Tesis Doctoral

The use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

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Anexo I

D. Jose Manuel Vergara Martín SECRETARIO DEL DEPARTAMENTO DE BIOLOGÍA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA,

Que el Consejo de Doctores del Departamento en su sesión extraordinaria tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada **"**The use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates" (Uso de la actividad AARS como índice de crecimiento para zooplancton e ictioplancton) presentada por la doctoranda Dña. Inmaculada Herrera Rivero y dirigida por los Doctores D. Santiago Hernández-León y Dña. Lidia Yebra Mora.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del Reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a 13 de Junio de dos mil catorce.

Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

Programa de Doctorado en Oceanografía

Bienio 2006/2008 con Mención de Calidad de la ANECA.

Título de la tesis:

"The use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates"

(Uso de la actividad AARS como índice de crecimiento para zooplancton e ictioplancton).

Tesis Doctoral presentada por Dña. Inmaculada Herrera Rivero para obtener el grado de Doctor por la Universidad de Las Palmas de Gran Canaria.

Dirigida por Dr. D. Santiago Hernández León

Dra. Dña. Lidia Yebra Mora

El/la Director/a **El/la Co-Director/a** **El/la Doctorando/a**

A mis padres

A la vida

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Aunque parezca un tópico el dinero no es todo, pero sí es necesario y por ello tengo que agradecer a los distintos organismos (Cabildo de Gran Canaria (2007-2009), a la Agencia Canaria de Investigación e Innovación de la Sociedad (2009-2013), al plan de ayudas de la Universidad de Las Palmas de Gran Canaria y a los proyectos Lucifer (CTM2008-03538/MAR)) y Procomex (CONACyT #2006-62152) por la financiación que he obtenido y que me ha permitido llevar a cabo la tesis doctoral.

Otra parte importante de esta carrera llamada tesis es el papel que juegan los directores, y por ello agradecer al Dr. D. Santiago Hernández León y a la Dra. Dña. Lidia Yebra Mora, por creer en mis capacidades, por ser pacientes y por estar en las duras y en las maduras.

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Dejando de lado por un segundo la parte profesional necesito gritar lo que siento pero no con voz sino con palabras. No ha sido fácil llegar a este punto sobre todo durante esta última fase, en la que he pasado muchos momentos duros. Sin embargo aquí estoy ya escribiendo mis últimas palabras que considero las más importantes sobre todo en la parte personal. Durante estos arduos años, he tenido la oportunidad de conocer a muchas personas que me han ayudado, de una forma u otra, en este camino largo y difícil que ha sido hacer la tesis. Simplemente con un gesto de complicidad han hecho que cada paso dado merezca la pena.

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Un especial y emotivo agradecimiento a mi familia, a mis hermanos/as y mis sobrinos/as y sobre todo muchas gracias a mis padres, María Rosa y Amador por ser el motor de mi vida, por estar ahí siempre que les necesito, por saber decirme lo correcto en cada momento y por hacerme luchar por lo que vale la pena en la vida, MUCHAS GRACIAS.

"*Lo que sabemos es una gota de agua; lo que ignoramos es el océano*".

Isaac Newton

Abstract

Zooplankton play a key role in the marine environment and the global biogeochemical cycles. These organisms have a central position in the marine ecosystem, as they partially control primary producers, and are prey for commercially important species. Variability on the distribution, production and growth of zooplankton communities and populations are influenced by environmental changes. Therefore, climate change may impact on marine ecosystems through changes in zooplankton. In order to understand the variability of zooplankton production in response to environmental changes in the marine ecosystem, in this thesis we validated the activity of the aminoacyl-tRNA synthetases as index of growth rate in early stages of zooplankton. This has allowed us to study the growth of the planktonic community in the marine environment, both in surface and in deep waters. In addition we reviewed all published studies relating growth and AARS activity. We tested the possibility of obtaining a global equation that allows inferring somatic growth rate of the planktonic communities from their AARS activity. Our findings contribute to the advancement of the use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates.

Resumen

El zooplancton tiene un papel clave en el medio marino y en los flujos biogeoquímicos. Estos organismos tienen una posición central en el ecosistema marino ya que ejercen parcialmente un control sobre los productores primarios y son alimento de especies de interés comercial. La variabilidad en la distribución, producción y crecimiento de las comunidades y poblaciones de zooplancton se ven afectadas por cambios en el medio marino. Por tanto, el cambio climático puede impactar sobre el ecosistema marino a través de cambios en el zooplancton. Dada la necesidad de entender la variabilidad en la producción del zooplancton como respuesta a cambios ambientales en el ecosistema marino, en esta tesis, se ha validado la actividad de las enzimas aminoacil-ARNt sintetasas como índice de crecimiento en los primeros estadios del zooplancton. Esto nos ha permitido estudiar el crecimiento de la comunidad planctónica en el medio marino, tanto en superficie como en aguas profundas. Asimismo, hicimos una revisión de todos los estudios publicados que relacionan el crecimiento y la actividad AARS, con el fin de ensayar la posibilidad de obtener una ecuación global que permita inferir la tasa de crecimiento somático de las comunidades planctónicas a partir de su actividad AARS. Los resultados de esta tesis contribuyen al avance del uso de la actividad AARS como índice de crecimiento para zooplancton e ictioplancton.

Thesis Preview

This thesis entitled “The use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates” compiles different studies carried out in the frame of the research projects Lucifer (CTM2008-03538/MAR) and Procomex (CONACyT #2006-62152). These projects were granted to Dr. Santiago Hernández León and Dr. Jaime Färber-Lorda respectively.

The present study has been co-supervised by Dr. Santiago Hernández León (Universidad de Las Palmas de Gran Canaria) and Dr. Lidia Yebra Mora (Instituto Español de Oceanografía).

This Thesis was developed mainly in English to apply for the European Doctor Mention (BOULPGC. Art.1 Chap.4, November 5th 2008). The general structure begins with an Introduction, followed by Objectives, Original Scientific Contributions, Results, General Discussion and Conclusions, to end with Future Research. In addition, a Spanish section is included, as required by the PhD Thesis Regulations from the University of Las Palmas de Gran Canaria (BOULPGC. Art.2 Chap.1, November 5th 2008).

Presentación de la tesis

La presente tesis titulada "Uso de la actividad AARS como índice de crecimiento para zooplancton e ictioplancton", resulta de la recopilación de una serie de trabajos encuadrados dentro de los proyectos de investigación Lucifer (CTM2008-03538/MAR) y Procomex (CONACyT #2006-62152) dirigidos por los doctores Santiago Hernández León y Jaime Färber-Lorda respectivamente.

El estudio que aquí se presenta ha sido codirigido por el Dr. Santiago Hernández León (Universidad de Las Palmas de Gran Canaria) y por la Dra. Lidia Yebra Mora (Instituto Español de Oceanografía).

Esta tesis se ha desarrollado mayoritariamente en inglés con el fin de poder optar a la Mención Europea del Título de Doctor de acuerdo a la normativa de la Universidad de Las Palmas de Gran Canaria (BOULPGC. Art.1 Cap. 4, 5 de noviembre 2008). Además, sigue la estructura exigida por este Reglamento: Introducción, Objetivos, Contribuciones científicas originales realizadas, Resultados, Discusión general, Conclusiones y Futuras Líneas de Investigación. También se presenta una sección en castellano, requerida por el Reglamento de Elaboración, Tribunal, Defensa y Evaluación de Tesis Doctorales de la Universidad de Las Palmas de Gran Canaria (BOULPGC. Art.1 Cap. 4, 5 de noviembre 2008).

Contents/ Índice

I. Introduction

Introduction

Zooplankton are small organisms that inhabit the water column and are transported by water masses. These organisms can be divided according to their size (Sieburth et al., 1978). Hence, microzooplankton comprises very diverse organisms between 0.02 and 0.2 mm, such as ciliates, copepod nauplii or meroplanktonic larvae. Mesozooplankton, between 0.2 and 20 mm in length, include copepods, cladocerans and appendicularians among others; and macrozooplankton, whose organisms are larger than 20 mm, correspond mainly to fish larvae, decapods or euphausiacea. Zooplanktonic organisms are also classified according to their life cycle, being known as holoplankton, those that are planktonic during their entire life cycle (e.g. copepods, ostracods, cladocerans) (Longhurst, 1985), and meroplankton, those organisms that are planktonic only for a part of their life cycle, usually eggs and larvae, such as fish, echinoderms, cnidarians or molluscs (Raymont, 1983).

Plankton studies are of great importance for humans because they are the base of the marine trophic webs. Zooplankton have a central role in the marine ecosystem, (1) exercising a partial control on primary producers, (2) being prey for higher trophic levels, and (3) representing an important link between the classic trophic chain and the microbial loop. Moreover, zooplankton (4) export particulate organic matter (POM), used by bacteria, and (5) excrete nutrients that can be recycled by phytoplankton (Fig. 1). In addition, zooplankton include all the larval stages of nektonic and benthic organisms. Therefore, the development of the exploitable marine resources also depends on the variability of zooplankton production.

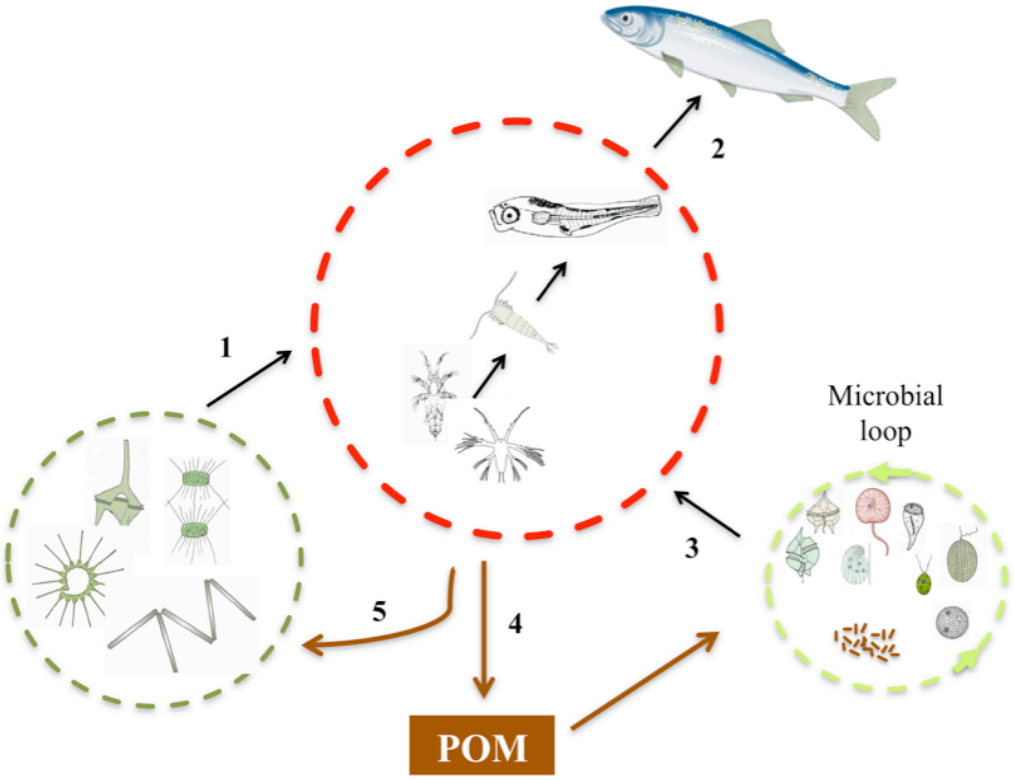

Figure 1. The importance of zooplankton in the marine ecosystem

The variability of the planktonic community is determined by abiotic and biotic factors. The former factors affect at different scales (Dickey and Bidigare, 2005), from the global scale to meso- and microscale. Physical forcing (Bonnet et al., 2005) including mesoscale structures like eddies and fronts that concentrate biomass (Yebra et al., 2005), enhance metabolism (Hernández-León et al., 2002; Landry et al., 2008) and vertical migration (Isla et al., 2004), and increase the active flux mediated by migrant zooplankton to deep waters (Yebra et al., 2005) promote this variability. At microscale, turbulence affects the predator-prey encounter rates, and therefore feeding rates (Kiørboe and Saiz, 1995; Visser et al., 2009). Also temperature (Parrilla et al., 1994; Drinkwater, 2005), salinity (Hirst and Lucas, 1998), light (Hernández-León, 2008; Hernández-León et al., 2001, 2004, 2010),

nutrients (Duarte et al., 2006), oxygen concentration (Teuber et al., 2013) and carbon dioxide (González-Dávila et al., 2006) affect directly to the planktonic community.

The biomass of the planktonic community is also affected by biotic factors, which regulate their organisms metabolism. This is influenced by food distribution and abundance (phyto- and microplankton), as it will determine their growth (Vidal, 1980; Hirst et al., 2003; Lin et al., 2013). Besides, biomass is also affected by mortality by predation (Hirst and Kiørboe, 2002; Runge et al., 2004; Maar et al., 2014). All these factors determine the population dynamics and the community structure, which also affect the carbon fluxes mediated by zooplankton in the ocean.

Given the central position of zooplankton in the trophic web, the study of zooplankton production is essential to evaluate the fluxes of energy and matter through pelagic ecosystems. The assessment of zooplankton production will increase our understanding and capacity to develop models coupling physical and biological variables with the goal of evaluating the impacts of zooplankton population dynamics over higher trophic levels.

In this sense, zooplankton production is defined as:

$$P = (B_1 - B_0) + M \qquad \text{(eq.1)}$$

where P is the zooplankton production, B_1 and B_0 are the biomass in time 1 and 0 respectively, and M is the mortality. Thus, biomass at a given time is:

$$B_1 = B_0 + (B_0 \cdot g) - (B_0 \cdot m) \qquad \text{(eq.2)}$$

being g and m the growth and the mortality rates, respectively.

There are several direct methods to assess zooplankton growth and production estimations (Omori and Ikeda, 1984; Runge and Roff, 2000). Among these methods the most common is the one developed by Heinle (1966) who, following the growth concept, measured the variations of weight over time. Another method used for *in situ* growth

estimation is the artificial cohort method (Dowing and Rigler, 1984). Growth is calculated following the development of a cohort, and it is based on the study of size and/or developmental stages frequency of the population. A third method used for growth estimations is based on the study of the molting rate (Hutching et al., 1995), but its use is only feasible in crustaceans and its application is restricted mainly to copepods. Besides, all these methods require incubating the organisms from several days up to months.

Because of the tedious procedure to measure growth, some authors proposed the egg production rate (Kiørboe and Johansen, 1986; Bergreen et al., 1988; Uye and Sano, 1995). This method assumes that egg production rate of females is equivalent to copepodites growth rate. However, other authors observed that is not true in different species (Peterson et al., 1991; Saiz et al., 1997; Campbell et al., 2001; Hirst and McKinnon, 2001). This method is simple but it also implies incubations and it is only applicable to adult females. Moreover, it has been observed that egg production rates underestimate growth when females are under food limitation (Richardson and Verheye, 1999; Rey-Rassat et al., 2002, 2004).

All these methods have the same drawback, they require incubations that are time consuming and are difficult to carry out on board research vessels. In addition, one of the main problems of direct estimations is the inability to reproduce the food conditions in the marine environment, with the risk of appearance of artefacts. Hence, measuring growth rate *in vitro* and *in situ* may be different. Moreover, these methods are applied frequently on a single species and/or developmental stage promoting errors in the estimation of zooplankton production when these growth rates are extrapolated to the whole community. These methodological limitations preclude the routine measurement of zooplankton growth during research cruises. Thus, there is a deficit of information that impedes the assessment of production at oceanic scale.

In order to meet the needs of biological oceanography, in the last decades the study of zooplankton production has been focused on the search for an effective method to determine growth rates avoiding incubations. In this sense, several indirect methods have been developed, among which there are numerical models and biochemical indices.

One of the well-known models is the one developed by Huntley and Lopez (1992) which estimates growth as a function of biomass and temperature. Recently, growth and egg production rates have been modelled as function of individual biomass, temperature and chlorophyll *a* (Hirst and Lampitt, 1998; Hirst and Bunker, 2003; Hirst et al., 2003). Other models are the West Brown Enquist (WBE, West et al., 1997) based on the distribution of nutrients and other resources necessary for the life of organisms, or the exponential model (e.g. Fox, 1970; Frost, 1972; Escribano and McLaren, 1992) based on the estimation of growth in relation to time and considering the effect of biotic and abiotic factors. Nevertheless, these models have their limitations, because they can only be applied on populations of copepods actively growing. In addition, their application on mixed populations in the ocean may induce considerable errors in the growth estimations (Hirst and Lampitt, 1998; Runge and Roff, 2000; Hirst and Forster, 2013).

At the same time, biochemical indices have started to be used in growth studies due to the advantages that these methods have over the direct methods. One of the advantages is that these methods do not require incubation of organisms allowing freezing the organisms immediately after their capture to be analysed later in the laboratory. In addition, laboratory analyses are economic, reproducible and allow obtaining results immediately, spending less time than using direct methods. However, these indices must be calibrated against the direct measurement of growth prior to their application in the field.

The first attempt to develop a suitable index of growth was the measurement of RNA and DNA (Sutcliffe, 1970; Dagg and Littlepage, 1972) and it has been in use until present

with different results. In general, this ratio was found to be a poor predictor of growth (Ota and Landry, 1984; Anger and Hirche, 1990; Wagner et al., 2001). However, some authors found significant relationships in fishes (Peck et al., 2003) and crustaceans (Yebra et al., 2011). However, the latter authors observed that this relationship differed between naupliar and copepodite stages, precluding its use in mixed populations.

There have also been proposed several indirect growth indices based on the measurement of the activity of diverse enzymes. Among them, DNA polymerase enzyme (Sapienza and Mague, 1979) belongs to a group of enzymes related to the processes of DNA replication in the cell. Its activity was validated as index of growth rate of the mysis *Praunus flexuosus*. Nevertheless, it has not been validated for other planktonic species (Runge and Roff, 2000). Also, aspartate transcarbamylase (ATC, Bergeron and Buestel, 1975; Bergeron, 1990, 1993, 1995), was proposed as a suitable growth index because it catalyses the first step of pyrimidine nucleotide formation, a fundamental constituent of nucleic acids. Although it was considered to be an index of growth in crustaceans, it has limitations because it is narrowly related to the moulting processes and not with somatic growth (Hernández-León et al., 1995, 2002). Another enzyme, the chitobiase is essential in recycling processes of chitin in crustaceans. Their exoskeleton is formed by chitin, a polymer formed by N-acetylglucosamine (Stevenson, 1985). This enzyme is directly related with the moulting processes necessary for growth (Oosterhuis et al., 2000; Sastri and Roff, 2000; Sastri and Dower, 2006, 2009). Furthermore, nucleoside-diphosphate kinase enzyme (NDPK, Berges et al., 1990), takes part in the assignment of energy in the growth processes. However, no relationship has been found between this enzyme activity and growth rate (Berges and Ballantyne, 1991).

In the last decades, the use of the aminoacyl-tRNA synthetases activity (AARS) as index of growth rate has also been developed. These enzymes catalyse the first step of the

protein synthesis, that is, the aminoacylation or activation and union of aminoacids to the transfer RNA (Novelli, 1967; Kisselev and Favorova, 1974; Schimmel and Söll, 1979). Consequently, AARS activity is directly related to protein synthesis (Parker et al., 1974; Neidhart et al., 1975; Johnson et al., 1977), being a limiting factor of this process (Larrinoa and Heredia, 1991; Sir Deshpande and Toogood, 1995). Likewise, positive relationships have been found between protein synthesis and growth rate in marine metazoans like cephalopods and fish (Houlihan et al., 1990; Sveier et al., 2000). Therefore, it is possible to consider AARS activity as a good candidate to be used as proxy for growth rate on zooplankton. Chang et al. (1984) developed a continuous spectrophotometric assay, non radioactive, to measure AARS activity.

The method of Chang et al. (1984) is very simple, the aminoacylation consumes adenosine triphosphate (ATP) and releases adenosine monophosphate (AMP) and pyrophosphate (PPi). Adding a pyrophosphate reagent to the sample homogenate, it is possible to measure continuously AARS activity, based in the release of pyrophosphate during the aminoacylation of transfer RNA, determined as the oxidation of NADH to NAD^+ (O'Brien, 1976):

$$\text{aminoacid} + \text{ATP} + \text{tRNA} \rightarrow \text{aminoacyl-tRNA} + \text{AMP} + PP_i \qquad \text{(eq.3)}$$

The decrease in absorbance at 340 nm is proportional to the enzymatic activity, which is purportedly related to somatic growth. Chang et al. (1984) assayed AARS activity adding substrates, registering in this way the potential activity of the enzymes. Enzyme activity is normally studied in substrate saturation conditions but cell experience different concentrations in the nature (Hernández-León and Gómez, 1996; Båmstedt, 2000). For this reason, enzyme activity should be measured based on their endogenous substrates (without substrate addition) reflecting the physiological condition of the cell *in vivo*.

Yebra and Hernández-León (2004) adapted the Chang et al. (1984) method to be applied on zooplankton without substrate addition. Thus, the authors analysed the capacity of zooplankton to synthesize their own proteins without addition of aminoacids and avoiding the overestimation due to the substrates saturation. This method has been applied successfully to the study of growth in juvenile and adult calanoid copepods (Yebra et al., 2005, 2006), in euphausiid larvae (Guerra, 2006) and recently, in nauplii and juvenile of cyclopoid copepods in the laboratory (Yebra et al., 2011).

Bearing in mind that (i) the abundance and growth of zooplankton and ichthyoplankton early stages are indispensable in zooplankton production studies, and (ii) zooplankton production is a determinant factor in the evolution and management of the stocks of many fish of commercial interest, it is of vital importance to validate the AARS method as an index of growth. In this thesis, we studied the relationship between growth rate and AARS activity on nauplii of the calanoid copepod *Paracartia grani* (**Chapter 1**) and on larvae of the small pelagic fish *Clupea harengus*, of commercial interest (**Chapter 2**). In these works we studied the effect of temperature and food concentration on growth and AARS activity, and also validated this method as an index of somatic growth rate.

The following step was the application of the AARS method to the study of the planktonic community in the marine environment. In **Chapter 3**, using this method and other indirect indices such as the gut fluorescence (GF) and the electron transport system activity (ETS), we were able to follow the epipelagic mesozooplankton population in the Canary waters and to infer the effects of the environmental variables on the zooplankton metabolism during the period of study.

One of the main advantages of the indirect methods is the possibility of freezing the organisms captured and to assay their enzyme activity later in the laboratory. These methods allow us to obtain an instantaneous snapshot of zooplankton metabolism in the moment of the

capture. The application of indirect methods has allowed studying zooplankton metabolism in deep waters (Seibel, 2011; Teuber et al., 2013). In this thesis, we present the first application of the AARS method to the study of zooplankton growth in relation to an oxygen minimum zone (OMZ). In **Chapter 4** we determined the effect of the OMZ on the metabolism of euphausiid *Euphausia distinguenda* at 400 m depth in the subtropical Pacific.

Finally, the main aim of the research in relation to zooplankton production has been the search for a standardized index that allow us to estimate zooplankton production at oceanic scale, as it is done with ^{14}C for primary production estimations. In this sense, in **Chapter 5**, we assayed the possibility of finding a suitable relationship between the growth rate of the planktonic community and their AARS activity, in order to facilitate the study of the community evolution. To carry out this point, we made a review using all relationships published between AARS activity and somatic growth together with the relationships presented in this thesis in order to search for a global equation that allows inferring the daily growth rate of planktonic communities from their AARS activity.

Objectives and Outline of this Thesis

The thesis has been structured following the normative for a PhD thesis as a compendium of publications. The main objective of the present thesis was to improve our knowledge on the use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates conducting laboratory and field studies.

In the general introduction we provided a general review of zooplankton and ichthyoplankton metabolism importance.

The results of this thesis are presented as five scientific articles organized in five central chapters, as follow:

1. In Chapter 1, we studied the effect of food concentration on the growth of *Paracartia grani* nauplii. In addition, we validated, in the laboratory, the enzymatic aminoacyl-tRNA synthetases (AARS) activity as proxy of growth rate for early stages of copepods.

2. In Chapter 2, we studied the effect of food concentration on the growth of Atlantic herring (*Clupea harengus*) larvae. In addition, we validated, in the laboratory, the enzymatic aminoacyl-tRNA synthetases (AARS) activity as proxy of growth rate for early stages of fish.

3. In Chapter 3, we studied the effect of temperature on zooplankton metabolism.

4. In Chapter 4, we studied the effect of oxygen concentration (oxygen minimum zone) on euphausiids metabolism.

5. In Chapter 5, we pursued the possibility of finding a general relationship between the zooplankton community growth rate and its aminoacyl-tRNA synthetases activity, in order to facilitate the study of the whole community evolution.

In the general discussion, we relate the results obtained from the preceding chapters, evaluate the initial goals of the thesis, and discuss the meaning of these findings in the context of zooplankton and ichthyoplankton growth rate. To conclude, we highlight the main conclusions of the thesis.

Original Scientific Contributions

1. **Herrera, I**., Yebra, L., Hernández-León, S., 2012. Effect of temperature and food concentration on *Paracartia grani* nauplii growth and protein synthesis rates. *Journal of Experimental Marine Biology and Ecology* 416-417, 101-109.

2. **Herrera, I**., Borchardt, S., Santana del Pino, A., Peck, M.A., Yebra, L., Hernández-León, S. Aminoacyl-tRNA synthetases (AARS) activity as an index of Atlantic herring (*Clupea harengus*) larvae growth. In preparation to be submitted to *Journal of Fish Biology*.

3. **Herrera, I**., López-Cancio, J., Yebra, L., Hernández-León, S. The effect of a strong warm year on subtropical mesozooplankton biomass and metabolism. Submitted to *Journal of Plankton Research.*

4. **Herrera, I**., Antezana, T., Giraldo, A., Beier, E., Yebra, L., Hernández-León, S., Färber-Lorda, J. Potential grazing, respiration and growth of *Euphausia distinguenda* in relation to the oxygen minimum zone at the Eastern Tropical Pacific off Mexico. In preparation to be submitted to *PlosOne.*

5. Hernández-León, S., Yebra, L., **Herrera, I.**, Bécognée, P. The use of aminoacyl-tRNA synthetases (AARS) activity as an index of zooplankton growth. In preparation to be submitted to *Marine Biology*.

II. Results

Chapter 1

Effect of temperature and food concentration on ***Paracartia grani*** nauplii growth and protein synthesis rates

Inma Herrera, Lidia Yebra and Santiago Hernández-Léon

J. Exp. Mar. Biol. Ecol. 416-417, 101-109; 2012.

Abstract

The *in situ* activity of the enzymes aminoacyl-tRNA synthetases (AARS) and the growth rates of naupliar stages of the planktonic marine copepod *Paracartia grani* were measured in the laboratory under different temperature and food concentrations. We assessed the effect of these parameters on growth and protein synthesis rates of *P. grani* nauplii. Growth and protein synthesis rates of *P. grani* nauplii depended on temperature and food concentration. AARS activity is valid as an index of somatic growth for *P. grani* nauplii when growth is not limited by food availability. However, the relationship between protein-specific AARS activity and nauplii growth varied according to food availability levels. The degradation of proteins during starvation and/or the ß-oxidation of fatty acids affected the relationship between specific AARS activity and growth rates. The results presented here add to previous studies showing that the AARS activity is a useful tool for estimating somatic growth of this and other key copepod species. Nevertheless, further research is required to elucidate the validity of AARS activity as a universal proxy for growth.

Keywords: AARS, food concentration, growth, *Paracartia grani*, protein metabolism, temperature.

1. Introduction

The assessment of zooplankton production is a milestone in oceanography. Zooplankton is the main link between the primary producers and fisheries, and they are also important in the flux of energy and matter in the ocean. From this point of view, it is of interest to know which changes may produce temperature and food concentration variations on the growth rates of early developmental stages of key zooplankton species.

There are an increasing number of approaches to estimate growth rates in planktonic organisms such as copepods (Runge and Roff, 2000). Traditionally, the direct method (Heinle, 1966), based on length or weight increases, and the egg production rate method (EPR, Marshall and Orr, 1955) have been applied to assess copepod growth rates. EPR is currently the most used method to estimate copepod growth (Hirst et al., 2003) and it is rather sensitive to changes in environmental variables (Saiz et al., 1997). However, the assumption that EPR is comparable to the growth rates of the juveniles is often invalid (Hirst and Bunker, 2003). Also, adult females may lose or gain weight whilst producing eggs, and as such, EPR may not accurately represent growth of the female (Hirst and McKinnon, 2001). In addition, EPR measurements are labor consuming and involve a risk of introducing artifacts due to the handling of the animals (Jones, 1980).

In recent years, the use of biochemical methods as indices of growth in copepods has increased. These methods allow the assessment of zooplankton production on field collected organisms with less laboratory manipulation and are mainly based on either biomass ratios (e. g. RNA/DNA, Dagg and Littlepage, 1972; RNA/protein, Saiz et al., 1998; Wagner et al., 2001; Gorokhova, 2003) or the activity of enzymes involved in the process of growth (e.g. nucleoside diphosphate kinase NDPK, Berges et al., 1990; aspartate transcarbamylase ATC, Bergeron and Buestel, 1979; Biegala and Bergeron, 1998; chitobiase, Oosterhuis et al., 2000;

Sastri and Roff, 2000). An enzymatic method, recently developed as index of copepod somatic growth, is based on the activity of the aminoacyl-tRNA synthetases (AARS, Yebra and Hernández-León, 2004). These enzymes catalyse the first step of the protein synthesis and their activity is significantly related to somatic growth in freshwater and marine crustaceans (*Daphnia magna*, Yebra and Hernández-León, 2004; *Calanus helgolandicus*, Yebra et al., 2005; *Calanus finmarchicus*, Yebra et al., 2006; *Euphausia superba*, Guerra, 2006).

In order to assess the effect of temperature and food concentration on their somatic growth and protein synthesis rates (AARS activity), as well as the relationship between both variables, we studied *Paracartia grani* nauplii. Copepods of the family *Acartiidae* are common in coastal and estuarine habitats worldwide (see Rosamma and Rao, 1985). They are mainly adapted to the high food concentrations normally found in estuaries and upwelled waters (Paffenhöfer and Stearns, 1988). As they are the principal link in the marine food web in some areas, there are many studies on growth of the genus *Acartia* (e.g. Durbin and Durbin, 1978; Landry, 1978; Klein Breteler and Gonzalez, 1982; Berggreen et al., 1988; Saiz et al., 1998; Bersano, 2000; Gorokhova, 2003; Leandro et al., 2006). However, their nauplii growth rates have rarely been described (Durbin and Durbin, 1978; Berggreen et al., 1988; Calbet and Alcaraz, 1997; Leandro and Tiselius, 2006).

In this work, we focused on the effect of temperature and food quantity on the growth and AARS activity of *Paracartia grani* (Sars, 1904) nauplii. In order to assert the use of this enzyme as a proxy for growth rates in the ocean, a parallel response of its activity and rates should be expected.

2. Material and Methods

2.1. Parental cultures

Paracartia grani Sars 1904 (*Copepoda*: *Calanoida*) and *Oxyrrhis marina* (heterotrophic *dinoflagellate, equivalent spherical diameter, ESD = 16.9 μm*) were obtained from continuous cultures maintained at the Institute of Marine Sciences (ICM, Barcelona, Spain). They were kept in 20 L transparent plastic tanks and 2 L pyrex bottles respectively, at 20ºC with a 12:12 h photoperiod. *P. grani* and *O. marina* were fed with *Rhodomonas baltica* (Cryptophyceae, ESD = 8 *μm*), grown at 20ºC on f/2 medium (Guillard 1975). Every 24 h, the eggs of *P. grani* were collected and refrigerated (4ºC) until used for experiments (between 2-30 days).

2.2. Experiments at different temperatures

Organisms were acclimated at different temperatures using six water baths (Table 1). In each of them we introduced a plastic container with 10 L of filtered seawater.

Table 1. *Paracartia grani* nauplii somatic growth (d^{-1}) and protein synthesis rates [$spAARS_s$ (nmol PPi·mg $prot^{-1}\cdot h^{-1}$) and individual $AARS_s$ (nmol PPi·$ind^{-1}\cdot h^{-1}$)] at different temperatures (ºC). n is the number of either individuals sized or samples analysed.

T (ºC)	Somatic growth (d^{-1}) (r^2, n)	$spAARS_s$ ± SE (n) (nmPPi·mg $prot^{-1}\cdot h^{-1}$)	individual $AARS_s$ ± SE (n) (nmPPi·$ind^{-1}\cdot h^{-1}$)
12	0.28 (0.990, 382)	24.35 ± 0.69 (9)	0.003 ± 0.000 (9)
16	0.41 (0.987, 480)	49.13 ± 7.04 (9)	0.006 ± 0.001 (9)
19.8	0.54 (0.987, 188)	50.26 ± 1.81 (8)	0.007 ± 0.002 (8)
24	0.70 (0.970, 299)	96.79 ± 5.08 (6)	0.019 ± 0.007 (6)
26	0.85 (0.973, 430)	102.23 ± 19.72 (6)	0.016 ± 0.005 (6)
28	0.85 (0.962, 597)	106.44 ± 13.32 (9)	0.017 ± 0.007 (9)

Once the water reached the desired temperature we added the previously refrigerated eggs to each container and allowed 16 hours for them to hatch. Each group of nauplii (~2 nauplii mL^{-1}) was grown under food saturating conditions (Calbet and Alcaraz, 1997). The nauplii were fed with *Oxyrrhis marina* (1,000-1,300 cells·mL^{-1}; 220-286 µg C·L^{-1}, assuming 215.8 pg C·$cells^{-1}$ from Klein Breteler and Schogt, 1994). Food concentration was measured daily with a Multisizer Coulter Counter. Every 12-24 h (depending on the experimental temperature) we took an aliquot of 100 mL from the nauplii culture and fixed it with Lugol's acid solution for abundance and individuals' length measurement. Three replicates of approx. 1,000 individuals were sampled daily and frozen immediately in liquid nitrogen (-196 ºC) for AARS activity assays. Sampling continued for 4-6 days until the nauplii reached the stage VI (NVI).

2.3. Experiments under different food concentrations

Nauplii of *P. grani* were acclimated at 20ºC in seven water baths. Eggs were allowed to hatch during 24h, and a similar amount of nauplii (~2 nauplii mL^{-1}) were incubated in 10 L plastic containers. Each group of nauplii was grown under different concentrations of *Oxyrrhis marina* (Table 2). Food concentration was measured daily with a Multisizer Coulter Counter. Every 24 h we took three aliquots of 50 mL from the nauplii culture and fixed them with Lugol's acid solution for abundance and individuals' length measurement. Three replicates of approximately 1,000 individuals were sampled and frozen immediately in liquid nitrogen (-196 ºC) to assess AARS activity. Sampling continued for 5-8 days, until the nauplii reached stage NVI.

Table 2. *Paracartia grani* nauplii somatic growth (d^{-1}), protein synthesis rates [$spAARS_s$ (nmol PPi·mg $prot^{-1}$·h^{-1}) and individual $AARS_s$ (nmol PPi·ind^{-1}·h^{-1})] at different food concentrations (µg C·L^{-1}). n is the number of either individuals sized or samples analysed.

Food concentration (µg C·L^{-1})	Somatic growth (d^{-1}) (r^2, n)	$spAARS_s$ ± SE (n) (nmPPi·mg $prot^{-1}$·h^{-1})	individual $AARS_s$ ± SE (n) (nmPPi·ind^{-1}·h^{-1})
0	-0.01 (0.604, 454)	59.60 ± 5.18 (12)	0.003 ± 0.000 (12)
11	0.03 (0.677, 678)	59.02 ± 12.24 (10)	0.004 ± 0.001 (10)
55	0.34 (0.945, 740)	46.51 ± 4.43 (12)	0.004 ± 0.001 (12)
110	0.49 (0.935, 459)	46.03 ± 6.84 (12)	0.005 ± 0.001 (12)
220	0.64 (0.989, 299)	34.80 ± 11.03 (11)	0.006 ± 0.001 (11)
440	0.68 (0.991, 482)	32.24 ± 3.12 (9)	0.004 ± 0.001 (9)
880	0.61 (0.945, 465)	34.29 ± 4.60 (10)	0.006 ± 0.001 (10)

2.4. Growth calculations

Organisms fixed in Lugol's were photographed using a camera connected to a dissecting microscope at 40x magnification. Prosome length (µm) was measured from pictures with Image/J software. Individual biomass of *Paracartia grani* nauplii was estimated from the length-dry weight (dw) equation given by Durbin and Durbin (1978) for *Acartia clausi*:

$W = 19.04 \cdot L^{2.849}$, $r^2 = 0.98$

where W is body weight in µg dw and L is prosome length in mm.

Dry weight (dw) was converted to carbon (C) assuming a carbon/dry weight ratio of 0.40 (Postel et al., 2000). Weight-specific growth rates (G. d^{-1}) were calculated as the slope of ln(weight) increases over time.

The temperature quotient (Q_{10}) of growth rates and AARS activities was calculated as: $Q_{10} = (M1 / M2)^{10 / (T2 - T1)}$, where M2 and M1 are the rates of the studied processes at

temperatures T2 and T1 (ºC), respectively. In order to use a 10ºC range, we calculated the Q_{10} between 16 and 26ºC.

2.5. AARS activity assay

Frozen samples were homogenized in Tris-HCl buffer (20 mM, pH 7.8) and centrifuged (10 min, 0ºC). AARS activity was assayed following the method of Yebra and Hernández-León (2004), slightly modified as follows: 250 µL of each sample supernatant was added to a mixture containing 200 µL of pyrophosphate (PPi) reagent (P-7275, from Sigma) and 300 µL of Milli-Q water at room temperature. The absorbance of the reaction mixture was monitored at 340 nm for 10 min at 25 ºC. The aminoacylation of the tRNA releases PPi, which produces an oxidation of NADH. This is registered as a decrease in absorbance (dA). The NADH oxidation rate ($dA \cdot min^{-1}$) was converted to PPi release rate (AARS activity, nmol $PPi \cdot mL^{-1} \cdot min^{-1}$) using the equation (1) in Yebra and Hernández-León (2004):

$$\text{nmol } PPi \cdot h^{-1} \cdot \text{sample } mL^{-1} = (dA \cdot min^{-1} \cdot 10^3 \cdot 60) \cdot (V_{rm} \cdot 6.22 \cdot 2)^{-1}$$

where V_{rm} is the volume of the reaction mixture in mL, 6.22 is the millimolar absorptivity of NADH at 340 nm and 2 is the number of moles of β-NADH oxidized per mole of PPi consumed.

