drop in individual AARS activity could point out a similar imbalance in the paralarval metabolic status. In that case, AARS activity could be used as metabolic or welfare biomarker, although further studies are necessary to confirm this hypothesis.

The lipid classes composition of the paralarvae in consistent to that obtained in the zoeae experiment and in other studies (Reis, 2016; Navarro and Villanueva, 2000, 2003). Interestingly the inert diet allowed the paralarvae to gain higher percentages of phosphatidylcholine (PC) and phosphatidylinositol at 14 days post-hatching. This could be seen as an advantage, taking in count that it is believed that the PC has great influence in growth and survival of fish paralarvae, even above other lipid classes (Rodriguez *et al.*, 2009). Even when PC was not the most abundant phospholipid, it may have had influence at the growth and survival of the paralarvae, by increasing the incorporation of important structural lipids in the paralarvae tissues.

4.3 Experiment 3

In this case, the egg stage was used as a growth marker to be related with AARS activity, showing a good correlation at both kinds of activity. In the case of individual AARSs *(*nmol PPi·ind^{-1}· h^{-1}) (Fig. 11A) it got values of r^2= 0.84 and p<0.01, while the spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) (Fig. 11B) obtained values of r^2= 0.80 and p<0.01.

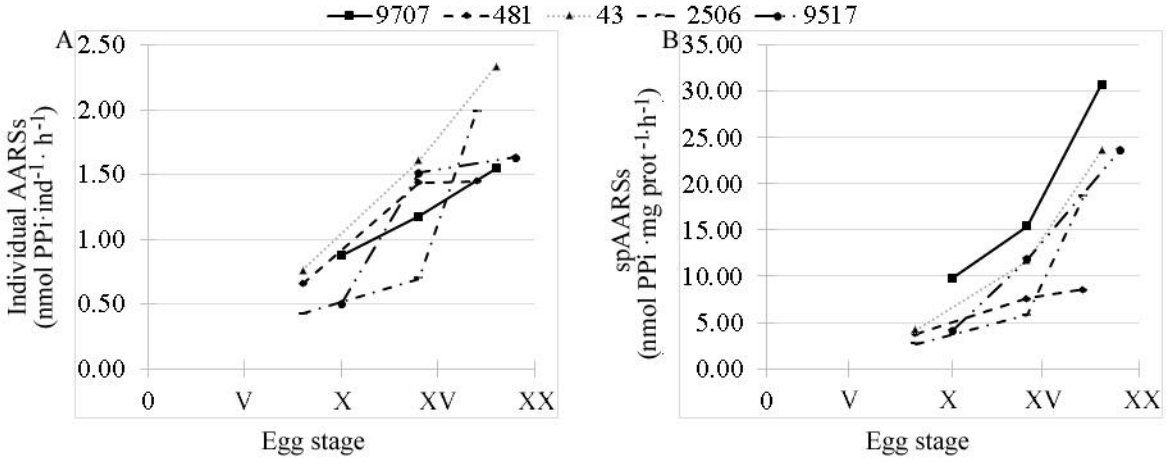

Figure 51. Relationship between Egg stage and A) Individual AARSs (nmol PPi·ind^{-1}· h^{-1}) and B) spAARSs (nmol PPi·mg prot^{-1}·h^{-1}) in eggs.

The general *O. vulgaris* eggs lipid class composition got cholesterol (CHO) as the main lipid component followed by phosphatidylcholine (PC) and phosphatidylethanolamine (PE). When the lipid class composition was compared between the different spawnings (Table 6) almost all the classes showed differences, and formed different groups, the only exceptions being for lysophosphatidylcholine (LPC), phosphatidylglycerol (PG) and sterol esters (SE). In particular, the lipid classes profile of the spawning of the female 9517, which had the lowest temperature (20.69 ± 0.61 °C) and a shorter mother's captivity time, tended to be separated from the rest of spawnings. This was particularly evident in the higher contents of PC, phosphatidylserine, and phosphatidylinositol, and the lower ones of PE, and TAG.

Table 6. Lipid classes composition (% of total lipids) of *O. vulgaris* eggs obtained from different spawnings.

