Doctoral Thesis

First evidences of the effect of polyunsaturated fatty acids in fish neural activity and their implications in behaviour

Tibiabin Benítez Santana
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GRUPO DE INVESTIGACIÓN EN ACUICULTURA

First evidences of the effect of polyunsaturated fatty acids in fish neural activity and their implications in behaviour

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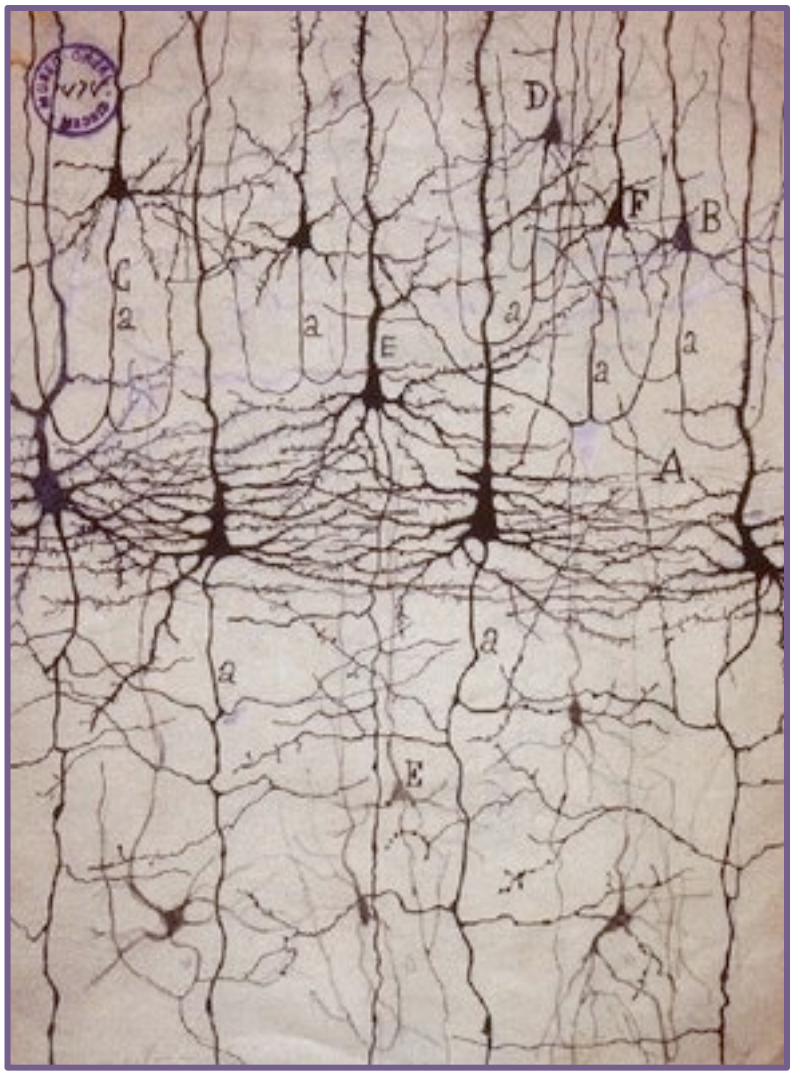
Directors:

Prof. Marisol Izquierdo & Prof. Reiji Masuda

A mi padre, Pepe AB.



“As long as our brain is a mystery, the universe, the reflection of the structure of the brain will also be a mystery”

Ramón y Cajal

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Este trabajo va dedicado a mi familia y en especial a mi padre. Él fue quien me animó a sumergirme en el mundo de la investigación y quien me enseñó que la perseverancia y el esfuerzo son el camino a seguir para lograr todo en esta vida. De todo corazón les agradezco a mi familia el apoyo mostrado durante estos años y sobre todo por su paciencia, comprensión y solidaridad con este proyecto. Sin su apoyo este trabajo nunca se habría escrito y, por ello, este trabajo es también el suyo.

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Un trabajo de investigación es siempre fruto de ideas, proyectos y esfuerzos previos que corresponden a otras personas. En este caso mi más sincero

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A todos, muchas gracias.

"La vida no es la que uno vivió, sino la que uno recuerda, y cómo la recuerda para contarla". Gabriel García Márquez (Vivir para contarla).

Con cariño,
Tibi.

Summary

It is well known that seed capture from the wild stocks is not a sustainable strategy for marine fish intensive aquaculture, being these practices restricted to other marine organisms as certain molluscs. In this sense, one of the main bottlenecks for the further development of marine fish aquaculture is the production of a good quality seed in adequate quantities. Larvae from most marine fish species are not fully developed when they hatch and certain structures in visual, immune, digestive, skeleton or nervous systems are yet incomplete in comparison to the adult. Thus, marine fish larvae are very sensitive to stress and disease and mortality rates often reach over 80 percentage of the population during this period. In this sense, knowledge on fish welfare is determinant for the success of larval rearing. The behaviour represents the reaction to the environment as fish perceives it and it is a key element of fish welfare. Description of the normal pattern of behaviour in fish larvae may constitute a powerful tool to study larval development, since delays in the appearance of those patterns or deviations from the typical conduct in certain individuals or patches of larvae may constitute an effective non-invasive indicator of health, development and maturity. However, only very few studies had been focused on different aspects of behaviour along fish larvae development before this thesis started and the factors affecting this behaviour. Certain types of behaviour are essential for larval survival, such as the escape response, which seems to strongly rely on the proper development of central nervous system. In turn, normal development of the brain could be affected by dietary deficiencies in certain nutrients, such as n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), since these fatty acids, in particular docosahexaenoic acid (DHA), are accumulated in neurons and play an important role in membrane functioning and fluidity regulation. A pair of large, identifiable neurons in the hindbrain, the Mauthner cells (M-cells), initiates the escape

response, also known as the fast-start or startle response. Therefore, this thesis tested for the first time in science the hypothesis that deficiencies in n-3 LCPUFA may affect fish larvae behaviour, central neural system content in fatty acids and neuron functioning.