AARS activity was corrected for the *in situ* temperature of each experiment by applying an activation energy of 8.57 $kcal \cdot mol^{-1}$ (Yebra et al., 2005) to the Arrhenius equation in order to obtain the *in situ* activity ($AARS_s$).

Protein content of the samples was measured following the Lowry et al. (1951) method adapted for micro-assay by Rutter (1967), using Bovin Serum Albumin as standard (A-4503, from Sigma).

3. Results

3.1. Effect of temperature on nauplii rates

Weight-specific growth rates (slope of each regression line in Fig. 1), varied from 0.28 to 0.85 d^{-1} between 12 and 28ºC (Table 1).

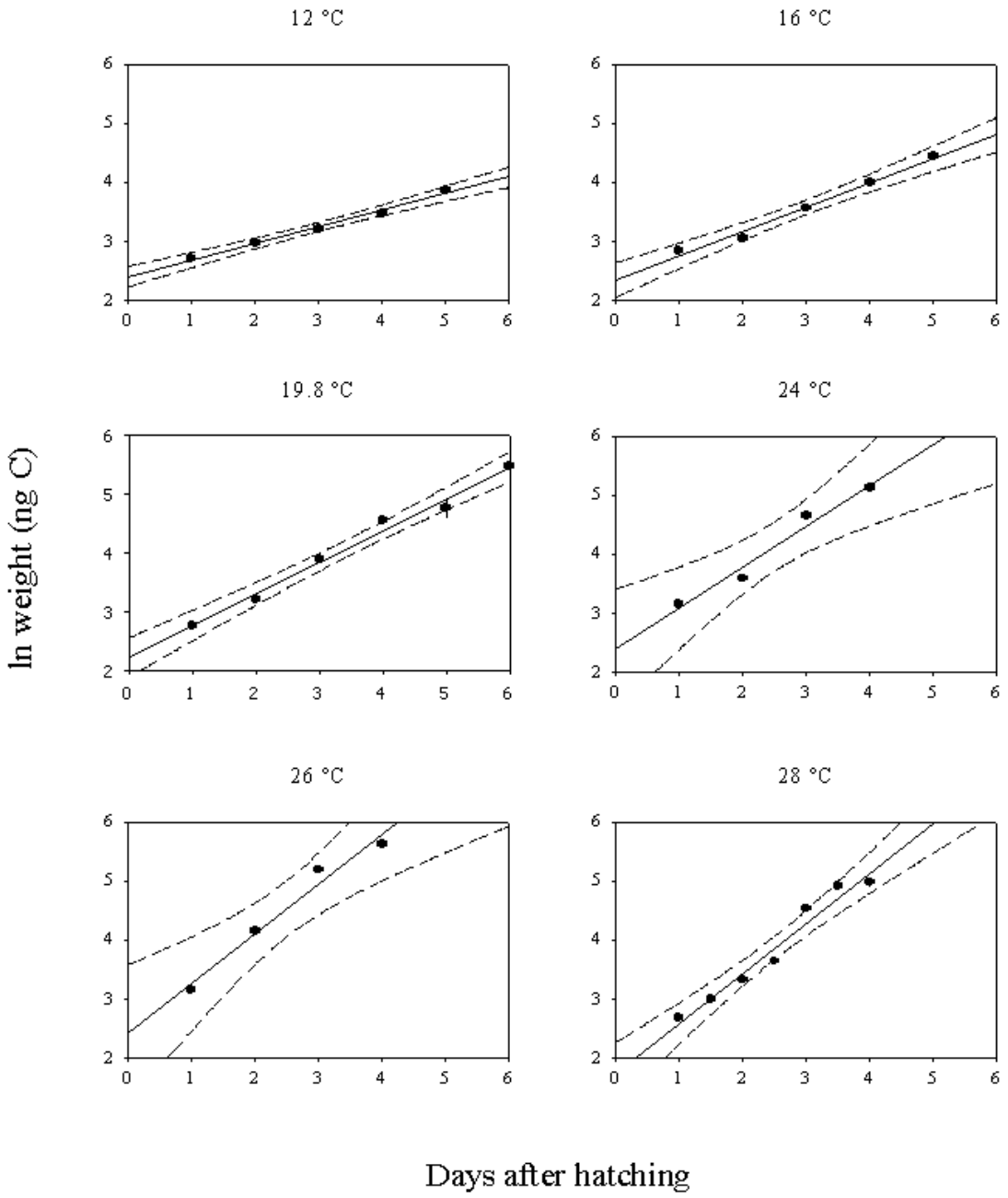

Figure 1. *Paracartia grani* nauplii. Carbon content (ng C) increases at different temperatures.

The protein-specific $AARS_s$ ($spAARS_s$, Table 1) ranged from 24.35 to 106.44 nmol PPi ·mg $prot^{-1}\cdot h^{-1}$ and the individual $AARS_s$ increased from 0.003 to 0.019 nmol $PPi\cdot ind^{-1}\cdot h^{-1}$.

Temperature (ºC) had a significant positive effect on growth rates (G, d^{-1}) of nauplii (Fig. 2A):

$$G = -0.188 + 0.038 \cdot T,\ r^2 = 0.984,\ p < 0.001 \quad \text{(eq.1)}$$

Also $spAARS_s$ (nmol $PPi\cdot mg\ prot^{-1}\cdot h^{-1}$) and individual $AARS_s$ (nmol $PPi\cdot ind^{-1}\cdot h^{-1}$) were significantly affected by temperature (Figs. 2B, 2C):

$$spAARS_s = -42.51 + 5.44 \cdot T,\ r^2 = 0.942,\ p < 0.001 \quad \text{(eq.2)}$$

$$AARS_s \cdot ind^{-1} = -0.010 + 0.001 \cdot T,\ r^2 = 0.852,\ p < 0.001 \quad \text{(eq.3)}$$

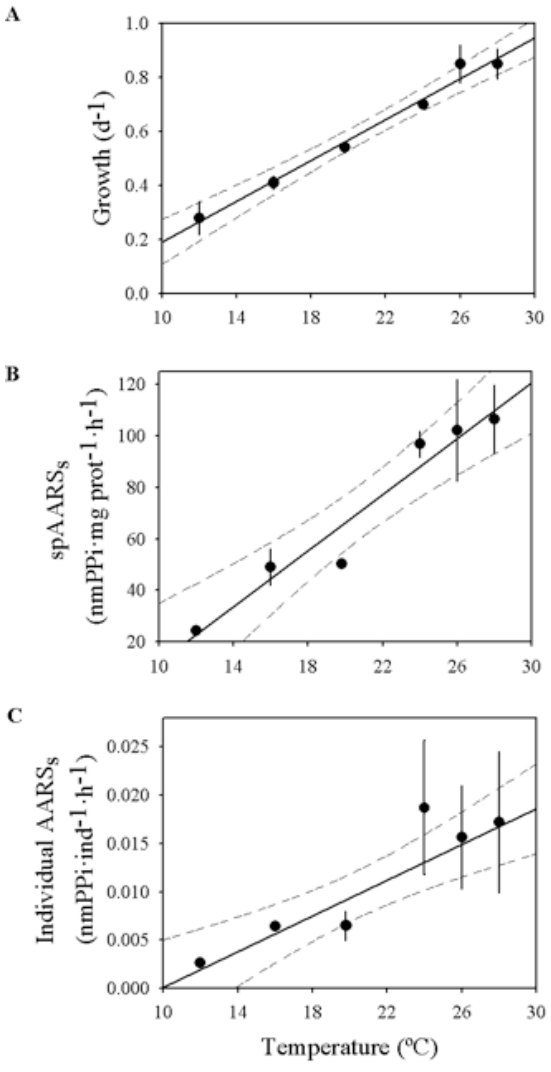

Figure 2. *Paracartia grani* nauplii. Effect of temperature on A) growth (d^{-1}), B) $spAARS_s$ (nmol $PPi\cdot mg\ prot^{-1}\cdot h^{-1}$), C) individual $AARS_s$ (nmol $PPi\cdot ind^{-1}\cdot h^{-1}$).

The Q_{10} value obtained within the 16 and 26ºC range was the same for growth rate and specific $AARS_s$ (2.1) and was 2.4 for individual $AARS_s$.

3.2. Effect of food concentration on nauplii rates

Weight-specific growth rates (slope of regression lines in Fig. 3) varied from -0.01 to 0.68 d^{-1} with increasing food concentrations (Fig. 4A) and the average $spAARS_s$ ranged between 32.24 and 59.60 nmol PPi·mg $prot^{-1}·h^{-1}$ (Table 2). The average $AARS_s$ per individual increased from 0.003 to 0.006 nmol PPi·$ind^{-1}·h^{-1}$ (Table 2) and presented high variability within experiments. The assumption that the aliquots frozen for biochemical assays contained a fixed amount of 1,000 nauplii was not always correct, as observed on the protein content of the sample replicates (data not shown). This was mostly noted in the growth experiment conducted at a food concentration of 440 µg $C·L^{-1}$, which was excluded from fit calculations in Fig. 4C.

Growth rates (d^{-1}) relative to food concentrations (C, µg $C·L^{-1}$) followed a saturation curve (Ivlev's equation, 1955) expressed by the function:

$$G = 0.65 \cdot (1 - e^{(-0.013 \cdot C)}),\ r^2 = 0.986,\ p < 0.001 \qquad \text{(eq.4)}$$

where 0.65 is the maximum growth rate (d^{-1}) and 0.013 is a constant that indicates the rate at which growth approaches the maximum rate. Naupliar growth became saturated at a food concentration level of 220 µg $C·L^{-1}$ (Fig. 4A).

Individual $AARS_s$ (nmol PPi·$ind^{-1}·h^{-1}$) activities also increased with increasing food concentration (Fig. 4C), following a logarithmic model:

$$AARS_s·ind^{-1} = 0.0032 + 0.0004 \ln(C),\ r^2 = 0.907,\ p < 0.05 \qquad \text{(eq.5)}$$

In contrast, specific $AARS_s$ (nmol PPi·mg $prot^{-1}·h^{-1}$) exhibited three different values in relation to food concentration (Fig. 4B). Specific AARS showed maximum values (59.31±0.29) from 0 to 11 µg $C·L^{-1}$, while between 55 and 110 µg $C·L^{-1}$ the average

$spAARS_s$ was 46.27±0.24. Above 220 µg $C \cdot L^{-1}$ $spAARS_s$ remained low and rather constant (33.78±0.78).

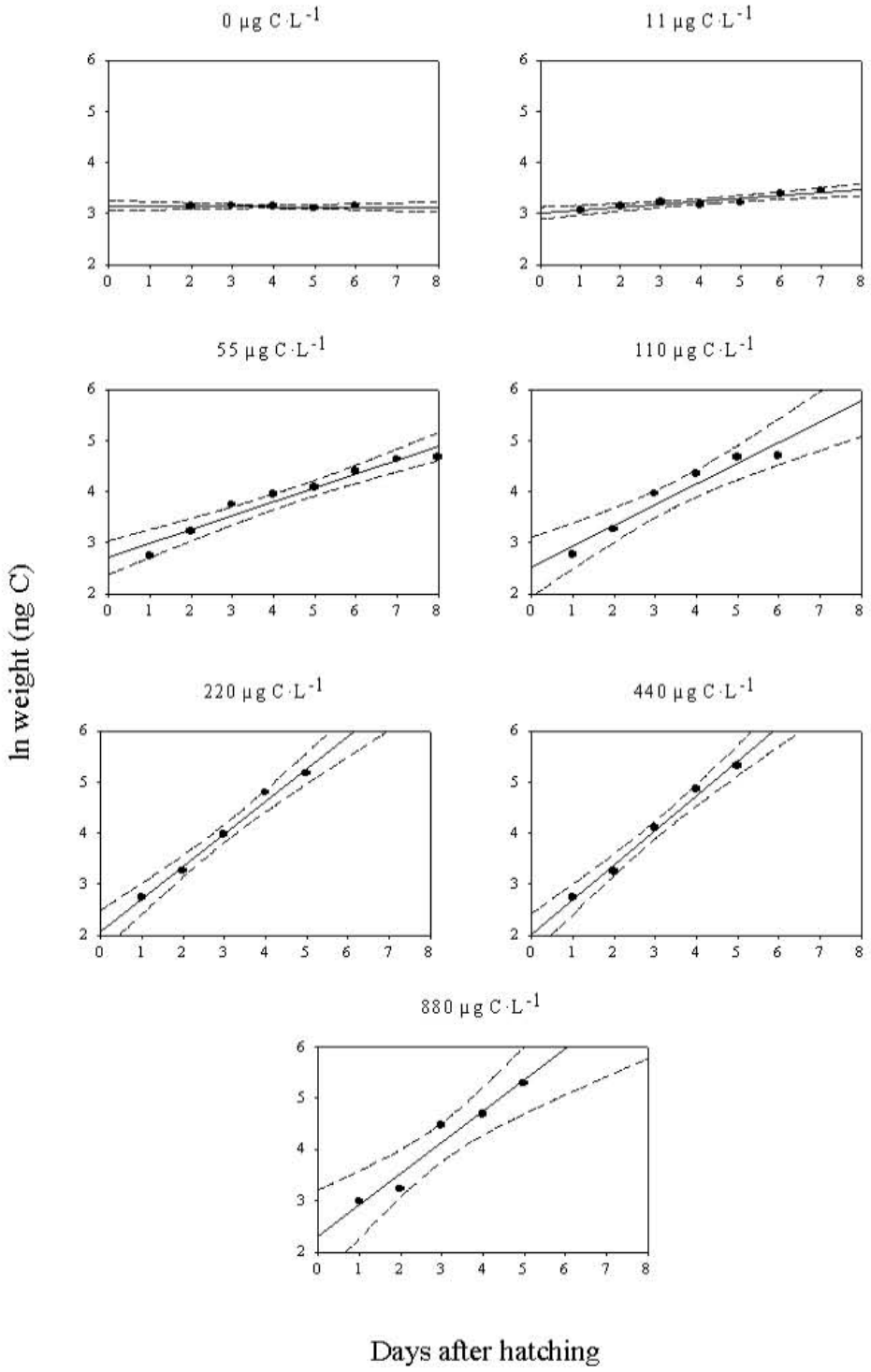

Figure 3. *Paracartia grani* nauplii. Carbon content (ng C) increases under different food concentrations.

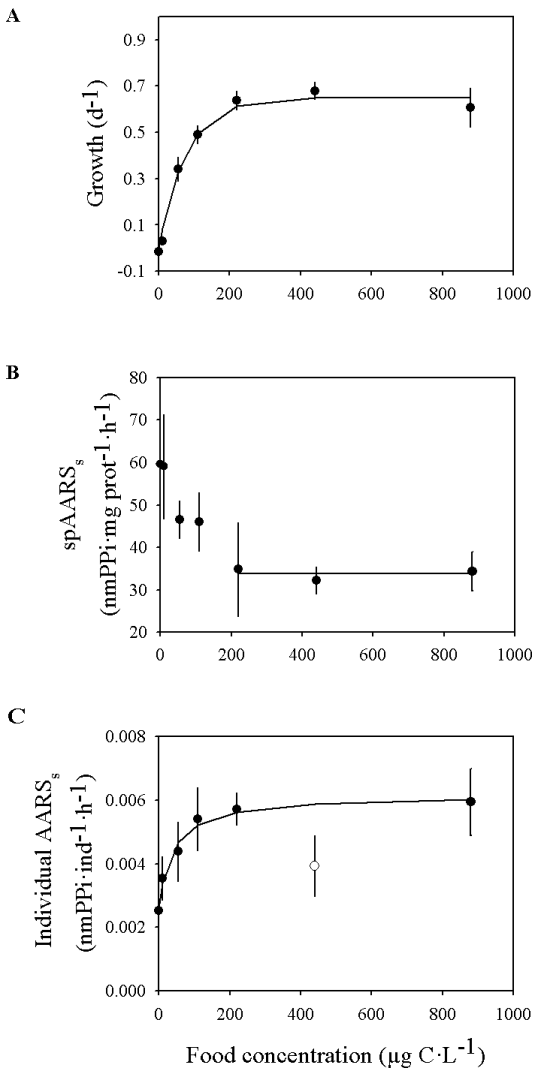

Figure 4. *Paracartia grani* nauplii. Effect of food concentration on A) growth (d^{-1}), B) $spAARS_s$ (nmol PPi·mg $prot^{-1}·h^{-1}$), C) individual $AARS_s$ (nmol PPi·$ind^{-1}·h^{-1}$); open circle: value not included in fit (see text).

3.3. Relationship between nauplii growth and protein synthesis rates

Positive significant relationships were found between growth rates (d^{-1}) and both $spAARS_s$ (nmol PPi·mg $prot^{-1}·h^{-1}$) and individual $AARS_s$ (nmol PPi·$ind^{-1}·h^{-1}$) activities under food saturating conditions within the 12-28ºC range (Fig. 5):

$$G = 0.13 + 0.007 \cdot spAARS_s,\ r^2 = 0.945,\ p < 0.001 \quad \text{(eq.6)}$$

$$G = 0.25 + 31.65 \cdot AARS_s \cdot ind^{-1},\ r^2 = 0.833,\ p < 0.001 \quad \text{(eq.7)}$$

A positive relationship between growth rates (d^{-1}) and individual $AARS_s$ (nmol PPi·ind^{-1}·h^{-1}) was also found within the 0 - 4,000 cels·mL^{-1} (0 - 880 µg C·L^{-1}) food concentration range (Fig. 6B):

$$G = -0.59 + 204.46 \cdot AARS_s \cdot ind^{-1},\ r^2 = 0.951,\ p < 0.001 \qquad \text{(eq.8)}$$

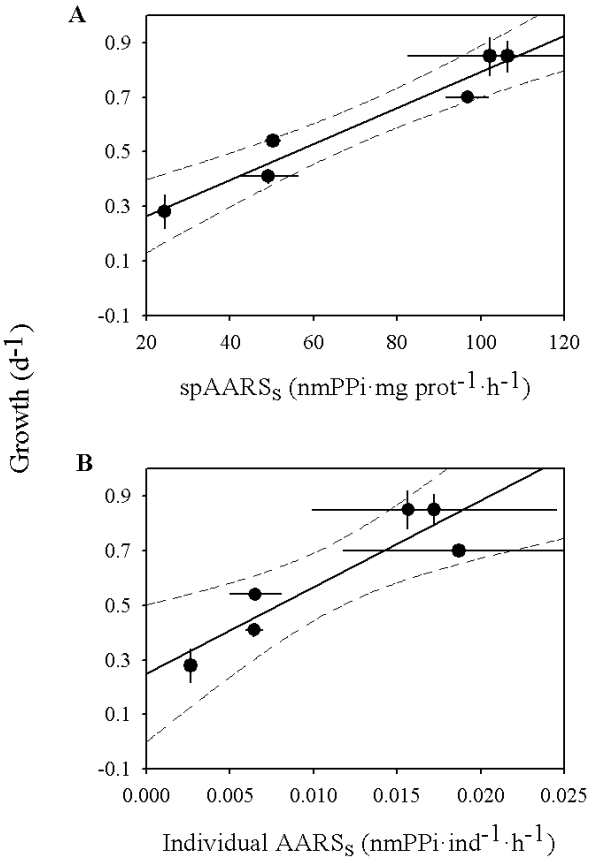

Figure 5. *Paracartia grani* nauplii. Relationship between growth rates (d^{-1}) and A) specific $AARS_s$ activities (nmol PPi·mg $prot^{-1}$·h^{-1}), B) individual $AARS_s$ (nmol PPi·ind^{-1}·h^{-1}) at different temperatures (ºC).

However, the relationship between growth rates (d^{-1}) and $spAARS_s$ activities (nmol PPi·mg $prot^{-1}$·h^{-1}) was negative (Fig 6A):

$$G = 1.49 - 0.025 \cdot spAARS_s,\ r^2 = 0.958,\ p < 0.0001 \qquad \text{(eq.9)}$$

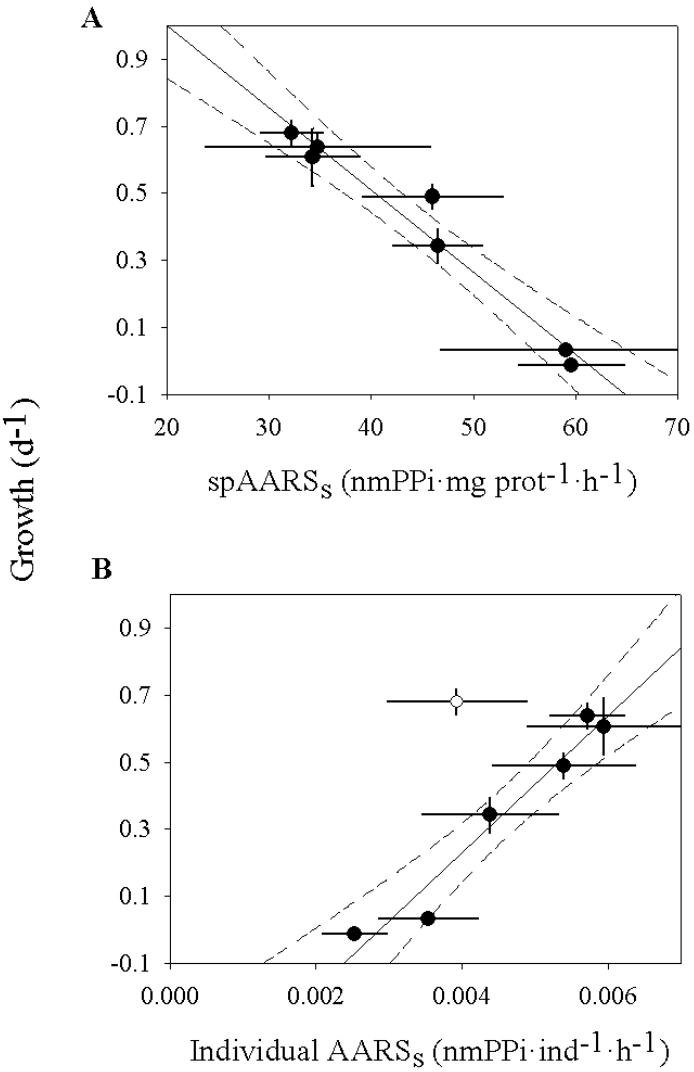

Figure 6. *Paracartia grani* nauplii. Relationship between growth rates (d^{-1}) and A) specific $AARS_s$ activities (nmol PPi·mg $prot^{-1}·h^{-1}$), B) individual $AARS_s$ (nmol PPi·$ind^{-1}·h^{-1}$) under different food concentrations (µg $C·L^{-1}$); open circle: value not included in fit (see text).

Specific $AARS_s$ activities (nmol PPi·mg $prot^{-1}·h^{-1}$) also showed a negative relationship with nauplii individual biomass (µg proteins · ind^{-1}), presenting higher $spAARS_s$ activities and lower individual biomass in starved organisms, and lower enzyme activities and higher protein content in the nauplii growing at food saturating levels (Fig 7A):

$$spAARS_s = 64.3 – 165.0 \cdot \text{individual biomass}, r^2 = 0.490, p < 0.0001 \quad \text{(eq.10)}$$

The relationship between daily growth rates and $spAARS_s$ in relation to the levels of food availability showed three different relationships (Fig 7B):

Starvation level (0-11 µg $C \cdot L^{-1}$):

$$G = -0.141 + 0.0024 \cdot spAARS_s,\ r^2 = 0.458,\ p = 0.095 \quad \text{(eq.11)}$$

Intermediate level (55 - 110 µg $C \cdot L^{-1}$):

$$G = -0.027 + 0.008 \cdot spAARS_s,\ r^2 = 0.146,\ p = 0.351 \quad \text{(eq.12)}$$

Saturation level (>220 µg $C \cdot L^{-1}$):

$$G = -0.57 + 0.038 \cdot spAARS_s,\ r^2 = 0.799,\ p = 0.003 \quad \text{(eq.13)}$$

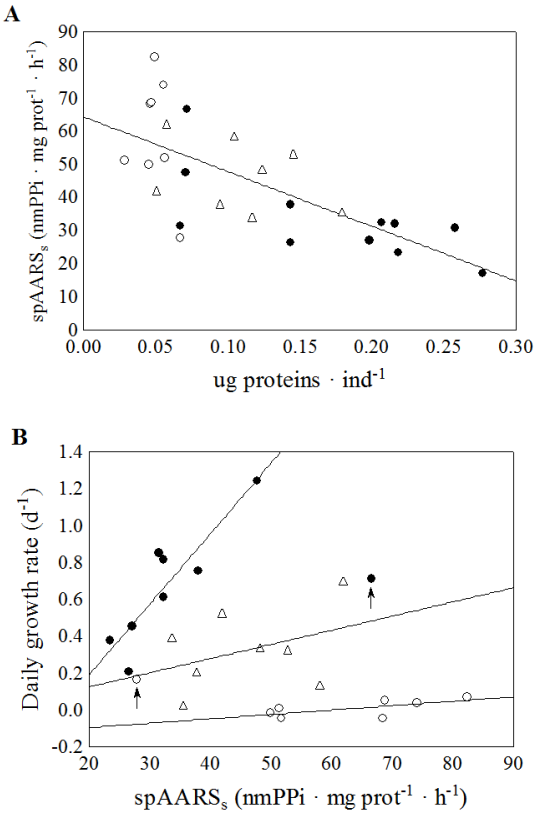

Figure 7. *Paracartia grani* nauplii. A) Relationship between specific $AARS_s$ activities (nmol PPi·mg $prot^{-1} \cdot h^{-1}$) and individual biomass (µg proteins · ind^{-1}); B) Relationships between daily growth rates (d^{-1}) and specific $AARS_s$ activities (nmol PPi·mg $prot^{-1} \cdot h^{-1}$) under different food concentrations (open circles: starvation level, triangles: intermediate level, filled circles: saturation level); arrows: values not included in fit.

4. Discussion

We assessed the effect of temperature and food concentration on somatic growth (calculated from length measurements) and protein synthesis rates (AARS activity) of *Paracartia grani* nauplii. A strong relationship was observed between growth rate and specific AARS activity at saturating food concentration and at a wide range of temperatures. However, this strong relationship was not observed at different food levels, except for those incubated at saturation. High specific AARS activities were found at low growth rates under limiting food concentration and low individual biomass. Early nauplii stages do not feed but develop consuming lipid reserves. Whether this feature influences the relationship between growth rates and specific AARS activity seems the key to understanding the high activity observed in the present and other works (e.g., Holmborn et al., 2009) at low levels of food and growth.

4.1 Effect of temperature

Acartia species present isochronal development and exponential growth when reared at *ad libitum* food concentrations, and it has been shown that both growth and development rates depend on temperature (Sekiguchi et al., 1980; Klein Breteler and Schogt, 1994; Leandro et al., 2006, Leandro and Tiselius, 2006). Temperature had a positive effect on the naupliar growth and on the protein synthesis rates of these species when they were fed *ad libitum*. This naupliar growth rates were similar to those of other *Acartia* species nauplii (Table 3). The results obtained by Berggreen et al. (1988) and Leandro and Tiselius, 2006 for *Acartia tonsa* agree with the rates of *P. grani* observed by Calbet and Alcaraz (1997) and those found in the present work within the 10-18ºC temperature range. However, at 22ºC *A. tonsa* grew faster (Leandro and Tiselius, 2006) than *P. grani*. This might be due to the

different quality of the food supplied (Table 3), but also to the different responses that these species may have with increasing temperature.

Table 3. Summary of published *Acartia spp.* nauplii growth rates under saturating food levels.

Temperature (ºC)	Growth rate (d^{-1})	Species	Food type	Reference
10	0.19	*Acartia clausi*	*Rhodomonas* sp.	Leandro et al., 2006
10	0.19	*Acartia tonsa*	*Rhodomonas* sp.	Leandro and Tiselius, 2006
12	0.28	*Paracartia grani*	*Oxyrrhis marina*	This work
15	0.27	*A. clausi*	*Rhodomonas* sp.	Leandro et al., 2006
15	0.37	*A. tonsa*	*Rhodomonas* sp.	Leandro and Tiselius, 2006
16	0.41	*P. grani*	*O. marina*	This work
16	0.41	*A. tonsa*	*Rhodomonas baltica*	Berggreen et al., 1988
18	0.46	*P. grani*	*R. baltica*	Calbet and Alcaraz, 1997
18	0.45	*A. tonsa*	*R. baltica*	Berggreen et al., 1988
18	0.46	*A. clausi*	*Rhodomonas* sp.	Leandro et al., 2006
18	0.54	*A. tonsa*	*Rhodomonas* sp.	Leandro and Tiselius, 2006
19.8	0.53	*P. grani*	*O. marina*	This work
20	0.42	*A. clausi*	*Rhodomonas* sp.	Leandro et al., 2006
20	0.61-0.68	*P. grani*	*O. marina*	This work
22	0.42	*A. clausi*	*Rhodomonas* sp.	Leandro et al., 2006
22	0.88	*A. tonsa*	*Rhodomonas* sp.	Leandro and Tiselius, 2006
24	0.70	*P. grani*	*O. marina*	This work
26	0.85	*P. grani*	*O. marina*	This work
28	0.85	*P. grani*	*O. marina*	This work

For example, *A. tonsa* is distributed worldwide (Kouwenberg, 2011), while *P. grani* is found in coastal NE Atlantic and Mediterranean Sea waters (Walter and Boxshall, 2011). Also, the temperature quotients (Q_{10}) observed for both growth and protein synthesis rates in *P. grani* (2.1 between 16-26 ºC) were lower than the Q_{10} values reported by Leandro and Tiselius, 2006 (3.66 between 10-22 ºC). However, the use of Q_{10} values calculated across different temperature ranges could result in errors when comparing the temperature effects on physiological rates, as the Q_{10} has been shown to be temperature dependent, decreasing when temperature rises (Almeda et al., 2010).

4.2 Effect of food availability

As expected, growth rates of *Paracartia grani* depended on the food availability. At low food concentration a low growth rate was observed in *P. grani* nauplii. This fact might be

explained by a decreased efficiency of food capture at low food concentration, as observed for other congeneric species (Paffenhöfer and Stearns 1988*: Acartia tonsa*), suggesting their adaptation to the high food environments in which they are mainly found (estuarine areas and coastal waters, Alcaraz, 1977; Villate, 1982). At high food concentrations growth rate became stable for *P. grani* nauplii*,* as was previously observed for this (Calbet and Alcaraz, 1997: *Acartia grani*) and other species (Berggreen et al., 1988: *A. tonsa*) at the same temperature 18 ºC, but feeding on a different food type (*Rhodomonas baltica*). There are several studies on the effect of the quality and food concentration on the egg-production rates, growth and development of the genus *Acartia* (Berggreen et al., 1988, Stottrup and Jensen, 1990, Hassett, 2004, Calliari and Tiselius, 2005). However, the effect of the food type on the growth of early stages of copepods is scarcely studied. The prey used in this study (*Oxyrrhis marina*) is considered a high-quality food for *Acartia* species (Klein Breteler and Schogt, 1994, Kleppel et al., 1998), although other studies used different prey (*Rhodomonas baltica or a* mixture of the diatom *Thalassiosira weissflogii* and the *cryptophyte Rhodomonas* sp.). It has been shown that diet modification affects *Acartia* species growth, development, production, and nutritional composition (Ismar et al., 2008; Teixeira et al., 2010). Nevertheless, the growth rate of naupliar stages of *P. grani* at 18 ºC (0.50 d^{-1}, calculated from eq.1) was comparable to those observed on nauplii of this and other congeneric species (Table 3) despite the different prey supplied as food. This fact suggests that food quality might not strongly affect nauplii growth rates of these species under saturating food concentrations.

4.3 Relationship between somatic growth and protein synthesis rates

Somatic growth and protein synthesis rates in *P. grani* nauplii were affected by temperature and food concentration. We found positive relationships between somatic growth

and protein synthesis rates of nauplii feeding at *ad libitum* food concentrations. This is in agreement with the significant correlations previously observed between somatic growth rates and specific $AARS_s$ activities in copepodites and adults of other calanoid species (Yebra et al., 2005; Yebra et al., 2006), both in laboratory and field experiments.

Protein-specific and individual $AARS_s$ activities of *Paracartia grani* nauplii showed patterns similar to growth at different temperatures when feeding *ad libitum*. We also observed a similar pattern between growth rates and individual $AARS_s$ in relation to food availability. However, contrary to expectations, the relationship between specific $AARS_s$ activities and growth rates was negative (Fig. 6A). *P. grani* nauplii specific $AARS_s$ activities also showed a negative relationship with individual biomass (Fig. 7A), and presented three different relationships with growth, corresponding to organisms either starved, under food saturating conditions or growing at intermediate food levels (Fig. 7B). Under starvation (0 - 11 μg $C \cdot L^{-1}$), *P. grani* nauplii metabolic activities were maintained at the expense of accumulated endogenous energy reserves, resulting in either negative or almost nil growth rates (-0.01 - 0.03 d^{-1}) and low individual $AARS_s$, but very high specific $AARS_s$ activities. On the contrary, nauplii feeding on saturating food concentrations showed a clear relationship between growth rates and specific $AARS_s$ activity. This observed variability could be due to one or, most probably, different factors combined, such as i) food limitation of growth and protein synthesis rates, ii) the relative importance of the protein turnover rates in relation to the '*the novo*' protein synthesis rates of the nauplii, which would lead to anomalously high $spAARS_s$ values on organisms under starvation, as previously observed in *Acartia bifilosa* females by Holmborn et al. (2009), and *Oithona davisae* nauplii by Yebra et al. (2011); iii) the body size of the nauplii (as suggested by the relationship observed between $spAARS_s$ activity and individual biomass) and iv) the different developmental stages reached by the nauplii in each food treatment. In this sense, under low food conditions nauplii may not reach

their first feeding stage, and the pre-feeding stages might have different protein metabolism and growth pathways that the feeding ones. Protein growth is defined as the change in mass of the protein pool and can be either positive or negative depending on the relative balance between protein synthesis and degradation (see Fraser and Rogers, 2007). Thus, a first case to explain the observed results could be related to the fact that in actively feeding organisms (above 55 µg $C \cdot L^{-1}$ in this study), rates of protein synthesis would be greater than protein degradation and hence somatic growth will occur. However, in starved organisms (0-11 µg $C \cdot L^{-1}$ in this study) proteins are degraded faster than they are synthesized (Hawkins, 1985). Previous studies on the effect of the frequency of feeding on both overall growth and protein metabolism in the European lobster showed elevated protein synthesis rates but also elevated degradation, resulting in high turnover and reduced growth (Mente et al., 2001). Then, the observed high protein-specific AARS values could be explained by the coupling of the nauplii protein mass decrease due to starvation (or insufficient food for growth) and their high turnover rates. A second case could be methodological as non-feeding organisms may use their lipid reserves to survive. The ß-oxidation of fatty acids could produce PPi during the assay which would interfere in our method because we measure AARS activity as PPi release rate. This precludes the application of this method on organisms living on the degradation of their own lipid reserves. These problems should be tested by measuring enzyme activity using radioactive substrates. In any case, this mismatch between specific AARS activity and growth rates due to artificially induced low food concentrations would not likely occur in the field for this species, considering that typical particulate organic carbon values observed in their coastal habitats lay above those experimental food levels (Huntley and Boyd, 1984; Gardner et al., 2006; Duforet-Gaurier et al., 2010).

Summarizing, both growth and protein synthesis rates of *Paracartia grani* nauplii depended on temperature and food concentration. AARS activity is valid as index of somatic

growth for *P. grani* nauplii when growth is not limited by food availability. However the degradation of proteins during starvation and/or the use of lipids as fuel in pre-feeding nauplii affected the relationship between specific AARS activity and growth rates. The results presented here add to previous studies showing that the $AARS_s$ activity is a useful tool for estimating somatic growth in copepods. Nevertheless, further investigations are required to elucidate the possible effects of size, lipids and protein metabolism in order to use AARS activity as a universal proxy for growth.

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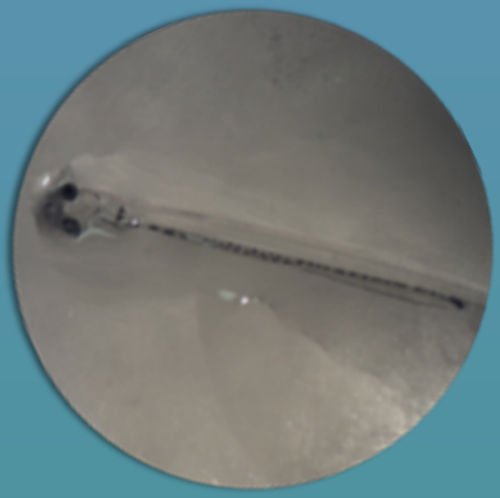
Chapter 2



Aminoacyl-tRNA synthetases (**AARS**) activity as an index of Atlantic herring (***Clupea harengus***) larvae growth

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Abstract

Specific growth rates (SGR) and specific aminoacyl-tRNA synthetases (AARS) activities of herring (*Clupea harengus*) larvae were studied in laboratory experiments. The larvae were kept at 7 and 17ºC feeding *ad libitum*, and at 12ºC were offered three different food concentrations of nauplii and copepodites of *Acartia tonsa* (0.1, 0.3 and 2 prey·mL^{-1}). Both SGR and AARS activities showed significant differences between 7ºC and 12-17ºC. Furthermore, SGR and AARS activities followed a similar pattern in relation to food concentration, with no statistically significant differences between treatments at 0.1 and 0.3 prey·mL^{-1} levels, while at the 2.0 prey·mL^{-1} food level both variables increased significantly. In addition, AARS activities and specific growth rates were influenced by protein degradation under food deprivation. Nevertheless, SGR and AARS activity were significantly correlated.

Keywords: AARS, feeding levels, growth, herring larvae, temperature.

1. Introduction

Atlantic herring (*Clupea harengus*) is one of the most abundant and commercially important fish species in the North Atlantic (Brandt and McEvoy, 2006; Overholtz and Link, 2007). This species plays an important role in the trophodynamics of many systems including the Baltic Sea, where it can exert a top-down control upon the crustacean zooplankton community (Hansson et al., 1990; Arrhenius and Hansson, 1993). Recruitment of spring-spawning Baltic Sea herring is thought to be regulated by processes occurring during the early larval period. Therefore, obtaining robust estimates of the nutritional status and growth rates of herring larvae may not only help to reveal processes affecting recruitment but also to help forecast year-class success.

