The European Zoological Journal

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tizo21

Engraulis encrasicolus larvae from two different environmental spawning areas of the Central Mediterranean Sea: first data on amino acid profiles and biochemical evaluations

F. Falco , M. Barra , G. Wu , M. Dioguardi , P. Stincone , A. Cuttitta , M. Torri , A. Bonanno & M. Cammarata

To cite this article: F. Falco , M. Barra , G. Wu , M. Dioguardi , P. Stincone , A. Cuttitta , M. Torri , A. Bonanno & M. Cammarata (2020) *Engraulis encrasicolus* larvae from two different environmental spawning areas of the Central Mediterranean Sea: first data on amino acid profiles and biochemical evaluations, The European Zoological Journal, 87:1, 580-590, DOI: 10.1080/24750263.2020.1823493

To link to this article: https://doi.org/10.1080/24750263.2020.1823493

Published online: 02 Oct 2020.

The European Zoological Journal, 2020, 580–590
Vol. 87, No. 1, https://doi.org/10.1080/24750263.2020.1823493

Engraulis encrasicolus larvae from two different environmental spawning areas of the Central Mediterranean Sea: first data on amino acid profiles and biochemical evaluations

F. FALCO[1], M. BARRA[2], G. WU[3], M. DIOGUARDI[4], P. STINCONE[5], A. CUTTITTA[2], M. TORRI[2], A. BONANNO[2], & M. CAMMARATA[4]*

[1]*Marine Biological and Biotechnological Resources Institute (IRBIM), National Research Council (CNR), Italy,* [2]*Institute for the Study of Anthropic Impacts and Sustainability in the Marine Environment (IAS), Units of Capo Granitola (TP) and Naples, Italy,* [3]*Department of Animal Science, Texas A&M University, TX, USA,* [4]*Department of Earth and Marine Sciences, University of Palermo, Italy, and* [5]*Laboratório de Bioquímica e Microbiologia Aplicada, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Brazil*

(Received 17 November 2019; accepted 9 September 2020)

Abstract
Early life stages of marine fish populations may be strongly affected by environmental factors. Changes in the physical environment or the availability of food resources could lead to stress-related physiological responses affecting larval fitness, growth and survival. In the present study, we determined, for the first time, amino acid composition (AAC), lipid, and carbohydrate content, as well as alkaline phosphatase and peroxidase activities in larvae from the European anchovy *Engraulis encrasicolus*. Fishes were caught in two different spawning areas of the Strait of Sicily, characterized by different environmental conditions, including a coastal upwelling with a lower temperature (Adventure Bank; 20.22 ± 0.38°C) and a thermohaline front with a higher temperature (Maltese Bank 23.10 ± 0.25°C). The results showed that the two groups of larvae, in their early life, had similar nutritional status. However, compared with the samples from the Maltese Bank, the specimens collected in the Adventure Bank area exhibited higher alkaline phosphatase activity, lower concentrations of aspartate plus asparagine, threonine, and arginine but a higher concentration of leucine, highlighting different patterns of amino acid metabolism. Collectively, these results indicated that AAC analysis could represent an additional valid tool to evaluate the link between physiological responses and environmental conditions at early life stages.

Keywords: *Eye Amino Acid composition, habitat conditions,* Engraulis encrasicolus *larvae, enzyme, lipids, carbohydrates*

Introduction

Aquatic organisms react to changes in environmental conditions through adaptive mechanisms that allow them to cope with real or perceived environmental variations to maintain their normal physiological state. Changes in temperature, salinity, and hydrodynamic conditions beyond naturally occurring ranges as well as food limitations are stressors (i.e., conditions threatening or disturbing the dynamic equilibrium of organisms) that strongly affect metabolic and biochemical processes and induce stress-related responses (Somero & Hochachka 1976; Somero et al. 1983). In this context, the physical and chemical characteristics of the marine environment, as well as its intrinsic variability, present significant challenges to tissue proteins, whose structures and biological activities are dependent on non-covalent ("weak") chemical bonds which are readily disrupted by changes in temperature, hydrostatic pressure, and solute composition (Somero 2003). It is well known that wild fish are exposed to large and rapid alterations in environmental temperatures and, for this reason, they developed

*Correspondence: M. Cammarata, Department of Heart and Marine Science DISTEM, Marine Immunobiology Laboratory, University of Palermo, Viale delle Scienze Ed. 16, Palermo, Italy. Email: matteo.cammarata@unipa.it

a variety of physiological responses to cope with these changes (Wu et al. 2019). The stress measure should provide insight into the homeostasis and survival of fish when faced with noxious stimuli. In ectothermic organisms, physiological rates can be adjusted to compensate for some changes in temperature (Sopinka et al. 2016). In fish, thermal acclimation is generally determined by metabolic changes, during which an initial period of thermal stress is followed by gradual compensation (Faggio et al. 2014). When a stable metabolic level is achieved, the animal is considered to be fully acclimated (Maricondi-Massari et al. 1998). In previous studies, it was found that amino acids play an important role during salinity acclimation, either as energy sources or as important osmolytes for cell volume regulation. The osmotic acclimation to different environmental salinities induced changes at osmoregulatory and metabolic levels, as well as the activation of the stress system (Arjona et al. 2007, 2008). Moreover, stress indicators are now used as objective indices of the wellbeing status of fish (Iwama 2007) and of the general condition of wild populations (Madliger & Love 2014).

Amino acids are the building blocks of proteins and regulate metabolic processes in the body (Wu et al. 2013). Generally, all cells have a basal requirement for amino acids to support physiological processes such as protein synthesis (Wu 2013). Recent studies have shown that amino acids (e.g., glutamate, glutamine, and aspartate) are major metabolic fuels in the proximal intestine, liver, kidney, and skeletal muscle of fish, such as hybrid-striped bass (Jia et al. 2017) and largemouth bass (Li et al. 2020). Interestingly, the most abundant amino acids in vertebrate's eyes are glutamate (for γ-aminobutyrate synthesis; GABA) and glycine. (Massey & Miller 1988; Redburn 1998; Thoreson & Witkovsky 1999). In addition to peptide-bound amino acids, eyes also contain free amino acids, including aspartate, asparagine, glutamate, glutamine, glycine, serine, proline, homocysteine, and taurine.

According to the preliminary study of Riveiro et al. (2011), the eyes could be the best fish tissue to discriminate among species through the amino acid composition (AAC) analysis. This is because animals adapt to the different photic environments that are often exposed to the dissolved substances depending on temperature and salinity. Consequently, the resulting changes could affect eye pigments due to possible alterations in amino acid metabolism and the AAC within the opsin (Douglas et al. 1998).

The AAC is becoming an important tool in ecological studies as it is able to indicate changes and/or adaptations in organism's physiology that results from different processes such as environmental variations (Riveiro et al. 2003). Arjona et al. (2007), Arjona et al. (2008) showed how osmotic acclimation to different salinities induced changes at osmoregulatory and metabolic levels, as well as the activation of the stress system which imply higher energy requirement. This latter requirement must be fueled by nutrients and their metabolites such as glucose, lactate, or amino acids (Sangiao-Alvarellos et al. 2003; Polakof & Soengas 2008). Temperature changes produce immediate effects on eurythermal aquatic animal physiology, due to the high rate of heat exchange with water (Stevens & Sutterlin 1976) and affect the metabolism of amino acids in fish. In particular, activities of glutamate-oxaloacetate and glutamate-pyruvate transaminases are highly responsive to temperature change (Jürss 1979). A common effect of a decrease in environmental temperature is an increase in the syntheses and activities of metabolic enzymes to compensate for the effects of low thermal energy on enzyme-catalyzed reactions (Somero 2004; Chebaani et al. 2014; Cordero et al. 2016; Reyes-Becerril et al. 2011).