Chip	**9517**	**2506**	**43**	**481**	**9707**
∑ Polar lipids	**55.25 ± 0.01^{c}**	**31.35 ± 0.91^{ab}**	**28.75 ± 1.80 ^{a}**	**42.18 ± 10.10^{b}**	**36.34 ± 4.40^{ab}**
Lysophosphatidylcholine	2.21 ± 0.01	2.96 ± 1.71	2.37 ± 0.68	3.16 ± 1.53	2.20 ± 1.30
Phosphatidylcholine	30.08 ± 0.03^{c}	9.52 ± 2.64^{ab}	6.63 ± 1.18 ^{a}	14.34 ± 3.30^{b}	13.78 ± 1.46^{b}
Phosphatidylserine	5.69 ± 0.01^{b}	2.05 ± 0.93 ^{a}	1.67 ± 0.30 ^{a}	3.34 ± 0.96 ^{a}	2.59 ± 1.02^{a}
Phosphatidylinositol	6.39 ± 0.01^{b}	2.10 ± 0.88 ^{a}	1.37 ± 0.79 ^{a}	2.23 ± 0.83 ^{a}	3.13 ± 2.46 ^{a}
Phosphatidylglycerol	0.59 ± .01	1.21 ± 0.19	1.04 ± 0.36	3.33 ± 3.73	2.32 ± 1.56
Phosphatidylethanolamine	10.29 ± 0.00^{a}	13.51 ± 1.03^{bc}	15.67 ± 1.24^{c}	15.78 ± 0.93^{c}	12.31 ± 0.99^{b}
∑ Neutral lipids	**44.75 ± 0.01^{a}**	**68.65 ± 0.91^{bc}**	**71.25 ± 1.80^{c}**	**57.82 ± 10.10^{b}**	**63.66 ± 4.40^{bc}**
Cholesterol	30.37 ± 0.01^{a}	45.23 ± 3.11^{ab}	48.20 ± 0.68^{b}	42.28 ± 11.80^{ab}	43.97 ± 3.32^{ab}
Free fatty acids	6.94 ± 0.01^{c}	3.75 ± 0.72^{b}	2.06 ± 0.10 ^{a}	1.84 ± 0.09^{a}	4.10 ± 0.48^{b}

Triacylglycerols	3.94 ± 0.01[a]	13.23 ± 1.67[bc]	15.33 ± 1.95[c]	10.92 ± 2.52[b]	11.72 ± 0.99[bc]
Sterol esters	3.51 ± 0.00	6.44 ± 1.77	5.66 ± 0.49	2.79 ± 1.58	3.88 ± 2.08

Data are represented as means ± SD (n=3). Different letters in superscript within the same row represent significant differences ($p<0.05$).

When the spawning lipids composition was compared taking in count the egg stage (Table 7) no differences were found denoting the great variability between the different spawning mentioned before (Table 6).

Table 7. Lipid classes composition (% of total lipids) from *O. vulgaris* eggs at different egg stages.

Egg stage	**X**	**XIV**	**XIX**
∑ **Polar lipids**	**36.62 ± 11.03**	**39.27 ± 15.08**	**39.28 ± 12.55**
Lysophosphatidylcholine	2.65 ± 0.86	1.37 ± 0.48	3.22 ± 1.87
Phosphatidylcholine	14.04 ± 9.39	16.63 ± 11.33	12.76 ± 5.41
Phosphatidylserine	2.97 ± 1.81	3.93 ± 3.25	2.72 ± 1.53
Phosphatidylinositol	2.59 ± 2.20	3.69 ± 4.17	2.57 ± 0.90
Phosphatidylglycerol	1.09 ± 0.43	1.00 ± 0.37	4.27 ± 3.23
Phosphatidylethanolamine	13.28 ± 1.97	12.64 ± 3.27	13.73 ± 2.72
∑ **Neutral lipids**	**63.338 ± 11.03**	**60.73 ± 15.08**	**60.72 ± 12.55**
Cholesterol	42.00 ± 6.94	42.07 ± 10.52	40.38 ± 10.05
Free fatty acids	3.96 ± 2.09	3.47 ± 1.70	3.26 ± 1.34
Triacylglycerols	12.31 ± 5.01	10.88 ± 5.51	11.59 ± 1.11
Sterol esters	5.12 ± 1.84	4.32 ± 1.11	5.50 ± 1.69

Data are represented as means ± SD (n=5). Different letters in superscript within the same row represent significant differences ($p<0.05$).