Atlantic herring is a well-known species (see review by Geffen, 2009) and previous studies have obtained larval growth rates by applying a variety of techniques, including otolith micro-structure analysis (Moksness and Wespestad, 1989; Campana and Moksness, 1991; Suneetha et al., 1999; Johanneseen et al., 2000). Folkvordet al. (2004) suggested that most of the variability in growth rates of early stages of Atlantic herring could be explained by temperature. Some previous studies (Johannessen et al., 2000; Folkvordet al., 2004; Fox et al., 2003) have examined growth rates within a limited range of temperatures (8-12ºC), but recent time-series analyses of larval cohorts of spring-spawning herring by Oeberst et al. (2009) indicates that 12-15 mm larvae normally experience much warmer temperatures (15 to 19°C). Unfortunately, no laboratory studies have examined growth rates or attempted to calibrate growth indices for herring at such higher temperatures.

Proxies for growth in fish larvae have focused on the ratio RNA:DNA, which gives a measure of the protein synthetic capacity of the cell (Buckley, 1984; Ferron and Leggett, 1994; Clemmesen and Doan, 1996; Buckley et al., 1999). Growth rate has a close relationship with the protein synthesis (Love, 1970) and the RNA content per cell varies with the amount of protein synthesis. Because DNA is relatively constant within a cell, the RNA content is normally standardized using DNA (Clemmensen, 1987). The RNA:DNA ratio has been used as a proxy for growth rate and condition in fish larvae (Buckley, 1979; Westerman and Holt, 1994; Clemmensen, 1996). Besides that, there are other biochemical indices related to growth rate based on enzymatic activities such as lactate deshydrogenase (LDH), or citrate synthase (CS). All of these methods have constraints, some are too time consuming, and others require large samples or are valid only for particular larval stages.

During the last decade the activity of the enzyme aminoacyl-tRNA synthetases (AARS), which catalyze the first step of protein synthesis, has been used in a variety of aquatic organisms. Here, we use AARS activity because the results obtained in previous works showed its suitability as an index of growth rate in freshwater and marine crustaceans, such as *Daphnia magna* (Yebra and Hernández-León, 2004), *Calanus helgolandicus* (Yebra et al., 2005) *Euphausia superba*, (Guerra, 2006), *Calanus finmarchicus* (Yebra et al., 2006) *Oithona davisae* (Yebra et al., 2011) and *Paracartia grani* (Herrera et al., 2012). The objective of this work was to study whether AARS could be used to explain changes in growth of Atlantic herring larvae reared at different temperatures and at different prey levels. This study specifically included experiments testing the growth response of larvae at warmer water temperatures, feeding on calanoid copepods to better match the conditions experienced in the field by the Baltic spring-spawning larvae.

2. Materials and Methods

Adult herring (*Clupea harengus*) was obtained from fisherman in Greifswald (Germany) on 22 April 2009 and transported on ice to the Elbe Aquarium (University of Hamburg). A total of 42 female fish (total length (mean±SD): 28.70 ± 1.07 cm, wet weight: 197.20 ± 2.63 g) and 21 male fish (total length: 28.50 ± 1.07 cm, wet weight: 202.80 ± 216.63 g) were used for strip-spawning eggs and milt, respectively. Eggs from female fish were extruded into small glass plates and fertilized with activated milt from males. Herring eggs were also gathered by SCUBA divers in the Bay of Greifswald on 25th and 29th April 2009. Eggs attached to seagrass (*Zostera marina*) and fennel pondweeds (*Potamogeton pectinatus*) were collected at Gahlkower Haken at a water temperature of 17.3ºC and a salinity of 6.3. These eggs were transported to the Elbe Aquarium and hatched at the same *in situ* temperature conditions. When collected, these eggs were already in an advanced stage of development. The temperature and salinity conditions of egg incubation varied between 7 to 12ºC and 17 to 18, respectively. Once the larvae reached the necessary length we proceeded with the experiments. Temperature and salinity were measured every day during the experiments (Cond 315i meter, TetraCon 325 Probe: WTW, Woburn, MA, USA).

Growth experiments to test the effect of temperature were conducted in temperature controlled rooms at 7 and 17ºC, using 2 tanks per treatment during 18 days, with a standard length range of larvae between 10 and 24 mm. The experimental tanks had a volume of 60 L and they were gently aerated. The outlet pipes of the *ad libitum* treatment tanks were fitted with a large pipe with holes covered by 35 µm mesh net in order to retain copepods and larvae in the tank. The water inlet tube was adjusted to produce a fine trickle of water

(approx. 0.3 $L \cdot min^{-1}$ during 2 $h \cdot d^{-1}$), resulting in every second day water exchange of approximately 30%.

Every day, the herring larvae were fed with *Rhodomonas baltica* and nauplii of *Acartia tonsa.* Late nauplii and early copepodites were added to the diet when larvae were able to consume larger prey, based on age and length. Atlantic herring larvae ($n \leq 10$) were collected every fifth and second day in the 7 and 17ºC treatments and the larvae were photographed individually for length measurements. Each larvae was transferred without water into a 1.5 mL eppendorf vial and immediately stored in liquid nitrogen (-196 ºC) for biochemical analysis.

Growth experiments to test the effect of food concentration were conducted at 12ºC using 6 tanks with 200 larvae/tank and 2 tanks per food level (0.1, 0.3 and 2 $prey \cdot mL^{-1}$) during 18 days. The standard length range of larvae was between 9 and 21 mm. The larvae were fed with nauplii and copepodites of *Acartia tonsa*, every day. Food concentration was measured daily with a binocular microscope. Every third day we collected 10 larvae per tank, measured individual length and immediately stored in liquid nitrogen (-196 ºC) for later analysis.

Larval food deprivation experiments were conducted to assess the time-course of changes in the index of growth during starvation. A short-term trial was conducted with larvae from an *ad libitum* culture at 17ºC. Larvae were deprived of food in two replicate tanks, using another tank as a control for *ad libitum* feeding, using *Acartia tonsa* nauplii as prey. A total of 8 larvae per tank were sampled every 6 hours for 36 hours. Medium-term trials were also conducted at 7ºC and 17ºC, using 8 tanks, three replicates per (unfed) treatment plus one control (fed *ad libitum* with *Acartia tonsa*). A total of 21 larvae were collected every 24 hours (17°C) for 5 days or every 48 hours (7°C) for 6 days. During

sampling, each larvae was photographed and transferred without water into a 1.5 mL eppendorf vial and immediately stored in liquid nitrogen (-196 ºC) for biochemical analysis.

Standard length (Ls ± 0.1 mm) was measured from the tip of the mouth to the end of the notochord using an image analysis system (Optimas 6.0). Length measurement was converted to dry weight using the relationship between dry weight (dw, µg) and length (mm) given by Borchardt (2010). Specific growth rates (SGR, day^{-1}) were calculated as the slope of the relationship between natural logarithm of dry weight (µg dw) and day post hatching (DPH) (Table 1).

Table 1. Atlantic herring larvae specific growth rates (SGR, d^{-1}) and specific AARS activities (spAARS, nmol PPi·mg $prot^{-1}·h^{-1}$) at different temperatures (ºC) and food levels (prey·mL^{-1}); 'n' is the number of either individuals sized or samples analyzed (see Figs. 1 and 3).

T (ºC)	**Food level (prey·mL^{-1})**	**SGR (d^{-1}) (r^2, n)**	**spAARS ± SE (n) (nmol PPi·mg $prot^{-1}·h^{-1}$)**
7	*ad libitum*	0.06 (0.88, 90)	114.71 ± 13.87 (50)
7	*ad libitum*	0.06 (0.95, 89)	98.42 ± 7.91 (47)
17	*ad libitum*	0.09 (0.98, 153)	114.73 ± 8.71 (76)
17	*ad libitum*	0.07 (0.82, 220)	115.84 ± 11.11 (71)
12	2	0.17 (0.99, 75)	133.40 ± 16.27 (30)
12	2	0.16 (0.98, 75)	169.21 ± 19.36 (30)
12	0.3	0.08 (0.95, 75)	129.61 ± 8.40 (26)
12	0.3	0.11 (0.89, 75)	162.70 ± 10.77 (37)
12	0.1	0.14 (0.98, 59)	140.48 ± 9.38 (23)
12	0.1	0.11 (0.93, 45)	170.93 ± 13.71 (30)

In the laboratory, frozen samples were homogenized in 500 µL Tris-HCl buffer (20 mM, pH 7.8) with an eppendorf micropestle and centrifuged (5000 rpm, 10 min, 0ºC). AARS activity was assayed at 25ºC following the method of Yebra and Hernández-León (2004), modified by Yebra et al. (2011) and adapted to microplate readings as follow: 50 µL of the samples supernatant was added to a mixture containing 40 µL of pyrophosphate (PPi) reagent (Sigma, P-7275) and 60 µL of Milli-Q water. The absorbance of the reaction mixture was monitored at 340 nm for 10 min on a SAFAS flx-xenius spectrofluorometer with microwell plates. The aminoacylation of the tRNA releases PPi, which produces an oxidation of NADH. This is registered as a decrease in absorbance (dAbs). The NADH oxidation rate ($dAbs \cdot min^{-1}$) was converted to PPi release rate (AARS activity, nmol $PPi \cdot h^{-1}$) using the next equation (1):

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

where $dAbs \cdot min^{-1}$ 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, V_{rm} is the volume of the reaction mixture (mL), V_s 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 microwell plate and V_{hom} is the volume of homogenized (mL). The AARS activity was corrected for the *in situ* temperature of each experiment by applying an activation energy of 8.57 kcal · mol^{-1} (Yebra et al., 2005) to the Arrhenius equation in order to obtain $AARS_{in\ situ}$ activity.

Protein content was assayed using a Sigma-Aldrich protein assay kit (BCA-1 and B 9643). In this method, proteins reduce alkaline Cu(II) to Cu(I) in a concentration-dependent

manner (Lowry et al., 1951). Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562 nm (Smith et al., 1985). Bovine Serum Albumin (BSA) was used as the protein standard. Absorbance of the solution was measured at 562 nm using a SAFAS flx-xenius spectrofluorometer.

2.7. Statistical Methods

STATISTICA 6.0 (Statsoft, Inc.) and R (R Development Core Team 2009) were used for statistical analyses. The effect of temperature and food level on dry weight and specific AARS activity were tested by a linear model with random effect on replication, where the replicates were nested in temperature and food level treatment groups respectively.

The model for temperature followed the next form:

$$\log(dw) = \beta_{0,7} + \beta_{0,12} I_{12} + \beta_{0,17} I_{17} + b_0 + (\beta_{1,7} + \beta_{1,12} I_{12} + \beta_{1,17} I_{17} + b_1)\text{temp} + \varepsilon \qquad \text{(eq.1)}$$

being $b_0 \approx N(0,\sigma_0)$, $b_1 \approx N(0,\sigma_1)$, $\varepsilon \approx N(0,\sigma\varepsilon_k)$. In this model I_{12} and I_{17} are dummy variables such that $I_k = 1$ when temperature is k degrees (k = 12, 17) and 0 otherwise. The terms b_0 and b_1 represent the random variation in intercept and slope due to the replicates. In this way $\beta_{0,7}$ and $\beta_{1,7}$ represent the intercept and slope of log (dw) versus time when temperature is 7ºC (reference); $\beta_{0,12}$ and $\beta_{1,12}$ represent variation in intercept and slope with respect to the reference values when temperature is 12ºC; and $ß_{0,17}$ and $\beta_{1,17}$ the respective variation respect to the reference when temperature is 17ºC. In the residual term ε a heteroscedastic model has been considered, with variance $\sigma\varepsilon_k$ depending on the temperature level and replicate. The same model was used for the different food levels. A significance level of $\alpha = 0.05$ was considered for the statistical analyses.

3. Results

3.1. Effect of temperature on fish larvae

The relationship between dry weight (µg dw) and day post hatching (8-40 DPH) of *C. harengus* larvae is shown in Fig. 1 at the 3 experimental temperatures tested (7, 12, 17ºC) including both replicates per experiment.

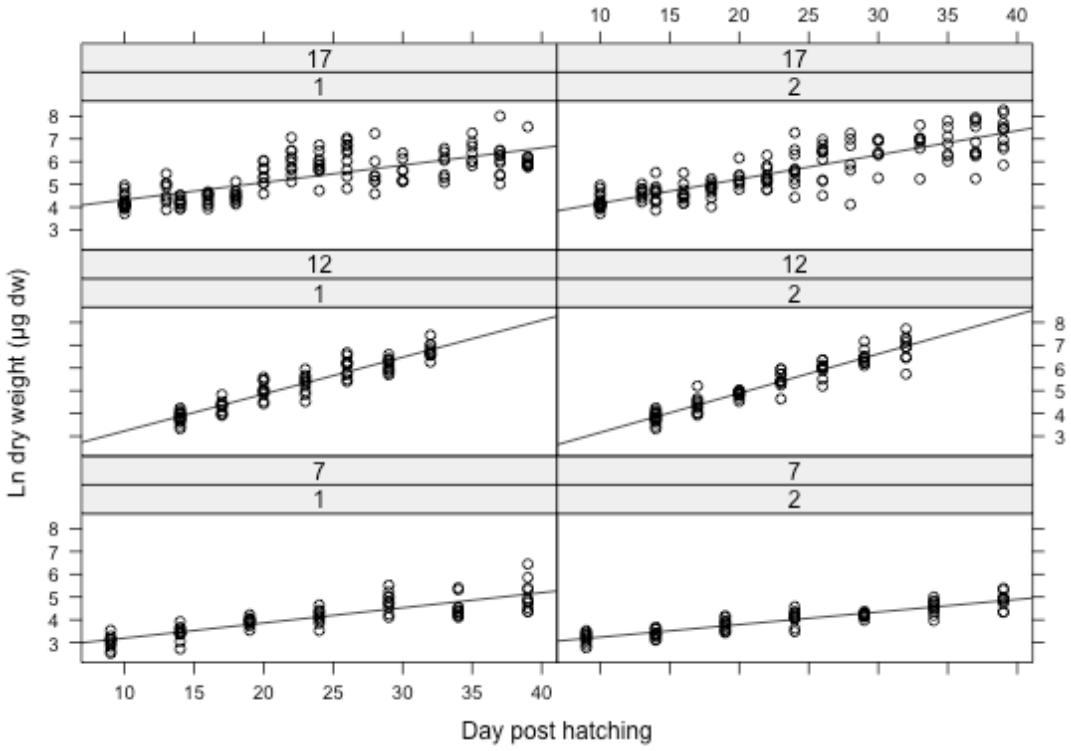

Figure 1. Relationship between dry weight (µg dw) of *C. harengus* and day post hatching at different temperatures (ºC).

All regression slopes were highly significant ($p<0.05$). Specific growth rates (SGR, day^{-1}) considered as the slope of these linear regressions are shown in Table 1. SGR varied from 0.06 to 0.17 day^{-1} between 7 and 17°C (Fig. 2).

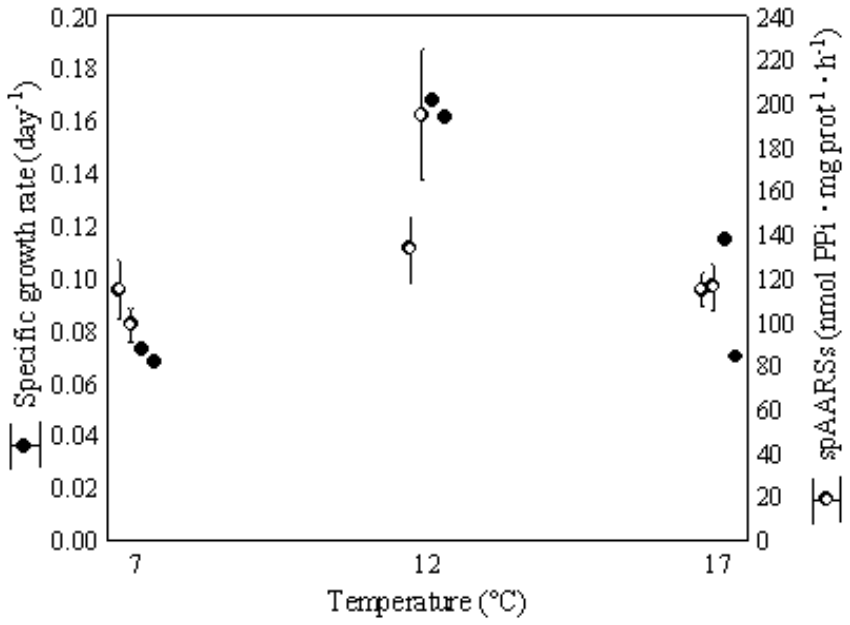

Figure 2. Effect of temperature on SGR (day^{-1}) and spAARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$).

The SGR at 7ºC showed significant differences with the SGR at 12 and 17ºC ($p<0.05$). The model used to analyze the results showed no differences among replicates at each temperature. Specific AARS activity (spAARS, Table 1) ranged from 98.42 to 169.21 nmol PPi·mg $prot^{-1}·h^{-1}$, and showed a similar pattern to SGR. There were statistically significant differences between average AARS activities at different temperatures ($p<0.05$).

3.2. Effect of food concentration on fish larvae

The relationship between dry weight (µg dw) and day post hatching (14-32 DPH) of *C. harengus* larvae is shown in Fig. 3 for the 3 experimental food levels tested (0.1, 0.3, 2 prey·mL^{-1}), including both replicates per experiment.

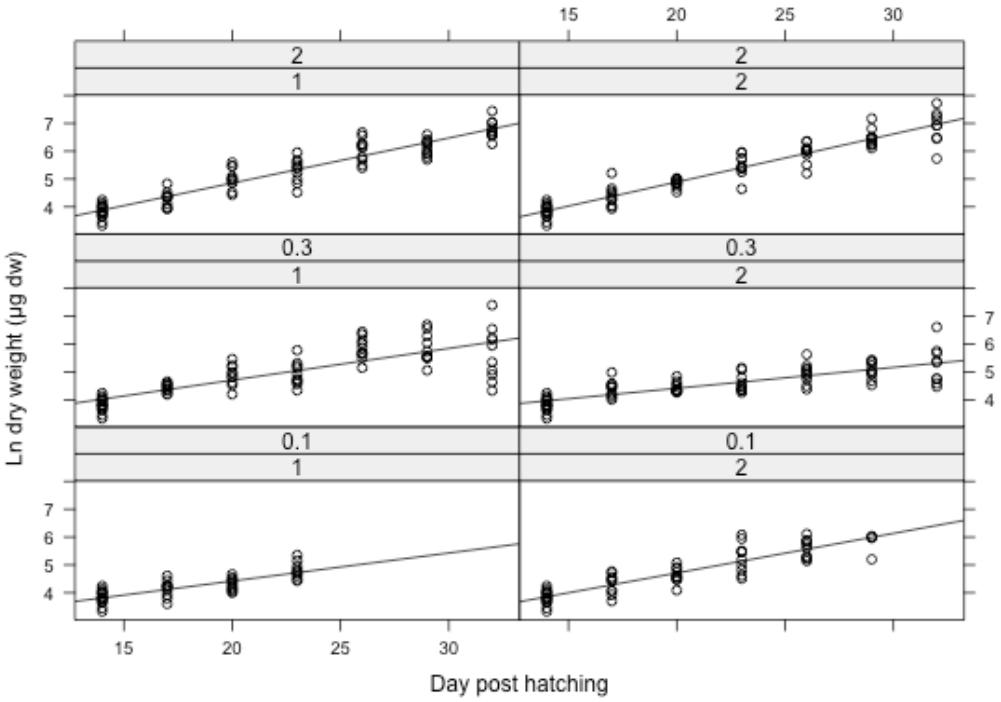

Figure 3. Relationship between dry weight (µg dw) of *C. harengus* and day post hatching under different food levels (prey·mL^{-1}).

All regressions were highly significant ($p<0.05$). The specific growth rates considered as the slope of these linear regressions are shown in Table 1. SGR varied from 0.08 to 0.17 day^{-1} (Fig. 4).

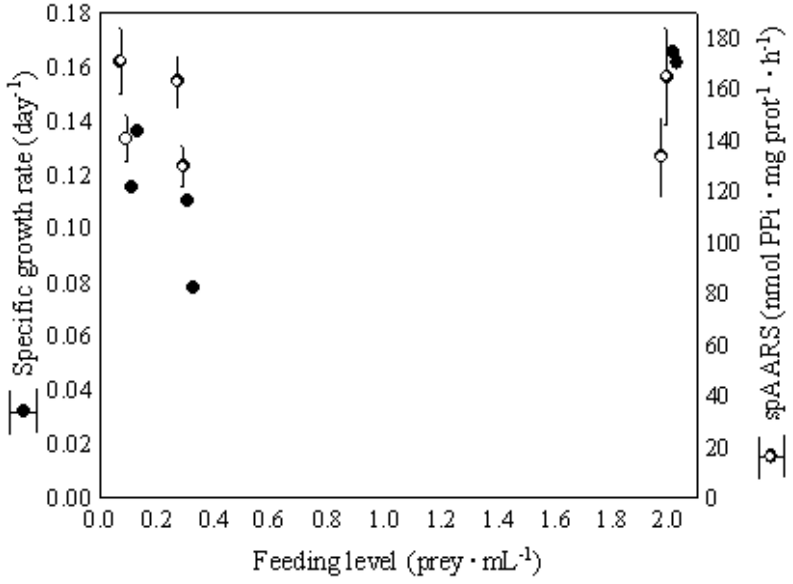

Figure 4. Effect of food concentration on SGR (day^{-1}) and spAARS activity (nmol PPi·mg prot^{-1}·h^{-1}).

The SGR at 0.1 and 0.3 prey·mL^{-1} food levels showed significant differences in relation to SGR at 2.0 prey·mL^{-1} ($p<0.05$). The model used to analyze the results showed no differences among replicates at each food concentration. Average spAARS ranged from 129.61 to 169.21 nmol PPi·mg prot^{-1}·h^{-1} (Table 1) showing a similar pattern to SGR for the different food concentrations. AARS values at 0.1 and 0.3 prey·mL^{-1} showed significant differences in relation to 2 prey·mL^{-1} ($p<0.05$). High values of specific growth matched the high values of spAARS at the same food level.

3.3. Relationship between growth rates and specific AARS activities

A positive relationship between specific growth rates (d^{-1}) and spAARS activities (Fig. 5A) was observed at different temperatures.

$$\text{Specific growth rate} = -0.1031 + 0.0017 \cdot \text{spAARS} \ (r^2=0.711;\ p<0.05) \qquad \text{(eq.2)}$$

However, specific growth rates (d^{-1}) and spAARS activities under different food levels (Fig. 5B) showed a slightly positive relationship but the slope was not significant ($p=0.78$).

$$\text{Specific growth rate} = 0.0876 + 0.0003 \cdot \text{spAARS} \ (r^2=0.022) \qquad \text{(eq.3)}$$

Using all data at different temperatures and under different food levels, specific growth rates (d^{-1}) explained 42.5% of spAARS activity variance ($p<0.05$) (Fig. 5C).

$$\text{Specific growth rate} = -0.0343 + 0.0011 \cdot \text{spAARS} \ (r^2=0.425) \qquad \text{(eq.4)}$$

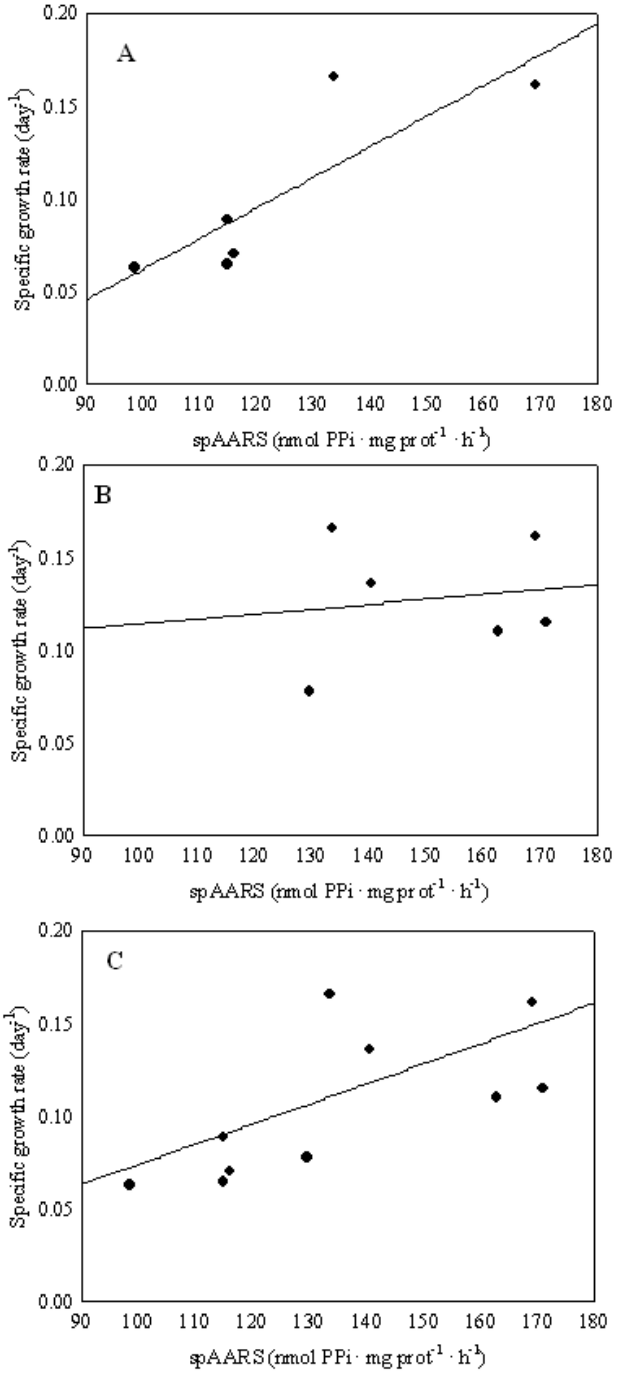

Figure 5. Relationships between growth rates (day^{-1}) and specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$) A: at different temperatures (ºC), B: under different food concentrations and C: all data pooled.

The relationship between specific AARS activity and individual biomass (μg protein·ind^{-1}) followed a negative exponential pattern (Fig. 6) at different temperatures (Fig. 6A), as well as under different food levels (Fig. 6B). This relationship showed that small

larvae (<100-200 µg protein·ind^{-1}) had the largest spAARS values; likewise lower and constant values were observed in larger individual biomass.

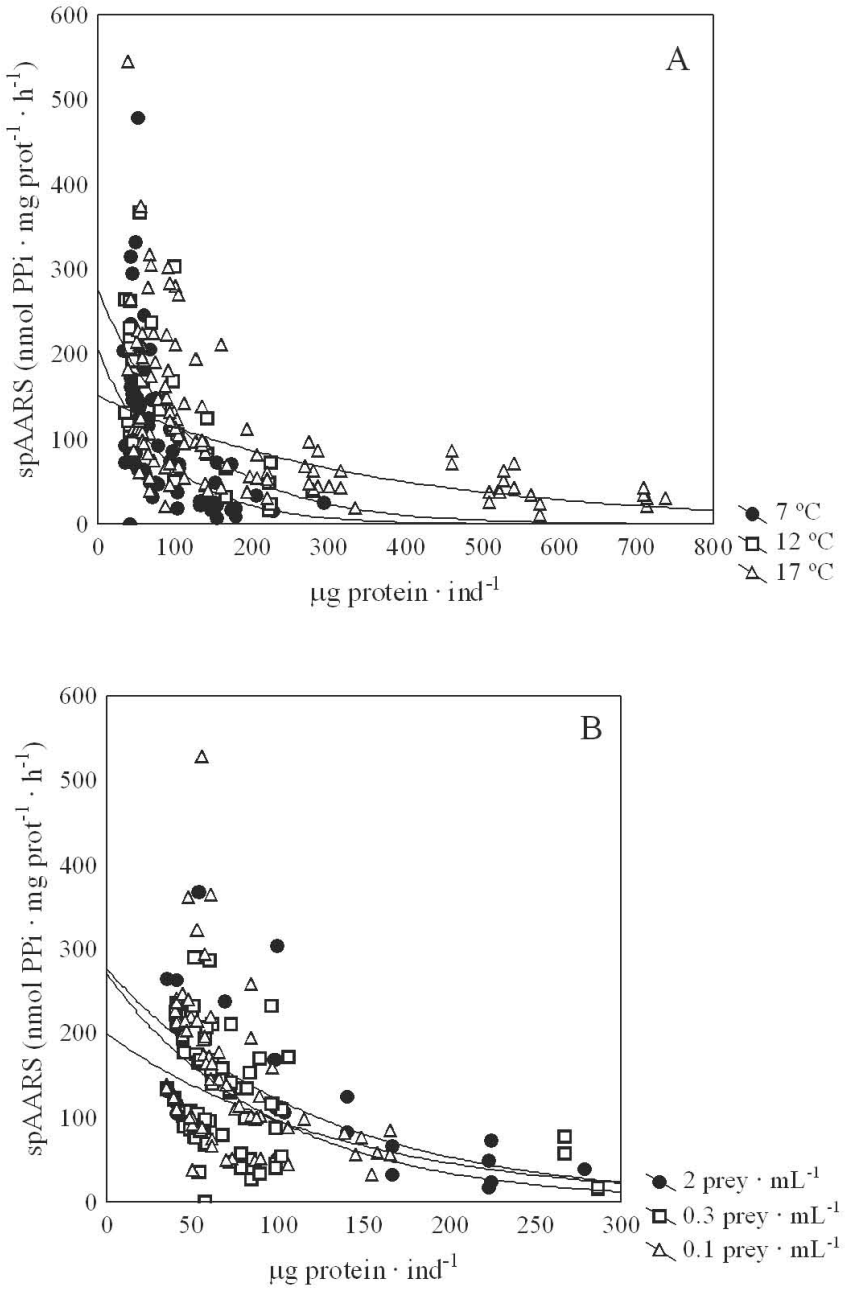

Figure 6. Relationship between specific AARS activity (nmol PPi·mg prot^{-1}·h^{-1}) and individual biomass (µg protein·ind^{-1}) A: at different temperatures (ºC), B: under different food concentrations (prey·mL^{-1}).

3.4. Effect of food deprivation on fish larvae

SGR of the starved larvae ranged from -0.15 to 0.07 day^{-1} (Table 2).

Table 2. Atlantic herring larvae specific growth rate (SGR, d^{-1}) and spAARS (nmol PPi·mg $prot^{-1}·h^{-1}$) under food deprivation; 'n' is the number of either individuals sized or samples analyzed. Data in italics correspond to experiments of 36 hours duration.

T (ºC)	**SGR (d^{-1}) (r^2, n)**	**spAARS ± SE (n) (nmol PPi·mg $prot^{-1}·h^{-1}$)**
7	0.01 (0.39, 46)	238.51 ± 20.90 (24)
7	0.01 (0.46, 43)	245.72 ± 24.49 (24)
7	0.01 (0.37, 56)	253.29 ± 20.35 (26)
17	0.07 (0.73, 73)	307.82 ± 20.98 (26)
17	*-0.15 (0.31, 60)*	*132.37 ± 12.91 (30)*
17	*0.05 (0.04, 64)*	*138.64 ± 17.29 (23)*

The relationship between spAARS and individual biomass (µg protein·ind^{-1}) of unfed larvae showed a similar trend to the one observed for fed larvae (Fig. 7**)**.

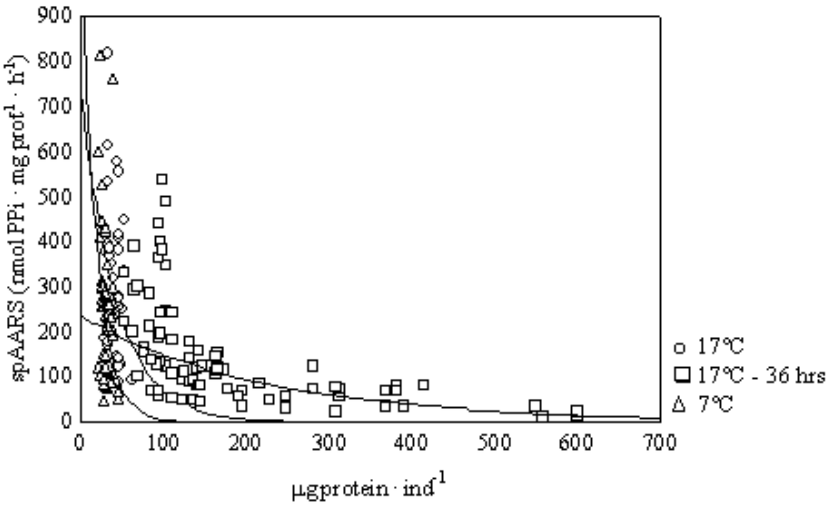

Figure 7. Relationship between specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$) and individual biomass (µg protein·ind^{-1}) under food deprivation.

4. Discussion

We assessed the effect of temperature and food concentration on specific growth rates and specific AARS activities of *Clupea harengus* larvae. A positive relationship between SGR and AARS activity was observed at different temperatures under *ad libitum* food concentrations. However, this relationship was not significant at different food levels. Besides, under food deprivation, high specific AARS activities were found at low growth rates

Specific growth rates estimated in this study were similar to the observed by other authors (Suneetha et al., 1999; Folkvord et al., 2000; Johannessen et al., 2000; Arrhenius and Hansson, 1996; Kiørboe and Munk, 1986, Table 3).

Table 3. Review of herring larvae growth rates under different food levels and temperatures.

T°C	Food concentration (prey·mL^{-1})	Standard length (mm)	SGR (% d^{-1})	Reference
7	*ad libitum*	10-24	6.10	this study
8	*ad libitum*		7.1	Suneetha et al., 1999
8	0.04	12-19	4-6	Folkvord et al., 2000
8	1.2	15-23	5-8	Folkvord et al., 2000
8	0.02 - 0.04	9-24	1.5	Johannessen et al., 2000
8	1.2 - 2.0	9-24	7	Johannessen et al., 2000
11	*ad libitum*		6.6	Suneetha et al., 1999
12	0.1	9-21	12.20	this study
12	0.3	9-19	9.40	this study
12	2	9-21	16.70	this study
17	*ad libitum*	10-22	9.20	this study

Furthermore, the specific growth rates of these fish larvae also depended on food availability. At low food concentration the growth rates observed were lower. The variability of the growth rates due to the food concentration was higher than in previous observations, showing that growth rate is affected by the amount of food concentration in the field (Kiørboe and Munk, 1986; Johannessen et al., 2000; Folkvord et al., 2000, Table 3).

AARS activities were also positively affected by food concentration. However, when *C. harengus* larvae were starved, they showed high specific AARS activities coupled with low or negative growth rates. This high enzyme activity under starvation was also observed on nauplii and adult stages of calanoid copepods (*Acartia bifilosa*, Holmborn et al., 2009; *Paracartia grani*, Herrera et al., 2012). Herrera et al. (2012) suggested that organisms under food deprivation were sustained at the expense of accumulated endogenous energy reserves, resulting in either negative or almost nil growth rates despite the high specific AARS activities (Table 2). The negative SGR in starved fish larvae could be explained by protein degradation (Love, 1980). This catabolism was previously described in *Clupea harengus* larvae under food deprivation (Ehrlich, 1974). The effect of starvation on metabolism is more remarkable in early stages than in juveniles, probably due to the lower lipid reserves in juveniles (Gadomskia and Petersen, 1988; Richard et al., 1991). Starved larvae lost weight due to metabolic costs, presenting minimum levels of muscle fibre, which represent 60 to 70% of their body weight (Machado et al., 1988). It has been observed that muscle mass of fish larvae was the only variable showing differences between starved and fed organisms (Martin and Wright, 1987; Ferron and Leggett, 1994; Catalán, 2003; Pliego Cortés, 2005). This is because, in fish larvae, body weight is very sensitive to food deprivation due to the fast protein degradation (Love, 1980). It is also known that *C. harengus* larvae consume lipid reserves during starvation (Ehrlich, 1974). Whether this characteristic influences the relationship between growth rates and specific AARS activity under food deprivation

conditions might be the key to understand the unexpected high AARS activities observed in organisms with low growth rates (Holmborn et al., 2009; Herrera et al., 2012).

On the other hand, when larvae were not food limited, both SGR and AARS activity showed a positive relationship with temperature. A significant correlation was found between AARS activities and specific growth rates. This is accordance with previous works in different zooplankton species such as *Daphnia magna* (Yebra and Hernández-León, 2004), *Calanus helgolandicus* (Yebra et al., 2005), *C. finmarchicus* (Yebra et al., 2006), *Oithona davisae* (Yebra et al., 2011), *Euphausia superba* (Guerra, 2006) and *Paracartia grani* (Herrera et al., 2012).

In summary, specific growth rates and specific AARS activities of *C. harengus* larvae depend on temperature and food concentration as expected. In addition, AARS activities and specific growth rates were influenced by protein degradation under food deprivation. Even so, SGR and AARS activities were positively correlated.

Acknowledgments

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Chapter 3

The effect of a strong warm year on subtropical mesozooplankton biomass and metabolism

Inma Herrera, José López-Cancio, Lidia Yebra and Santiago Hernández-Léon

Abstract

The winter of 2010 was the warmest of the last 30 years in the subtropical oceanic waters north off the Canary Islands. In this year, surface temperature was always above 19ºC, promoting a strong stratification and quite low values of chlorophyll during winter-spring. The late winter bloom, typical in these waters, was not observed. During the normal timing of the bloom, February-March, the indices of mesozooplankton grazing (gut fluorescence) and respiration (ETS activity) showed low values compared to previous years. However, relatively high mesozooplankton biomass (dry weight) was observed during the post bloom period (April-June). This paradox was explained by the input of dust deposition from the Sahara desert. These inputs are suggested to be indirectly responsible for the sustained growth rates (AARS activity) observed throughout the study period. Our findings show how climatic warming and dust events may interact affecting the intensity of the winter-spring bloom in subtropical waters.

Keywords: AARS, biomass, dust deposition event, ETS, GF, lunar cycle, zooplankton

1. Introduction

Zooplankton grazing, respiration and growth in the open ocean have an important role in the ocean's biogeochemical cycles. However, physiological information on zooplankton in the subtropical oligotrophic gyres remains rather limited (Welschmeyer and Lorenzen, 1985; Harrison et al., 2001; Huskin et al., 2001; Woodd-Walker et al., 2002). The Canary Islands are located in the subtropical gyre and exhibit oligotrophic characteristics (De León and Braun, 1973). These waters are characterized by a quasi-permanent thermocline caused by a strong surface heating through the year that restrains the pumping of nutrients to surface layers. During winter, the atmospheric cooling erodes the thermocline allowing a small enrichment of nutrients in the euphotic zone promoting the so-called late winter bloom (Ryther and Menzel, 1960). This process produces an increase in primary production and zooplankton biomass. This winter bloom is well known in the Canary Island waters from the stand point of plankton biomass and production (De León and Braun, 1973; Arístegui et al., 2001; Hernández-León et al., 1998, 2004, 2010; Moyano et al., 2009; Schmoker et al., 2012). The bloom starts when temperature falls below 19ºC in the upper 100 m, promoting chlorophyll values above 0.5 mg Chl $a \cdot m^{-3}$, and it is normally observed during February and March (Hernández-León et al., 2004, 2010).