In fish larvae, the high amino acid content is necessary to fulfil their nutritional demand once the yolk sac is absorbed (Rayner et al. 2017). Some dietary amino acids are used for either the synthesis of proteins or other purposes such as ATP production (catabolized) and/or transamination into other amino acids (Houlihan et al. 1995).

The innate immune factors in the skin mucus of fish are affected by the ecological and physiological parameters such as salinity, pH, handling stress, developmental stage and seasonal cycle (Lebedeva 1999; Subramanian et al. 2008; Magnadottir 2010). Temperature is known as a principal factor, affecting both innate and acquired immune responses in fish (Hernández & Tort 2003; Nikoskelainen et al. 2004; Bowden et al. 2007; Pascoli et al. 2011).

In this regard, the enzymatic activities of peroxidase and alkaline phosphatase can be used as biochemical indicators of oxidative stress (Ross et al. 2000), because they correlate with metabolic rate and environmental food availability. Moreover, variations in enzymatic activities may not always be related to food availability per se, but occur as a result of changes in other environmental variables (Iger & Wendelaar Bonga 1994; Yang & Chen 2003; Dahlhoff 2004; Shi et al. 2015). Abolfathi et al. (2020) showed that activities of hydrolytic enzymes were directly correlated with thermal changes in the mucus of rainbow trout.

In the present work, we investigated the effect of different environmental regimes on the AAC and enzyme activity in *Engraulis encrasicolus* (Linnaeus 1758) larvae collected along the southern coast of

Sicily. We collected the specimens in two specific areas characterized by different environmental conditions. In the study area, Basilone et al. (2013) identified two main recurrent spawning areas for anchovy in the southern coast of Sicily: a first area over the Adventure Bank (AB; Figure 1), characterized by upwelling events and a lower temperature, as well as a second area over the Maltese Bank (MB; Figure 1), influenced by the presence of thermohaline fronts with a higher temperature (Bonanno et al. 2014). The early life stages of such species are strongly affected by environmental conditions influencing their fitness, growth, and survival.

Differences in stress levels were investigated by determining the activities of enzymes, alkaline phosphatase, and peroxidase, while the possible presence of food limiting factors was assessed by analyzing lipids and carbohydrates, representing indicators of energy reserves in the metabolic status of animals (Wang et al. 2012; Wu 2018).

Materials and methods

Description of the sampling areas

The study area (Figure 1) is located in the Sicily channel which connects the two main basins of the Mediterranean Sea. Here the surface water movement is mainly influenced by the Atlantic Ionian Stream (AIS), producing a complex mesoscale surface circulation, characterized by distinctive oceanographic features such as a coastal upwelling in the western part, cyclonic, and anti-cyclonic vortexes, and a thermohaline front located in the easternmost sector of the study area (Bonanno et al. 2006). In particular, the AIS, which is the most energetic stream of the Modified Atlantic Water (MAW), is directed eastward and, due to a topographic effect, forms two large meanders, while approaching the southern Sicilian coast over the Adventure and Maltese banks. Along its path, AIS produces a coastal upwelling that is a permanent feature in the westernmost part of the study area in summer (Bonanno et al. 2014). Conversely, when crossing the Maltese Bank, the AIS flows parallel to the thermohaline front positioned south of Cape Passero, making this sector an area characterized by a higher temperature and more stable environmental conditions than the Adventure Bank (Bonanno et al. 2014).

In order to assess differences in primary production between the two sampling sites, weekly images of surface chlorophyll concentration (CHL_{sat}, mg/m^3), during the annual 15 days period of surveys were downloaded from the "Copernicus" data portal (http://marine.copernicus.eu/). Downloaded images were temporally averaged to obtain the average CHL_{sat} values during the considered period. The differences between CHL_{sat} and the conductivity, temperature, and depth (CTD) values in each year of the study were obtained by means of spatial overlap.

Biological and environmental sampling

The sampling activities were performed during three summers (2012, 2013, and 2015) and were carried out in strict accordance with Directive 2010/63/EU of the European Parliament and of the European Council of 22 September 2010 on the protection of animals used for scientific purposes, within the work plan of the regional project MIPAF–FAO, with the approval of the Institute for Coastal and Marine Environment (IAMC), Detached Units of Capo Granitola, Naples, Italy. The general objective of BANSIC oceanographic campaign, has been the relationship between oceanography structure and spatial biology phenomena, based on the specimens of the first levels of the trophic chain. The ichtyoplankton was sampled using Bongo 40 net with a mouth diameter of 40 cm and mesh size 200 μm (Basilone et al. 2013). The maximum sampling depth was 100 m as anchovy larvae are mainly found in the upper 100 m of the water column with a higher density in the first 20 m (Palomera et al. 2007).

The fish larvae identified as anchovy were collected individually to make sure they were still alive and were euthanized with tricaine methanesulfonate, also known as MS-222, at the concentration of 200 mg/L in deionized water, with the pH of the solution being adjusted to 7.4 through the addition of sodium bicarbonate (Parisi et al. 2017). **T**he larval eyes from the dead fish were taken with a tweezers and individually collected and introduced in HCl 6 N at 114°C for 24 h for the amino acid analysis or stored in frozen at −20°C. Thus, whole body without eyes were then fixed in alcohol and the main morphological measurements as standard length (SL), head length (HL), body depth (BD), eye diameter (ED) and anal length (AL: distance from the anteriormost part to the anterior limit of the anal aperture) were taken by means of an optical microscope (Cuttitta et al. 2015).

Collected samples were preserved in buffered 70% alcoholic solution at 4°C for the analyses of AAC, lipids and carbohydrates. For the determination of total lipid content, we also used alcohol conservation solutions, and the reported values of lipid content referred to the sum of both larval body extracts and