A table of correlations was done for the eggs, grouping the data obtained for each spawning. Taking this in count the only variables that showed a significant correlation were the proteins ($mg \cdot ind^{-1}$) with two of the lipid classes (PE and FFA) (Table 8), but after the Bonferroni's correction, which stablished $p<0.007$ as the significant value, none significant differences were detected. When data were grouped accordingly to the females that laid the eggs, a lot of significant correlations were found between time of captivity before laying the eggs and the lipids as, the total PL fraction, PC, PS, PI, PE, NL, CHO, FFA, TAG and SE. Other variable that seems to clearly affect the lipid classes composition of the eggs is the temperature (°C), which in this case got a correlation with the captivity time of $r^2= 0.81$ and $p<0.01$, and which showed a significant correlation with all the same lipids that the captivity time did. The last variable checked was the female initial weight when they arrived at the institute, but this got only a significant correlation with PE and FFA.

Table 8. Pearson's correlation values for variables of eggs.

					Mother's characteristics	
	Proteins ($mg \cdot ind^{-1}$)	Ind.AARSs (nmol $PPi \cdot ind^{-1} \cdot h^{-1}$)	spAARSs (nmol $PPi \cdot mg\ prot^{-1} \cdot h^{-1}$)	Temp. (°C)	Captivity time (days)	Initial weight (kg)
Temp. (°C)	0.209	-0.034	-0.133	1	.814	0.322
	0.454	0.903	0.636		**0.000**	0.241
Polar lipids	-0.089	0.050	0.090	-.838	-.872	-0.088
	0.751	0.860	0.751	**0.000**	**0.000**	0.756

Lysophosphatidylcholine	0.279	0.172	-0.074	0.285	0.171	0.346
	0.313	0.539	0.794	0.302	0.541	0.207
Phosphatidylcholine	-0.172	-0.138	-0.008	-.879	-.927	-0.227
	0.541	0.625	0.978	**0.000**	**0.000**	0.415
Phosphatidylserine	-0.181	0.076	0.120	-.861	-.855	-0.259
	0.519	0.788	0.670	**0.000**	**0.000**	0.351
Phosphatidylnositol	-0.409	0.193	0.399	-.798	-.810	-0.400
	0.130	0.491	0.141	**0.000**	**0.000**	0.139
Phosphatidylglycerol	0.091	0.138	0.085	-0.067	-0.149	0.407
	0.747	0.625	0.764	0.812	0.597	0.133
Phosphatidylethanolamine	.523	0.198	-0.121	.629	.752	.535
	0.045	0.480	0.667	**0.012**	**0.001**	**0.040**
Neutral lipids	0.104	-0.051	-0.102	.833	.881	0.060
	0.712	0.856	0.717	**0.000**	**0.000**	0.832
Cholesterol	0.064	0.028	-0.032	.703	.749	0.210
	0.820	0.920	0.910	**0.003**	**0.001**	0.453
Free fatty acids	-.528	-0.207	0.123	-.603	-.737	-.578
	0.043	0.458	0.662	**0.017**	**0.002**	**0.024**
Triacylglycerols	0.299	-0.071	-0.150	.833	.911	0.183
	0.280	0.801	0.593	**0.000**	**0.000**	0.513
Sterol esters	0.106	-0.052	-0.194	.529	.520	-0.321
	0.706	0.855	0.489	**0.043**	**0.047**	0.243

The p-value in bold indicates a significance below 0.05 and the ones in bold and underlined indicates significance after Bonferroni correction.

Other data that were found to have an interesting correlation, even when not satisfying Bonferroni's correction were both, the proteins (mg $\cdot ind^{-1}$) and the lipid class PE (r^2= 0.52, p= 0.04); and the proteins (mg $\cdot ind^{-1}$) with FFA (r^2= -0.52, p=0.04) as shown in Figure 12.