Micro- and mesozooplankton biomass also bloom during the late winter bloom as a consequence of the increased primary production (Arístegui et al., 2001; Schmoker et al., 2012). In mesozooplankton the timing of the bloom is also related to the lunar cycle (Hernández-León, 1998; Hernández-León et al., 2001, 2002, 2004, 2010). This relationship is explained by the effect of moonlight on diel vertical migrants (DVM) behaviour. During the illuminated period of the lunar cycle the migrants avoid the shallower layers (0-100 m) during the night. This promotes an increase in epipelagic zooplankton abundance and

biomass due to the lower predatory pressure of migrants. However, the magnitude of the mesozooplankton bloom is related to climate as mixing during winter is stronger and lasts longer during cold years. In this sense, Schmoker and Hernández-León (2013) showed the mesozooplankton bloom to last three months during a cold year and only one month during a warm year.

On the other hand, the proximity of the Canary Islands to the African continent makes this archipelago a suitable place to study Saharan dust deposition effects on ocean productivity. Dust increases the availability of carbon, nitrogen, silica and iron, among other nutrients (Duarte et al., 2006), promoting blooms of phyto- and zooplankton (Hernández-León et al., 2004, 2010; Giovagnetti et al., 2012). Besides that, we know the effect of dust storms on bacteria, unicellular diazotrophs and *Trichodesmiun* (Benavides et al., 2013), as well as on primary production and microzooplankton in the Canary Island waters (Hernández-León et al., 2004; Franchy et al., *submitted*).

The objective of the present work was to study the mesozooplankton biomass and metabolism north off the Canary Islands from February to June 2010. Mesozooplankton biomass was estimated as dry weight, while gut fluorescence (GF), electron transport system (ETS), and aminoacyl-tRNA synthetases (AARS) activities where used as indices of grazing, respiration and growth, respectively. According to remote sensing data (www.oceanmotion.org/html/resources/ssedv.htm), the year 2010 presented the warmest winter of the last 30 years in the study area. The knowledge of the effect of a warming ocean on plankton productivity is of paramount importance in future scenarios, especially in the Canary Current where a general warming has been observed since 1986 (Arístegui et al., 2009; Demarcq, 2009). Hence, we emphasized this aspect by comparing with previous studies of the late winter bloom in the area.

2. Materials and Methods

Sampling took place from February to June 2010 in the oceanic waters north off Gran Canaria Island (Fig. 1). Four oceanographic stations, separated 10 miles, were monitored from coast to open ocean on board the RV Atlantic Explorer. Vertical profiles (0-300 m) of temperature, conductivity and fluorescence were obtained using a CTD Sea-Bird Electronics with SBE-25 probe. Samples for chlorophyll *a* concentration were taken at 20 m depth with a 4 L Niskin bottle, and used for calibration of the fluorescence profiles data from the fluorometer (Turner Self-Contained Underwater Fluorescence Apparatus, SCUFA) attached to the rosette and CTD. Unfortunately, CTD data from February were not available due to a CTD failure. However, Las Palmas de Gran Canaria port provided sea surface temperature information from a buoy close to our sampling area (Fig. 1).

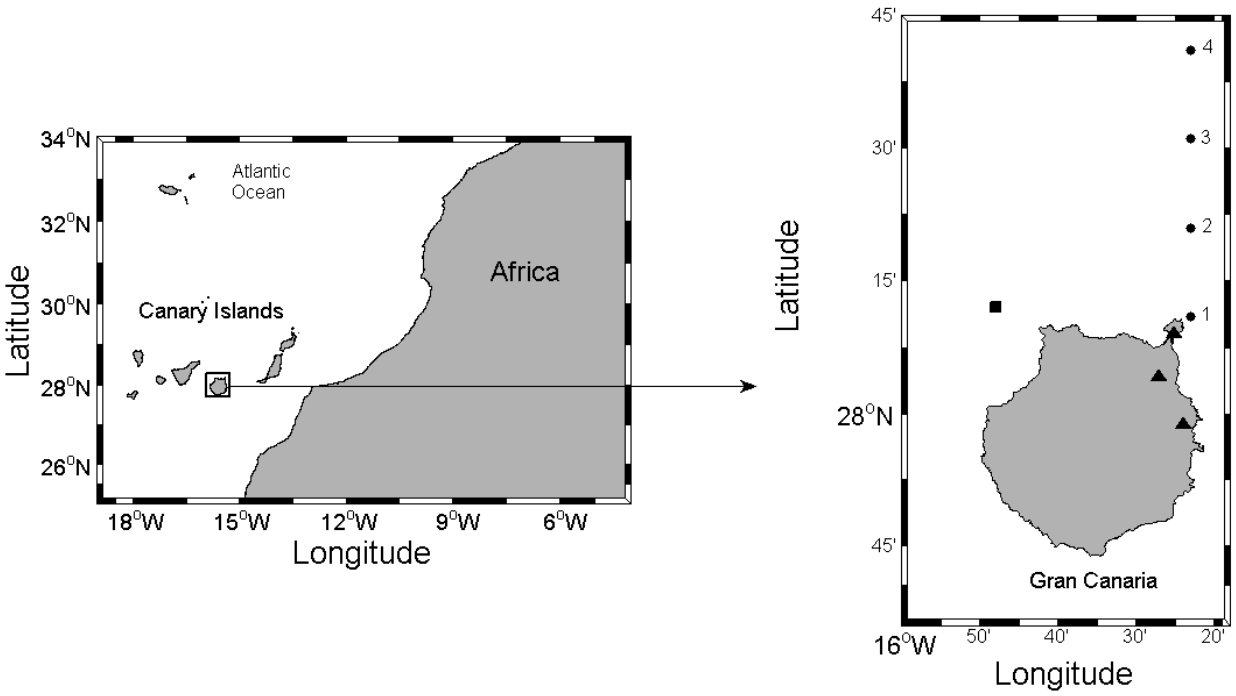

Figure 1. Location of the four oceanographic stations (circles), three atmospheric stations (triangles), and the buoy position (square) during the study period.

Atmospheric total suspended particulate matter was collected onto Whatman GF/A fiberglass filters using a high volume sampler pumping system (MCV) at a flow rate of 50 $m^3 \cdot h^{-1}$. Each sampling period started at 08:00 h and lasted 24 h. The collectors were placed 10 m above the ground at three stations to the north of Gran Canaria Island (Fig. 1). For iron (Fe) analysis, the filters were processed with nitric and hydrochloric acid, according to the Beyer modified method (López Cancio et al., 2008).

Zooplankton was sampled in vertical hauls (0-200 m; 0.6 $m \cdot s^{-1}$) using a double WP-2 net (UNESCO 1968, 100 μm mesh size) fitted with a TSK flowmeter. One cod-end was fractionated into different size categories (100-200, 200-500, 500-1000 and >1000 μm) and immediately frozen in liquid nitrogen (-196ºC) for later analysis in the laboratory. The sample from the second cod-end was fixed in formalin (1% in seawater), kept at 4ºC for 24 h and divided in two subsamples in the laboratory with a Folsom plankton splitter for taxonomy and dry weight (DW) measurements (Lovegrove 1966), respectively.

At the laboratory, frozen samples were homogenized in Tris-HCl buffer (20 mM, pH 7.8) and a subsample of 0.2 mL was taken to determine gut fluorescence (GF, chlorophyll *a* plus phaeopigments), following the procedure given by Parsons et al. (1984) and Arar and Collins (1997). Pigments were extracted for 24 h in 10 mL of 90% acetone at 4ºC in the dark (Parsons et al., 1984). Chlorophyll *a* and phaeopigments were measured fluorometrically on a Turner Design fluorometer 10-AU digital, calibrated with pure Chl *a* (C-6144, Sigma-Aldrich) as suggested by Yentsch and Menzel (1963).

The remaining sample was centrifuged (10 min, 0ºC) and subsamples where taken to measure (1) the electron transport system (ETS) activity according to Packard et al. (1971) taking into account the modifications of Kenner and Ahmed (1975), and (2) aminoacyl-tRNA synthetases activity (AARS) following the method of Yebra and Hernández-León (2004) modified by Yebra et al. (2011). AARS activity was calculated using the next equation:

$$\text{AARS activity (nmol PPi} \cdot \text{h}^{-1}) = \left(\frac{\frac{\text{dAbs}}{\text{min}} \cdot 10^3 \cdot 60 \cdot \text{V}_{\text{rm}}}{\text{V}_\text{s} \cdot 6.22 \cdot 2 \cdot 1} \right) \cdot \text{V}_{\text{hom}}$$

where $dAbs \cdot min^{-1}$ 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, V_{rm} is the volume of the reaction mixture (mL), V_s is the volume of sample in the assay cuvette (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, 1 is the pathlength correction for the cuvette width (cm) and V_{hom} is the total volume of sample homogenate (mL).

Biomass was measured as proteins following the method of Lowry et al. (1951) adapted for micro-assay by Rutter (1967) and using Bovine Serum Albumin (BSA) as standard. The enzymatic activities were recalculated for the *in situ* temperature using the Arrhenius equation and the corresponding activation energies for ETS (15 kcal · mol^{-1}, Packard et al., 1975) and for AARS (8.57 kcal · mol^{-1}, Yebra et al., 2005).

An analysis of variance (ANOVA) was used to compare the evolution of each variable (chlorophyll *a,* biomass, GF, ETS and AARS) over time at the four stations sampled. There were no differences between the different stations (ANOVA, $p>0.05$), except for the chlorophyll *a* at the more coastal station (Table 1). Hence, we pooled the zooplankton data of all stations. For chlorophyll *a*, we averaged the three more oceanic stations.

Table 1. Variance analysis (ANOVA) comparing differences between stations for each variable: chlorophyll *a* (mg Chl *a* · m^{-3}), mesozooplankton biomass (mg DW · m^{-2}), specific GF (µg pigment · mg $prot^{-1}$), specific ETS (µL O_2·mg $prot^{-1}$·h^{-1}) and specific AARS (nmol PPi · mg prot-1·h-1).

Variable	F	p-value	F crit
Chl *a* (stations 1-4)	6.72	<0.05	2.78
Chl *a* (stations 2-4)	0.69	>0.05	3.25
Biomass	0.35	>0.05	2.80
GF	2.79	>0.05	2.79
ETS	0.86	>0.05	2.79
AARS	0.62	>0.05	2.79

In addition, interannual variability of environmental (temperature) and biological (chlorophyll, zooplankton biomass) variables was evaluated during the normal timing of the bloom (January-March) and afterwards (April-June), comparing our data with the literature. In order to compare the zooplankton biomass of this study (0-200 m) with Moyano and Hernández-León (2011) values (0-100 m depth), we used the relationship log-mg DW (0-100)=0.0869+0.9452·log-mg DW (0-200), (R^2=0.894, p<0.001, Hernández-León, unpubl.). To assess the differences between the different years, an analysis of variance (ANOVA) was performed using the statistical package R. ANOVA assumptions were verified using Levene's test for homogeneity of variance. When a variable showed significant differences, Scheffe's multiple range test was performed to determine differences among the average temperature, chlorophyll *a* and biomass for each year. When conditions for normality were not satisfied, the Kruskall-Wallis ANOVA by ranks was used to determine whether the differences were significant.

3. Results

Temperature and salinity showed rather high values in the upper 100 m (Fig. 2) during all the period sampled. Temperature was always higher than 19ºC during winter (Fig. 2a), preventing mixing during late winter.

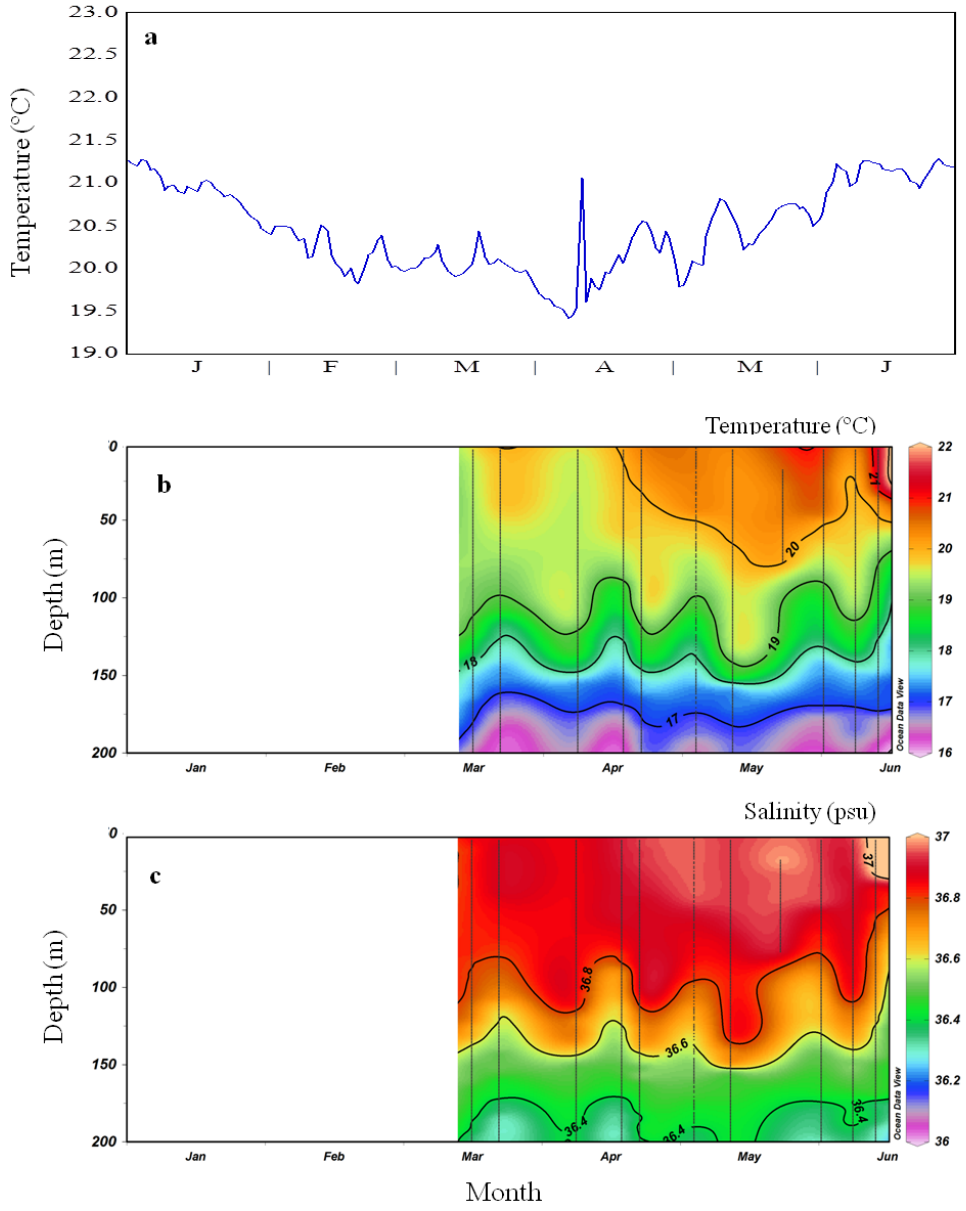

Figure 2. (a) Sea surface temperature from the oceanic buoy, (b) vertical sections showing the evolution of temperature (ºC) and (c) salinity at station 3. Dotted lines indicated the intervals of sampling time.

Additionally, two important events of Saharan dust took place over the islands during January and March (Fig. 3a). Other weak events were measured on 22nd February, 11th and 29th April, 17th May, and 4th June. Atmospheric total suspended particulate matter (TSM) reached over 500 µg·m^{-3} on 18th March, and consequently an increment of iron concentration was observed (Fig. 3a). Chorophyll *a* showed no clear response to these events during the period studied (Fig. 3b and c).

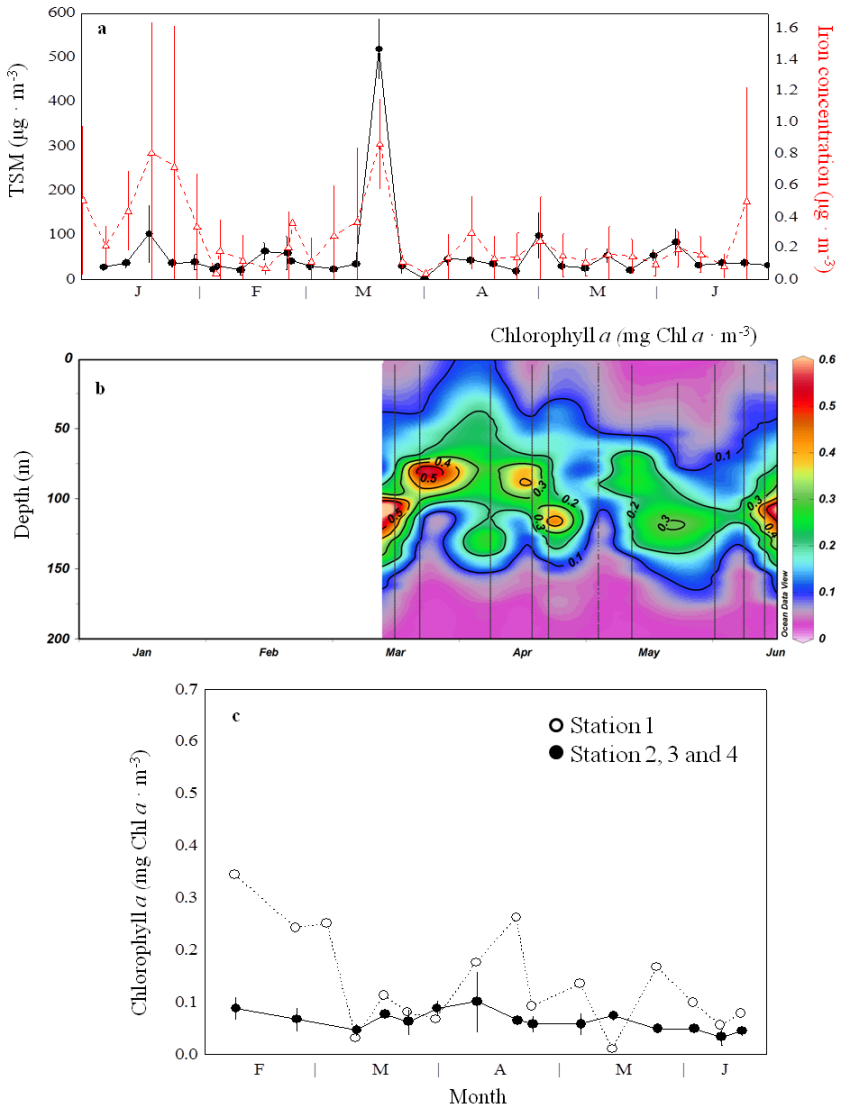

Figure 3. (a) Atmospheric total suspended particulate matter and iron concentration; (b) vertical section showing the evolution of chlorophyll *a* at station 3; (c) average values of chlorophyll *a* (mg Chl*a* · m^{-3} ± SE, at 20 m); dashed line showed chlorophyll *a* values at station 1 and solid line average values for the other three oceanic stations sampled.

Size fractionated mesozooplankton biomass showed a high variability throughout the period of study (Fig. 4). At all sampled stations the >1000 µm size fraction dominated the biomass, followed by the 200-500 µm, 500-1000 µm and 100-200 µm size fractions.

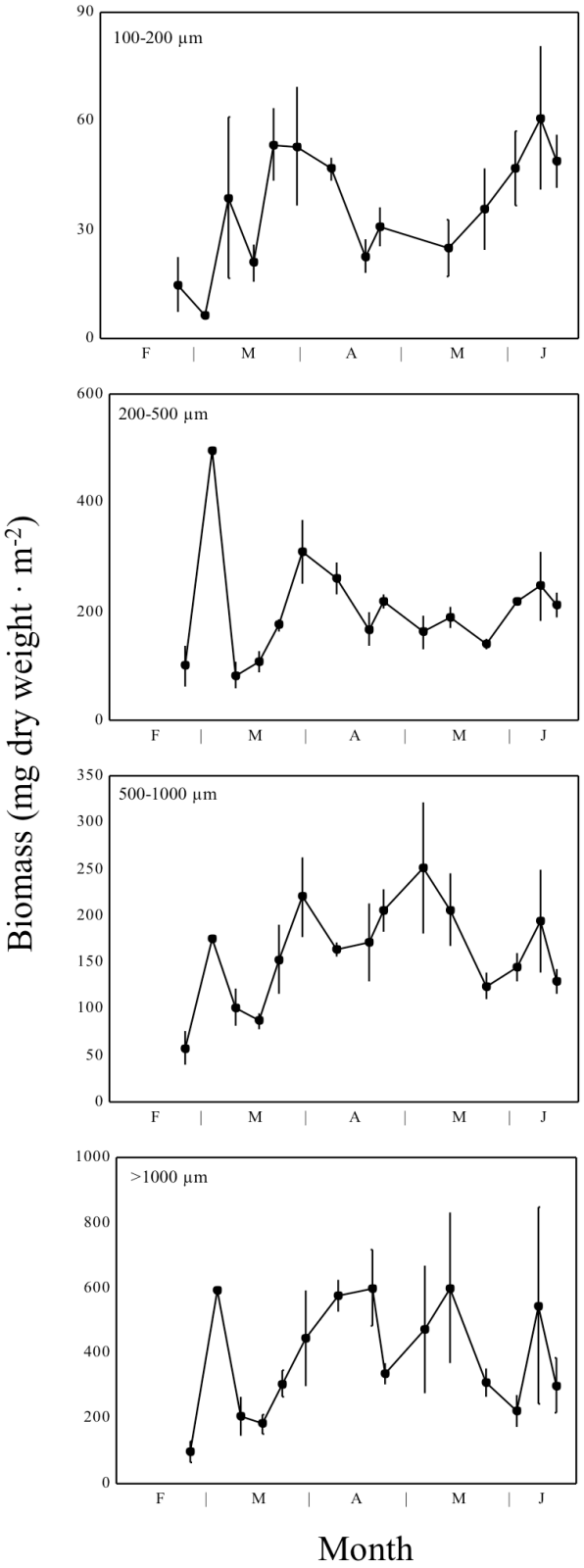

Figure 4. Average biomass values (mg dry weight·m^{-2} ± SE, 0-200 m) for the different size fractions at the four stations sampled.

Specific gut fluorescence showed higher values in the smaller size fraction, as expected (Fig. 5).

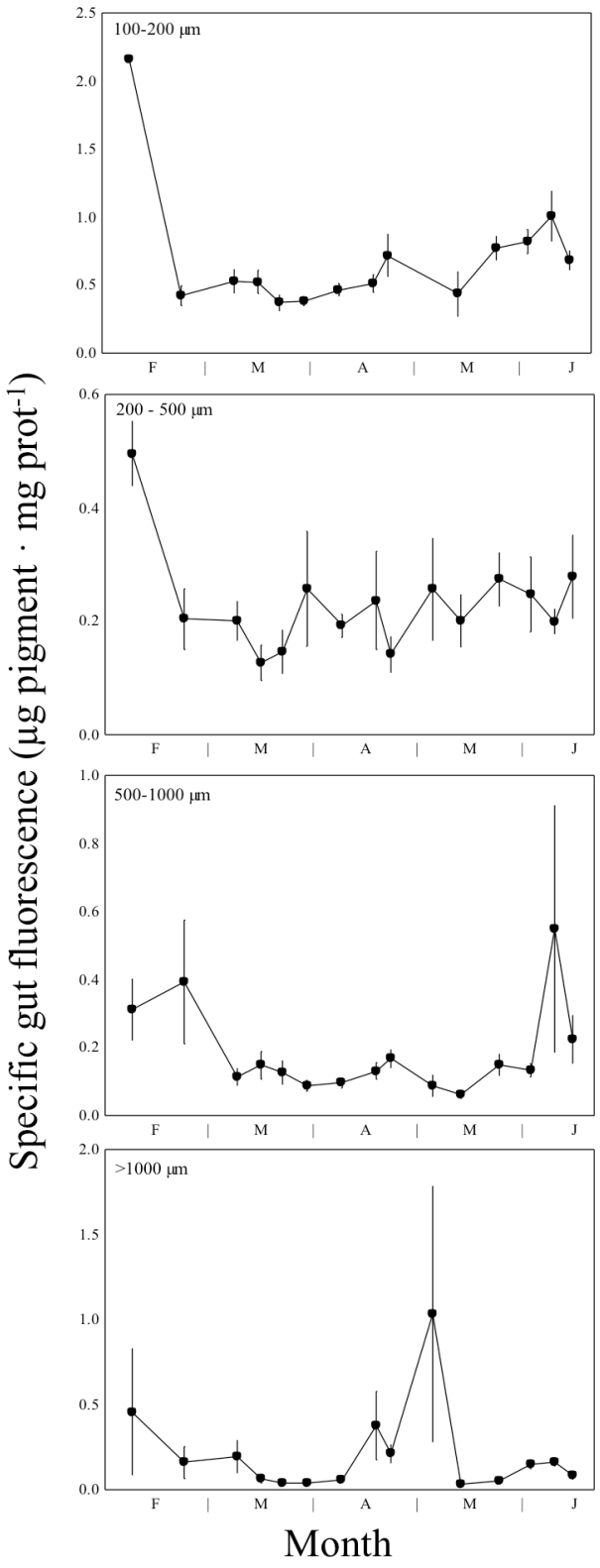

Figure 5. Average specific gut fluorescence (µg pigment·mg prot^{-1} ± SE, 0-200 m) for the different size fractions at the four stations sampled.

It sharply decreased in all size fractions during February, and remained variable but low until June in the 100-200 µm and 500-1000 µm fractions. Opposite to this, the >1000 µm fraction showed a peak in May, which was 2-fold higher than observed during the winter period. Specific ETS activity showed several increases during the period of study (Fig. 6).

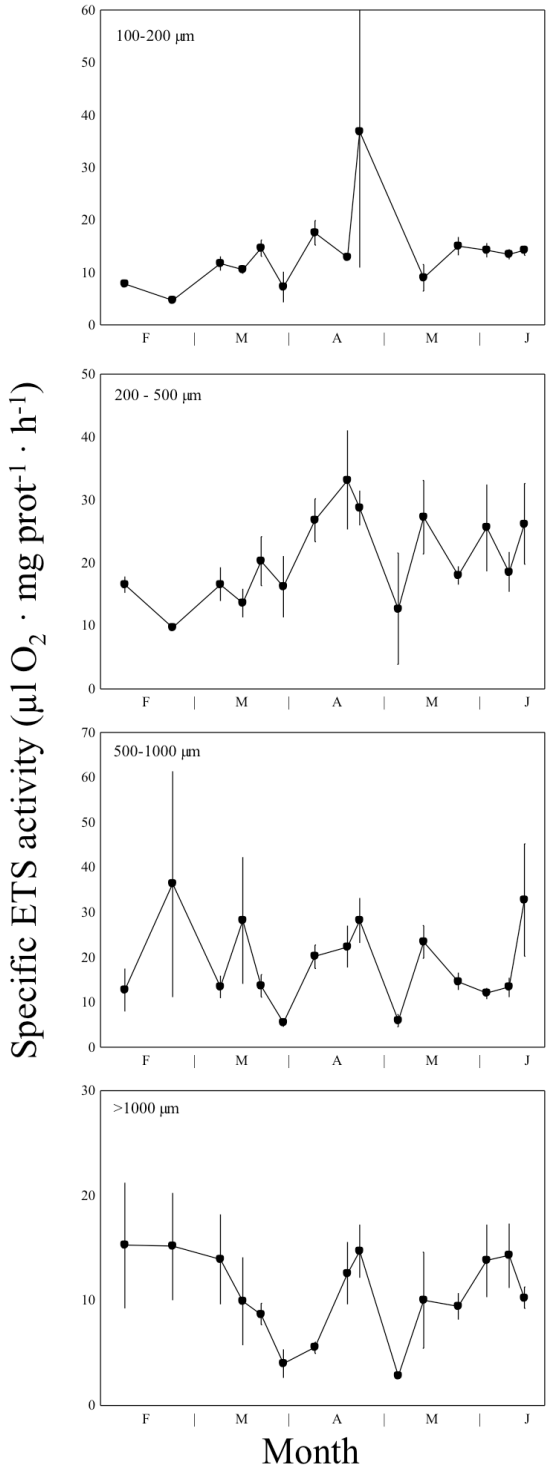

Figure 6. Average specific ETS activity (µL O_2·mg $prot^{-1}$·h^{-1} ± SE, 0-200 m) for the different size fractions at the four stations sampled.

All size fractions showed high specific values in April, although the larger fractions (>500 µm) also displayed high ETS activities during February and June. Zooplankton size fractions showed monthly specific AARS activity increases during the whole study, with higher values in the 500-1000 and the 200-500 µm fractions (Fig. 7).

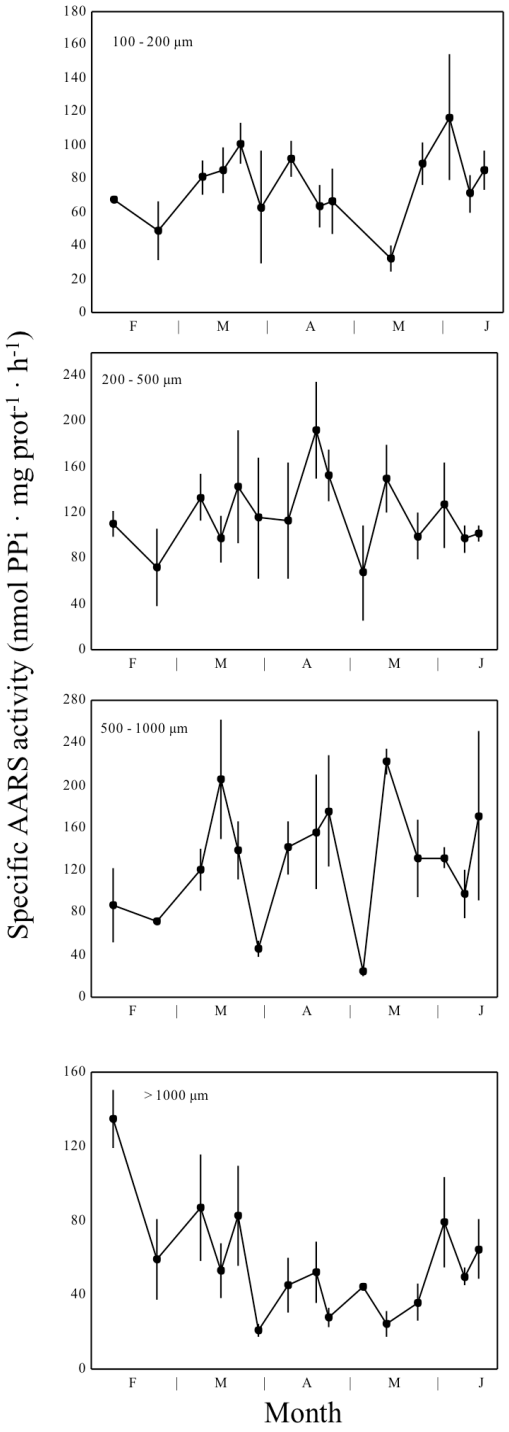

Figure 7. Average specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$ ± SE, 0-200 m) at the four stations sampled.

Throughout the area of study, total mesozooplankton biomass was characterized by values ranging from 355.98 to 1316.25 mg DW · m^{-2} (Fig. 8a). Mesozooplankton showed a first biomass increase at the end of February coincident with the illuminated phase of the lunar cycle (Fig. 8a). By the end of March biomass also increased matching the increasing lunar illumination, and remained relatively high thereafter. Besides, the biomass increase in March started right after the strongest event of iron deposition recorded during our study. The average specific GF showed values lower than 0.5 μg pigment · mg $prot^{-1}$, except in February that presented a peak of 0.63 μg pigment · mg $prot^{-1}$ (Fig. 8b). GF showed no pattern related to the lunar cycle or any increase with the input of dust deposition. Average specific ETS activity showed a large increase in April (26.24 μL O_2·mg $prot^{-1}$·h^{-1}) and other small increments during March, May and June (Fig. 8c). Average values of specific AARS activity showed increases in March (115.11 nmol PPi·mg $prot^{-1}$·h^{-1}) and in April (123.80 nmol PPi·mg $prot^{-1}$·h^{-1}) coinciding with high levels of Saharan dust deposition, and 7 and 9 days respectively before the illuminated phase of the lunar cycle (Fig. 8d). High values of AARS activity were also found in May and June.

Interannual variability of environmental and biological variables was evaluated during the normal timing of the bloom (January-March) and afterwards (April-June). During the first period, ANOVA results indicated that average temperature (20-30 m) differed significantly (ANOVA, $p<0.05$) among years. Multiple comparison tests (Scheffe) indicated a significant difference in average temperature between 2010 and 2005 ($p<0.05$). After the bloom, significant differences in average temperature were also observed (Scheffe's test) between 2007 and 2010 ($p<0.05$). The interannual variability in the average chlorophyll *a* showed statistically significant differences among years (Kruskal-Wallis, $p<0.05$), with decreasing chlorophyll concentrations towards 2010 (Table 2). Average mesozooplankton biomass, despite the lower values during 2010, showed not significant differences (ANOVA, $p>0.05$)

during the bloom period. Afterwards (April-June), the ANOVA test showed significant differences in average biomass (ANOVA, $p<0.05$). Although the ANOVA indicated an effect of year on mesozooplankton biomass, Scheffe's multiple range test indicated no significant differences between years, except between 2005 and 2007 ($p<0.05$).

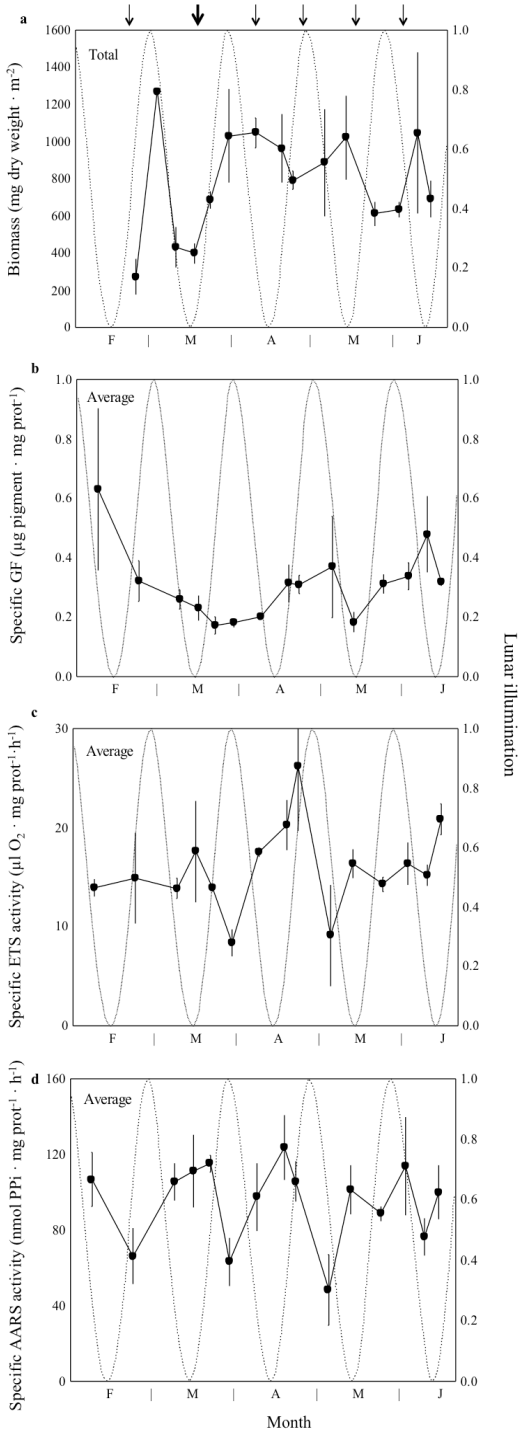

Figure 8. (a) Total mesozooplankton biomass values (mg dry weight·m^{-2} ± SE, 0-200 m); (b) average specific gut fluorescence (µg pigment · mg $prot^{-1}$ ± SE, 0-200 m); (c) average specific ETS activity (µL O_2·mg $prot^{-1}$·h^{-1} ± SE, 0-200 m); and (d) AARS activity (nmol PPi·mg $prot^{-1}$·h^{-1} ± SE, 0-200 m) at the four stations sampled; thin dashed line shows the lunar illumination, arrows shows the dust deposition events during sampling; the arrow in bold shows the main dust event.

Table 2. Interannual variability of environmental and biological variables was evaluated during normal timing of the bloom (January-March) and afterwards (April-June). Average temperature (T; 20-30 m, °C ± SD), average chlorophyll *a* (Chl *a*; 20-30 m, mg Chl *a* · m^{-3}± SD) and average zooplankton biomass (Biomass; 0-100 m, mg DW · m^{-2}± SD) in 2005-2007 by Moyano and Hernández-León, 2011; and in 2010, this study.