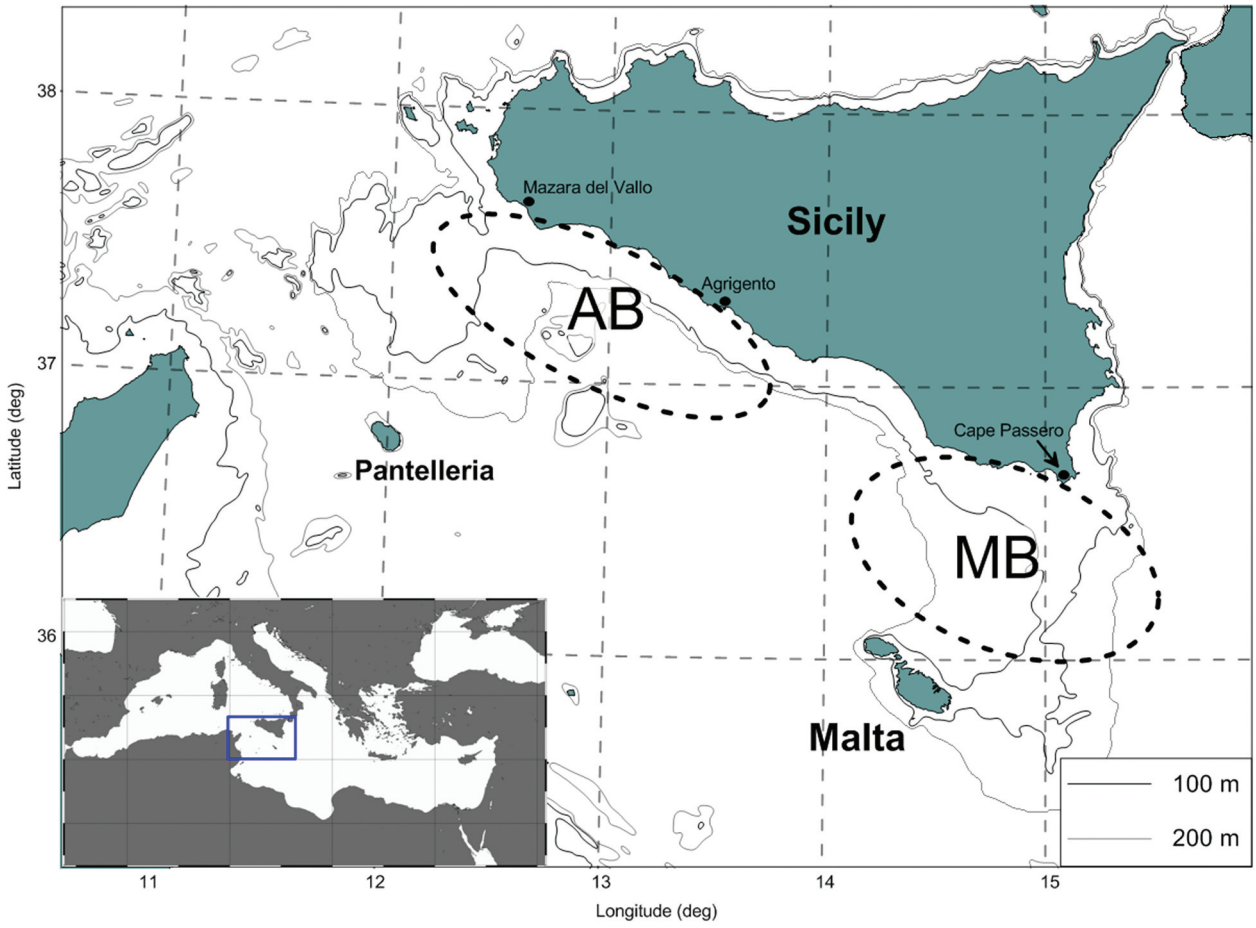

Figure 1. Study area; black and gray lines are the 100 m and 200 m isobaths. The Adventure Bank (AB) and the Maltese Bank (MB) areas were indicated by dashed ellipses.

conservation solution content. Samples for the measurement of enzyme activities were frozen at −80°C.

The SL of each specimen was also evaluated in the laboratory in order to evaluate possible differences in length frequency distribution (LFD) of larvae between the two considered sampling areas and among surveys.

Temperature and salinity data along the water column were determined by means of a Seabird CTD probe (SBE 9/11 plus). The collected downcast data were quality checked and processed according to the Mediterranean and Ocean Data Base instructions, using the SEASOFT-Win32 SBE Data Processing. The mean values of temperature and salinity were computed for the upper layer of the water column (0–20 m).

Biochemical analysis

The AAC, as well as lipid and carbohydrate content, were evaluated for each larval specimen. In particular, AAC (relative abundance, %) was evaluated in larval fish eyes (Falco et al. 2016) by means of high-performance liquid chromatography, as described by Dai et al. (2014). For the hydrolysis of protein in eyes, we used the method described by Wu et al. (1999) and Riveiro et al. (2011). The following amino acids were analyzed: aspartate plus asparagine (ASP + ASN), glutamate plus glutamine (GLU + GLN), serine (SER), glycine (GLY), histidine (HIS), arginine (ARG), threonine (THR), alanine (ALA), tyrosine (TYR), valine (VAL), lysine (LYS), isoleucine (ILE), leucine (LEU), phenylalanine (PHE), and methionine (MET). Lipids and carbohydrate as well as alkaline phosphatase and peroxidase analyses activities were determined with sub-samples of larvae. In particular, the method described by Dubois et al. (1956), involving phenolic sulphuric acid, was used to analyze the carbohydrate content, while lipid content was analyzed by using the sulfophosphovanillin method (Zollner & Kirsch 1962). In both methods, a *Shimadzu* spectrophotometer (UV- mini 1240) was

used, and wavelengths were 485 nm for carbohydrate and 530 nm for lipid analysis. As the analysis was conducted with whole-larvae samples, values were normalized to the larval weight. The enzymatic activities were determined in a pool of about 5 larval fish. Specifically, the fish were homogenized in a phosphate-buffered solution (pH 7.0), centrifuged at 12000 x g for 30 min at 4°C, and the resulting supernatant were diluted with water to obtain a similar protein concentration. The phosphatase activity was measured in a solution consisting of 30 μL of the larval extract with 170 μL p-nitrophenyl phosphate 4 mmol L^{-1} (Sigma) in ammonium bicarbonate buffer containing 1 mmol L^{-1} $MgCl_2$, pH 7.8 (Ross et al. 2000). The peroxidase activity was evaluated, according to Quade and Roth (1997) with slight modifications. In larval extracts samples were measured by using 50 μL 3,3′,5,5′ tetramethylbenzidine solution (Sigma) as substrates and 30 μL of the sample diluted with 120 μL of Hank's buffer and, after 15 min, the reaction has been stopped with 100 μL of a 0.5 M H_2SO_4. The produced yellow color was read at 450 nm. One OD arbitrary unit was defined as the amount of enzyme-producing an absorbance change of 0.01 and the activity expressed as OD unit mg-1 protein.

Statistical methods

The differences in AAC, lipids, carbohydrate, or enzymatic activity of larvae as well as the differences in the environmental conditions between the AB and MB area were statistically evaluated by *multivariate analysis* (Kruskal Wallis ANOVA test) (Kruskal & Wallis 1952). In particular, for each comparison, normality and homoscedasticity (the two basic assumptions of t-test) were evaluated by means of the Shapiro-Wilk and Levene's tests, respectively. If such assumptions were not verified, the non-parametric Mann–Whitney test was used.

Because the AA profile of larvae changes considerably during larval development (Conceição et al. 1998), in all statistical analyses we considered only the specimens belonging to a common length range (2 mm ≤ SL ≤ 12 mm). Furthermore, as the LFD showed great variability among survey and sites, AAC, lipid, and carbohydrate measurements were averaged according to survey, area and 1 mm length classes, therefore avoiding possible biases linked to differences in the LFD as well as pseudo-replication problems, typical of multistage sampling design, inflating the Type I error rate in statistical tests.

Data on enzyme activities (Table II) were statistically analyzed for a total of 29 larvae from both BM and BA and dividing in a seven pool of about 5 larval fish for each pool. The Mann–Whitney U test was used for highlight significant differences.