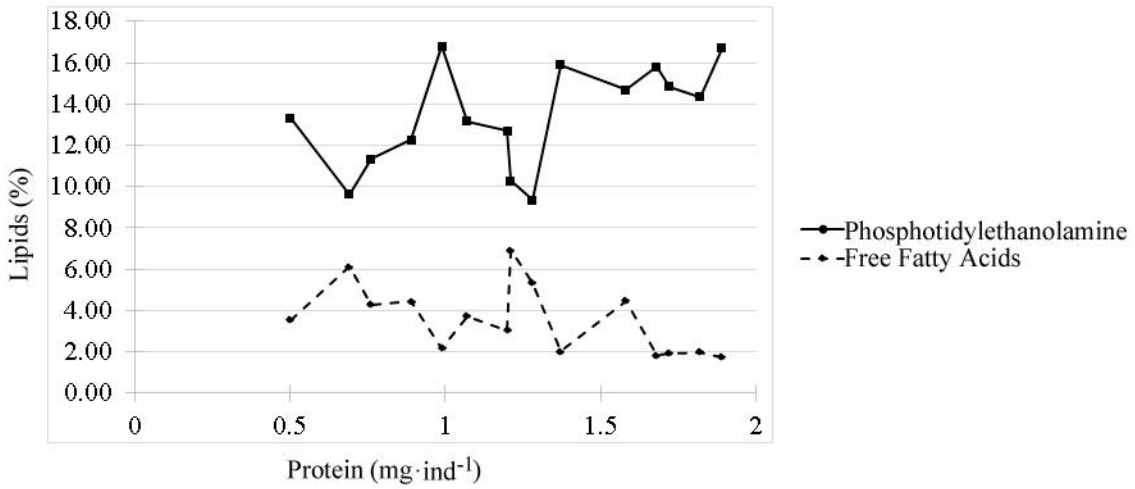

Figure 62. Relationship between Protein (mg ·ind^{-1}) and Lipids (%) in eggs.

When the egg stage was correlated with the AARS activity a good correlation was found with both measures of activity, individual AARSs (nmol PPi·ind^{-1}· h^{-1}) and spAARSs activity (nmol PPi·mg prot^{-1}·h^{-1}). This could be because, in contrast with paralarvae, the eggs just develop and growth, without getting affect by lack of food, being this last point a confirmed factor that affects the AARS activity in zooplankton (Herrera *et al.*, 2012).

In the case of the lipid class composition, and as seen in the paralarvae of the other experiments, CHO was the most abundant lipid. In spite of the differences regarding if PE is the second most abundant lipid class, followed by PC, or the opposite, the general composition of the eggs is quite similar to the one found in *O. vulgaris* paralarvae (Reis, 2016; Navarro and Villanueva, 2000, 2003) and *S. officinalis* eggs (Sykes *et al.*, 2009). The high phospholipid content of the eggs could mean that they use the reservoir of PL for a next structural use at the paralarvae phase (Rodriguez *et al.*, 2009). The high amount of PE could be due to this DHA-rich phospholipid has been found as very abundant in the fishes' retina and brain, keeping its balance between fluency and rigidity (Rodriguez *et al.*, 2009). The same could also happen in the octopus eggs, when the eyes and the brain represent a high percent of their biomass and cognitive vision and predation strategies will become essential. In similar ways, the correlation of proteins with PE in the eggs could be explained because both of them are important components in most of the embryonic and paralarval tissues (Rodriguez *et al.*, 2009).The present experiment was not aiming at proving the effect of temperature in the eggs lipid class composition, but as it has been proved, the temperature can influence the time the eggs spend to develop, and may also cause lower weight in the paralarvae that hatch from eggs developed at high temperatures (18°C) (Nande *et al.*, 2016). In this study the eggs developed at a range between 19.9°C and 21.04°C proving that even small changes of temperature can affect the eggs lipid composition. But a study done by Reis *et al.* (2013) did not found differences in lipid composition of eggs reared at different temperatures (16, 19, 23°C) so more studies focusing on this topic are necessary.

About the influence the mother can have in the eggs quality, a study done by Farias *et al.* (2011) with the red octopus (*Enteroctopus megalocyathus*), proved the effect of different diets in the females fecundity. However, this diet was proved for 3 months before the egg laying, whereas in our case just one female was fed so long with the diet provided at the IEO. Also, a study done by

Quintana *et al.* (2015), proved that the diet of the females of *O. vulgaris* greatly influences the protein content and lipid composition of eggs and hatchlings. No studies have been performed to elucidate how long does it take for the females to get the benefits from a diet and pass them to their offspring and how the weight of the female can affect, but the results of this work aims to the possibility of these being new variables to take in count for new essays.

5. CONCLUSIONS

1. This thesis provides the first approach to the use of AARS activity and lipid class composition as biomarker of the condition and growth of *Octopus vulgaris* eggs and paralarvae. Being possible tools to improve the rearing and control the growth of this species.

2. The data obtained suggest a correlation between the individual AARS activity and dry weight, which point out the use of AARS activity as growth biomarker. However further studies are necessary to confirm this relationship.

3. The lipid class composition found was similar to the ones registered before for the species, confirming the importance of some lipids such as cholesterol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol for the correct development of the organisms; which must be taken in count when the diet of the organisms is planned.

4. The lack of correlation between AARS activity and lipid classes composition, suggest that other is not metabolic linkage between both parameters, however further studies are needed to discard it.

5. The high correlation found between the eggs lipid composition and the female's captivity period as well as the environmental temperature suggest a relevant effect of the maternal conditions in the eggs and paralarvae condition.

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