		2005	2006	2007	2010	Average
During Bloom	**T ºC**	18.52 (± 0.48)	18.83 (± 0.51)	19.11 (± 0.55)	19.78 (± 0.08)	18.84 (± 0.58)
January-March	**Chl *a***	0.56 (±0.20)	0.30 (±0.07)	0.25 (±0.05)	0.07 (±0.01)	0.36 (± 0.20)
	Biomass	1228.64 (±874.41)	1531.53 (±719.67)	1178.65 (±1369.76)	465.38 (±122.91)	1240.38 (± 949.51)
Post-Bloom	**T ºC**	19.87 (± 0.75)	19.99 (± 0.77)	19.07 (± 0.46)	20.30 (±0.43)	19.86 (± 0.74)
April-June	**Chl *a***	0.33 (±0.11)	0.20 (±0.04)	0.18 (±0.05)	0.07 (±0.03)	0.21 (± 0.13)
	Biomass	1381.01 (±824.83)	943.86 (±221.40)	661.16 (±435.46)	723.59 (±167.49)	975.93 (± 606.45)

4. Discussion

2010 was an atypical year, it showed the warmest winter sea surface temperature of the last 30 years (www.oceanmotion.org/html/resources/ssedv.htm), which also coincided with the minimum NAO recorded since 1950 (http//www.cpc.ncep.noaa.gov/data/teledoc/nao_ts.shtml). A low NAO index has been related with high temperatures and low trade winds during winter in the NW African upwelling zone (26-25 ºN), including the Canary Island region (Cropper et al., 2014). Hence, the winter-spring period studied did not show the typical conditions that produce the so-called late winter bloom in this latitude. Consistent with low chlorophyll values, Franchy et al. (*submitted*) found extremely low values of pico-, nano- and microplankton biomass during the same period of study. Temperature and salinity were higher than previously observed in the Canary Islands waters in winter (see Moyano and Hernández-León, 2011; Schmoker and Hernández-León, 2013). The interannual variability also showed that 2010 was the warmest year of the four years compared (Table 2), presenting surface values higher than 19ºC. However, despite the high temperatures, strong stratification, and low chlorophyll values, Franchy et al. (*submitted*) observed relatively high

values of primary production coinciding with heavy dust deposition events during this winter-spring period. Such events were more intense than in previous years, especially in March (http://earthobservatory.nasa.gov/) and promoted an increased Fe deposition as well as an increase in diatoms (Franchy et al., *submitted*). However, the maximum value of chlorophyll *a* found in this study (0.25 $mg \cdot m^3$) was considerably lower than the usually found in the area (Arístegui et al., 2001; Hernández-León et al., 2004, 2010; Neuer et al., 2007; Moyano and Hernández-León, 2011; Schmoker et al., 2012). In addition, relatively high chlorophyll *a* values were observed during cold years suggesting that warm temperatures restricted the vertical nutrient flux to the surface layers (Cianca et al., 2007), limiting the phytoplankton growth.

Maximum total zooplankton biomass values were recorded during the first month reaching values higher than 1000 mg DW $\cdot$ m^{-2}, similar to that observed by Hernández-León et al. (2004). Also, during the whole study the total biomass presented rather high values (over 600 mg DW $\cdot$ m^{-2}). In this sense, the pattern described by Arístegui et al. (2001) and Hernández-León et al. (2004) showed two clear periods. During the first one, from January to March, maximum biomass values were recorded coinciding with maximum mixing. However, during our study, biomass showed values lower than expected because of the strong stratification observed. The second period, from April to June, characterized by higher temperatures, always showed relatively lower biomass values compared to the bloom period (Hernández-León et al., 2004; Moyano and Hernández-León, 2011**)**. In our interannual comparison, the post-bloom period during 2010 also showed higher temperatures, denoting higher stratification. However, average zooplankton biomass for this period was within the range of previous years, despite the stronger stratification. This suggests that dust deposition allowed a surplus production (Franchy et al., *submitted*) and slightly higher mesozooplankton biomass than that expected based only on hydrographic factors.

Additional evidence of the lack of winter bloom during 2010 was the low values of the indices of grazing (GF) and respiration (ETS activity). Specific GF during the whole period showed values as low as previously reported for the post-bloom period (April to June) in these subtropical waters (Hernández-León et al., 2004). The latter authors showed maximum GF values from January to March, and thereafter values decayed by 2-fold. Although size fractionated GF values in 2010 were lower compared to previous years, the expected pattern of decreasing GF values as size fraction increases was maintained. Similarly, the specific ETS activity also showed low values compared to previous years in the same area. Hernández-León et al. (2004) observed the maximum specific ETS values coinciding with the mixing period prior to the zooplankton biomass outburst, whereas in our work, specific ETS values only increased coinciding with increments of zooplankton biomass. In addition, specific AARS activity showed relatively high values, suggesting that the recurrent dust input events allowed for sustained growth rates during both the bloom and post-bloom periods. Unfortunately, there are no prior zooplankton growth studies during the late winter bloom to compare with. Hence, we could not ascertain the possible effects of warming over the zooplankton production in the area.

Besides, mesozooplankton biomass increased in parallel to the moon illumination during February-March, the usual timing of the late winter bloom, as previously seen in subtropical waters (Hernández-León, 1998; Hernández-León et al., 2001, 2002, 2004, 2010). However, no clear match with the lunar cycle was observed thereafter, probably due to the slightly high biomass stock maintained during most of the post-bloom period. The higher than expected biomass is suggested to be a consequence of the increased primary production observed by Franchy et al. (*submitted*). Thus, it seems that dust depositions during the post-bloom period would enhance the transfer of energy and matter to the mesozooplankton. However, the way this transfer occurs is not clear, as chlorophyll and specific GF values

remained low.

In summary, the high warm winter temperature and the strong stratification of the water column precluded the typical late winter bloom during that year. On the contrary, an intense frequency of dust deposition events promoted an increase of primary production and a higher than expected mesozooplankton biomass during the post-bloom period (April to June). Our findings show some clues about how under a warming environment scenario the frequency of dust events may gain importance in shaping the intensity of the winter-spring bloom in subtropical waters in the near future.

Acknowledgements

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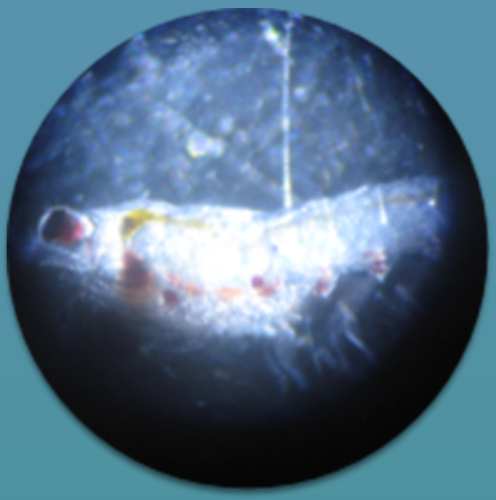
Chapter 4



Potential grazing, respiration and growth of ***Euphausia distinguenda*** in relation to the oxygen minimum zone at the Eastern Tropical Pacific off Mexico

Inma Herrera, Tarsicio Antezana, Alan Giraldo, Emilio Beier, Lidia Yebra, Santiago Hernández-León and Jaime Färber-Lorda

Abstract

Euphausiids play an important role in the carbon cycling of pelagic ecosystems. They perform diel vertical migrations promoting a transport of organic matter from the euphotic zone to deep layers. The oxygen minimum zone (OMZ) in tropical oceans affects the diversity, distribution and physiological processes of marine organisms. In order to study the relationship between the physiological processes of *Euphausia distinguenda* and the OMZ, we collected samples of euphausiids in the Eastern Tropical Pacific off Mexico (ETPM) during November 2009. We assessed the indices of potential grazing (specific gut fluorescence, GF), respiration (specific electron transport system activity, ETS) and growth (specific aminoacyl-tRNA synthetases activity, AARS) and the individual protein content. *E. distinguenda* showed diel vertical migration behavior descending during the day into the OMZ and ascending to the surface at night. Specific ETS and AARS activities showed high values during the night in superficial highly oxygenated waters. Both proxies for growth and respiration were significantly reduced within the OMZ, suggesting that the oxygen minimum zone conditions the euphausiid physiological processes.

Keywords: AARS, ETS, *Euphausia distinguenda*, GF, individual protein content, OMZ

1. Introduction

The so-called oxygen minimum zones (OMZs, Fiedler and Talley, 2006; Kessler 2006; Fernández-Álamo and Färber-Lorda, 2006) are water masses extending from 100 to 900 m in depth where oxygen concentrations are lower than 0.1 $mL \cdot L^{-1}$ due to low water mass ventilation (Karstensen et al., 2008). These OMZs are found close to tropical and subtropical upwelling zones, which are also characterized by high primary production rates in the euphotic zone (Fiedler and Talley, 2006). This high productivity generates a huge export of organic matter, which promotes a rather high bacterial respiration rate (Sues, 1980; Williams, 1981). These factors promote anoxic conditions in deep waters, which are important for marine organisms, as oxygen is essential for aerobic metabolism.

All major zooplankton groups are constrained by the presence of OMZs (Brinton, 1967, 1979; Tarling et al., 2000; Taki, 2008; Antezana, 2002, 2009). Diel vertical migration (DVM) is also affected by OMZs. It is well known that OMZs act as a barrier for many of these organisms, and as a refuge from predators (Fernández-Álamo and Färber-Lorda, 2006). Some vertical migrators are metabolically adapted to survive in the OMZ (Seibel, 2011; Antezana, 2002b). Under low oxygen conditions these organisms reduce their metabolic rates, allowing their survival in the OMZ, but impeding their growth (Maas et al., 2012).

Many euphausiid species perform diel vertical migrations, which play an important role in the transport of organic matter from the euphotic zone to deep layers (Brinton, 1979). Diel vertical migration of euphausiids is influenced by physical factors such as temperature, density, changes in water masses, light intensity and oxygen concentrations (Brinton, 1967; Tarling et al., 2000), as well as by biotic factors such as predation pressure and food abundance and quality (Antezana 2002a; Fernández-Álamo and Färber-Lorda, 2006).

Euphausia distinguenda is one of the most abundant species in the OMZ at the Eastern Tropical Pacific off Mexico (ETPM), contributing between 88 and 90% of the total euphausiid abundance in coastal waters (Ambriz-Arreola et al., 2012), identified as strong migratory and oxygen-deficiency adapted organism (Brinton, 1979, Antezana 2002b). Nonetheless, physiological processes of *E. distinguenda* have never been investigated despite the possible trophic importance of this species in the pelagic ecosystem of the Eastern Tropical Pacific off Mexico (ETPM), area characterized by a huge and extensive oxygen minimum zone (OMZ).

The physiological processes of these migratory organisms have important biogeochemical implications. Active transport of organic matter by organisms that feed at the surface but metabolize and excrete at depth ranges between 20 and 40 % of the particle export to deep layers (Bianchi et al., 2013). Furthermore, physiological responses of organisms to OMZs can influence the active flux. Based on that, our study focused on the analysis of potential grazing, respiration and growth rates of *E. distinguenda* in the ETPM. We measured the variability of the specific gut fluorescence (GF), electron transport system activity (ETS), aminoacyl-tRNA synthetases activity (AARS) and the individual protein content of this species in relation to the OMZ.

2. Material and Methods

Sampling was carried out from 18th to 30th of November 2009 on board the B/O "El Puma" in the ETPM (Fig. 1). Vertical profiles of conductivity, temperature and oxygen from 0 to 500 m were obtained using a CTD Sea-Bird Electronics Inc., 2003. Data from 38 CTD casts (transect B: 20 casts; transect D: 18 casts) defined the physical structure of the water column, especially the extent of the Oxygen Minimum Zone (OMZ).

In the two transects studied, a Multiple Net (MOCNESS; 200 µm mesh) was deployed and different depth layers were sampled (0 - 50, 50 - 100, 100 - 150, 150 - 200, 200 - 300, 300 - 400 and 400 - 500 m). The MOCNESS stations were named B20, B16, B12, B11 (Transect B) and D16, D12, D4, D3 (Transect D). The B11 and D3 stations were sampled both during day and night within a period of 24 and 48 h respectively, whereas in the other stations a unique cast was done (Fig. 1).

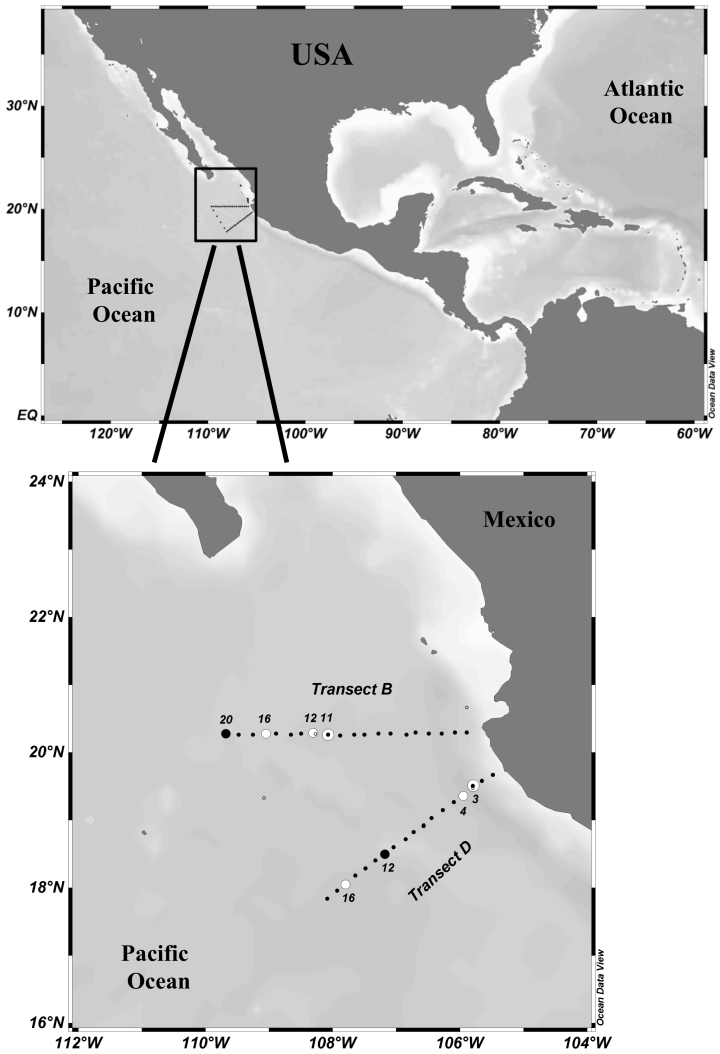


Figure 1. Location of transects sampled from 18th to 30th November 2009 in the Eastern Tropical Pacific off Mexico.

On board, the family of euphausiids was identified and 10 individuals of *Euphausia distinguenda* were selected from each sampling. Euphausiid abundance was determined per each sampling depth. The organisms length was estimated using a stereomicroscope (range standard length was 6-15 mm), and the stomach was observed, considering it full or empty. Once identified, the euphausiids were immediately frozen in liquid nitrogen for biochemical analysis.

In the laboratory, frozen samples were homogenized with Tris-HCl buffer (pH=7.8). Gut fluorescence (GF), was measured as the sum of chlorophyll *a* plus phaeopigment concentrations, following the procedure given by Parsons et al. (1984) and Arar and Collins (1997). The electron transport system (ETS) activity, as a measurement of potential respiration, was assayed using the method of Packard et al. (1971) modified by Kenner and Ahmed (1975). ETS activity was corrected for the *in situ* temperature using the Arrhenius equation with activation energy of 15 kcal·mol^{-1} (Packard et al., 1975).

The aminoacyl-tRNA synthetases (AARS) activity, as growth index, was measured using the method of Yebra and Hernández-León (2004) modified by Yebra et al. (2011). AARS activity was calculated using the equation given by Herrera et al. (Chapter 3 in this volume) and corrected for the *in situ* temperature using the Arrhenius equation with an activation energy of 8.57 kcal · mol^{-1} (Yebra et al., 2005). The individual protein content was measured using the method of Lowry et al. (1951) adapted for micro-assay by Rutter (1967), using Bovine Serum Albumin as standard.

Statistical analyses were done in STATISTICA 7.0 (Statsoft, Inc.) and in R (R Development Core Team 2009). To assess the differences between station, night and day period, and depth an analysis of variance (ANOVA) was performed. ANOVA assumptions were verified using Levene's test for homogeneity of variance. When a variable showed significant differences, Scheffe's multiple range test was performed to determine differences

among the average for each variable.

3. Results

Temperature (0-500 m) varied between 27.7 and 10ºC, with a thermocline located between 70 and 100 m depth (Fig. 2).

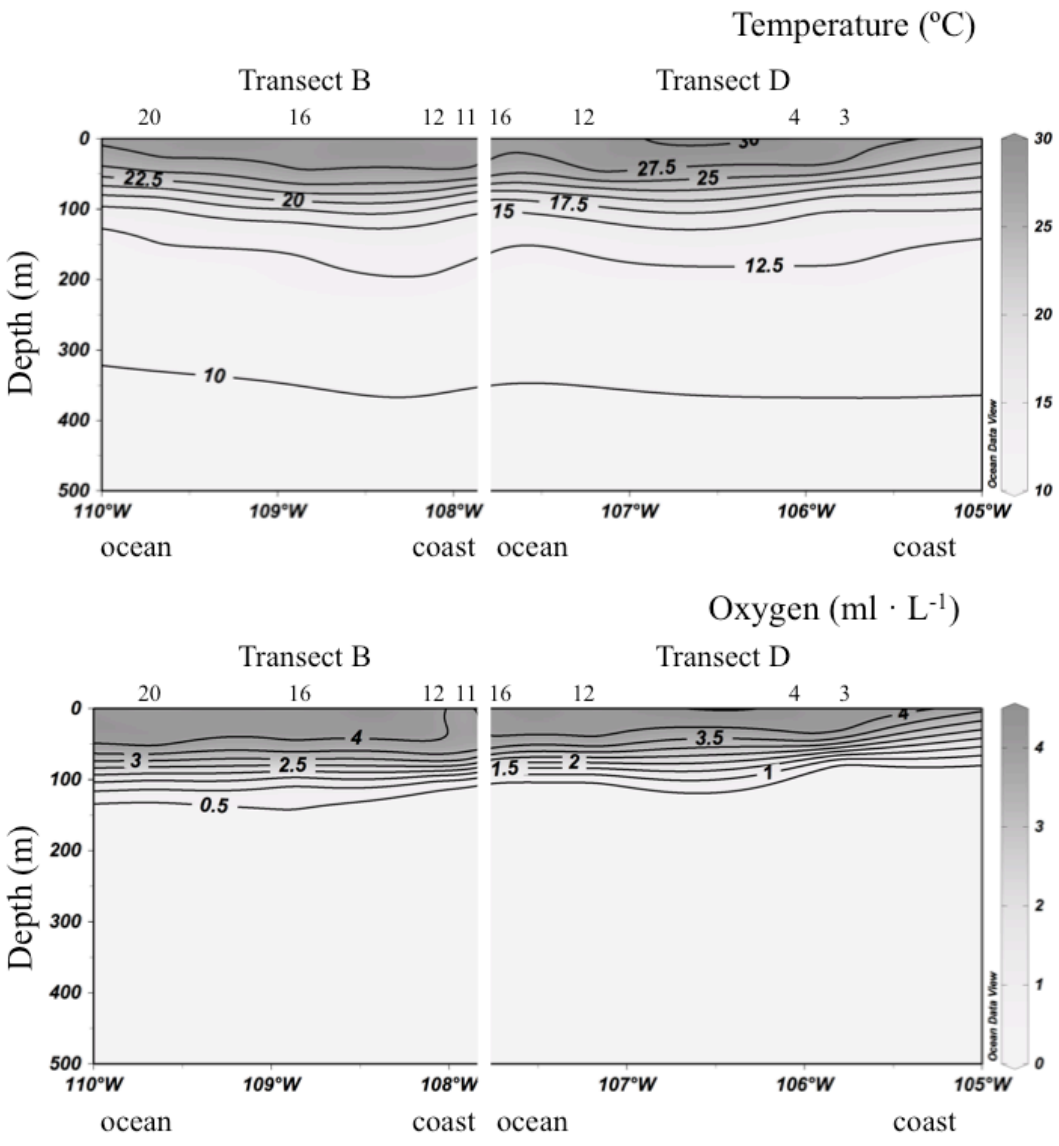


Figure 2. Vertical sections of temperature (ºC) and oxygen concentration (mL $O_2 \cdot L^{-1}$) from 0 to 500 m depth in each sampled transect.

An abrupt decrease of the dissolved oxygen concentration was observed between 75 and 100 m depth. Oxygen concentration varied between 4.19 and 0.03 mL $O_2 \cdot L^{-1}$ in the water column (Fig. 2). There was a quite strong oxycline located between 100-200 m, beneath which the oxygen concentration was below 1 mL $O_2 \cdot L^{-1}$. Extreme hypoxic conditions (<0.1 mL $O_2 \cdot L^{-1}$) occurred between 150 and 500 m.

Total euphausiid abundance showed a clear difference between day and night, with the mean value during the night (21:00-6:00 hrs) in the first 50 m being 0.40±0.37 ind·m^{-3} and a mean value of 2.30±2.71 ind·m^{-3} during the day (8:00-19:00 hrs) at different depths (Table 1, Fig. 4a). The percentage of species showed clearly that *Euphausia distinguenda* was the most abundant euphausiid species, between 40-100% of the sampled organisms (Table 1).

Individual protein content showed a normal distribution (Fig. 3) with values varying from 0.13 to 1.89 mg prot · ind^{-1} (mean 0.50 ± 0.26). Individual protein biomass did not show statistical significant differences ($p>0.05$) between day (0.59±0.02 mg prot · ind^{-1}) and night times (0.61±0.04 mg prot · ind^{-1}) for each station (Fig. 4b). However, during nighttime, all organisms of *E. distinguenda* were found above the oxycline at 50 m, but through the day they were found in different layers (150 - 200, 200 – 300 and 300 – 400 m, Fig. 4a).

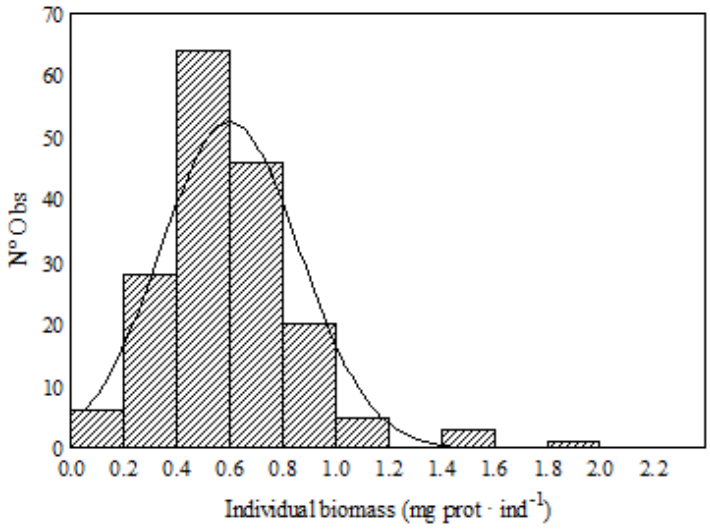


Figure 3. Histogram of individual *E. distinguenda* biomass (mg prot · ind^{-1}).

Table 1. Total euphausiid abundance (ind · m^{-3}) and percentages for each species collected during the cruise.

Station	Date	Cast Final Time (h)	Depth range (m)	Oxygen (ml ·L^{-1})	Ind./ Sample	Filtered Volume (m^3)	Abundance (ind · m^{-3})	*Euphausia distinguenda* (%)	*Euphausia diomedeae* (%)	*Euphausia tenera* (%)	*Nematocelis gracilis* (%)	*Stylocheiron carinatum* (%)	*Euphausia eximia* (%)
B11	21/11/2009	21:00	0-50	3.934	80	404.0	0.20	100.00					
B11	22/11/2009	8:00	200-300	0.042	372	286.0	1.30	88.17	7.53	4.30			
B12	22/11/2009	19:00	200-300	0.042	279	260.0	1.07	80.29	8.60				
B16	23/11/2009	10:14	300-400	0.041	776	118.0	6.58	61.86	36.08		2.06		
B20	24/11/2009	2:11	0-50	3.200	160	178.0	0.90	40.00			40.00	5.00	15.00
D16	25/11/2009	11:50	300-400	0.038	400	119.0	3.36	90.00	10.00				
D12	26/11/2009	2:30	0-50	3.200	232	286.6	0.81	86.21	13.79				
D4	27/11/2009	12:30	200-300	0.042	368	246.0	1.50	80.43	13.04	6.52			
D3	27/11/2009	16:00	200-300	0.048	904	108.0	8.37	95.58	4.42				
D3	27/11/2009	18:30	100-150	0.067	280	348.5	0.80	100.00					
D3	28/11/2009	4:23	0-50	3.200	144	422.7	0.34	77.78	22.22				
D3	28/11/2009	11:00	200-300	0.048	184	392.2	0.47	100.00					
D3	28/11/2009	17:18	200-300	0.048	208	247.3	0.84	88.46	3.85	3.85	3.85		
D3	29/11/2009	2:58	0-50	3.934	16	221.0	0.07	100.00					
D3	29/11/2009	5:16	0-50	3.200	24	361.8	0.07	100.00					
D3	29/11/2009	10:38	200-300	0.048	48	162.7	0.30	50.00	33.33	16.67			
D3	29/11/2009	12:52	200-300	0.048	168	223.2	0.75	95.24		4.76			

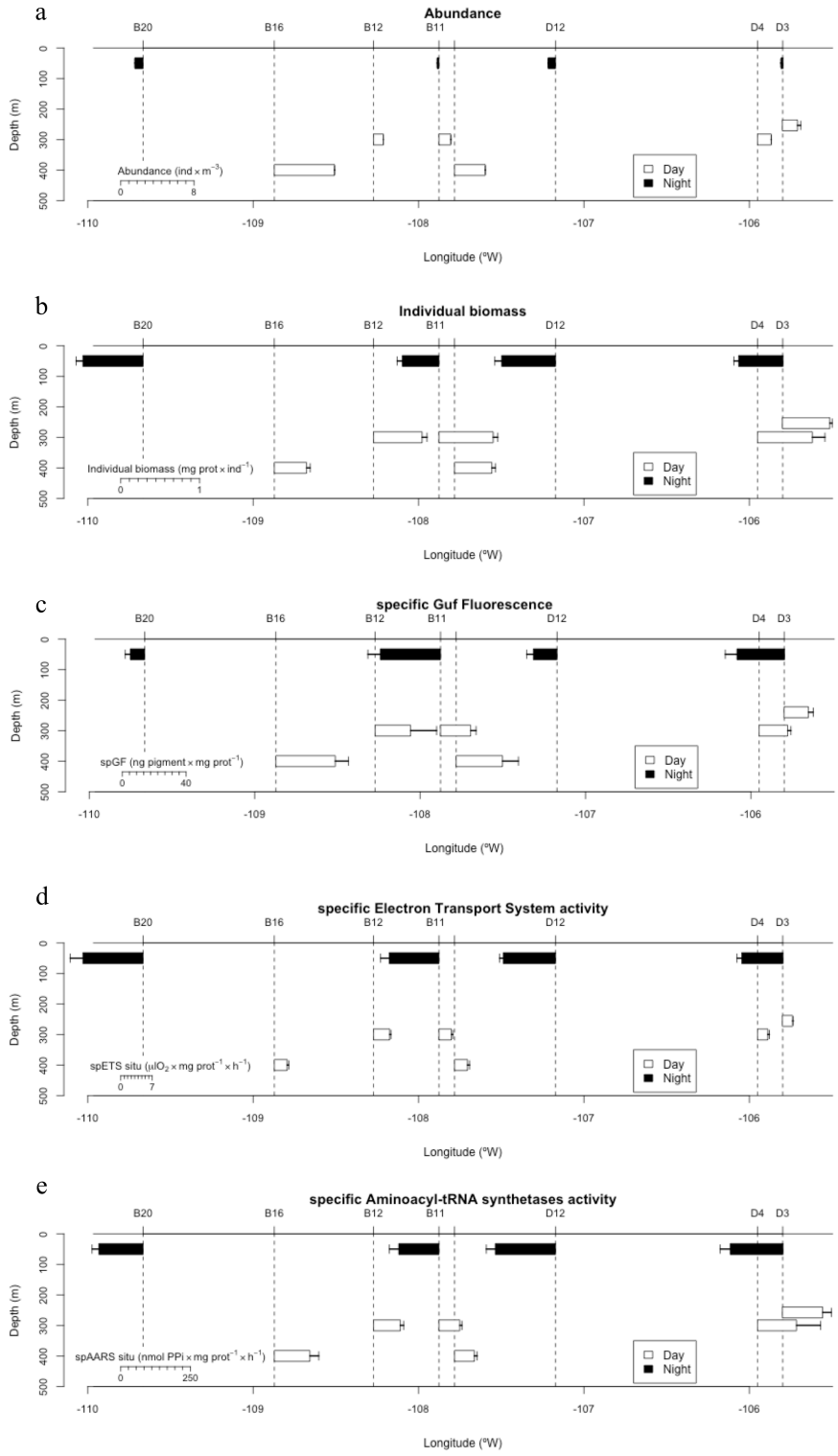


Figure 4. Spatial distribution, 0-500 m depth, of a) average abundance (ind · m^{-3} ± SE from table 1); b) average individual biomass (mg prot · ind^{-1} ± SE); c) average specific gut fluorescence (ng pigment · mg $prot^{-1}$ ± SE); d) average specific electron transport system activity (µL O_2·mg $prot^{-1}$·h^{-1} ± SE) and e) average specific aminoacyl-tRNA synthetases activity (nmol PPi·mg $prot^{-1}$·h^{-1} ± SE), for each transect where N (night station), D (day station).

Specific gut fluorescence (GF) vertical profiles did not show statistical significant differences between day and night periods ($p>0.05$; Fig. 4c). We observed similar values during night (23.47±3.48 ng pigment · mg prot^{-1}) and day (22.48±3.05 ng pigment · mg prot^{-1}). The vertical profile of average specific GF (Fig. 5a) did not show statistical significant differences and remained practically constant with depth. Pooling all stations together we found that the average GF also showed a high variability with time with not significant differences between day and night ($p>0.05$: Fig. 6a).

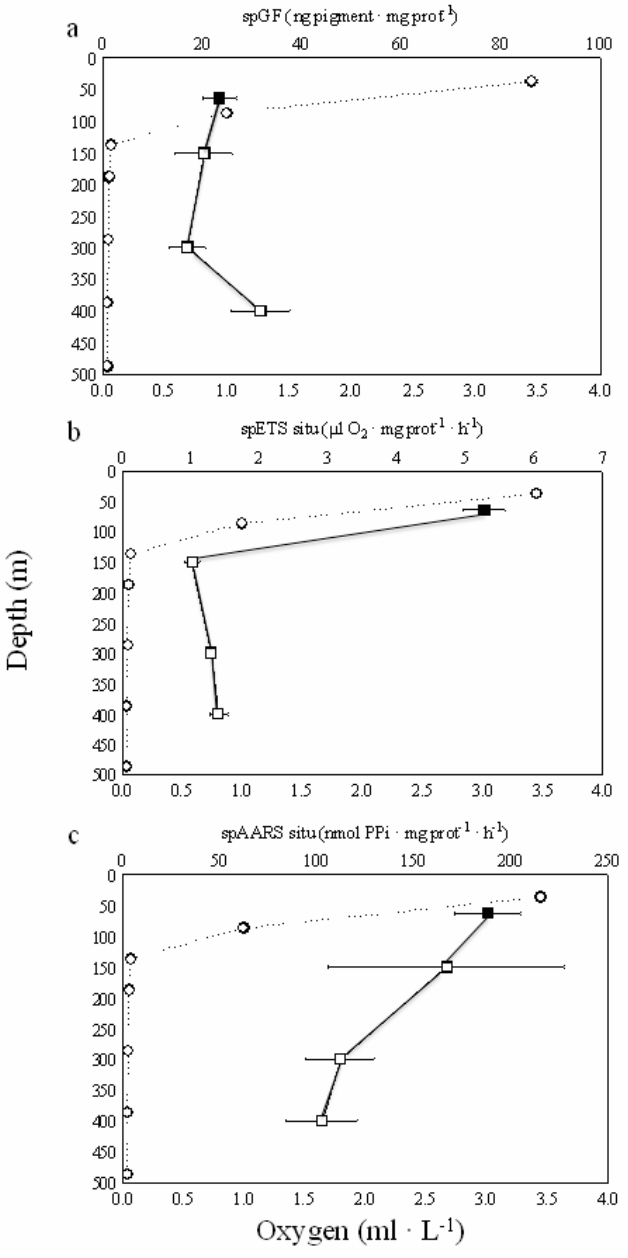


Figure 5. Vertical profiles of a) average specific gut fluorescence (ng pigment·mg prot^{-1} ± SE); b) average specific electron transport system activity (µL O_2·mg prot^{-1}·h^{-1} ± SE); c) average specific aminoacyl-tRNA synthetases activity (nmol PPi·mg prot^{-1}·h^{-1} ± SE) and oxygen concentration (mL O_2·L^{-1}). Full squares (night station), open squares (day station).

On the contrary, specific ETS activity vertical profiles showed significant differences ($p<0.05$) between day and night periods (Fig. 4d). We observed higher values during the night (5.28±0.33 µL O_2·mg $prot^{-1}$·h^{-1}) than by day (1.28±0.06 µL O_2·mg $prot^{-1}$·h^{-1}). The vertical profile of average specific ETS activity (Fig. 5b) showed statistically significant differences ($p<0.05$). ETS decreased gradually with depth, similar to the oxygen concentration (mL O_2·L^{-1}). The variations over time of average ETS activities also showed the same pattern (Fig. 6b), presenting statistical significant differences ($p<0.05$).

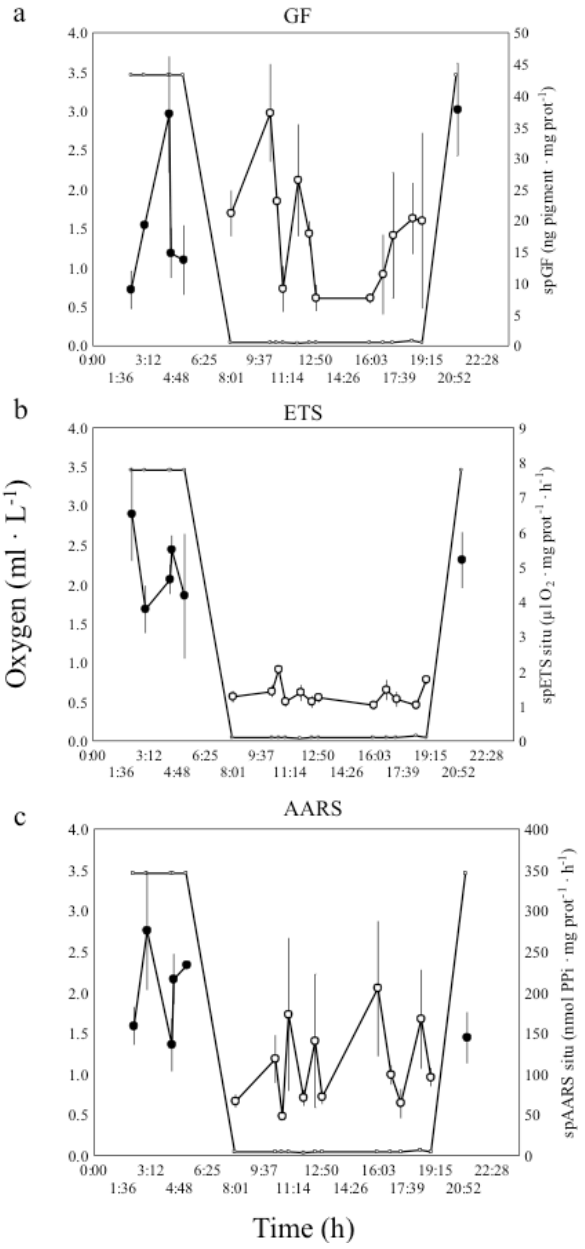


Figure 6. Time variation of a) average specific gut fluorescence (ng pigment·mg $prot^{-1}$ ± SE); b) average specific electron transport system activity (µL O_2·mg $prot^{-1}$·h^{-1} ± SE); c) average specific aminoacyl-tRNA synthetases activity (nmol PPi·mg $prot^{-1}$·h^{-1} ± SE); and oxygen concentration (mL O_2·L^{-1}) per time (h). Full squares (night station), open squares (day station).

Similarly, specific AARS activity showed high values during the night (187.88±18.02 nmol PPi·mg prot^{-1}·h^{-1}) over 50 m, and lower values during the day (116.76±15.11 nmol PPi·mg prot^{-1}·h^{-1}, Fig. 4e) with statistical significant differences ($p<0.05$). The vertical profile of average specific AARS activity (Fig. 5c) also showed significant differences between 50 m and the deeper samples, decreasing gradually with depth. However, when pooling all stations together, the average specific AARS activities over time (Fig. 6c) presented a high variability, showing not significant differences between sampling times ($p>0.05$).

4. Discussion

The spatial distribution of temperature and oxygen did not show differences along the transects, following the typical pattern of the Eastern Tropical Pacific off Mexico (Fiedler and Talley, 2006; Kessler, 2006). *Euphausia distinguenda* was the most abundant euphausiid species in the sampled area, as observed in previous studies by Ambriz-Arreol et al. (2012). Färber-Lorda (*in prep*.) observed clear vertical migrations of euphausiids during the same cruise. This is a common observation, as it is known that euphausiids have high swimming and strong vertical migration capacities, being able to reach 200 m depth in about one hour (Ritz, 1994).

In the current study the values of specific GF suggested that *E. distinguenda* did not show significant grazing differences between day and night. Antezana (2002b) found slight or no differences in the stomach content of *Euphausia mucronata* collected in the OMZ during daytime and in surface layers at nighttime. In addition the GF values observed in *E. distinguenda* were lower than values found by Hernández-León et al. (2013) on *Euphausia superba* in a similar size range. Euphausiids show different feeding strategies and for instance, *Euphausia pacifica* changes their feeding preferences from phytoplankton to

zooplankton during DVM (Nakagawa et al., 2001, 2002). Moreover, Hernández-León et al. (2001) suggested that *E. superba* fed on phytoplankton during the day, and change to carnivore feeding during nighttime. The rather low values of GF in *E. distinguenda* observed here should be related to the common observation of euphausiids feeding on micro- and mesozoplankton (Hernández-León et al., 2001, 2013).

Our results also showed that potential respiration (ETS activity) was significantly higher at the surface and decreased when the oxygen concentration decreased with increasing depth. King et al. (1978) found similar results and suggested that it was due to a decrease of individual biomass. On the contrary, Teal and Carey (1967) suggested that metabolism depended on temperature; the organisms (*Euphausia mucronata*) decreased the respiration rate when temperature decreased. According to Torres and Childress (1983), DVM is energetically expensive. Staying in the cold deep during the day is associated with low food concentrations, low growth and low metabolism (Lampert, 1989), and low oxygen (Seibel, 2011). In addition, Teuber et al. (2013) also found that copepod ETS activities were higher above the OMZ and decreased in the core and below OMZ while increasing their anaerobic metabolism (lactate dehydrogenase activity, LDH). Recently, Seibel (2011) suggested that oceanic organisms (such as Euphausiids) could survive in the OMZ by two situations: First, they would suppress their metabolism, which would be very much like hibernation in other organisms (e.g. *Dosidicus gigas*, *Euphausia eximia*). Second, converting food into energy normally requires more oxygen concentration; however these organisms would use a different process, anaerobic glycolysis, allowing them to use only a low oxygen concentration. Seibel (2011) also found that these organisms could survive in hypoxic waters from hours up to days, using the oxygen available with anaerobic metabolic pathways.