Results

The analysis of the environmental dataset highlighted the presence of significant differences in temperature and CHL_{sat} while no significant differences were detected in the salinity of the environmental water. In particular, a significantly ($W = 15468$, p-value < 0.01) higher temperature was recorded over the Maltese Bank, while CHL_{sat} values were significantly higher ($W = 13848$, p-value < 0.01) than those for the Adventure Bank (Table I).

The LFD highlighted differences in SL ranges, with the observed ranges being 2–20 mm in the MB area and 2–12 mm in the AB one.

Overall, the amino acid, lipid, and carbohydrate content for the larval nutritional status were evaluated in 29 specimens caught in the AB area and 63 in the MB from the years of 2012 to 2015. The results showed higher percentages of GLU+GLN, ASP +ASN (AB), LEU and LIS (MB), while lower average percentages were recorded for MET and TYR (MB) (Table II). Significant differences between the two sampling sites were found for ASP+ASN ($t = 3.2751$, $df = 33$, p-value = 0.002), THR ($W = 79$, p-value = 0.041), ARG ($t = 2.9197$, $df = 33$, p-value = 0.006) and LEU ($t = 4.7343$, $df = 33$, p-value = 4.028e-05). In particular, ASP+ASN, THR and ARG values were lower in the AB area

Table I. Basic statistics (median and Quartile Range) by area (AB: Adventure Bank; MB: Maltese Bank) related to the considered environmental parameters. Absolute differences between the median values in the two areas are reported in the last row.

	Temperature (°C)		Salinity (PSU)		CHLsat (mg/m^3)	
Area	Median	QR	Median	QR	Median	QR
AB	20.22	18.8–21.2	37.9	37.8–38.1	0.08	0.06
MB	23.1	22.1–24.4	38	37.7–38.2	0.05	0.04
Median difference	2.88		−0.1		−0.07	

Table II. Basic statistics (Minimum, Mean, Maximum and Standard Deviation) of AAC, lipids, carbohydrates and enzymes in analyzed samples. The AAc is expressed as percentage (%) of the total analyzed amino acids in the acid hydrolysates of larvae, and carbohydrates and lipid content as μg/ml of tissue homogenate. Values for alkaline phosphatase and peroxidase activities are expressed as **OD unit**/mg protein. Asterisk beside the biochemical and amino acids indicate statistically significant differences ($P < 0.05$).

	Adventure Bank		Maltese Bank	
	Mean	Std. Dev.	Mean	Std. Dev.
ASP+ASN*	11.19	3.58	9.77	3.28
GLU+GLN*	13.08	3.28	12.27	3.33
SER	5.70	2.63	5.26	2.58
HIS	3.84	2.08	3.98	2.41
GLY*	6.38	2.41	5.39	2.35
THR	5.06	1.67	5.03	1.83
ARG*	8.74	2.17	7.98	2.05
ALA	7.20	3.54	7.29	3.74
TYR	3.04	0.95	3.16	1.05
MET*	2.69	5.51	3.41	7.38
VAL	5.81	1.78	5.80	2.26
PHE	6.63	1.46	6.34	1.37
ILE	5.62	1.20	5.91	1.48
LEU*	8.76	2.67	10.01	2.72
LYS*	8.15	4.57	9.80	5.23
Carbohydrate	25.26	30.31	18.91	13.29
Lipid	12.07	7.18	15.78	14.38
Alkaline Phosphatase*	34.65	32.90	20.62	27.56
Peroxidase	6.66	5.70	9.00	8.40

than those in the MB area (Figure 2). On the contrary, LEU was higher in the AB area than in the MB area (Figure 2).

Lipids and carbohydrates of larvae (Table II) did not show significant differences between the two sampling areas.

There were significant differences (t = 4.29, df = 12, p-value = 0.001) in alkaline phosphatase activity between the AB and MB areas (Figure 2), while no significant difference was detected in peroxidase activity between the two sampling areas.

Discussion

The adaptation phenomenon that leads to the development of complex eyes, like those of mammals and teleost fish, results from different factors, such as the influence of specific environmental changes on the biochemical composition of the tissue structure of a living (Nissling & Vallin 1996; Guisande et al. 1998). Thus, changes in the AAC and nutrient content of the eyes may be useful indicators of the responses of aquatic animals to alterations in the environment (Riveiro et al. 2000, 2003).

Animals adapt to their habitat environments to survive when faced with specific environmental pressures. Importantly, changes in AAC and the density patterns of pelagic and mesopelagic fish larvae may provide evidence for the oceanographic phenomenon in different areas of the Central Mediterranean Sea. The animals attempt to adapt to their habitats for survival in response to specific environmental pressures. We proposed that the AAC, nutrient content, and the density pattern of pelagic and mesopelagic fish (Cuttitta et al. 2006; Bonanno et al. 2013) are also affected by the oceanographic phenomenon in the Sicily channel. Our study investigates the effect of environmental-related stressors on the physiology of anchovy larvae through the AAC analysis. Although the AAC in the eyes of pelagic fishes is species-specific (Riveiro et al. 2011; Falco et al. 2016), differences in some AAs were detected between larvae from the AB and MB areas. Moreover, aquatic animals of species respond consistently to climate changes, i.e., a specie is adapted equally well as other species to different environmental conditions (Garland & Kelly 2006). Nonetheless, intra-species variations as a result of environmental changes were observed in the present study in agreement with other studies (Guisande et al. 1998; Riveiro et al. 2000, 2003). Among the considered environmental factors that are able to induce physiological stress, no significant differences were observed in the salinity of the water, while significant differences were recorded for the temperature and CHLsat. The latter factors are reported as the environmental parameters that characterize the AB and differentiate the area from the MB, probably due to the upwelling events as reported by (Bonanno et al. 2014). Interestingly, lipid or carbohydrate content in the larvae did not differ between the individuals collected from the AB and MB areas. Because of our limited analysis of nutrient metabolism in the larvae, the present study does not permit us to exclude a possible effect of the primary production (CHL) on the observed AAC variation between the two areas. However, the observed differences in the anchovy larvae AAC may be considered to be induced by the different temperature regimes of the two groups of larvae. The influences of the water temperature on most of the biochemical and physiological processes make it the most important factor for the survival of organisms inhabiting the aquatic environment (Reynolds & Casterlin 1979). Due to the indirect observations, more data are necessary for a definitive conclusion. Early life stages of marine fish are particularly sensitive to environmental stressors, due to