Studies about aerobic and anaerobic metabolic processes in crustaceans (Teuber et al., 2013; Yannicelli et al., 2013), medusae (Thuesen et al., 1994), jumbo squid, *Dosidicus gigas*

(Rosa and Seibel, 2010), polychaete (Quiroga et al., 2007) showed that these organisms could survive in the OMZ by using anaerobic metabolic pathways. Anaerobic glycolysis involves transformation of glucose to pyruvate, and the further conversion from pyruvate to lactate. This pathway takes place in complete oxygen's absence or in limited supply of it. For the anaerobic metabolic pathway is necessary less energy, only 2 ATP, whilst for the aerobic metabolic pathway are necessary 38 ATP molecules (Lenhinger, 1975, 1977).

There are no previous studies about the growth rate of zooplankton related to the OMZ. Nevertheless, it is an important physiological process that might be affected by oxygen levels in water. Specific AARS activity in *E. distinguenda* showed a similar relationship with the oxygen concentration than the ETS. This suggests that the AARS of *E. distinguenda* was also conditioned by the OMZ. The AARS enzymes catalyze the first step of the protein synthesis. The contribution that protein synthesis makes to the oxygen consumption of different marine fish species has been estimated in previous works (Houlihan, 1991; Smith and Houlihan, 1995). The enzymatic activities rely on ATP production. Not enough ATP production due to low oxygen concentration produced low values of protein synthesis rate (AARS activity) and consequently reduced translation step (Anderson et al., 2009), supporting the diminished spAARS activities observed for *E. distinguenda* within the OMZ. Translation is the second step of protein synthesis. Messenger RNA (mRNA) produced by transcription is decoded by a ribosome complex to produce a specific protein (Crick, 1958). Anderson et al. (2009) suggested that a translational suppression in hypoxia conditions would inhibit or reduce the protein synthesis of the organism, as well as the AARS activity. Nevertheless, when the oxygen concentration starts to increase the protein synthesis recovers its levels, as observed by Koumenis et al. (2002). This would allow *E. distinguenda* to rapidly resume their protein synthesis activity when migrating to oxygenate waters above the OMZ.

In summary, our results suggest that the oxygen minimum zone conditions the euphausiid physiological processes. Both proxies for respiration and growth rates were significantly reduced within the OMZ. However, the metabolism of euphausiids might be effective using both aerobic and anaerobic pathways, thus allowing this species to inhabit within the OMZ during daytime. Further research would be needed to fully comprehend the effects of hypoxia on the *in situ* aerobic and anaerobic metabolism in euphausiids and its relationships with the mechanisms regulating respiration and growth of migrant organisms in the OMZ.

Acknowledgments

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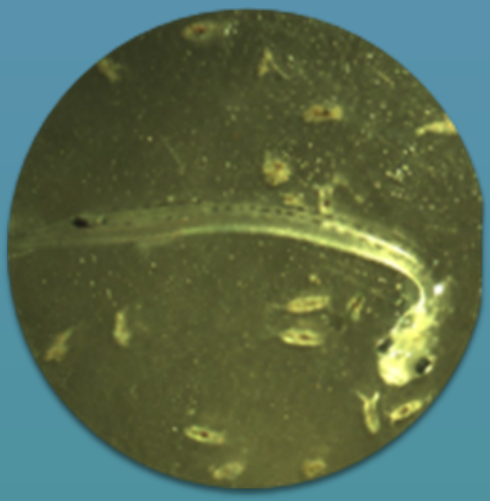
Chapter 5



The use of aminoacyl-tRNA synthetases (AARS) activity as an index of zooplankton growth

Santiago Hernández-León, Lidia Yebra, Inma Herrera, Pierrick Bécognée

Abstract

The search for an index of growth in zooplankton and fish larvae has been a challenge for decades. In order to match physical, chemical, or even other biological measurements, the actual estimation of growth is rather tedious to carry out on board oceanographic vessels. Therefore, different enzymatic (e.g., DNA polymerase, aspartate transcarbamylase, and aminoacyl-tRNA synthetases activities) and non-enzymatic (e.g., radiochemical methods, RNA/DNA ratio among others) were assayed and correlated to growth with quite different results. Here, we review the experiments performed to relate the activity of the enzyme aminoacyl-tRNA synthetases (AARS) to growth rates in freshwater (*Daphnia magna*) and marine (*Calanus finmarchicus*, *Calanus helgolandicus*, *Oithona davisae*, *Euphausia superba*, *Paracartia grani*) zooplankton, and fish larvae (*Clupea harengus*). In order to further investigate this relationship between AARS activity and growth rate, we allowed to grow *Sparus aurata* larvae to grow at a constant rate in order to account for differences in specific AARS activities during their development. The result of these experiments showed quite high enzymatic values during the first phases of development, corresponding to low growth rates. The same pattern was observed in other fish larvae (*Clupea harengus*) as well as in different species of copepods. High protein degradation during the first phases of development is suggested to promote the mismatch between growth and specific AARS activity. However, these two parameters were significantly correlated, thereafter shedding some light to the use of these enzymes activity as a proxy for growth rate.

Keywords: AARS, growth, zooplankton

1. Introduction

Zooplankton growth estimation in nature is a rather difficult task because the assessment of changes in body weight with time need long incubations (days) under simulated conditions of temperature and food. Those conditions are quite difficult to maintain in the laboratory. Moreover, there is a requirement for a large collection of animals in order to prolong the experiment to promote statistical confidence. Those problems are almost irresolvable in oceanography for the routine work at sea. Therefore, the search for a proxy of zooplankton growth has been a challenge for decades. The use of the egg production method, although it gives valuable information, is a poor tool because of the problems of relating body weight changes with reproduction of adult females. Growth rates for females and other naupliar and copepodite stages are quite different for a given species (Hutchings et al., 1995). The artificial cohort (Kimmerer and McKinnon, 1987) and the physiological (Le Borgne, 1982) methods also require long and tedious incubations, preventing their use in oceanography in order to match physical and chemical data output.

Several methods were assayed to find an index of growth in zooplankton. The measurement of RNA and DNA was the first attempt to develop a suitable index (Sutcliffe, 1970; Dagg and Littlepage, 1972) and it has been in use until present with different results. In general, this ratio was found to be a poor predictor of growth (Ota and Landry, 1984, Anger and Hirche, 1990, Wagner et al., 2001). However, some authors found predictive relationships in fishes (Peck et al., 2003) and crustaceans (Yebra et al., 2011). However, the latter author observed that this relationship differed between naupliar and copepodite stages, precluding its use in mixed populations.

The release and degradation of chitobiase have also been proposed as a growth index (Oosterhuis et al., 2000; Sastri and Roff, 2000) showing good relationships and being quite

sensitive. However, organisms cannot be preserved for later analysis and the procedure is not routinely used in oceanography because it is rather tedious for the work at sea.

Sapienza and Mague (1979) proposed the use of the activity of the enzyme DNA polymerase, and concomitantly Bergeron and Buestel (1979) the measurement of aspartate transcarbamylase (ATC). Unfortunately, the former enzyme was not the subject of later work and ATC activity also gave some contradictory results. Although Bergeron and Alayse-Danet (1981) and Bergeron (1982) found strong relationships between growth and ATC in the mantle and gonad of mollusks and fish larvae, Alayse-Danet (1980) and Hernández-León et al. (1995) found poor relationships in *Artemia* and copepods. The enzymatic approach in oceanography has some drawbacks as activities are measured at cell substrate saturation, something not expected to occur in nature. The results found in other enzymatic approaches such as the electron transfer system (ETS) and glutamate dehydrogenase (GDH) activities as proxies of respiration and ammonia excretion (see Hernández-León and Gómez, 1996, Hernández-León and Torres, 1997) were quite similar to the one found by Hernández-León et al. (1995) for ATC activity. Different relationships appeared depending on the nutritional status of organisms in the environment, therefore influencing the cell substrate saturation level.

Yebra and Hernández-León (2004) proposed the measurement of the activity of the enzymes aminoacyl-tRNA synthetases (AARS) as index of *Daphnia magna* growth. Since this seminal paper, other calibrations between zooplankton growth and AARS activity were obtained in *Calanus helgolandicus* (Yebra et al., 2005), *Calanus finmarchicus* (Yebra et al., 2006), *Euphausia superba* (Guerra, 2006), *Oithona davisae* (Yebra et al., 2011), *Paracartia grani* (Herrera et al., 2012), and the larvae of *Clupea harengus* (Herrera et al., Chapter 2 in this volume). Different relationships were also observed in those papers but the latter authors suggested that degradation of proteins during starvation or the effect of fatty acids on the

measurement of AARS activity could promote the absence of a close relationship with growth. Rather high AARS activity values were found at low growth rates but also those large values were observed in early stages of copepods. These organisms could promote an important synthesis of proteins as observed from their high AARS activity, but these proteins are also metabolized and not used to build body structures. Alternatively, oxidation of fatty acids could produce pyrophosphate (PPi), which is the end product of the reaction in the AARS assay, thus interfering the enzymatic activity assessment. Thus, the importance of young individuals affecting the relationship between growth and AARS activity should be confirmed.

In the present work, we review the existing calibrations between growth rates and AARS activity after a decade of effort. Because of the high activities at low growth rates observed in previous studies (see Herrera et al., 2012; Herrera et al., Chapter 2 in this volume), we seek to test if similar results were observed in previous calibrations. We also test experimentally the latter observation. We allowed fish larvae to grow at high food levels, at relatively high and constant growth rates in order to evidence the increased activities in small individuals as observed in previous studies.

2. Material and methods

We reviewed the available relationships between growth rates and specific AARS activities for zooplankton (cladocerans and copepods) and fish larvae (see Introduction). AARS activities were always measured following the method of Yebra and Hernández-León (2004) and Yebra et al. (2011) recalculated using the equation given by Herrera et al. (Chapter 3 in this volume). Individual biomass is always expressed as protein content, as measured using the method of Lowry et al. (1951) adapted for micro-assay by Rutter (1967).

We also present here additional experiments using fish larvae (*Sparus aurata*) in order to test the variability of AARS activity in organisms growing at almost constant growth rates. Larvae of *S. aurata* were reared in the laboratory at 22±1^{o}C and 24 h photoperiod (100-500 lux) under high food concentration. Three days after hatching, near complete yolk sac consumption, rotifers (*Brachiounus plicatilis*) were provided as food for the fish larvae at saturated densities. *Artemia* nauplii were provided after day 16 jointly with rotifers. Fish larvae, rotifers and *Artemia* were cultured in phytoplankton medium to provide a rich food environment in order to attain high growth rates (Parra and Yúfera, 2001).

Every 1-3 days three to six fish larvae were gently captured from the tank and frozen in liquid nitrogen (-196^{o}C) for later analysis of individual biomass and specific AARS activity as the proxy for growth rate. AARS activity was also measured following the method of Yebra and Hernández-León (2004), and using the equation given by Herrera et al. (Chapter 3 in this volume). Individual biomass as dry weight was calculated from a length-weight relationship.

3. Results

A review of literature values of growth versus specific AARS activity showed a rather high scatter of data (Fig.1), and a significant but poor predictive regression among those parameters (growth= 0.040+0.0019·spAARS; r^2= 0.346; p< 0.001).

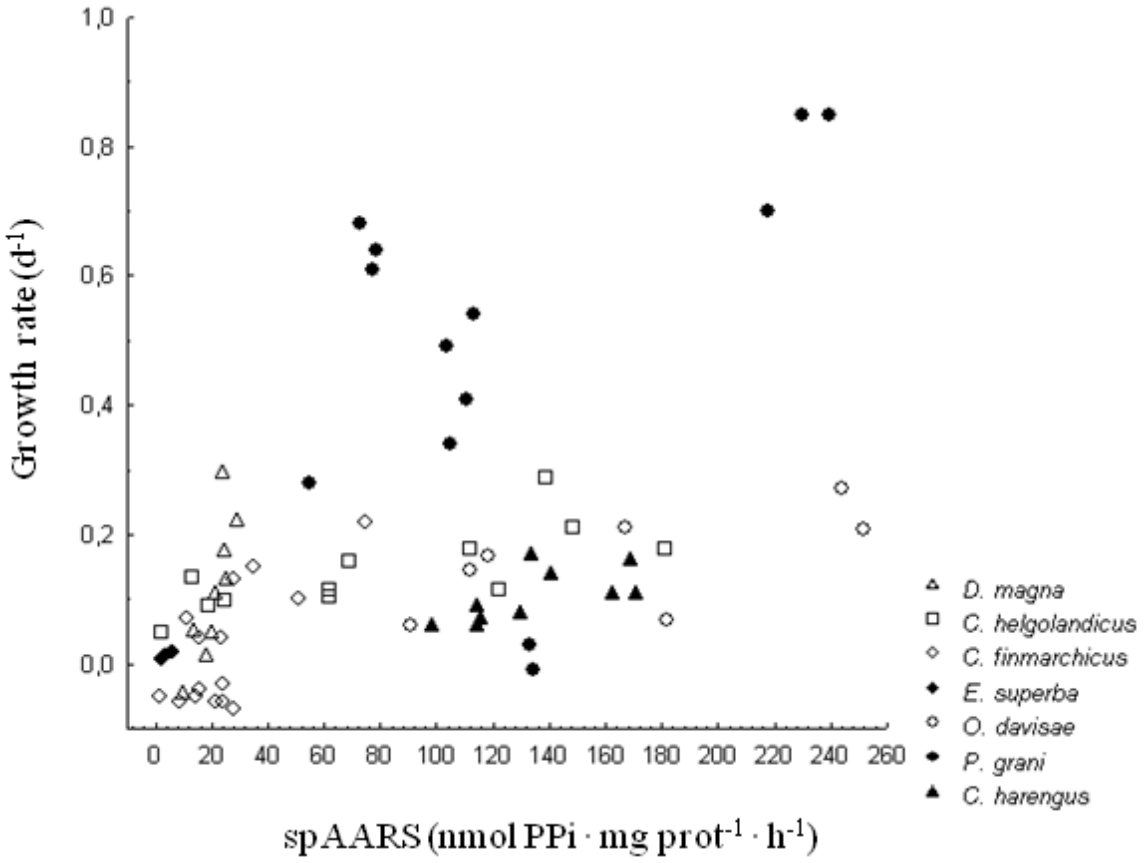


Figure 1. Relationship between growth rate (d^{-1}) and specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$) in the different calibrations performed taken form the literature.

Taking into account the experimental food level used in the experiments (as stated in the original publications), no relationship was observed between growth rates and specific AARS activity (Fig. 2). Nevertheless, low and medium food was only 20% of data precluding a thorough analysis. No pattern was also observed between low-medium and high food concentration.

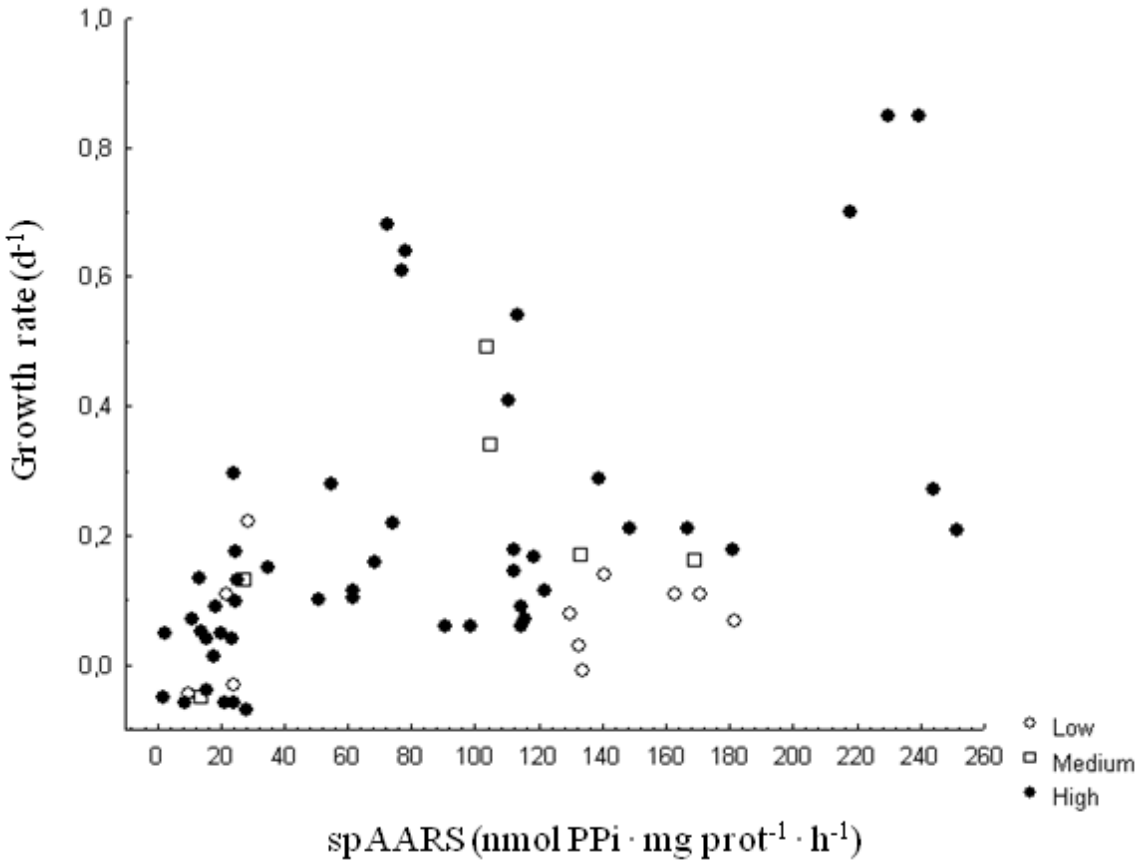


Figure 2. Growth rate (d^{-1}) versus specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$) at low, medium, and high food levels.

However, differences were found in relation to species and/or experiments carried out. We compared the slopes of the significant relationships published between growth and specific AARS activity. The relationships for *C. finmarchicus*, *P. grani* nauplii under food saturation and *E. superba* were significantly similar (ANOVA, $p>0.05$) and grouped together showed a significant positive correlation ($r^2=0.937$; $p<0.0001$). Also the relationships for *C. helgolandicus*, *O. davisae* and *C. harengus* larvae were similar (ANOVA, $p>0.05$) and together showed a positive correlation between growth and AARS activity ($r^2=0.275$; $p<0.005$), although the slope was lower. The slopes of the relationships for *D. magna* and *P.*

grani nauplii at varying food concentrations were significantly different from the rest of experiments and were not merged with any of the former two groups (Fig. 3).

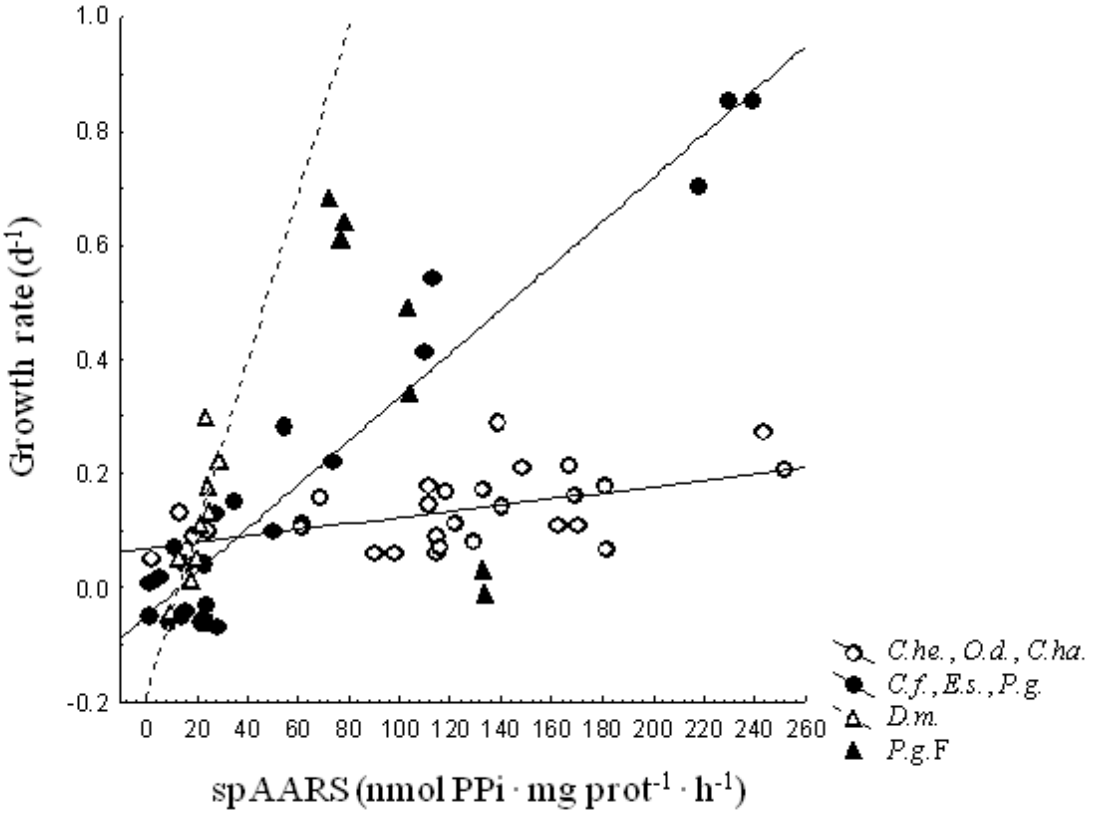


Figure 3. Relationships found between growth rate (d^{-1}) and specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$) after grouping significantly similar published calibrations. *C.he.*: *Calanus helgolandicus*, *O.d.*: *Oithona davisae*, *C.ha.*: *Clupea harengus*, *C.f.*: *Calanus finmarchicus*, *E.s.*: *Euphausia superba*, *D.m.*: *Daphnia magna*, *P.g.*: *Paracartia grani* under food saturation, *P.g.*F: *Paracartia grani* under varying food concentrations (relationship not shown in figure for clarity).

In order to test the high AARS activity sometimes observed at low growth rates (e.g., Fig. 3), larvae of *Sparus aurata* were induced to grow at constant and near maximum rates in two experiments. Individual biomass of fish larvae increased along 26 days at g= 0.132 d^{-1} (r^2=0.976; p<0.001) in experiment 1 (Fig. 4a), and at g= 0.139 d^{-1} (r^2=0.975; p<0.001) for experiment 2 (Fig. 4b). AARS was measured in both experiments and despite the constant growth, quite high specific AARS activity values were observed during the first 12-15 days

after hatching (Fig. 4). Thereafter, specific AARS activity became almost constant in both experiments. Small larvae (<100 µg dry weight·individual^{-1}) showed the largest specific AARS activity values, having lower and rather constant values at higher individual biomass (Fig. 5).

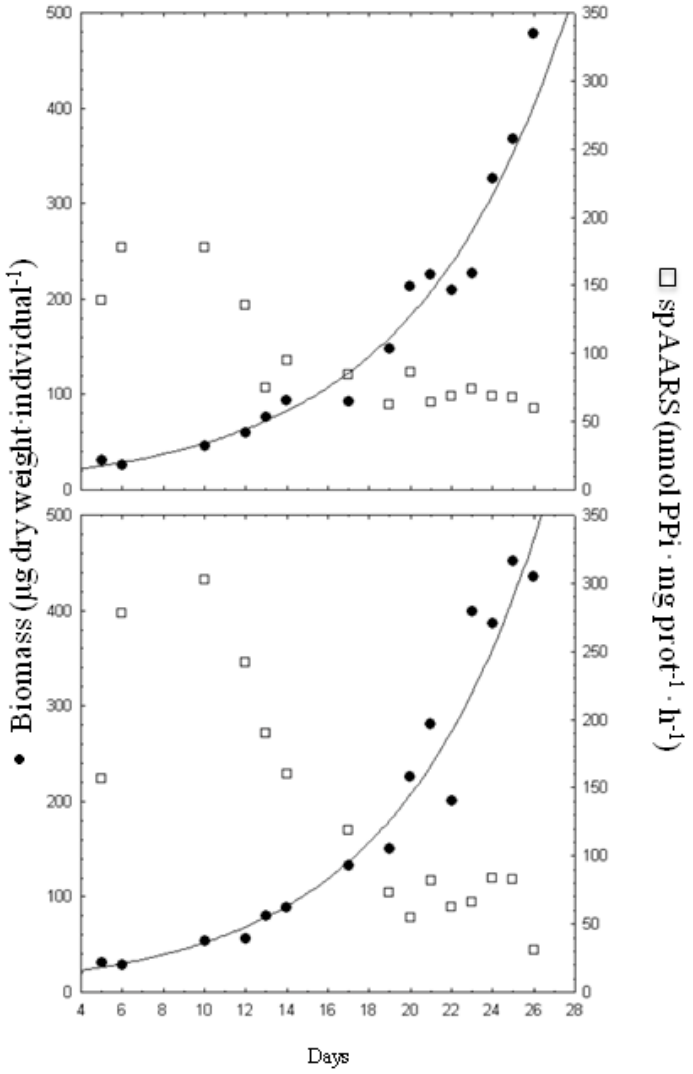


Figure 4. Individual biomass (µg dry weight·individual^{-1}) and specific AARS activity (nmol PPi·mg prot^{-1}·h^{-1}) of *Sparus aurata* larvae during development. Observe the high specific AARS activities during the first 12-17 days of life in organisms <100 µg dry weight·individual^{-1}.

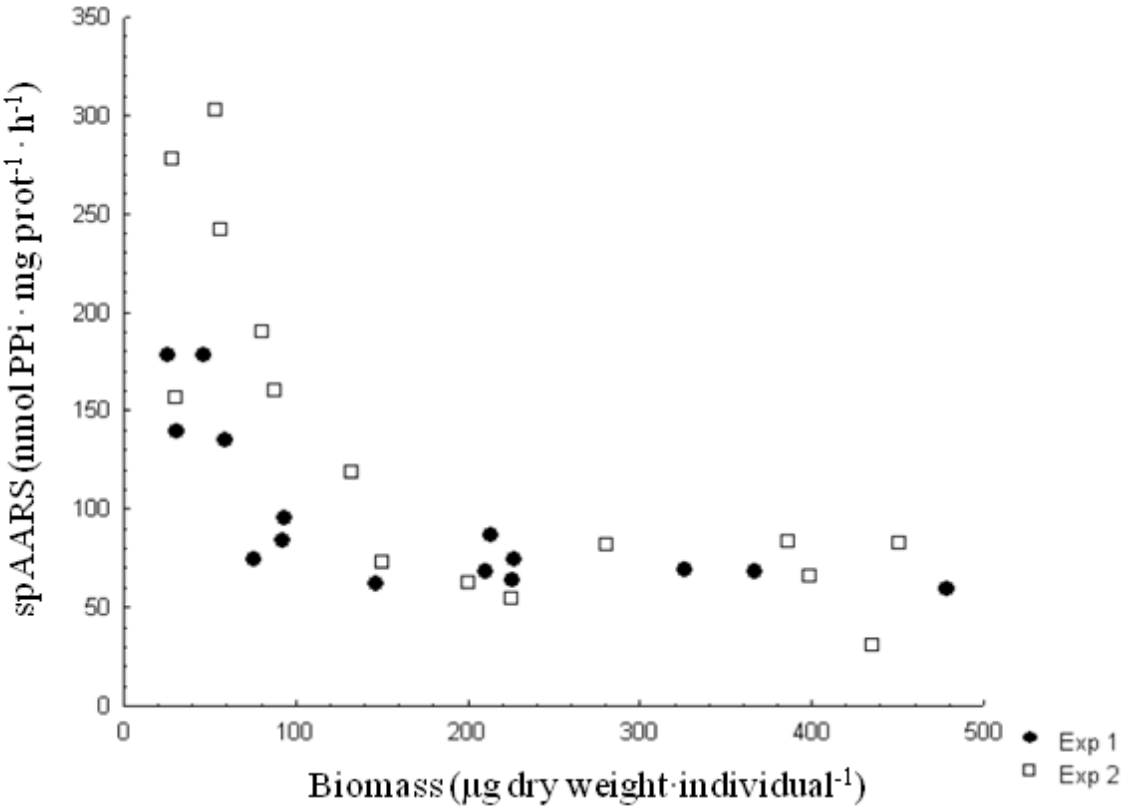


Figure 5. Individual biomass (µg dry weight·individual^{-1}) of *S. aurata* larvae versus specific AARS activity (nmol PPi·mg prot^{-1}·h^{-1}). Observe the high activities in organisms <100 µg dry weight·individual^{-1}, and the constant AARS at higher values of individual biomass.

To illustrate this, we represented growth rates versus AARS activities for small and large individuals of fish larvae and the CI to CV stages of the copepod *C. helgolandicus* (Fig. 6). In both cases, specific AARS activity changed from high values in small organisms at the onset of development to low values as they increase in biomass, and at almost constant growth rates.

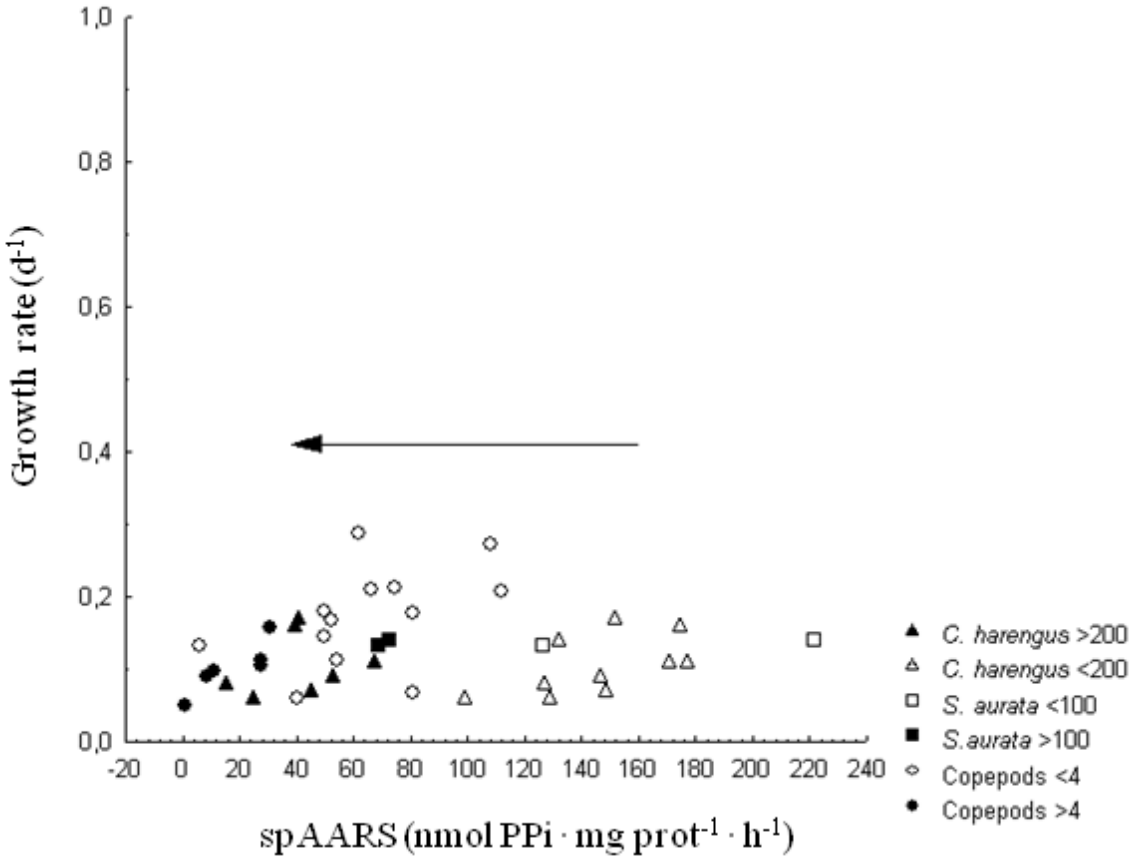


Figure 6. Relationship between growth rates (d^{-1}) and specific AARS activities (nmol PPi·mg $prot^{-1}·h^{-1}$) during development in fish larvae, and *Calanus helgolandicus* from copepodite I to V. The arrow indicate development from low to higher biomass per individual. Dark symbols indicate large organisms (>200 µg protein·$individual^{-1}$ for *C. harengus*; >100 dry weight·$individual^{-1}$ for *S. aurata*; and >4 µg protein·$individual^{-1}$ for *C. helgolandicus*).

Values of specific AARS activity in experiments of *Daphnia magna*, *Calanus helgolandicus*, *Calanus finmarchicus*, *Oithona davisae*, *Paracartia grani* nauplii and *Clupea harengus* larvae also showed quite variable specific AARS activity values for small individuals. After growing to more than 25% of their biomass, specific AARS activity showed lower values and reduced scatter, following the same pattern observed for *S. aurata* larvae (Fig. 7). Most of the individuals promoting the lower slope regression in Fig. 3 were

young individuals for naupliar or copepodite stages with biomass lower that 25% of their maximum (Fig. 7).

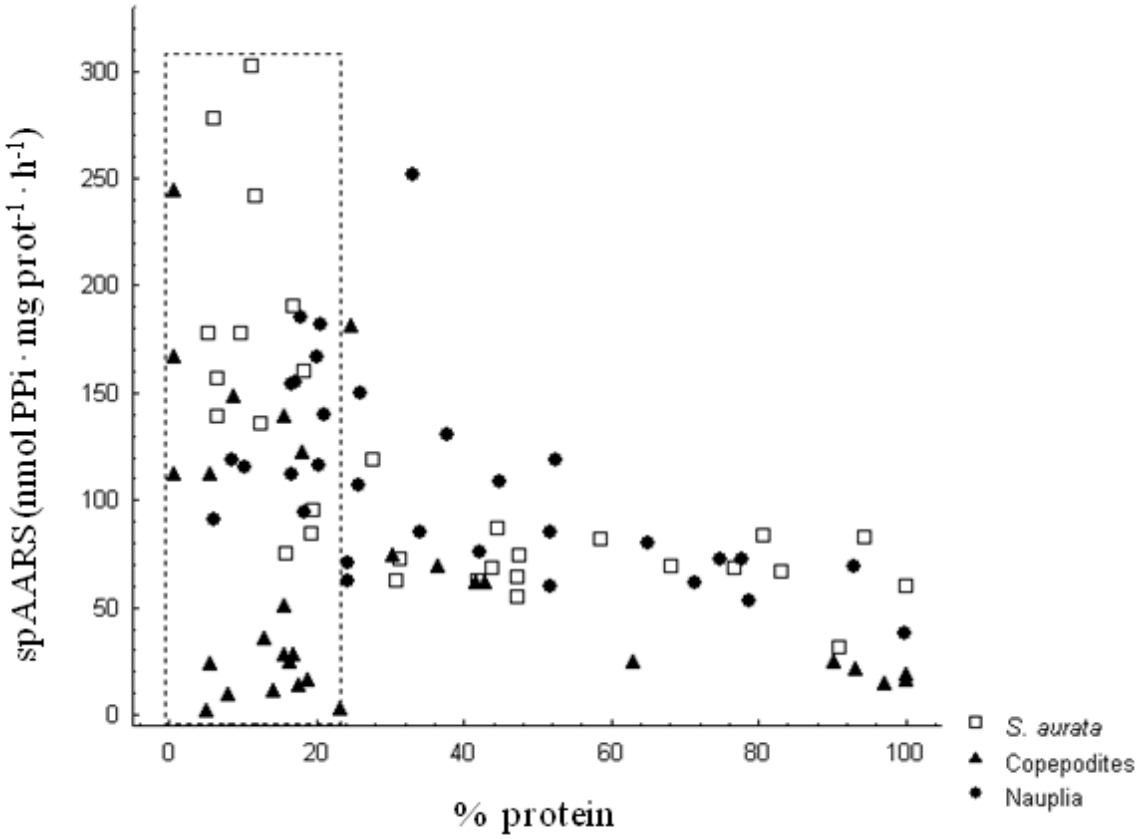


Figure 7. Standardized biomass (percentage of maximum biomass for fish larvae, naupliar or copepodite stages) for the different organisms used in the calibrations between growth rates and specific AARS activity. Observe the high variability of enzyme activities for organisms below 25% of their maximum biomass. Also observe the decrease in specific activities at increasing individual biomass.

Taking this into account, the data in Figs. 1 and 6 were grouped according to their individual biomass percentage (Fig. 8). Both correlations found were statistically significant, but the relationship comprising the large individuals (>25% final biomass) was stronger (growth=-0.0117+0.0038·spAARS, r^2=0.738; p<0.001), than the relationship observed for the small individuals (<25% final biomass; growth=0.0082+0.0009·spAARS, r^2=0.358; p<0.001).

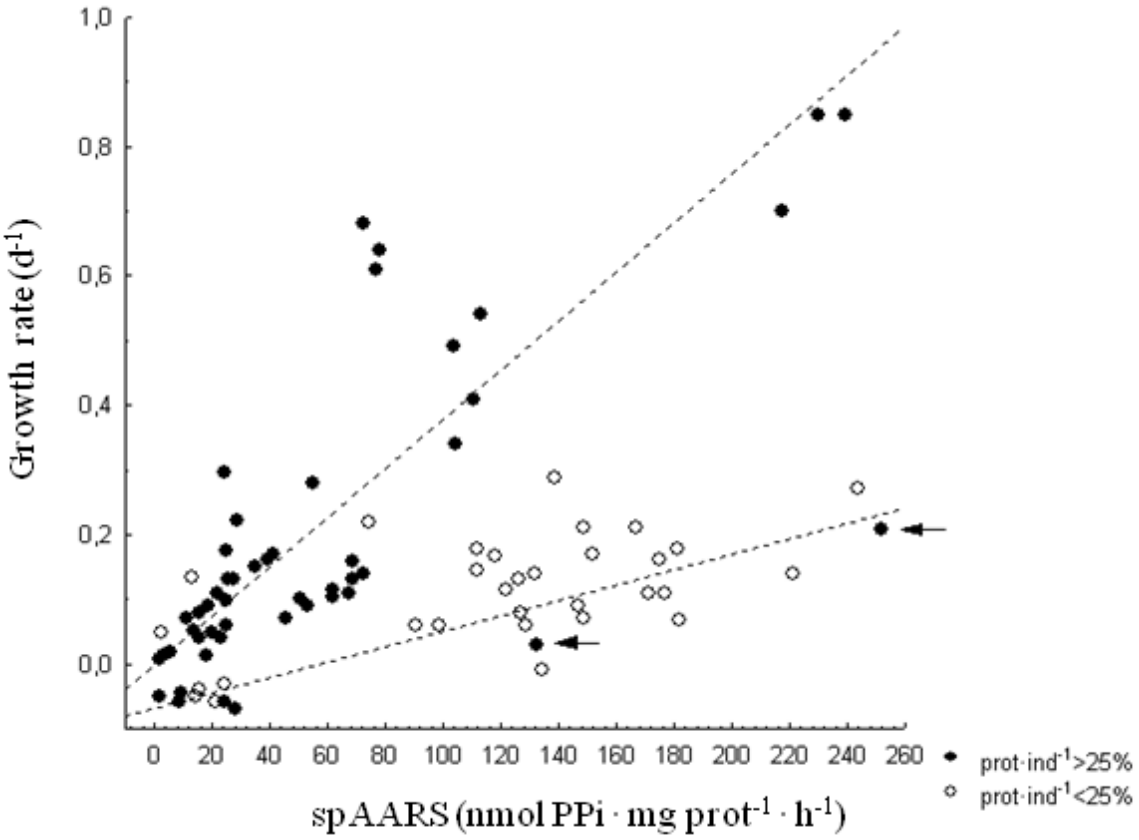


Figure 8. Growth rate (d^{-1}) versus specific AARS activity (nmol PPi·mg $prot^{-1}·h^{-1}$) in organisms below and above 25% of maximum biomass for the different organisms. Arrows indicate outliers not considered in the relationships given.

4. Discussion

We reviewed the available calibrations between growth rates and specific AARS activity (Fig. 1) and no predictive relationship was obtained. Herrera et al. (2012) suggested that food level could also explain the relationship between growth and the enzyme activity. However, this relationship was not observed in our work (Fig. 2). Nevertheless, low and medium food levels were only 20% of the data in our review, emphasizing the need for more experiments under food limitation.

Taking into account the different relationships published, most of the species and/or experiments could be merged in two significant relationships (Fig. 3). From these, the lower slope relationship showed high specific activities at rather low growth rates and corresponded to *Oithona davisae*, *Calanus helgolandicus* and *Clupea harengus* species. Similarly, Herrera et al. (2012) reported high specific activities at low growth rates for early stages of *Paracartia grani* nauplii at low food concentrations. Holmborn et al. (2009) also observed anomalously high specific AARS activities under starvation (low growth rates expected) in *Acartia bifilosa* females.

Yebra et al. (2011), Herrera et al. (2012; Chapter 2 in this volume) discussed the relative importance of protein turnover rates in relation to the *de novo* protein synthesis rates, which would lead to anomalously high specific AARS activity values on organisms under low food as observed in *P. grani* by Herrera et al. (2012; Chapter 2 in this volume). A high level of protein degradation to aminoacids will prevent the increase of the protein pool in the cell and therefore growth. Yebra et al. (2011) and Herrera et al. (2012; Chapter 2 in this volume) also discussed a methodological artifact related to the measurement of AARS promoting the low-growth and high-activity (LGHA) data. Non-feeding or young organisms may use lipid reserves to survive. The β-oxidation of fatty acids releases pyrophosphate (PPi) and these reactions could interfere the AARS assay as it is based on the measurement of PPi release during the aminoacylation of tRNA, which is assessed as the oxidation of NADH by PPi. It is known that early stages of marine organisms use lipids because its oxidation produces an important amount of energy. This is the case of crustacean nauplii and fish larvae. In fact, the latter organisms consume the yolk sac and the oil droplet during the first days of life and later are normally fed with other organisms rich in lipids such as rotifers and *Artemia* nauplii. Thus, the lipid metabolism at the feeding onset of those early stages could promote the formation of PPi and the rather high activity values found.

Our experiments in fish larvae feeding *ad libitum* showed rather high growth rates (0.132 and 0.139 d^{-1}) compared to growth of larvae of this species at different food concentrations (Fernández-Díaz and Yúfera, 1997; Parra and Yúfera, 2001). However, despite the constant rates observed, quite high specific activities were observed during the first 10-17 days of life (Fig. 4). Crustaceans and fish larvae showed a common pattern of variable AARS activities clearly related to the early stages of organisms (Figs. 5, 6 and 7). Thus, the experiments performed in fish larvae should give some insight into the processes promoting the LGHAs as the physiology of these organisms during their early development is rather well known.

Lipids are important during the development of fish larvae. First, the larvae use the content of the yolk sac to provide energy before the onset of feeding. In *S. aurata* this process is completed in 4-5 days after hatching, and the complete oil droplet absorption takes place at about 4 days after. Our LGHA values increased after day 5 (yolk sac completely exhausted) and remained high until day 12-17 (Fig. 4), beyond the complete absorption of the oil droplet. In any case, through the development of the larvae, lipid-rich rotifers and *Artemia* nauplii were provided during all the study. Rotifers were provided after day 3, and *Artemia* after day 16. A lipid-rich diet is common in fish larvae cultures in order to provide enough food quality. Thus, the possible interference of stored lipids producing PPi in our AARS assays should be observed through the entire period studied. However, the LGHAs only occurred between day 5 and 12-17 after hatching. In this sense, the study of nitrogen excretion in fish larvae suggests that protein is the major catabolic substrate, with little, if any, energy stored in, or produced from, lipid reserves (Torres et al., 1996). These authors found rather low O:N ratios in well fed and 24 hours starved larvae, indicating a protein fuel source in both cases. The slightly lower O:N ratios observed for starved larvae suggested an even stronger dependence on tissue proteins. Moreover, relatively high trypsin activity has been observed in

fish larvae after the yolk sac consumption and days 14-16 after hatching (Cahu et al., 1998, Lazo et al., 2000), just the period of high AARS activity observed in the present work. Hence, the LGHA observed in this and previous studies would not be a methodological artifact induced by lipids catabolism.

On the other hand, growth is the net result of protein synthesis and protein degradation (Conceiçao et al., 1997). AARS activity measures the rate of protein synthesis and not the net protein deposition in the cell. Therefore, it is plausible that the relationship between growth rate and AARS activity could be masked by the variability in the balance between protein synthesis and degradation. Our measurements of AARS activity in fish larvae started just after the yolk sac absorption (day 4 post-hatch), at the feeding onset of *S. aurata* larvae. It is known that feeding in larvae increases progressively, reaching a maximum by day 12 post-hatch (Parra and Yúfera, 2001), also increasing respiration (Yúfera and Darias, 2007). The result is a sharp decrease of assimilation and gross growth efficiency (K_1=growth/ingestion) from the onset of feeding towards day 12-14 post-hatch (Parra and Yúfera, 2001). The high assimilation at constant growth rates implies a similar decrease in protein synthesis, as protein deposition for growth is protein synthesis minus protein degradation.

Thus, our higher AARS activity in young organisms (<25% of maximum individual biomass, Fig. 7) at constant growth rates in *S. aurata* larvae, and at low growth rates in crustaceans is suggested to be the effect of high protein synthesis at the onset of feeding in those organisms rather than a methodological artifact during the AARS assay. Because AARS activity is a measurement of protein synthesis in the cell, its use as a proxy for protein deposition should be considered with caution because it could promote the above mentioned error. In ecosystems where clear cohorts could be identified, recognition of these young organisms could be an alternative to avoid this problem, as the relationship obtained is also

significant for these individuals. However, high diversity and continuous reproducing organisms is normally the rule in the pelagic system. Although the proportion of the biomass of young organisms should be small in the different size fractions normally sampled, hiding the high activities related to protein synthesis, the error related to protein synthesis and deposition will remain. Despite these shortcomings, growth and AARS activity were positively correlated, and the AARS method could provide reliable estimations of growth rates in mixed zooplankton.

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IV. General discussion

General discussion

The results included in this thesis have been discussed in detail in their respective chapters. The purpose of this general discussion is to relate the main results obtained in the different studies.

Validation of the AARS method as index of growth rate on zooplanktonic larvae

The early stages of zooplankton, such as copepod nauplii and fish larvae, are of great importance in the marine environment because they can influence the species recruitment success, and the distribution and abundance of the populations. In addition, these early stages are probably the most abundant metazoans in the ocean (Fryer, 1986). The first part of this thesis is focused on the validation of the AARS method as index of growth in these organisms.

To study the relationship between planktonic larvae growth rate and their AARS activity we used the nauplii of the calanoid copepod *Paracartia grani* as representative of invertebrate zooplankton. Calanoid copepods represent between 55 and 99% of the plankton in the ocean (Mauchline, 1998). Similarly, for the validation of the AARS method as index of growth in vertebrate zooplankton, the larvae of the small pelagic fish *Clupea harengus* was chosen. This clupeid is one of the most captured in North Atlantic and North Pacific waters, being of great importance due to the role that they play in the marine ecosystem and in the fisheries (Cushing, 1975).

Specific growth and AARS activity of *P. grani* nauplii were positively affected by temperature within 10º to 18ºC. The growth rates of *P. grani* obtained from length-weight relations (**Chapter 1**) were similar to the previously found for *P. grani* (Calbet and Alcaraz, 1997) and for other *Acartia* species (Berggreen et al., 1988; Leandro and Tiselius, 2006).

Similarly, growth and specific AARS activity of *Clupea harengus* larvae were positively affected by the temperature in a range between 7-17°C (**Chapter 2**). The growth values estimated based on length-weight relations were similar to the obtained by other authors for the same species (Suneetha et al., 1999; Folkvord et al., 2000; Johannessen et al., 2000; Arrhenius and Hansson, 1996; Kiørboe and Munk, 1986). In both species AARS activity was significantly correlated with growth rate, reflecting variations on temperature.

The copepod nauplii and fish larvae growth rates also depended on the food availability. At low food concentration, low growth rates were observed in these organisms. In the case of *P. grani* nauplii, this may be explained by a decrease in the efficiency of the capture of food at low concentrations, as it was observed for the copepod *Acartia tonsa* (Paffenhöfer and Stearns, 1988). This suggests their adaptation to environments with high food availability (estuarine areas and coastal waters; Alcaraz, 1977; Villate, 1982). However at high food concentrations the growth remained constant for *P. grani* nauplii, as it was previously observed in this (Calbet and Alcaraz, 1997: *Acartia grani*) and other species (Berggreen et al., 1988: *A. tonsa*). Nevertheless, the effect of the food type in the growth of early stages of copepods has been poorly studied. The prey used in our study (*Oxyrrhis marina*) is considered to be a high quality food for *Acartia* species (Klein Breteler and Schogt, 1994; Kleppel et al., 1998), although other studies have used different food types (*Rhodomonas baltica* or mixtures of diatoms *Thalassiosira weisflogii* and *cryptophyte Rhodomonas sp.*). All these changes in the diet of *Acartia* species affect their growth, development, production and nutritional condition (Ismar et al., 2008; Teixeira et al., 2010). In addition, the growth of *P. grani* nauplii (**Chapter 1**) was comparable to the results observed in nauplii of this and other species. This suggests that the quality of the food might not affect the growth of the nauplii of this species under food saturation conditions.

In the case of *Clupea harengus* larvae, the growth rate was directly affected by prey

density (**Chapter 2**). The differences on growth related to food concentration agree with observations done by Folkvord et al. (2000) and confirm food concentration as one of the main factors affecting these larvae growth rate (Kiørboe and Munk, 1986; Johannessen et al., 2000; Folkvord et al., 2000).

Moreover, food concentration affected directly AARS activity of *P. grani* nauplii as well as of *C. harengus* larvae. Paradoxically, we observed higher values of specific AARS activity when the growth of these organisms was limited by starvation or very low food concentrations. This increase of specific AARS activity suggests that the organisms under starvation survived at the expense of its own lipid reserves, and/or through protein degradation (protein catabolism) (Love, 1980). The implications of starvation on metabolism are more pronounced in early stages of life than in advanced ones, probably since the latter stages have less energy reserves (Gadomskia and Petersen, 1988; Richard et al., 1991). Weight loss on starved larvae due to metabolic cost shows minimum levels of muscle mass, which represents 60-70% of their body weight (Machado et al., 1988). It has been observed that muscle mass was the only variable that showed significant differences between fed and starved larvae (Martin and Wright, 1987; Ferron and Leggett, 1994; Catalán, 2003; Pliego Cortés, 2005). This shows that muscle mass is very sensitive to the lack of food, since the larvae rapidly degrades muscle proteins (Love, 1980).

The results obtained in **Chapters 1** and **2** confirm that AARS activity should be suitable as an index of growth rate in early stages of the species studied. This agrees with the significant relationships previously observed between somatic growth and specific AARS activity in other zooplanktonic species such as the cladoceran *Daphnia magna* (Yebra and Hernández-León, 2004), the copepods *Calanus helgolandicus*, *C. finmarchicus* and *Oithona davisae* (Yebra et al., 2005, 2006, 2011) or the euphausiid *Euphausia superba* (Guerra, 2006).

The validation of this enzymatic method as index of growth rate in zooplanktonic larvae would facilitate to a great extent the study of planktonic organisms growth rate both in the laboratory and in the ocean, as well as the factors that modulate the production of zooplankton communities.

Use of AARS activity as index of growth rate in the ocean

The use of indirect methods allows determining the metabolic rates of zooplankton at oceanic scale due to the fact that incubations are not needed, allowing freezing the organisms captured, analysing its activity later in the laboratory. In addition, the laboratory analyses are economic, reproducible, and the results are obtained spending less time than by using direct methods.

Examples of indirect indices widely used in zooplankton are the gut fluorescence (GF, Parsons et al., 1984) as index of potential grazing, and the electron transport system activity (ETS, Packard et al., 1971) as index of potential respiration. In the second part of this thesis we applied these indices together with the AARS method to obtain a wide image of the metabolism of the zooplankton communities.

The first application of this set of indirect methods allowed us to study the evolution of the zooplankton community north off Gran Canary Island (Canary Islands) during the winter-spring bloom in 2010. This year was characterized to show the warmest winter of the last 30 years. The typical late-winter bloom was not observed due to the high surface temperature. In addition, potential grazing and respiration also showed values much lower than previous years. However, a higher relatively biomass of zooplankton was observed during the post-bloom period (**Chapter 3**). These higher biomass values were explained by the input of atmospheric dust from the Sahara desert, which increased the levels of nutrients in the water column, increasing primary production (Franchy et al., submitted), and

zooplankton biomass. This was also reflected in the zooplankton AARS activity, showing high values throughout the study (**Chapter 3**). This suggests that the recurrent dust input events and their effect on primary producers promoted sustained growth rates during both the bloom and post-bloom periods. Given that this was the first application of AARS activity to the assessment of zooplankton growth during the late winter bloom, we have no prior data to compare with other cold years. However, the relationships found in the laboratory between AARS activity and temperature suggests that a warming of the marine environment would have marked effects on the zooplankton production in the area.

Besides, previous studies suggest that both biomass and growth of the zooplankton community are associated with the lunar illumination in the oceanic waters of the Canary current (Hernández-León, 1998; Hernández-León et al., 2001, 2002, 2004, 2010). Similar to what was observed in lakes (Gliwicz, 1986), the vertical migrants have developed strategies to avoid predation. When there is new moon (the dark period of the lunar cycle) these organisms rise to the surface and feed on the non-migratory zooplankton, whilst during the full moon (the clear period of lunar cycle), the migratory species do not reach the first 100 meters of the water column to avoid predation. This allows non-migratory zooplankton to grow and to increase in abundance (Hernández-León, 1998; Hernández-León et al., 2001) and biomass (Hernández-León et al., 2002, 2004).

The variability in AARS activity of the zooplankton community (**Chapter 3**) did not clearly show this relationship with the lunar cycle, probably due to the effect of the intense dust input events. However, before the increase in biomass due to the dust events, we observed that AARS activity showed two increases coinciding with the crescent moon, about eight days before the full moon, as predicted by Hernández-León et al. (2010) model. The use of the AARS method, due to its simplicity, could also facilitate the incorporation of growth studies in future time series following natural zooplankton communities. This would allow to

further increase the knowledge of the relationships between zooplankton production and environmental factors, such as the light inciding into the surface, which varies for example with the lunar cycle.

A significant advantage of the use of indirect methods over direct methods is that the application of the former ones does not restrict to the superficial waters. As incubations are not needed, the organisms can be captured at depth and frozen rapidly on board. In this way, they preserve the properties of their environment up to the moment of their analysis in the laboratory.

Nowadays, due to the possible acidification of the ocean and the expansion of the oxygen minimum zones (OMZ), it is vital to know how the vertical migrants are affected by OMZs and how oxygen concentrations in the marine system affect the zooplankton metabolism and growth. However, due to limitations of the direct methods, there are no studies on the growth of vertical migrant organisms in relation to these OMZ.

In this thesis, we used the AARS activity, in combination with the GF and the ETS activity, to perform the first study of the growth of vertical migrants in relation to the OMZ in the Tropical Pacific off Mexico. Specific AARS activity on the euphausiid *Euphausia distinguenda* showed a pattern similar to the found for potential respiration (ETS). Thus, AARS activity values were higher at the surface, decreasing significantly with depth and with oxygen concentration. Our results (**Chapter 4**) suggest that AARS activity of *E. distinguenda* was affected by the OMZ. The enzymes AARS catalyse the first step of the protein synthesis. The contribution of the protein synthesis to the oxygen consumption has been previously studied in marine fishes (Houlihan, 1991; Smith and Houlihan, 1995). Enzyme activity depends on the production of ATP. Thus, if the production of ATP is not enough, because of low oxygen concentrations, low values of protein synthesis (AARS activity) are found and thereby the second step of protein synthesis, translation, is also reduced (Anderson et al.,

2009). This fact coincides with the decrease of AARS activity observed for *E. distinguenda* within the OMZ (**Chapter 4**). Anderson et al. (2009) suggested that translation suspension in hypoxia conditions could inhibit or reduce the protein synthesis of organisms as well as the AARS activity. However, when the oxygen concentration increases also the protein synthesis increase its levels (Koumenis et al., 2002). This allowed that *E. distinguenda* quickly increased its protein synthesis and its AARS activity when migrating towards oxygenated waters above the OMZ. Thus, the metabolism regulation of these organisms would be an adaptive advantage in the presence of OMZ in its vertical migrations.

Towards a global equation to estimate zooplankton production from AARS activity

In the last section we pursue the possibility of finding a general relationship between the planktonic community growth rate and its AARS activity, facilitating the study of the whole community evolution. We gathered a review of all published relationships between AARS activity and somatic growth rate and added the data presented in this thesis (**Chapter 5**). We did not found an overall equation relating somatic growth rate and AARS activity. However, when data from experiments, were splitted according to the relative individual biomass, two significant relationships were observed. One pooled the experiments corresponding to organisms larger than 25% of their final biomass. This equation included both freshwater (*Daphnia magna*) and marine species (*Calanus finmarchicus*, *Paracartia grani* and *Euphausia superba*). A second regression grouped the experiments carried out with organisms at the onset of their development (individual biomass <25% of its final biomass). This was mainly the case of the marine copepods *Calanus helgolandicus* and *Oithona davisae*, and the larvae of fish *Clupea harengus* and *Sparus aurata*. In these organisms we observed anomalously high specific AARS activities at rather low growth rates. This mismatch agrees with the results observed for *Paracartia grani* nauplii (**Chapter 1**), *C.*

harengus larvae (**Chapter 2**) and *Acartia bifilosa* females under food deprivation (Holmborn et al., 2009). It is known that early stages of zooplankton use lipids as fuel because its oxidation produces an important amount of energy. For example fish larvae consume the yolk sac and the oil droplet during the first days of life and later prey upon other organisms rich in lipids such as rotifers, *Artemia* nauplii and copepods. It was suggested that the lipid metabolism at the feeding onset of those early stages could promote the formation of PPi, interfering in the AARS assay, overestimating the AARS activity (Herrera et al., 2012, **Chapters 1 and 2**). However, the data presented in **Chapter 5** show that *S. aurata* larvae just after hatching, despite its growth metabolizing fat from the yolk sac, did not show AARS values as high as days later, when they started actively to feed and their growth depended on food availability and not on endogenous reserves. This confirms that high AARS values found in early developmental stages did not correspond to a methodological artefact involving lipid metabolism but by opposite, we should look for the answer in the protein metabolism.

Somatic growth is the net result of protein synthesis minus protein degradation (Conceiçao et al., 1997). AARS activity measures the rate of protein synthesis and not the net protein deposition in the cell. Thus, a high protein degradation rate during the first phases of development in planktonic organisms would cause the mismatch observed between growth rates and AARS activity in young organisms. Despite these shortcomings, growth and AARS activity were positively correlated, thereafter allowing the use of the AARS method as index for the estimation of growth in zooplankton and ichthyoplankton.

Conclusions

The main conclusions obtained in this thesis are:

1. Growth and AARS activity of *Paracartia grani* nauplii and *Clupea harengus* larvae depend on temperature and food concentration.

2. Protein degradation during starvation affects the relationship between AARS activity and somatic growth on *P. grani* nauplii and *C. harengus* larvae.

3. The increase in temperature in the Canary Island waters affects the biomass and the specific AARS activity of the zooplankton community.

4. *Euphausia distinguenda* AARS activity and potential respiration are affected by the oxygen concentration in the OMZ.

5. Combining data of nauplii, juveniles and adults of zooplankton as well as fish larvae two main relationships between AARS activity and somatic growth were found. The equations augur that in the near future it may be possible to determine zooplankton production using the AARS index.

Future research

On the basis of the results obtained in this thesis there are several aspects concerning biological oceanography that would need to be solved in the near future:

- In order to find a general equation for its application at oceanic scale, further studies are needed to understand the relationships between somatic growth rate and AARS activity (e.g. zooplankton under starvation).

- Also, validation of the AARS method on zooplankton groups other than crustaceans and fish larvae should be conducted if we aim to assess production of zooplankton communities using AARS activity as index of growth rate.

V. Spanish summary/

Resumen en español

Introducción

Introducción

El zooplancton está formado por pequeños organismos que habitan en la columna de agua y que son transportados por las masas de agua. Se pueden dividir según el tamaño de los organismos (Sieburth et al., 1978). Así, el microzooplancton comprende organismos muy diversos de talla entre los 0.02 y 0.2 mm, como p.e. ciliados, nauplios de copépodos o larvas meroplanctónicas. El mesozooplancton, entre 0.2 y 20 mm de longitud, incluye especies de copépodos, cladóceros o apendicularias entre otros; y el macrozooplancton, cuyos organismos son mayores de 20 mm, corresponde principalmente a larvas de peces, decápodos o eufausiáceos. Los organismos que componen el zooplancton también se clasifican según su ciclo vital, conociéndose como holoplancton aquellos que en todos los estadios de su ciclo biológico viven formando parte del plancton (p.e. copépodos, ostrácodos, cladóceros) (Longhurst, 1985), y meroplancton, aquellos que sólo pasan algunas etapas de su vida como parte del plancton, generalmente la de huevos y larvas, como peces, equinodermos, cnidarios o moluscos (Raymont, 1983).

El estudio del plancton es de vital importancia para el hombre dado que es la base de la mayoría de las redes tróficas marinas. El zooplancton tiene un papel central en el ecosistema marino ya que (1) ejerce un control parcial sobre los productores primarios y (2) es alimento de niveles tróficos superiores, al tiempo que (3) representa un importante nexo de unión entre la cadena trófica clásica y el bucle microbiano. Además, (4) exporta materia orgánica particulada (MOP) que utilizan las bacterias y (5) excreta nutrientes que pueden ser aprovechados por los organismos fitoplanctónicos (Fig. 1). Además, el zooplancton incluye todos los estadios larvarios de los organismos nectónicos y bentónicos, por lo que el desarrollo de los recursos marinos explotables depende en gran medida de la variabilidad de la producción zooplanctónica.

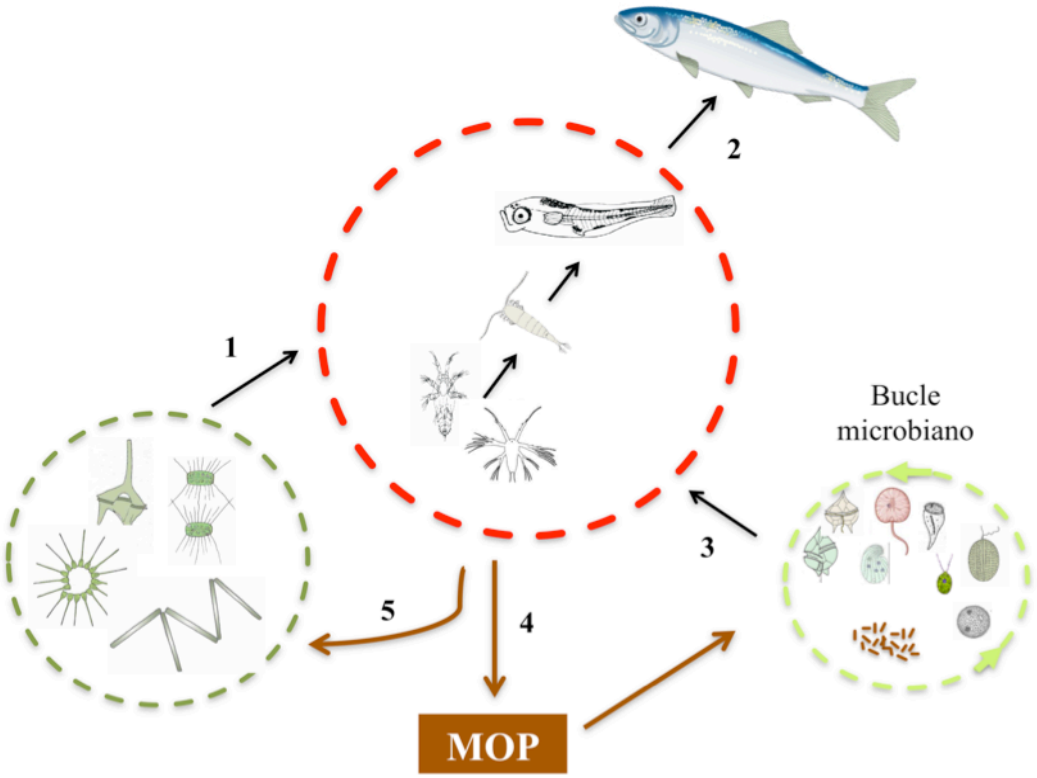

Figura 1. Importancia del zooplancton en el ecosistema marino.

La variabilidad de las comunidades planctónicas viene condicionada por factores abióticos y bióticos. Los primeros factores afectan a diferentes escalas (Dickey y Bidigare, 2005), desde la escala global a la meso- y microescala. El forzamiento físico (Bonnet et al., 2005) incluyendo las estructuras de mesoescala como los remolinos y frentes que concentran biomasa (Yebra et al., 2005), potencian el metabolismo (Hernández-León et al., 2002; Landry et al., 2008) y la migración vertical (Isla et al., 2004), e incrementan el flujo activo mediado por el zooplancton migrador hacia aguas profundas (Yebra et al., 2005). A microescala, la turbulencia afecta a la tasa de encuentro entre el depredador y la presa y por tanto a las tasas de alimentación (Kiørboe y Saiz, 1995; Visser et al., 2009). También la temperatura (Parrilla et al., 1994; Drinkwater, 2005), la salinidad (Hirst y Lucas, 1998), la luz (Hernández-León,2008; Hernández-León et al., 2001, 2004, 2010), los nutrientes (Duarte et al., 2006), la

concentración de oxígeno (Teuber et al., 2013) y el dióxido de carbono (González-Dávila et al., 2006) afectan de manera directa a la comunidad planctónica.

La biomasa de la comunidad zooplanctónica se ve también afectada por factores bióticos que modulan el metabolismo de los organismos. Dicho metabolismo se ve influenciado por la distribución y abundancia del alimento (fito- y microplancton), ya que determinarán su crecimiento (Vidal, 1980; Hirst et al., 2003; Lin et al., 2013). Asimismo la biomasa se ve afectada por la mortalidad por depredación (Hirst y Kiørboe, 2002; Runge et al., 2004; Maar et al., 2014). El conjunto de estos factores determinará la dinámica de poblaciones y la estructura de las comunidades, lo que también afectará a los flujos de carbono mediados por el zooplancton en el océano.

Debido a la posición central del zooplancton en la red trófica, el estudio de la producción del zooplancton es imprescindible para la evaluación de los flujos de materia y energía a través de los ecosistemas pelágicos. La determinación de la producción zooplanctónica, aumentará nuestro entendimiento y capacidad para desarrollar modelos que acoplan parámetros físicos y biológicos con la finalidad de evaluar los impactos de la dinámica de poblaciones del zooplancton sobre los eslabones superiores de la cadena trófica.

En este sentido, la producción del zooplancton viene definida como;

$$P = (B_1\text{-}B_0)+M \qquad \text{(ec.1)}$$

donde P es la producción del zooplancton, B_1 y B_0 son la biomasa en el tiempo 1 y 0 respectivamente, y M es la mortalidad. Así, la biomasa en un tiempo dado es:

$$B_1 = B_0 + (B_0 \cdot g) - (B_0 \cdot m) \qquad \text{(ec.2)}$$

siendo g y m las tasas de crecimiento y mortalidad respectivamente.

Existen varios métodos directos para la estimación del crecimiento y la producción del zooplancton (Omori y Ikeda, 1984; Runge y Roff, 2000). Entre estos métodos el más común

es el desarrollado por Heinle (1966) en el que, siguiendo la definición de crecimiento, se miden las variaciones de peso en función del tiempo. Otro método utilizado para la estimación de crecimiento *in situ* es el método de la cohorte artificial (Dowing y Rigler, 1984). Con este método el crecimiento se calcula siguiendo el desarrollo de una cohorte, y está basado en el estudio de la frecuencia de tamaños y/o estadios de desarrollo en la población. Un tercer método utilizado para determinar el crecimiento se basa en el estudio de la tasa de muda (Hutchings et al., 1995), pero únicamente es factible su uso en crustáceos y su aplicación se restringe principalmente a copépodos. Además, todos estos métodos requieren incubaciones que duran desde días hasta meses, según los organismos.

Debido a los tediosos procedimientos para medir crecimiento, algunos autores propusieron la tasa de producción de huevos (Kiørboe y Johansen, 1986; Berggreen et al., 1988; Uye y Sano, 1995). Este método asume que la tasa de producción de huevos por las hembras es equivalente a la tasa de crecimiento de los copepoditos, aunque se ha observado que esto no se cumple en todas las especies (Peterson et al., 1991; Saiz et al., 1997; Campbell et al., 2001; Hirst y McKinnon, 2001). Este método es sencillo pero también implica incubaciones y solo es aplicable en hembras adultas. Además se ha observado que la tasa de producción de huevos subestima el crecimiento cuando las hembras sufren limitación por alimento (Richardson y Verheye, 1999; Rey-Rassat et al., 2002, 2004).

Todos estos métodos adolecen de un mismo inconveniente, requieren de incubaciones que conllevan gran cantidad de tiempo y son difíciles de llevar a cabo en campañas oceanográficas. Otro de los principales problemas de la estimación directa es la imposibilidad de replicar las condiciones de alimento reales en el medio marino por lo que las estimaciones del crecimiento *in vitro* pueden ser diferentes a las *in situ*. Además estos métodos se aplican frecuentemente sobre una única especie y/o estadio, potenciando errores en la estimación de la producción del zooplancton cuando se extrapolan estas tasas de crecimiento a toda la

comunidad. Estas limitaciones metodológicas impiden la medida rutinaria del crecimiento de zooplancton durante campañas oceanográficas, y por tanto, la determinación de la producción a escala oceánica.

Para cubrir las necesidades de la oceanografía biológica, en las últimas décadas el estudio de la producción zooplanctónica ha sido enfocado a la búsqueda de un método eficaz para determinar el crecimiento evitando incubaciones. En este sentido, se han desarrollado diversos métodos indirectos entre los que encontramos modelos numéricos e índices bioquímicos.

Uno de los modelos más conocidos es el publicado por Huntley y López (1992) con el cual se estima el crecimiento en función de la biomasa y la temperatura. Recientemente se han modelado las tasas de crecimiento y de producción de huevos como función de la biomasa individual, la temperatura y la clorofila *a* (Hirst y Lampitt,1998; Hirst y Bunker, 2003; Hirst et al., 2003). Otros modelos son por ejemplo el West Brown Enquist (WBE, West et al., 1997) basado en cómo se distribuyen los nutrientes y otros recursos necesarios para la vida de los organismos, o el modelo exponencial (p.e. Fox, 1970; Frost, 1972; Escribano y McLaren, 1992) basado en la estimación del crecimiento en función del tiempo y considerando el efecto tanto de factores bióticos como abióticos. Sin embargo, estos modelos tienen sus limitaciones, ya que sólo pueden aplicarse en poblaciones de copépodos que estén creciendo activamente y, además, su aplicación en poblaciones mixtas puede dar lugar a errores considerables en las estimaciones del crecimiento en el océano (Hirst y Lampitt, 1998; Runge y Roff, 2000; Hirst y Forster, 2013).

Paralelamente se ha comenzado a estudiar el crecimiento utilizando índices bioquímicos debido a las ventajas que estos tienen sobre los métodos directos. Entre las ventajas, destaca que no se necesitan incubaciones, esto permite congelar los organismos recién capturados y analizar su actividad a *posteriori* en el laboratorio. Además los análisis de

laboratorio son económicos, reproducibles y se obtienen resultados en menor tiempo que utilizando los métodos directos. Sin embargo, estos índices deben ser calibrados con medidas directas de crecimiento previamente a su aplicación en campañas oceanográficas.

El primer índice desarrollado para medir crecimiento fue la medida de ARN y ADN (Sutcliffe, 1970; Dagg y Littlepage, 1972) y se sigue usando en la actualidad con diferentes resultados. En general, la relación ARN/ADN es un pobre predictor del crecimiento (Ota y Landry, 1984; Anger y Hirche, 1990; Wagner et al., 2001). Sin embargo, algunos autores encontraron relaciones significativas en peces (Peck et al., 2003) y crustáceos (Yebra et al., 2011). Sin embargo, estos últimos autores observaron que esta relación difiere entre nauplios y copepoditos, impidiendo su uso en poblaciones mixtas.

También podemos encontrar distintos índices indirectos de crecimiento basados en la medida de la actividad de diferentes enzimas. Entre ellas, se encuentran la enzima ADN polimerasa (Sapienza y Mague, 1979) que pertenece a un grupo de enzimas relacionadas con los procesos de replicación del ADN en la célula. Se validó como índice de la tasa de crecimiento del misidáceo *Praunus flexuosus*. Sin embargo, no se ha validado para otras especies planctónicas (Runge y Roff, 2000). También, se ha utilizado la enzima aspartato transcarbamilasa (ATC, Bergeron y Buestel, 1979; Bergeron, 1990, 1993, 1995), que cataliza el primer paso de la formación de las bases pirimidínicas, constituyentes fundamentales de los ácidos nucleicos. Aunque se consideró como índice de crecimiento en crustáceos, tiene sus limitaciones ya que está estrechamente relacionado con los procesos de muda y no con el crecimiento somático (Hernández-León et al., 1995, 2002). Otra enzima, la quitobiasa es esencial en los procesos de reciclado de la quitina en los crustáceos. El exoesqueleto de los crustáceos está formado por quitina, un polímero formado por N-acetilglucosamina (Stevenson, 1985). Por ello, esta enzima está relacionada directamente con los procesos de muda que son necesarios para el crecimiento de los organismos (Oosterhuis et al., 2000;

Sastri y Roff, 2000; Sastri y Dower, 2006, 2009). Por otro lado, la enzima nucleosidil-difosfato quinasa (NDPK, Berges et al., 1990), participa en la asignación de energía en los procesos de crecimiento. Aún así, no se ha encontrado una relación entre la actividad de la enzima NDPK y el crecimiento (Berges y Ballantyne, 1991).

En la últimas décadas también se ha desarrollado el uso de la actividad de las enzimas aminoacil-ARNt sintetasas (AARS) como índice de crecimiento. Estas enzimas catalizan el primer paso de la síntesis de proteínas, es decir, la aminoacilación o activación y unión de los aminoácidos al ARN transferente (Novelli, 1967; Kisselev y Favorova, 1974; Schimmel y Söll, 1979). Por consiguiente, la actividad AARS está directamente relacionada con la síntesis de proteínas (Parker et al., 1974; Neidhart et al., 1975; Johnson et al., 1977), siendo un factor limitante de dicho proceso (Larrinoa y Heredia, 1991; SirDeshpande y Toogood, 1995). Asimismo, se han encontrado relaciones positivas entre la síntesis de proteínas y el crecimiento en metazoos marinos como cefalópodos y peces (Houlihan et al., 1990; Sveier et al., 2000). Por tanto, podemos considerar la actividad de la AARS como un buen candidato para utilizarse como índice del crecimiento en zooplancton.

Chang et al. (1984) desarrollaron un ensayo espectrofotométrico continuo no radiactivo para medir la actividad de las enzimas AARS. Este es sencillo pues la aminoacilación consume adenosín trifosfato (ATP) y libera adenosín monofosfato (AMP) y pirofosfato (PPi). Añadiendo un reactivo de pirofosfato al homogeneizado de la muestra podemos medir en continuo la actividad AARS, en base a la liberación de pirofosfato durante la aminoacilación del ARN transferente, determinada como oxidación del NADH a NAD^+ (O'Brien, 1976):

$$\text{aminoácido} + \text{ATP} + \text{ARNt} \rightarrow \text{aminoacil-ARNt} + \text{AMP} + PP_i \qquad \textbf{(ec.3)}$$

La disminución de absorbancia a 340 nm es proporcional a la actividad enzimática, que a su vez se relaciona con el crecimiento somático de los organismos. Chang et al. (1984)

realizaron las medidas de la actividad AARS añadiendo sustratos, registrando de este modo la actividad máxima potencial máxima de las enzimas. Esta actividad normalmente se estudia en condiciones de saturación de sustrato, aunque no significa que las células se encuentren así en la naturaleza (Hernández-León y Gómez, 1996; Båmstedt, 2000). Por ello, la actividad enzimática debería ser medida en base a los sustratos endógenos (sin adición de sustratos) con la finalidad de reflejar la condición fisiológica de las células *in vivo*.

Yebra y Hernández-León (2004) adaptaron el método de Chang et al. (1984) para aplicarlo en zooplancton sin adición de sustratos. De este modo, los autores analizaron la capacidad del zooplancton para sintetizar sus propias proteínas sin adición de aminoácidos y con ello evitaron la sobreestimación debida a las condiciones de saturación de sustratos. Este método se ha aplicado con éxito al estudio del crecimiento en adultos y juveniles de copépodos calanoideos (Yebra et al., 2005, 2006), en larvas de eufausiáceos (Guerra, 2006) y, recientemente, en nauplios y juveniles de copépodos ciclopoideos en el laboratorio (Yebra et al., 2011).