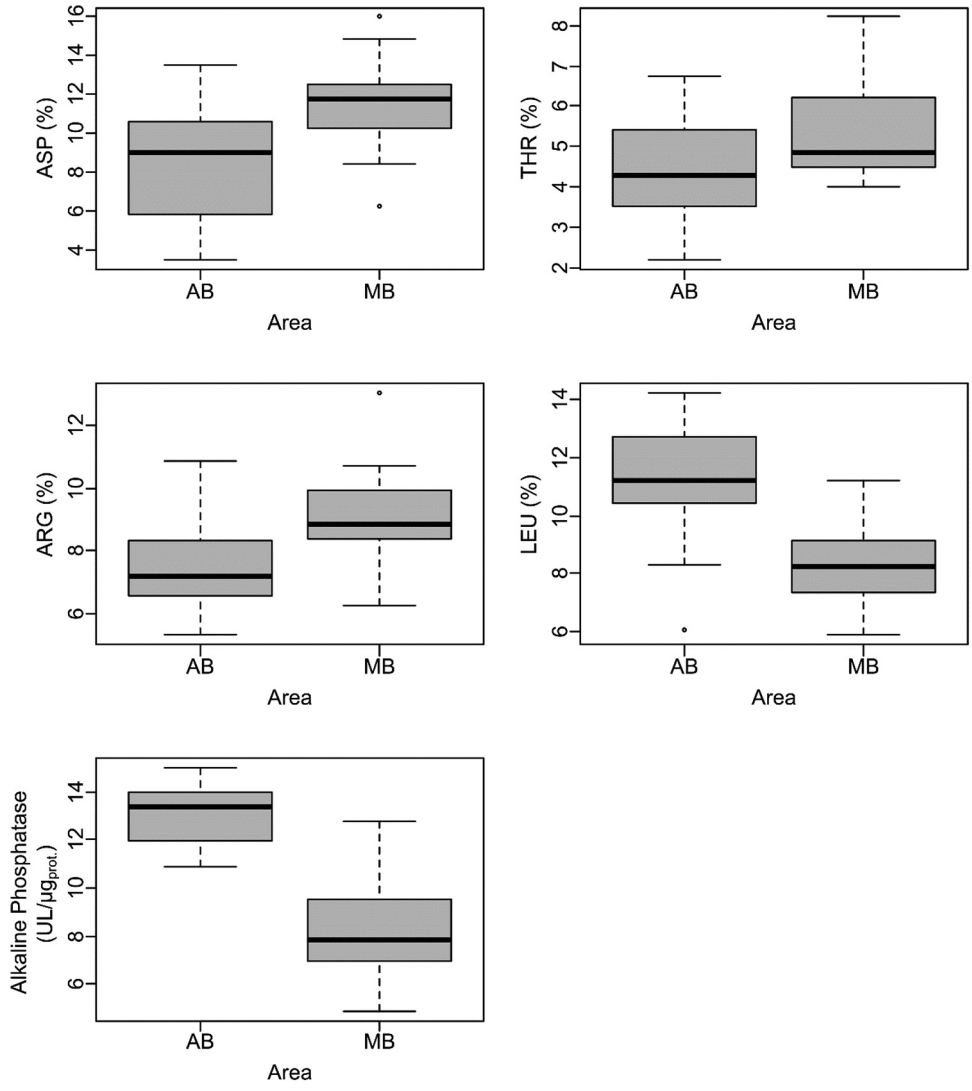

Figure 2. Boxplot of eyes AAs and larval whole-body enzymes found significantly different between the two areas.

either the lack or low functional capacity of some organs and to the high metabolic rates needed for ATP production as well as growth and development (Pimentel et al. 2015). The higher activity of alkaline phosphatase in the larvae from the AB area than those from the MB area suggests that specimens from the AB area are exposed to a higher level of stress. In agreement with this view, alkaline phosphatase was used as a stress biomarker in the skin mucus of fish (Ross et al. 2000). Moreover, changes in the alkaline phosphatase activity of the rod cells have been demonstrated in common carp (*Cyprinus carpio*) (Iger 1994) and rainbow trout in response to various stress conditions, such as the acidity, heavy metals, temperature, and pollution of water in rivers (Iger & Abraham 1997). Furthermore, results by Abolfathi et al. (2020) revealed that the activity of alkaline phosphatase was significantly decreased with increasing water temperature from winter to spring in their experimental groups. Alterations in enzyme activities are of central importance in the mechanism of adaptations to cold environments (Hochachka & Somero 1984) to compensate for the effects of low body temperature. Alkaline phosphatase is an important enzyme in metabolic processes in animals, including fish (Ram & Sathayanesan 1985), and plays a key role in the digestion, absorption, and transition of nutrients (Swarup 1981; Wu 2018). The observed increase in the enzymatic activity of alkaline phosphatase in the larvae from the AB area (Figure 2) is consistent with the finding of Das and Majhi (2015) that the activities of metabolic enzymes in fish are increased in response to a low environmental temperature.

Moreover, an increase in alkaline phosphatase activity in larval fish may indicate an increase in the synthesis of enzymes in response to cold stress (Yang & Chen 2003; Hassaan et al. 2019). According to Shi et al. (2015), in short-term cold stress, the profiles of enzymes activities such as alanine transaminase, aspartate transaminase and lactate dehydrogenase are substantially elevated in Nile tilapia.

A novel and important observation from the present study is that the AAC of the larvae differed between the AB and MB areas. Specifically, ASP+ASN, THR, and ARG showed lower concentrations in larvae collected in the AB area, while the opposite was true for LEU. The carbon skeletons of both THR and ARG are not synthesized by animal cells and these amino acids must be provided from the diet (Wu 2013; Wu et al. 2013). However, there was no difference in the lipid and carbohydrate content of the larvae between the AB and MB areas. In tissues of fish, lipids and carbohydrates are only minor metabolic fuels, as reported for hybrid striped bass (Jia et al. 2017) and largemouth bass (Li et al. 2020). Thus, our data should be interpreted to reflect the differences in the utilization of macronutrients in fish and further underscore the important role of amino acids in the survival adaptation of fish larvae to environmental changes. This notion is consistent with the suggestion that metabolic activity in fish can alter in response to energy demands and thermal stress (Wendelaar Bonga 1997; Portz et al. 2006). In addition, the utilization of food and performance of the fish decline in response to a high environmental temperature (Katersky & Carter 2005) because their metabolic and physiological functions are coordinated to maintain thermal equilibrium (Elliot 1981; Goldspink 1995; Beitinger et al. 2000), leading to an increase in intracellular proteolysis and a decrease in the intracellular synthesis of proteins (Carter et al. 2006). ARG is an indispensable AA for fish, which serves as a building block of tissue proteins and participates in several metabolic pathways, including nitric oxide, polyamine and creatine syntheses (Wu 2013). These metabolites are essential for cell growth and survival, as well as immune responses and reproduction (Wu et al. 2009). Aragao et al. (2008) found that the Senegalese sole living under stressful conditions have higher amino acid requirements for the synthesis of stress-related proteins. This could agree with our observation of the lower ARG concentration in the AB larvae. On the contrary, ASP is a major energy substrate in fish (Li et al. 2020), and this view is in agreement with Somero (2004).

LEU can stimulate muscle growth (Li et al. 2009; Wu 2009) and was found to be more abundant in the larvae from the AB area. We surmise that LEU is preserved possibly as a result of decreased catabolism, so that this functional amino acid (Wu 2013) can regulate intracellular protein synthesis (Ballantyne 2001; Wu et al. 2013). Future experiments are warranted to test this hypothesis and understand the mechanisms responsible for stress-related physiological responses to environmental stressors. In those new studies, the analysis of the AAC may provide insight into the physiological adaptation to environmentally related stressors. During the last decades, in order to develop effective management plans, considerable efforts have been made to understand the link between environmental conditions, spatial distribution, and population dynamics in some economically important fish species. Assessing the link between physiological and environmental conditions represents an important step to better define the relationship between environmental complexity and population dynamics.

Contributions

Conceptualization: MC, FF, AC, GW, Data curation: MB, PS, MT. Investigation and Formal analysis: MD, FF, PS, MB. Writing: MC, FF. Review and editing: FF, MB, GW, MD, PS, AC, MT, AB, MC.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Abolfathi M, Akbarzadeh A, Hajimoradloo A, Joshaghani HR. 2020. Seasonal changes of hydrolytic enzyme activities in the skin mucus of rainbow trout, *Oncorhynchus mykiss* at different body sizes. Developmental and Comparative Immunology 103:103499. DOI: 10.1016/j.dci.2019.103499.

Aragao C, Corte-Real J, Costas B, Dinis MT, Conceição LEC. 2008. Stress response and changes in amino acid requirements in Senegalese sole (*Solea senegalensis*Kaup 1858). Amino Acids 34:143–148. DOI: 10.1007/s00726-007-0495-2.

Arjona FJ, Vargas-Chacoff L, Martín Del Río MP, Flik G, Mancera JM, Klaren PHM. 2008. The involvement of thyroid hormones and cortisol in the osmotic acclimation of *Solea senegalensis*. General and Comparative Endocrinology 155:796–803. DOI: 10.1016/j.ygcen.2007.09.007.

Arjona FJ, Vargas-Chacoff L, Ruiz-Jarabo I, Martin Del Rio MP, Mancera J. 2007. Osmoregulatory response of Senegalese sole (*Solea senegalensis*) to changes in environmental salinity. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 148:413–421. DOI: 10.1016/j.cbpa.2007.05.026.

Ballantyne JS. 2001. Amino acids metabolism. In: Wright PA, Anderson PM, editors. Nitrogen excretion. Vol. 20 Fish physiology. San Diego: Academic Press. pp. pp 77–107.

Basilone G, Bonanno A, Patti B, Mazzola S, Barra M, Cuttitta A, McBride R. 2013. Spawning site selection by European anchovy (*Engraulis encrasicolus*) in relation to oceanographic conditions in the Strait of Sicily. Fisheries Oceanography 22:309–323. DOI: 10.1111/fog.12024.

Beitinger TL, Bennett WA, McCauley RW. 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. Environmental Biology of Fishes 58:237–257. DOI: 10.1023/A:1007676325825.

Bonanno A, Goncharov S, Mazzola S, Popov S, Cuttitta A, Patti B, Basilone G, Di Nieri A, Patti C, Aronica S, Buscaino G. 2006. Acoustic evaluation of anchovy larvae distribution in relation to oceanography in the Cape Passero area (Strait of Sicily). Chemistry and Ecology 22:S265–S273. DOI: 10.1080/02757540600670307.

Bonanno A, Placenti F, Basilone G, Mifsud R, Genovese S, Patti B, Di Bitetto M, Aronica S, Barra M, Giacalone G, Ferreri R, Fontana I, Buscaino G, Tranchida G, Quinci E, Mazzola S. 2014. Variability of water mass properties in the Strait of Sicily in summer period of 1998–2013. Ocean Science 10:759–770. DOI: 10.5194/os-10-759-2014.

Bonanno A, Zgozi S, Cuttitta A, El Turki A, Di Nieri A, Ghmati H, Basilone G, Aronica S, Hamza M, Barra M, Genovese S, Falco F, Knittweis L, Mifsud R, Patti B, Bahri T, Giacalone G, Fontana I, Tranchida G, Mazzola S. 2013. Influence of environmental variability on anchovy early life stages (Engraulis encrasicolus) in two different areas of the Central Mediterranean Sea. Hydrobiologia 701:273–287. https://link.springer.com/article/10.1007/s10750-012-1285–8

Bowden TJ, Thompson KD, Morgan AL, Gratacap RML, Nikoskelainen S. 2007. Seasonal variation and the immune response: A fish perspective. Fish & Shellfish Immunology 22:695–706. DOI: 10.1016/j.fsi.2006.08.016.

Carter CG, Bridle AR, Ketersky RS, Barnes JC, Hauler RC. 2006. Influence of nutrition and thermal stress on protein synthesis and degradation fish. In Proceeding of International Society for Fish Nutritional and Feeding. pp. 57. Biarritz, France.

Chebaani N, Guardiola FA, Sihem M, Nabil A, Oumouna M, Meseguer J, Esteban MA, Cuesta A. 2014. Innate humoral immune parameters in Tilapia zillii under acute stress by low temperature and crowding. Fish Physiology and Biochemistry 40:797–804. DOI: 10.1007/s10695-013-9886-3.

Conceição LEC, Ozório ROA, Suurd EA, Verreth JAJ. 1998. Amino acid profiles and amino acid utilization in larval African catfish (*Clarias gariepinus*): Effects of ontogeny and temperature. Fish Physiology and Biochemistry 19:43–58. DOI: 10.1023/A:1007738228055.

Cordero H, Cuesta A, Meseguer J, Esteban MÁ. 2016. Changes in the levels of humoral immune activities after storage of gilthead seabream (*Sparus aurata*) skin mucus. Fish & Shellfish Immunology 58:500–507. DOI: 10.1016/j.fsi.2016.09.059.

Cuttitta A, Guisande C, Riveiro I, Maneiro I, Patti B, Vergara A R, Basilone G, Bonanno A, Mazzola, S. 2006. Factors structuring reproductive habitat suitability of Engraulis encrasicolus in the south coast of Sicily. Journal of fish biology 68 (1): 264–275.

Cuttitta A, Patti B, Maggio T, Quinci EM, Pappalardo AM, Ferrito V, De Pinto V, Torri M, Falco F, Nicosia A, Musco M, Armeri GM, Placenti F, Tranchida G, Mifsud R, Bonanno A, Mazzola S. 2015. Larval population structure of *Engraulis encrasicolus* in the Strait of Sicily as revealed by morphometric and genetic analysis. Fisheries Oceanography 24:135–149. DOI: 10.1111/fog.12098.

Dahlhoff EP. 2004. Biochemical indicators of stress and metabolism: Applications for marine ecological studies. Annual Review of Physiology 66:183–207. DOI: 10.1146/annurev.physiol.66.032102.114509.

Dai ZL, Wu ZL, Jia SC, Wu G. 2014. Analysis of amino acid composition in proteins of animal tissues and foods as pre-column *o*-phthaldialdehyde derivatives by HPLC with fluorescence detection. Journal of Chromatography B 964:116–127. DOI: 10.1016/j.jchromb.2014.03.025.

Das SK, Majhi SK. 2015. Low water temperature induces stress and affects somatic growth in teleost *Channa stewartii* (Perciformes). Acquaculture Research 46:3088–3092. DOI: 10.1111/are.12448.

Douglas RH, Partridge JC, Marshall NJ. 1998. The eyes of deep-sea fish I: Lens pigmentation, tapeta and visual pigments. Progress in Retinal and Eye Research 17:597–636. DOI: 10.1016/S1350-9462(98)00002-0.

Dubois M, Gilles KA, Hamilton JK, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350–356. DOI: 10.1021/ac60111a017.

Elliot J. 1981. Some aspects of thermal stress on freshwater teleosts. In: AD Pickering editor. Stress and fish. London: Academic Press. pp. 209–245.