Teniendo en cuenta que (i) la abundancia y el crecimiento de los estadios tempranos de zooplancton e ictioplancton son elementos imprescindibles para el estudio de la producción zooplanctónica, y (ii) que esta producción es un factor determinante en la evolución y gestión de los stocks de especies de peces comercialmente explotables, es de vital importancia verificar la idoneidad de la aplicación del método AARS como un índice de crecimiento. En esta tesis, se ha estudiado la relación entre el crecimiento y la actividad AARS en nauplios del copépodo calanoideo *Paracartia grani* (**Capítulo I**) y en larvas del pequeño pez pelágico *Clupea harengus,* de interés comercial (**Capítulo II**). En estos trabajos estudiamos el efecto de la temperatura y la concentración de alimento sobre el crecimiento y la actividad AARS, y validamos este método como índice de crecimiento somático.

El siguiente paso fue la aplicación del método AARS al estudio de la comunidad

planctónica en el medio marino. En el **Capítulo III**, gracias a éste y otros índices indirectos como el índice de la fluorescencia en el tracto digestivo (GF) y la actividad del sistema de transporte de electrones (ETS), pudimos hacer un seguimiento de la población del mesozooplancton en aguas epipelágicas de Canarias e inferir los efectos que las variables ambientales ejercieron sobre el metabolismo del zooplancton durante el periodo de estudio.

Una de las principales ventajas de los métodos indirectos es, como se indicó anteriormente, la posibilidad de congelar los organismos recién capturados y analizar su actividad enzimática a *posteriori* en el laboratorio. Estos métodos nos permiten obtener una "fotografía" instantánea de su metabolismo en el momento de la captura. Con la aplicación de métodos indirectos se ha podido estudiar el metabolismo del zooplancton en aguas profundas (Seibel, 2011; Teuber et al., 2013). En esta tesis presentamos la primera aplicación del método AARS al estudio del crecimiento del zooplancton migrador en relación a una zona de mínimo de oxígeno (ZMO). En concreto, en el **Capítulo IV** se determinó el efecto de la ZMO en eufausiáceos a 400 m de profundidad en el Pacífico tropical.

Para finalizar, el principal objetivo de la investigación en los últimos años en relación a la producción del zooplancton ha sido la búsqueda de un índice estandarizado que nos permita estimar la producción zooplanctónica a escala oceánica, como se viene haciendo con el ^{14}C para la estimación de la producción primaria. En este sentido en el **Capítulo V**, ensayamos la posibilidad de encontrar una buena relación entre el crecimiento de la comunidad planctónica y su actividad AARS, para así facilitar el estudio de la evolución de las comunidades en su conjunto. Para llevar a cabo este punto, hemos aunado una revisión de todas las relaciones publicadas entre la actividad AARS y la tasa de crecimiento somático junto con las relaciones presentadas en esta tesis con el fin de buscar una ecuación global que permita inferir la tasa de crecimiento diario de las comunidades planctónicas a partir de su actividad AARS.

Objetivos de la Tesis

Según lo expuesto anteriormente, se pretenden alcanzar los siguientes objetivos:

1. Estudiar el efecto de la temperatura y de la concentración de alimento en el crecimiento de fases larvarias de zooplancton e ictioplancton (Capítulos I y II).

2. Validación en el laboratorio de la actividad AARS como índice de crecimiento en estadios larvarios de zooplancton e ictioplancton (Capítulos I y II).

3. Aplicación del método AARS, junto con otros índices metabólicos, al estudio de la comunidad zooplanctónica epipelágica en aguas de Canarias durante el bloom de invierno-primavera (Capítulo III).

4. Aplicación del método AARS, combinado con otros índices metabólicos, al estudio del efecto de la zona del mínimo de oxígeno en el Pacífico Tropical Mexicano sobre el metabolismo de eufausiáceos migradores (Capítulo IV).

5. Búsqueda de una relación global entre la actividad AARS y el crecimiento de la comunidad del zooplancton e ictioplancton (Capítulo V).

Contribuciones Científicas Originales

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Planteamiento y Metodología

Planteamiento y Metodología

Las metodologías específicas utilizadas en cada estudio de esta tesis se encuentran descritas rigurosamente en detalle en los capítulos respectivos. Los principales métodos utilizados se explican brevemente en esta sección.

Determinación de la biomasa

Para llevar a cabo esta tesis se ha determinado la biomasa como peso seco y como proteínas. El peso seco de los organismos se estimó mediante el método de Lovegrove (1966). Para determinar el peso seco de los organismos se transfiere una muestra a una microcápsula de aluminio prepesada y se mantiene en una estufa a 60ºC durante 24 horas. Transcurrido este tiempo se pesan los organismos en una microbalanza obteniendo el valor del peso seco mediante la diferencia entre las dos medidas.

La biomasa expresada como contenido en proteínas de las muestras se determinó utilizando el sobrenadante de las muestras homogeneizadas con una solución tampón (Tris-HCl, 20 mM, pH 7.8) mediante ultrasonido o varilla de teflón según fuera necesario en función del tipo de muestra. Posteriormente los homogeneizados se centrifugaron a 0 ºC durante 10 minutos a 5000 r.p.m. Para la determinación del contenido proteico en zooplancton se utilizó el método de Lowry et al. (1951) modificado para microanálisis por Rutter (1967), usando suero de albúmina bovina (BSA) como estándar. Tras centrifugar las muestras se tomó una alícuota de 0.2 mL y se añadió 1.0 mL de reactivo Rutter (formado por una solución A que contiene 20 g de Na_2CO_3 0.90M, 4 g de NaOH 0.45N, 0.2 g tartatro de Na-K 2% en 1L de agua y una solución B que contiene 0.5 g de $CuSO_4 \cdot 5\ H_2O$ en 100 mL

de agua). Pasados 10 min se añadió 0.1 mL de reactivo Folin1:1 a temperatura ambiente y en oscuridad. Transcurridos 40 min se hicieron las lecturas de absorbancia a 750 nm en un espectrofotómetro con cubetas de un centímetro de paso de luz.

Para la determinación del contenido proteico en ictioplancton se utilizó un kit de ácido bicinconínico (BCA-1, Sigma B-9643). En este método las proteínas se reducen en medio alcalino Cu (II) a Cu(I) de manera dependiente de la concentración (Lowry, 1951). El ácido bicinconínico es un agente reactivo cromogénico para Cu(I) formando un complejo púrpura con un máximo de absorbancia a 562 nm (Smith et al., 1985). Debido a esta propiedad, la absorbancia se midió a 562 nm, siendo ésta directamente proporcional a la concentración de proteína. En este método se utilizó también albúmina bovina (BSA) como estándar.

Contenido de pigmentos en el tracto digestivo

La fluorescencia del tracto digestivo (Gut Fluorescence; GF) se empleó como índice del contenido de pigmentos en el tracto digestivo del zooplancton. Para la determinación de la fluorescencia las muestras fueron homogeneizadas con una solución tampón (Tris-HCl, 20 mM, pH 7.8), a partir del homogeneizado se utilizó una alícuota de 0.2 mL para la determinación clorofila *a* más feopigmentos, siguiendo la metodología de Parsons et al. (1984) y Arar y Collins (1997). Los pigmentos fueron extraídos en 10 mL de acetona al 90% durante 24 h a -20ºC en oscuridad (Parsons et al., 1984). Transcurrido este tiempo los extractos se centrifugaron y las medidas de fluorescencia se realizaron a partir del sobrenadante en un fluorómetro, calibrado con clorofila *a* pura (C-6144, Sigma-Aldrich) según Yentsch y Menzel (1963).

Actividad del sistema de transporte de electrones (ETS)

Para determinar la actividad ETS se utilizó el método de Packard et al. (1971) modificado por Kenner y Ahmed (1975). La actividad ETS se midió tomando una muestra, la cual se homogeneizó durante 1 minuto, con una solución tampón (Tris-HCl, 20 mM, pH 7.8) mediante ultrasonido o varilla de teflón según fuera necesario, y luego se centrifugó a 0 ºC, durante 10 minutos a 5000 r.p.m. Una vez obtenido el homogeneizado, se mezclaron 0.5 mL del mismo con los sustratos (solución sustrato conteniendo 0.2% (v/v) de Tritón X-100, 50 mM de tampón fosfato sódico pH 8.0, 0.133 M de succinato disódico, 0.835 mM de NADH y 0.24 mM de NADPH) añadiéndole una solución INT 4 mM. Las muestras fueron inmediatamente incubadas en la oscuridad a 18ºC durante 20 min, tiempo tras el cual la reducción del INT se detuvo con una solución Quench formada por una parte de ácido fosfórico 0.1 M y otra de formaldehído al 36%. De igual manera se realizó un ensayo con cada muestra pero sin las coenzimas, como blanco para cada muestra. Tras el periodo de reacción se procede a la lectura de la absorbancia a 490 nm y 750 nm como línea base para corregir la turbidez.

El cálculo de la actividad ETS se realizó utilizando la siguiente ecuación:

$$\mu L\ O_2 \cdot h^{-1} = \left(\frac{60 \cdot\ V_{rm} \cdot\ COD}{1.42 \cdot 1 \cdot V_s \cdot\ T}\right) \cdot V_{hom}$$

donde, 60 = conversión de minutos a horas, V_{rm} = volumen de la reacción en mL, COD = diferencias entre absorbancias, 1.42 = absortividad milimolar ($L \cdot mmol^{-1} \cdot cm^{-1}$) del INT a 490 nm transformado a $L \cdot mL\ O_2^{-1} \cdot cm^{-1}$, 1 = longitud del paso de luz (cm) de la cubeta, V_s = volumen de la muestra en mL, T = tiempo de la reacción en min, V_{hom} = volumen del homogeneizado en mL.

Actividad aminoacil-ARNt sintetasas (AARS)

Para determinar la actividad AARS se utilizó el método de Yebra y Hernández-León (2004) modificado por Yebra et al. (2011). La actividad AARS se midió tomando una muestra, la cual se homogeneizó durante 1 minuto, con una solución tampón (Tris-HCl, 20 mM, pH 7.8) mediante ultrasonido o varilla de teflón según fuera necesario, y luego se centrifugó a 0 ºC, durante 10 minutos a 5000 r.p.m. Una vez obtenido el homogeneizado, se mezclaron 250 µL del sobrenadante con 300 µL de agua mili-Q y 200 µL de reactivo de pirofosfato (Sigma P-7275) en microcubetas de plástico PMMA y se introdujo en el espectrofotómetro para leer, durante 10 minutos, el descenso de absorbancia a 340 nm a 25 ºC. Esta absorbancia se convirtió en nmoles de PPi liberados con la ecuación:

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

donde, $dAbs \cdot min^{-1}$ = pendiente negativa de la absorbancia por minuto, 10^3 = conversión de µmol a nmol, 60 = conversión de minutos a horas, V_{rm} = volumen de la reacción (reactivo de PPi + agua + muestra) en mL, V_s = volumen de la muestra en mL, 6.22 = absortividad milimolar ($L \cdot mmol^{-1} \cdot cm^{-1}$) del NADH a 340 nm, 2 = número de moles de β-NADH oxidado por mol de PPi consumido, 1 = longitud del paso de luz (cm) de la cubeta, V_{hom} = volumen del homogeneizado en mL.

Los componentes del reactivo de pirofosfato usan el PPi liberado por las enzimas para oxidar el NADH (NADH a NAD^+), lo que se refleja en una disminución de absorbancia. Ésta es proporcional a la actividad de las enzimas aminoacil-ARNt sintetasas de los organismos.

Resultados

Resultados

CAPÍTULO I. Efecto de la temperatura y la concentración de alimento en el crecimiento y la síntesis de proteínas de los nauplios de *Paracartia grani*.

Se estudió el efecto de la temperatura y de la concentración de alimento en el crecimiento; asimismo también se validó el método enzimático AARS como índice del crecimiento en nauplios de *Paracartia grani*. Éstos se incubaron a seis temperaturas distintas (12-28ºC) en condiciones de saturación de alimento y a siete concentraciones de alimento (0-880 μg $C \cdot L^{-1}$) a 20ºC.

El crecimiento y la actividad de las enzimas AARS de los nauplios de *P. grani* dependen de la temperatura y de la concentración de alimento. La actividad AARS fue válida como índice de crecimiento somático para los nauplios de *P. grani* cuando el crecimiento no fue limitado por la disponibilidad de alimento. Sin embargo, la relación entre la actividad AARS y el crecimiento de los nauplios varió de acuerdo a las distintas concentraciones de alimento. La degradación de las proteínas durante la inanición (0 μg $C \cdot L^{-1}$) y/o la ß-oxidación de los ácidos grasos pudo afectar la relación entre la actividad específica de la AARS y al crecimiento. Los resultados obtenidos en este trabajo junto a trabajos previos muestró que la actividad AARS es una herramienta de gran utilidad para estimar el crecimiento somático de los nauplios de *P. grani* y de otras especies de copépodos.

CAPÍTULO II. Actividad Aminoacil-ARNt Sintetasas (AARS) como índice de crecimiento de larvas de arenque atlántico (*Clupea harengus*).

Se estudió el efecto de la temperatura y de la concentración de alimento en el crecimiento; asimismo también se validó el método AARS como índice de crecimiento en las etapas larvarias de arenque atlántico (*Clupea harengus*). Las larvas de arenque atlántico se incubaron a 7 y 17ºC en saturación de alimento y a 12ºC bajo tres concentraciones de alimento (0.1, 0.3 y 2 presas·mL^{-1}).

El crecimiento y la actividad de las enzimas AARS de las larvas de *Clupea harengus* dependió de la temperatura y de la concentración de alimento. Se mostraron diferencias significativas entre el crecimiento específico a las distintas temperaturas. Además el crecimiento a 12ºC mostró diferencias significativas en relación a la concentración de alimento. Se encontró un crecimiento mayor a 2 presas·mL^{-1} en relación con las otras dos concentraciones de alimento (0.1 y 0.3 presas·mL^{-1}). Asimismo, la relación positiva encontrada entre el crecimiento específico y la actividad AARS especifica mostró que este método es válido como índice de crecimiento para las larvas de arenque atlántico (*Clupea harengus*).

CAPÍTULO III. Efecto de un año cálido sobre la biomasa y metabolismo del mesozooplancton en aguas subtropicales.

La toma de muestras tuvo lugar a bordo del RV Atlantic Explorer, desde Febrero a Junio 2010 en aguas oceánicas al norte de la Isla de Gran Canaria. Los datos hidrográficos mostraron temperaturas por encima de los 19ºC durante todo el periodo de muestreo, provocando una fuerte estratificación que dio lugar a valores bajos de clorofila *a* en la zona.

El zooplancton obtenido con una red WP2 doble de 100 micras de abertura de malla, se congeló inmediatamente en nitrógeno líquido para posteriores medidas metabólicas y taxonómicas en el laboratorio. Se estimó la biomasa, como peso seco, y los índices de pastaje (GF), respiración (ETS) y crecimiento (AARS) para las distintas fracciones del mesozooplancton (100-200, 200-500, 500-1000 y > 1000 micras).

El año 2010 se caracterizó por mostrar el invierno más cálido de los últimos 30 años, lo cual originó resultados muy distintos a los encontrados previamente. El típico bloom de finales de invierno no se observó debido a las altas temperaturas y la consiguiente estratificación. Además los índices de pastaje y respiración mostraron valores menores a los observados en años anteriores. Sin embargo, se observó una alta biomasa de mesozooplancton durante el periodo de post-bloom. Esta paradoja podría explicarse por las entradas de polvo atmosférico desde el desierto del Sáhara. Estos aportes de polvo son responsables indirectamente de la elevada actividad AARS observada durante el estudio. Además, estos resultados mostraron cómo el calentamiento climático y los eventos de polvo pueden interactuar afectando directamente a la intensidad del bloom de finales de invierno en las aguas subtropicales.

CAPÍTULO IV. Procesos fisiológicos de *Euphausia distinguenda* en relación a la zona del mínimo de oxígeno en el Pacífico Oriental Tropical Mexicano.

El estudio se llevó a cabo durante la campaña oceanográfica PROCOMEX XI en noviembre 2009 en el Pacífico Oriental Tropical Mexicano a bordo del B/O El Puma. La zona de estudio se caracteriza por tener una zona de mínimo de oxígeno (ZMO) y una masa de agua que se extendió desde los 100 a los 900 metros de profundidad donde la concentración de oxígeno fue menor a 0.1 $mL \cdot L^{-1}$ debido a la poca ventilación.

La presencia de ZMOs en los océanos tropicales tiene consecuencias para la diversidad, distribución y procesos fisiológicos de los organismos marinos. Los eufausiáceos juegan un papel importante en el ciclo del carbono de los ecosistemas pelágicos. Estos organismos realizan migraciones verticales promoviendo un transporte de materia orgánica desde las aguas superficiales hacia aguas profundas. En este trabajo se estudió la relación entre los procesos fisiológicos (pastaje, respiración y crecimiento) de *Euphausia distinguenda* y la ZMO.

Se estimaron los índices de pastaje (GF), respiración (ETS) y crecimiento (AARS) así como el contenido proteico por individuo. El comportamiento de *E. distinguenda* se caracterizó por una migración vertical descendiendo y ascendiendo durante periodos día-noche, respectivamente. Las actividades específicas de ETS y AARS mostraron el mismo patrón, donde los valores más altos se encontraron durante la noche en aguas superficiales y oxigenadas mientras que los valores más bajos se hallaron durante el día en aguas más profundas y menos oxigenadas, dentro de la ZMO. Los resultados obtenidos sugieren que las zonas de mínimo de oxígeno condicionan los procesos metabólicos de los eufausiáceos.

CAPÍTULO V. Uso de la actividad Aminoacil-ARNt Sintetasas (AARS) como índice de crecimiento en zooplancton.

Se realizó una revisión de todos los datos disponibles en la literatura sobre la relación entre la actividad AARS y el crecimiento del zooplancton, y se añadieron las relaciones estudiadas en esta tesis, con el fin de buscar una ecuación global que permita inferir la tasa de crecimiento somático de las comunidades planctónicas a partir de la actividad de las enzimas AARS. Además se realizaron experimentos de laboratorio para estudiar esta relación en larvas de dorada (*Sparus aurata*), especie de gran interés comercial.

Agrupando todos los experimentos no se encontró una ecuación significativa entre actividad AARS y crecimiento. Sin embargo, cuando se dividieron los experimentos en base a la biomasa individual de los organismos, se observaron dos relaciones altamente significativas entre estas variables. Por un lado se agruparon los experimentos correspondientes a organismos con un peso mayor al 25% de su biomasa individual final. Esta ecuación incluye tanto especies de agua dulce (*Daphnia magna*) como marinas (*Calanus finmarchicus*, *Paracartia grani* y *Euphausia superba*). Una segunda correlación agrupó los experimentos realizados con organismos en sus primeros estadios del desarrollo (con un peso inferior al 25% de su biomasa individual final). Este fue principalmente el caso de los copépodos marinos *Calanus helgolandicus* y *Oithona davisae*, y de las larvas de peces *Clupea harengus* y *Sparus aurata*. En estos organismos se observaron valores de actividad AARS anormalmente altos en presencia de tasas de crecimiento bajas. Asimismo, los resultados mostraron que los valores altos de actividad AARS hallados en los estadios iniciales de diversas especies no responden a un artefacto metodológico relacionado con el metabolismo lipídico, sino que pueden deberse al desacople que existe en algunos casos entre la síntesis de proteínas (medida como actividad AARS) y su deposición como biomasa

(medido como crecimiento somático). De todos modos, el crecimiento y la actividad de las enzimas AARS están correlacionados positivamente, permitiendo el uso del método AARS como índice para la estimación del crecimiento en zooplancton e ictioplancton.

Discusión

Discusión

Los resultados incluidos en este trabajo se han discutido en detalle en sus respectivos capítulos. El propósito de esta discusión general es relacionar los principales resultados obtenidos en los diferentes estudios.

Validación del método AARS como índice de crecimiento en larvas zooplanctónicas

Los primeros estadios del zooplancton, como por ejemplo nauplios de copépodos y larvas de peces, son de gran importancia en el medio marino porque pueden influir en el éxito del reclutamiento de las especies, y en la distribución y abundancia de las poblaciones. Además, estos estadios larvarios son probablemente los metazoos más abundantes del océano (Fryer, 1986). Por ello, la primera parte de esta tesis se ha centrado en la validación del método AARS como índice de crecimiento en estos organismos.

Para el estudio de la relación entre el crecimiento de larvas planctónicas y su actividad AARS se tomaron a los nauplios del copépodo calanoideo *Paracartia grani* como organismos representativos del zooplancton invertebrado. Los copépodos calanoideos representan entre 55 y 99% del plancton en los océanos (Mauchline, 1998). De igual modo, para la validación del método de AARS como índice de crecimiento en zooplancton vertebrado se utilizaron larvas de *Clupea harengus*. Este *clupeído* es de los más capturados en aguas del *Atlántico norte* y del *Pacífico norte*, siendo de gran importancia por el papel que juegan en el ecosistema marino y en las pesquerías (Cushing, 1975).

El crecimiento y la actividad AARS específica de los nauplios de *P. grani* se relacionaron positivamente con la temperatura en un rango entre 10 y 18ºC. Las tasas de crecimiento de *P. grani* obtenidas mediante relaciones longitud-peso (**Capítulo I**) fueron similares a las encontradas previamente para esta especie (Calbet y Alcaraz, 1997) y para

otras del género *Acartia* (Berggreen et al., 1988; Leandro y Tiselius, 2006). Asimismo, el crecimiento y la actividad AARS específica de las larvas de *Clupea harengus* fueron afectados positivamente por la temperatura en un rango entre 7 y 17ºC (**Capítulo II**). Los valores de crecimiento estimados en base a relaciones talla-peso fueron similares a los hallados por otros autores para la misma especie (Suneetha et al., 1999; Folkvord et al., 2000; Johannessen et al., 2000; Arrhenius y Hansson, 1996; Kiørboe y Munk, 1986). Para ambas especies la actividad AARS estuvo correlacionada significativamente con las tasas de crecimiento, reflejando variaciones debidas a las distintas temperaturas.

Además, el crecimiento de los nauplios de copépodos y las larvas de peces también dependió de la disponibilidad de alimento. A menor concentración de alimento menor fue el crecimiento observado en los organismos. En el caso de *P. grani*, este hecho puede explicarse por una disminución en la eficiencia de la captura del alimento a bajas concentraciones, como se observó para el copépodo *Acartia tonsa* (Paffenhöfer y Stearns, 1988), sugiriendo que estarían más adaptados a ambientes con alta disponibilidad de alimento (áreas estuarinas y aguas costeras; Alcaraz, 1977; Villate, 1982). Sin embargo a altas concentraciones de alimento el crecimiento se estabilizó para los nauplios de *P. grani*, como se observó previamente en esta (Calbet y Alcaraz, 1997, *Acartia grani*) y en otras especies (Berggreen et al., 1988, *A. tonsa*). Sin embargo, el efecto del tipo de alimento en el crecimiento de los primeros estadios de los copépodos se ha estudiado muy poco. La presa utilizada en este trabajo (*Oxyrrhis marina*) se considera un alimento de alta calidad para las especies de *Acartia* (Klein Breteler y Schogt, 1994, Kleppel et al., 1998), aunque otros estudios utilicen distinto tipo de alimento (*Rhodomonas baltica* o mezclas de diatomeas *Thalassiosira weisflogii* y de criptofitas *Rhodomonas sp.)*. Todas estas modificaciones en la dieta de las especies de *Acartia* afectan al crecimiento, al desarrollo, a la producción y a la condición nutricional (Ismar et al., 2008; Teixeira et al., 2010). Sin embargo, a pesar de las diferencias

en la presa utilizada como alimento, el crecimiento de los nauplios de *P. grani* (**Capítulo I**) fue comparable a los resultados observados en nauplios de esta y otras especies. Esto sugiere que la calidad del alimento podría no afectar al crecimiento de los nauplios de esta especie en condiciones de saturación de alimento.

En el caso de las larvas de *Clupea harengus*, el crecimiento también se vio afectado directamente por la densidad de presas (**Capítulo II**). Las variaciones en el crecimiento debidas a la concentración del alimento coincide con las observaciones de Folkvord et al. (2000), y confirman la concentración de alimento como uno de los principales factores que influyen en el crecimiento de estas larvas (Kiørboe y Munk, 1986; Johannessen et al., 2000; Folkvord et al., 2000).

Asimismo, la concentración de alimento afectó directamente a la actividad AARS tanto de los nauplios de *P. grani* como de las larvas de *C. harengus*. Paradójicamente, se observaron valores mayores de actividad AARS específica cuando el crecimiento de estos organismos estaba limitado por situaciones de inanición o concentraciones muy bajas de alimento. Este aumento de la actividad AARS específica sugiere que los organismos en condiciones de inanición sobrevivieron a expensas de sus propias reservas lipídicas, y/o por la degradación de las proteínas (catabolismo proteico) (Love, 1980). Las implicaciones de la inanición sobre el metabolismo son más pronunciadas en los primeros estadios de vida que en estadios avanzados, probablemente debido a que los últimos tienen menos reservas energéticas (Gadomskia y Petersen, 1988; Richard et al., 1991). La pérdida de peso en estadios larvarios en inanición debido al coste metabólico presenta niveles mínimos de masa muscular, lo cual representa el 60-70% de su peso corporal (Machado et al., 1988). Se ha observado que la masa muscular fue la única variable que mostró diferencias significativas entre larvas alimentadas y en inanición (Martin y Wright, 1987; Ferron y Leggett, 1994; Catalán, 2003; Pliego Cortés, 2005). Esto muestra que la masa muscular es muy sensible a la

falta de alimento, debido a que la larva degrada rápidamente las proteínas del músculo (Love, 1980).

Los resultados obtenidos en los **Capítulos I y II** confirman que la actividad AARS es un método válido como índice de crecimiento en los primeros estadios de las especies estudiadas. Esto concuerda con las correlaciones significativas observadas previamente entre el crecimiento somático y la actividad AARS específica en otras especies zooplanctónicas como el cladócero *Daphnia magna* (Yebra y Hernández-León, 2004), los copépodos *Calanus helgolandicus*, *C. finmarchicus* y *Oithona davisae* (Yebra et al., 2005, 2006, 2011) o el eufausiáceo *Euphausia superba* (Guerra, 2006).

La validación de este método enzimático como índice de crecimiento en larvas zooplanctónicas facilitará en gran medida el estudio del crecimiento de estos organismos planctónicos tanto en el laboratorio como en el océano, así como de los factores que modulan la producción de las comunidades zooplanctónicas.

Uso de la actividad AARS como índice de crecimiento en el océano

La utilización de métodos indirectos permite determinar las tasas metabólicas del zooplancton a escala oceánica gracias a que no son necesarias incubaciones, permitiéndose congelar los organismos recién capturados y analizar su actividad a *posteriori* en el laboratorio. Además los análisis de laboratorio son económicos, reproducibles y se obtienen resultados en menor tiempo que utilizando métodos directos.

Ejemplos de índices indirectos ampliamente utilizados en zooplancton son la fluorescencia en el tracto digestivo (Gut Fluorescence, GF, Parsons et al., 1984) como índice de tasa de pastaje o alimentación herbívora, y la actividad del sistema de transporte de electrones (ETS, Packard et al., 1971) como índice de respiración potencial. En la segunda parte de esta tesis aplicamos estos índices junto con el método AARS para obtener una visión

lo más completa posible del metabolismo de las comunidades zooplanctónicas objeto de estudio.

Una primera aplicación de este conjunto de métodos indirectos permitió el estudio de la evolución de la producción de la comunidad zooplanctónica al norte de la isla de Gran Canaria durante el bloom de invierno-primavera en 2010. Ese año se caracterizó por ser el invierno más cálido de los últimos 30 años, obteniendo resultados muy distintos a los esperado. El típico bloom de finales de invierno no se observó debido a estas altas temperaturas. Además los índices de pastaje y respiración potencial mostraron valores menores a los observados en años anteriores. Sin embargo, una alta biomasa de la comunidad zooplanctónica se observó durante el periodo de post-bloom. Estos valores de biomasa se explican por la entrada de polvo atmosférico procedente del desierto del Sáhara, que aumentó los niveles de nutrientes en la columna de agua, aumentando la producción primaria (Franchy et al., enviado) y con ello aumentó la biomasa de la comunidad zooplanctónica. Esto también se reflejó en su actividad AARS, mostrando valores altos durante todo el estudio. Estos valores de AARS durante y después del bloom pueden deberse a las recurrentes entradas de polvo atmosférico. Dado que ésta es la primera aplicación de la actividad AARS al estudio del crecimiento del zooplancton en la zona de estudio, no poseemos información previa para contrastar con otros años menos cálidos. Sin embargo, las relaciones obtenidas en el laboratorio entre actividad AARS y temperatura sugieren que un calentamiento del medio marino tendrá marcados efectos en la producción del zooplancton.

Por otro lado, estudios previos muestran que tanto la biomasa como el crecimiento de la comunidad del zooplancton están relacionados con la iluminación lunar en las aguas oceánicas de la Corriente de Canarias (Hernández-León, 1998; Hernández-León et al., 2001, 2002, 2004, 2010), de forma similar a lo observado en lagos (Gliwicz, 1986). Los migradores verticales utilizan ciertas estrategias para evitar la depredación, cuando hay luna nueva

(periodo oscuro del ciclo lunar) estos organismos suben a la superficie y se alimentan del zooplancton no migrador, mientras que durante la luna llena (periodo iluminado del ciclo lunar), los migradores no alcanzan los 100 primeros metros de la columna de agua para evitar la depredación, permitiendo al zooplancton no migrador, crecer y aumentar su abundancia (Hernández-León, 1998; Hernández-León et al., 2001) y su biomasa (Hernández-León et al., 2002, 2004, 2010).

Las variaciones en la actividad AARS de la comunidad del zooplancton (**Capítulo III**) no mostraron claramente esta relación con el ciclo lunar, posiblemente debido al efecto de los intensos aportes de polvo sahariano. Sin embargo, antes del aumento de biomasa debido a los eventos de polvo, observamos cómo la actividad AARS mostró dos picos que coincidieron con la luna creciente, unos ocho días antes de la luna llena, como sugirió Hernández-León et al. (2010) en su modelo. La utilización del método AARS, por su sencillez, facilitará la incorporación del estudio del crecimiento en futuras series temporales de seguimiento de comunidades naturales de zooplancton, lo que permitirá profundizar en el conocimiento de la relación entre la producción del zooplancton y factores ambientales como la luz incidente en la superficie del océano, que varía por ejemplo con el ciclo lunar.

Una ventaja notable del uso de los métodos indirectos sobre los directos es que la aplicación de los primeros no se restringe a las aguas superficiales. Al no requerir incubaciones, los organismos pueden capturarse a gran profundidad y congelarse rápidamente en cubierta. De este modo conservan las propiedades características de su entorno hasta el momento de su análisis en el laboratorio.

Actualmente, debido a la posible acidificación del océano y la expansión de las zonas con valores mínimos de oxígeno (ZMO), es vital conocer cómo afectan estas zonas a los migradores verticales en el océano y cómo las concentraciones de oxígeno en el sistema marino pueden afectar al metabolismo y crecimiento del zooplancton. Sin embargo, debido a

las limitaciones de los métodos directos,no hay estudios sobre el crecimiento de los organismos migradores en relación a estas ZMO.

En esta tesis utilizamos la actividad AARS, en combinación con la fluorescencia en el tracto digestivo y la actividad ETS, para realizar el primer estudio del crecimiento de organismos planctónicos migradores en relación a la ZMO del Pacífico tropical Mexicano. La actividad AARS específica en el eufausiáceo *Euphausia distinguenda* mostró una relación similar a la encontrada para la respiración potencial (ETS). Es decir, los valores de la actividad AARS fueron mayores en superficie disminuyendo significativamente con la profundidad y con la concentración de oxígeno. Los resultados (**Capítulo IV**) sugieren que la actividad AARS de *E. distinguenda* se vio afectada por la ZMO. Las enzimas AARS catalizan el primer paso de la síntesis de proteínas. La contribución de la síntesis de proteínas al consumo de oxígeno se estudió previamente en especies de peces marinos (Houlihan, 1991; Smith y Houlihan, 1995). La actividad enzimática depende de la producción de ATP. Si la producción de ATP no es suficiente debido a la baja concentración de oxígeno, esto entorpece la síntesis de proteínas (actividad AARS) y con ello también reduce el segundo paso de la síntesis de proteínas, la traducción (Anderson et al., 2009). Este hecho coincide con la disminución de la actividad AARS observada para *E. distinguenda* en la ZMO (**Capítulo IV**). Anderson et al. (2009) sugieren que la suspensión de la traducción en condiciones de hipoxia podría inhibir o reducir la síntesis de proteínas de los organismos así como la actividad AARS. Sin embargo, cuando la concentración de oxígeno aumenta la síntesis de proteínas también aumenta sus niveles (Koumenis et al., 2002). Esto permitiría que *E. distinguenda* aumente rápidamente su actividad AARS, y por tanto su tasa de síntesis proteica, cuando realiza migraciones hacia aguas oxigenadas por encima de la ZMO. De este modo la regulación del metabolismo por parte de estos organismos sería una ventaja adaptativa para aprovechar la presencia de la ZMO en sus migraciones verticales.

Hacia una ecuación global para estimar producción zooplanctónica a partir de actividad AARS

En esta última sección ensayamos la posibilidad de encontrar una relación general entre el crecimiento de la comunidad de los organismos zooplanctónicos y su actividad AARS, para facilitar el estudio de la evolución de las comunidades en su conjunto. Hemos aunado una revisión de todas las relaciones publicadas entre la actividad AARS y la tasa de crecimiento somático y las relaciones estudiadas en esta tesis (**Capitulo V**). No se encontró una ecuación general relacionando la actividad AARS y el crecimiento somático. Sin embargo, cuando se dividieron los experimentos en base a la biomasa individual de los organismos, se observaron dos relaciones altamente significativas entre la actividad AARS y el crecimiento. Por un lado se agruparon los experimentos correspondientes a organismos con un peso mayor al 25% de su biomasa individual final. Esta ecuación incluye tanto especies de agua dulce (*Daphnia magna*) como marinas (*Calanus finmarchicus*, *Paracartia grani* y *Euphausia superba*). Una segunda correlación agrupó los experimentos realizados con organismos en sus primeros estadios del desarrollo (con un peso inferior al 25% de su biomasa individual final). Este fue principalmente el caso de los copépodos marinos *Calanus helgolandicus* y *Oithona davisae*, y de las larvas de peces *Clupea harengus* y *Sparus aurata*. En estos organismos se observaron valores de actividad AARS anormalmente altos en presencia de tasas de crecimiento bajas. Este desacople coincide con los resultados observados en nauplios de *Paracartia grani* (**Capítulo I**), larvas de *C. harengus* (**Capitulo II)** y en hembras adultas de *Acartia bifilosa* en condiciones de inanición (Holmborn et al., 2009). Es conocido que los primeros estadios del zooplancton utilizan los lípidos como fuente de energía. Por ejemplo, las larvas de peces consumen durante los primeros días de vida el saco vitelino y posteriormente comienzan a alimentarse de organismos ricos en lípidos (p.e. rotíferos, nauplios de Artemias y copépodos). Con anterioridad se postuló que el catabolismo

lipídico podría liberar PPi, y esto dar lugar erróneamente a valores altos de actividad AARS (ver Herrera et al., 2012; **Capítulos I y II**). Sin embargo, los datos presentados en el **Capítulo V** muestran como las larvas de *S. aurata* durante los primeros días después de la eclosión, aunque crecen a costa de metabolizar la grasa del saco vitelino, no presentan valores de actividad AARS tan altos como cuando comienzan a alimentarse activamente y su crecimiento depende de la ingesta de alimento y no de reservas endógenas. Esto demuestra que los valores altos de actividad AARS hallados en los estadios iniciales de diversas especies no responden a un artefacto metodológico relacionado con el metabolismo lipídico, sino que debemos buscar la respuesta en el metabolismo proteico.

El crecimiento somático es el resultado neto de la síntesis y degradación de las proteínas (Conceiçao et al., 1997). La actividad AARS mide la síntesis de proteínas y no la deposición neta de la proteína en la célula. Por tanto, una tasa elevada de degradación proteica durante las primeras fases del desarrollo en organismos planctónicos sería la causante del desacople observado entre las tasas de crecimiento y la actividad AARS. A pesar de estas limitaciones, el crecimiento y la actividad de las enzimas AARS están correlacionados positivamente, permitiendo el uso del método AARS como índice para la estimación del crecimiento en zooplancton e ictioplancton.

Conclusiones

Las principales conclusiones que se obtienen de esta tesis son:

1. El crecimiento y la actividad AARS de los nauplios de *Paracartia grani* y de las larvas de *Clupea harengus* dependen de la temperatura y de la concentración de alimento.

2. La degradación de las proteínas durante la inanición afecta la relación entre la actividad AARS y el crecimiento somático en los nauplios de *Paracartia grani* y en las larvas de *Clupea harengus*.

3. El aumento de la temperatura en las aguas de las Isla Canaria afecta a la biomasa y al crecimiento de la comunidad zooplanctónica.

4. El crecimiento y la respiración de *Euphausia distinguenda* se ven afectados por la concentración de oxígeno en la columna de agua.

5. Combinando datos de nauplios, juveniles y adultos de zooplancton, así como de larvas de peces, se obtuvieron dos correlaciones positivas entre la actividad AARS y el crecimiento somático. Estas ecuaciones generales auguran que en un futuro próximo se podría determinar la producción zooplanctónica empleando el índice AARS.

Futuras Líneas de Investigación

Partiendo de los resultados obtenidos en esta tesis hay varios aspectos concernientes a la oceanografía biológica que necesitarían ser resueltos en un futuro próximo:

- Para encontrar una ecuación general aplicable a escala oceánica es necesario realizar nuevos estudios que profundicen en el conocimiento de la relación entre crecimiento somático y actividad AARS (p.e. zooplancton en inanición).

- Además, deben realizarse validaciones del método AARS en otros grupos del zooplancton, distintos a crustáceos y larvas de peces, si pretendemos evaluar la producción de las comunidades de zooplancton usando la actividad AARS como índice de crecimiento.

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VI. References/ Referencias

(from Introduction, Discussion and Spanish Summary)

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Se acaba una etapa y comienza otra.

“Lo importante en este mundo no es donde nos encontramos, sino en que dirección vamos”.

Oliver Wendell Holmes

The use of AARS activity as a proxy for zooplankton and ichthyoplankton growth rates