Faggio C, Piccione G, Marafioti S, Arfuso F, Fortino G, Fazio F. 2014. Metabolic response to monthly variations of Sparus aurata reared in Mediterranean on-shore tanks. Turkish Journal of Fisheries and Aquatic Sciences 14:567–574.

Falco F, Barra M, Cammarata M, Cuttitta A, Jia S, Bonanno A, Mazzola S, Wu G. 2016. Amino acid composition in eyes from zebrafish (*Danio rerio*) and sardine (*Sardina pilchardus*) at the larval stage. SpringerPlus 5:519. DOI: 10.1186/s40064-016-2137-1.

Garland T, Kelly SA. 2006. Phenotypic plasticity and experimental evolution. Journal of Evolutionary Biology 209:2344–2361.

Goldspink G. 1995. Adaptation of fish to different environmental temperature by qualitative and quantitative changes in gene expression. Journal of Thermal Biology 20:167–174. DOI: 10.1016/0306-4565(94)00045-K.

Guisande C, Riveiro I, Sola A, Valdes L. 1998. Effect of biotic and abiotic factors in the biochemical composition of wild eggs and larvae of several fish species. Marine Ecology Progress Series 163:53–61. DOI: 10.3354/meps163053.

Hassaan MS, Nagar AGE, Salim HS, Fitzsimmons K, El-Haroun ER. 2019. Nutritional mitigation of winter thermal stress in *Nile tilapia* by propolis-extract: Associated indicators of nutritional status, physiological responses and transcriptional response of delta-9-desaturase gene. Aquaculture 511:734256. DOI: 10.1016/j.aquaculture.2019.734256.

Hernández A, Tort L. 2003. Annual variation of complement, lysozyme and haemagglutinin levels in serum of the gilthead sea bream *Sparus aurata*. Fish & Shellfish Immunology 15:479–481. DOI: 10.1016/S1050-4648(03)00024-X.

Hochachka PW, Somero GN. 1984. Biochemical Adaptation. Princeton: Princeton University Press. pp. 145–181.

Houlihan DF, Carter CG, McCarthy ID. 1995. Protein synthesis in fish. In: Hochachka P, Mommsen T, editors. Fish molecular biology and biochemistry. Vol. 4. Amsterdam: Elsevier Press. pp. 191–220.

Iger Y, Abraham M. 1997. Rodlet cells in the epidermis of fish exposed to stressors. Tissue & Cell 29:431–438. DOI: 10.1016/S0040-8166(97)80029-8.

Iger Y, Wendelaar Bonga SE. 1994. Cellular responses of the skin of carp (*Cyprinus carpio*) exposed to acidified water. Cell and Tissue Research 275:481–492. DOI: 10.1007/BF00318817.

Iwama GK. 2007. The welfare of fish. Diseases of Aquatic Organisms 75:155–158. DOI: 10.3354/dao075155.

Jia SC, Li XY, Zheng SX, Wu G. 2017. Amino acids are major energy substrates for tissues of hybrid striped bass and zebrafish. Amino Acids 49:2053–2063. DOI: 10.1007/s00726-017-2481-7.

Jürss K. 1979. Effects of temperature, salinity, and feeding on aminotransferase activity in the liver and whit muscle of rainbow trout (*Salmo Gairdner* Richardson). Comparative Biochemistry and Physiology, Part B 64:213–218. DOI: 10.1016/0305-0491(79)90163-9.

Katersky R, Carter CG. 2005. Growth efficiency of juvenile barramundi, *Lates calcarifer*, at high temperatures. Aquaculture 250:775–780. DOI: 10.1016/j.aquaculture.2005.05.008.

Kruskal WH, Wallis WA. 1952. Use of ranks in one criterion variance analysis. Journal of the American Statistical Association 47:583–621. DOI: 10.1080/01621459.1952.10483441.

Lebedeva NY. 1999. Skin and superficial mucus of fish: biochemical structure and functional role. In: Saksena DN editor. Ichthyology: recent research advances, pp. 177–193. Enfield, NH: Science Publishers.

Li P, Mai KS, Trushenski J, Wu G. 2009. New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquafeeds. Amino Acids 37:43–53. DOI: 10.1007/s00726-008-0171-1.

Li XL, Zheng SX, Wu G. 2020. Nutrition and metabolism of glutamate and glutamine in fish. Amino Acids 52:671–691. DOI: 10.1007/s00726-020-02851-2.

Madliger CL, Love OP. 2014. The need for a predictive, context-dependent approach to the application of stress hormones in conservation. Conservation Biology 28:283–287. DOI: 10.1111/cobi.12185.

Magnadottir B. 2010. Immunological control of fish diseases. Marine Biotechnology 12:361–379.

Maricondi-Massari M, Kalinin AL, Glass ML, Rantin FT. 1998. The effects of temperature on oxygen uptake, gill ventilation and ECG waveforms in the Nile tilapia, *Orechromis niloticus*. Journal of Thermal Biology 23:283–290. DOI: 10.1016/S0306-4565(98)00019-9.

Massey SC, Miller RF. 1988. Glutamate receptors of ganglion cells in the rabbit retina: Evidence for glutamate as a bipolar cell transmitter. The Journal of Physiology 405:635–655. DOI: 10.1113/jphysiol.1988.sp017353.

Nikoskelainen S, Bylund G, Lilius E-M. 2004. Effect of environmental temperature on rainbow trout (Oncorhynchus mykiss) innate immunity. Developmental and Comparative Immunology 28:581–592. DOI: 10.1016/j.dci.2003.10.003.

Nissling A, Vallin L. 1996. The ability of Baltic cod eggs to maintain neutral buoyancy and the opportunity for survival in fluctuating conditions in the Baltic Sea. Journal of Fish Biology 48:217–227. DOI: 10.1111/j.1095-8649.1996.tb01114.x.

Palomera I, Olivar MP, Salat J, Sabatés A, Coll M, García A, Morales-Nin B. 2007. Small pelagic fish in the NW Mediterranean Sea: An ecological review. Progress in Oceanography 74:377–396. DOI: 10.1016/j.pocean.2007.04.012.

Parisi MG, Mauro M, Sarà G, Cammarata M. 2017. Temperature increases, hypoxia, and changes in food availability affect immunological biomarkers in the marine mussel *Mytilus galloprovincialis*. Journal of Comparative Physiology B 187:1117–1126. DOI: 10.1007/s00360-017-1089-2.

Pascoli F, Lanzano GS, Negrato E, Poltronieri C, Trocino A, Radaelli G, Bertotto D. 2011. Seasonal effects on hematological and innate immune parameters in sea bass *Dicentrarchus labrax*. Fish & Shellfish Immunology 31:1081–1087. DOI: 10.1016/j.fsi.2011.09.014.

Pimentel MS, Faleiro F, Diniz M, Machado J, Pousão-Ferreira P, Peck MA, Pörtner HO, Rosa R. 2015. Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean. Plos One 10:0134082.

Polakof S, Soengas JL. 2008. Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout. Journal of Experimental Biology 211:1075–1086. DOI: 10.1242/jeb.014050.

Portz DE, Woodley CM, Cech JJ. 2006. Stress-associated impacts of short-term holding of fishes. Reviews in Fish Biology and Fisheries 16:125–170.

Quade MJ, Roth JA. 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology 58:239–248. DOI: 10.1016/S0165-2427(97)00048-2.

Ram R, Sathayanesan AG. 1985. Mercuric chloride, cythion and ammonium sulfate induced changes in the brain, liver and ovarian alkaline phosphatase content in the fish *Channa puntactus*. Environmental Ecology 3:263–268.

Rayner TA, Hwang JS, Hansen BW. 2017. Anticipating the free amino acid concentrations in newly hatched pelagic fish larvae based on recently fertilized eggs and temperature. Journal of Plankton Research 39:1012–1019. DOI: 10.1093/plankt/fbx058.

Redburn DA. 1998. Neurotransmitter systems in the outer plexiform layer of mammalian retina. Neuroscience Research. Supplement 8:127–S136. DOI: 10.1016/0921-8696(88)90012-6.

Reyes-Becerril M, Tovar-Ramírez D, Ascencio-Valle F, Civera-Cerecedo R, Gracia-López V, Barbosa-Solomieu V, Esteban MÁ. 2011. Effects of dietary supplementation with probiotic live yeast *Debaryomyces hansenii* on the immune and antioxidant systems of leopard grouper *Mycteroperca rosacea* infected with *Aeromonas hydrophila*. Aquaculture Research 42 (11):1676–1686. DOI: 10.1111/j.1365-2109.2010.02762.x.

Reynolds WW, Casterlin ME. 1979. Behavioral Thermoregulation and the "Final Preferendum" Paradigm. American Zoologist 19:211–224. DOI: 10.1093/icb/19.1.211.

Riveiro I, Guisande C, Franco C, Lago Do Lanzós A, Maneiro I, Vergara AR. 2003. Egg and larval amino acid composition as indicators of niche resource partitioning in pelagic fish species. Marine Ecology Progress Series 260:255–262. DOI: 10.3354/meps260255.

Riveiro I, Guisande C, Iglesias P, Basilone G, Cuttitta A, Giráldez A, Patti B, Mazzola S, Bonanno A, Vergara AR, Maneiro I. 2011. Identification of subpopulations in pelagic marine fish species using amino acid composition. Hydrobiologia 670:189–199. DOI: 10.1007/s10750-011-0663-y.

Riveiro I, Guisande C, Lloves M, Maneiro I, Cabanas JM. 2000. Importance of parental effects on larval survival in *Sardina pilchardus*. Marine Ecology Progress Series 205:249–258. DOI: 10.3354/meps205249.

Ross NW, Firth KJ, Wang A, Burka JF, Johnson SC. 2000. Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Diseases of Aquatic Organisms 41:43–51. DOI: 10.3354/dao041043.

Sangiao-Alvarellos S, Laiz-Carrion R, Guzman JM, Martin Del Rio MP, Miguez JM, Mancera JM, Soengas JL. 2003. Acclimation of *Sparus aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 285:897–907. DOI: 10.1152/ajpregu.00161.2003.

Shi G-C, Dong X-H, Chen G, Tan B-P, Yang Q-H, Chi S-Y, Liu H-Y. 2015. Physiological responses and HSP 70 m RNA expression of GIFT strain of N ile tilapia (*Oreochromis*

niloticus) under cold stress. Aquaculture Research 46:658–668. DOI: 10.1111/are.12212.

Somero G. 2004. Adaptation of enzymes to temperature: Searching for basic "strategies". Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 139:321–333. DOI: 10.1016/j.cbpc.2004.05.003.

Somero GN. 2003. Protein adaptations to temperature and pressure: Complementary roles of adaptive changes in amino acid sequence and internal milieu. Comparative Biochemistry and Physiology. Part B: Biochemistry & Molecular Biology 136:577–591. DOI: 10.1016/S1096-4959(03)00215-X.

Somero GN, Hochachka PW. 1976. Biochemical adaptations to temperature. In: R.C. Newell, editor. Adaptation to environment: Essays on the physiology of marine animals. London: Butterworths. pp. 125–190.

Somero GN, Siebenhaller JF, Hochachka PW. 1983. Biochemical and physiological adaptations of deep-sea animals. The Sea. Rowe GT.

Sopinka NM, Donaldson MR, O'Connor CM, Suski CD, Cooke SJ. 2016. Stress indicators in fish. Fish Physiology 35:405–462.

Stevens ED, Sutterlin AM. 1976. Heat transfer between fish and ambient water. Journal of Experimental Biology 65:131–145.

Subramanian S, Ross NW, MacKinnon SL. 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comparative Biochemistry and Physiology. Part B: Biochemistry & Molecular Biology 150:85–92. DOI: 10.1016/j.cbpb.2008.01.011.

Swarup G. 1981. Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatases. Journal of Biological Chemistry 256:8197–8201.

Thoreson WB, Witkovsky P. 1999. Glutamate receptors and circuits in the vertebrate retina. Progress in Retinal and Eye Research 18:765–810. DOI: 10.1016/S1350-9462(98)00031-7.

Wang J, Wu Z, Li D, Li N, Dindot SV, Satterfield MC, Bazer FW, Wu G. 2012. Nutrition, epigenetics, and metabolic syndrome. Antioxidant and Redox Signaling 17:282–301.

Wendelaar Bonga SE. 1997. The stress response in fish. Physiological Reviews 7:591–625. DOI: 10.1152/physrev.1997.77.3.591.

Wu F, Yang C, Wen H, Zhang C, Jiang M, Liu W, Lu X. 2019. Improving low temperature stress tolerance of tilapia, *Oreochromis niloticus*: A functional analysis of *Astragalus membranaceus*. Journal of the World Aquaculture Society 50:749–762. DOI: 10.1111/jwas.12586.

Wu G. 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids. 37(1):1–17. DOI: 10.1007/s00726-009-0269-0

Wu G. 2013. Amino acids: Biochemistry and nutrition. Boca Raton, Florida: CRC Press.

Wu G. 2018. Principles of animal nutrition. Boca Raton, Florida: CRC Press.

Wu G, Ott TL, Knabe DA, Bazer FW. 1999. Amino acid composition of the fetal pig. The Journal of Nutrition 129:1031–1038. DOI: 10.1093/jn/129.5.1031.

Wu G, Wu Z, Dai Z, Yang Y, Wang W, Liu C, Wang B, Wang J, Yin Y. 2013. Dietary requirements of "nutritionally nonessential amino acids" by animals and humans. Amino Acids 44:1107–1113. DOI: 10.1007/s00726-012-1444-2.

Wu GY, Bazer FW, Davis TA, Kim SW, Li P, Rhoads JM, Satterfield MC, Smith SB, Spencer TE, Yin YL. 2009. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168. DOI: 10.1007/s00726-008-0210-y.

Yang J-L, Chen H-C. 2003. Effects of gallium on common carp (*Cyprinus carpio*): Acute test, serum biochemistry, and erythrocyte morphology. Chemosphere 53:877–882. DOI: 10.1016/S0045-6535(03)00657-X.

Zollner N, Kirsch K. 1962. Über die quantitative Bestimmung von Lipoiden (Mikromethode) mittels der vielennatürlichen Lipoiden (allenbekannten Plasmalipoiden) gemeinsamen Sulfophosphovanillin-Reaktion. Zeitschriftfür Die Gesamte Experimentelle Medizin 135:545–561. DOI: 10.1007/BF02045455.