The results showed the first evidences of the effect of dietary LCPUFA on fish escape behaviour and its relation to the central nervous system. Considering that LCPUFA are indispensable for the normal development of the central nervous system, the importance of welfare of all farmed animals including fish, and the little information available in this field in teleosteans, the main objective of this thesis was to determine the effect of LCPUFA action in the M-cells activity and their implications in the behaviour in different species of fishes. For this purpose, diets with different fatty acids profiles were designed and tested. In order to determine the larva response to different stimulus (visual and sonorous), several behavioural parameters were studied including cruise swimming speed, burst swimming speed, percentage response rate, peak angle speed and time to peak bend angle. The nutritional properties of LCPUFA on M-cells were investigated in sea bream (*Sparus aurata*), sea bass *(Dicentrarchus labrax*) and zebrafish (*Danio rerio*).

The first part of this Thesis showed the specific effect of dietary essential fatty acids contents in live preys on gilthead sea bream larvae behaviour. The goal of this study was to determine the relation between behavioural responses to different stimulus along the larval gilthead sea bream development and these dietary nutrients (Study 1). Once this effect was demonstrated, the importance of LCPUFA on behaviour was further investigated applying, in one hand, specific microdiets to study larvae at a higher developmental stage and, in the other hand, newly developed techniques to study the implication of M-cells activity. Therefore, the aim of Study 2 was to better understand the effect of dietary n-3 LCPUFA on fish escaping behaviour and neural function in larval gilthead sea bream. In view of the enhanced escaping response in marine fish larvae fed increased n-3 LCPUFA in previous chapters, the purpose of the Study 3 was to determine the specific

effect of each n-3 LCPUFA, comparing the efficacy of different dietary docosahexaenoic and eicosapentaenoic acid contents in escaping behaviour and neuron activity of gilthead sea bream larvae. The next step was to determine on a freshwater species, the zebrafish, the effect of dietary n-3 LCPUFA levels on M-cells functioning in relation to escaping behaviour and neural function (Study 4). The last part was aimed to further investigate on M-cells, conducting a histological study of M-cells ontogeny using different antibodies (Chapter 5). Thus, this study goal was to describe the presence of calretinin and parvabumin in the M-cells of European sea bass larvae along ontogeny using immunohistochemical and immunofluorescence tecniques and adapting to marine fish the method used by other author in fresh water fish.

The results obtained in this Thesis showed for the first time the importance of n-3 LCPUFA, especially docosahexaenoic acid, for the normal function of M-cells and correct escaping behaviour in fish larvae.

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List of abbreviations

ARA	Arachidonic acid (20:4n-6)
CNS	Central nervous system
CR	Calretinin
DAH	Day after hatching
DHA	Docosahexaenoic acid (22:6n-3)
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (20:5n-3)
FO	Fish oil
H&E	Hematoxilin and eosin
HUFA	Highly unsaturated fatty acids
LA	Linoleic acid (18: 2n-6)
LNA	Linolenic acid (18:3n-3)
LSO	Linseed oil
LCPUFA	Long chain polyunsaturated fatty acids
M-cells	Mauthner cells
PBS	Phosphate bufferd saline
PUFA	Polyunsaturated fatty acids (equivalent to HUFA)
PV	Parvalbumin
RSO	Rapseed oil
SBO	Soybean oil
SL	Standar lenght

1. Introduction

1.1 Significance of fish lipid nutrition in larviculture

Aquaculture has a long history of more than 4,000 years despite its significant contribution to global human food production is only very recent. It is one of the most rapid developing animal production sectors in the world, growing at a rate higher than 5% per year during the last decade (Izquierdo, 2005). Specifically, the contribution of world-wide farm production of fish, crustaceans and molluscs has increased from 3.9% of total fishing production in 1970 to 43% in 2008 (68,329280 million metric tons), being expected to reach 50% of total fishing production by the year 2025 (FAO, 2008) (Figure 1.1). According to FAO previsions, aquaculture is a feasible complement to wild captures fisheries to satisfy the high demand of marine products. One of the main challenges for the further development of aquaculture is to meet the demand on mass production of high quality fry (Izquierdo and Fernandez-Palacios, 1997). In marine fish species, larval stages are particularly sensitive, and sudden and uncontrolled mortalities or reduced growth constrain the further development of production of those species (Watanabe *et al.*, 1983; Yúfera and Pascual, 1984; Sargent *et al.* 1997; Izquierdo *et al.*, 2000). Larval rearing success is mostly affected by first feeding regimes and the nutritional quality of starter diets (Izquierdo *et al.*, 2000). Among the nutritional requirements of larvae, lipids are recognized as one of the most important nutrients affecting growth and survival of fish larvae (Watanabe *et al.*, 1983). The use of dietary lipids by the larvae, directly or indirectly, is affected by a sequence of morphological and physiological changes that occur during larval development. Thus, to ensure a better growth and a high survival rate, a reliable diet that meet the nutritional requirements of larvae, both qualitatively and quantitatively is essential (Kolkovski *et al.*, 1993; Sargent *et al.*, 1997). In the last years, more attention has been paid to study the importance of dietary lipids in marine fish larvae (Izquierdo *et al.*, 2003), since they

are essential for fish growth and development (Watanabe 1982; Sargent *et al.*, 1999a).

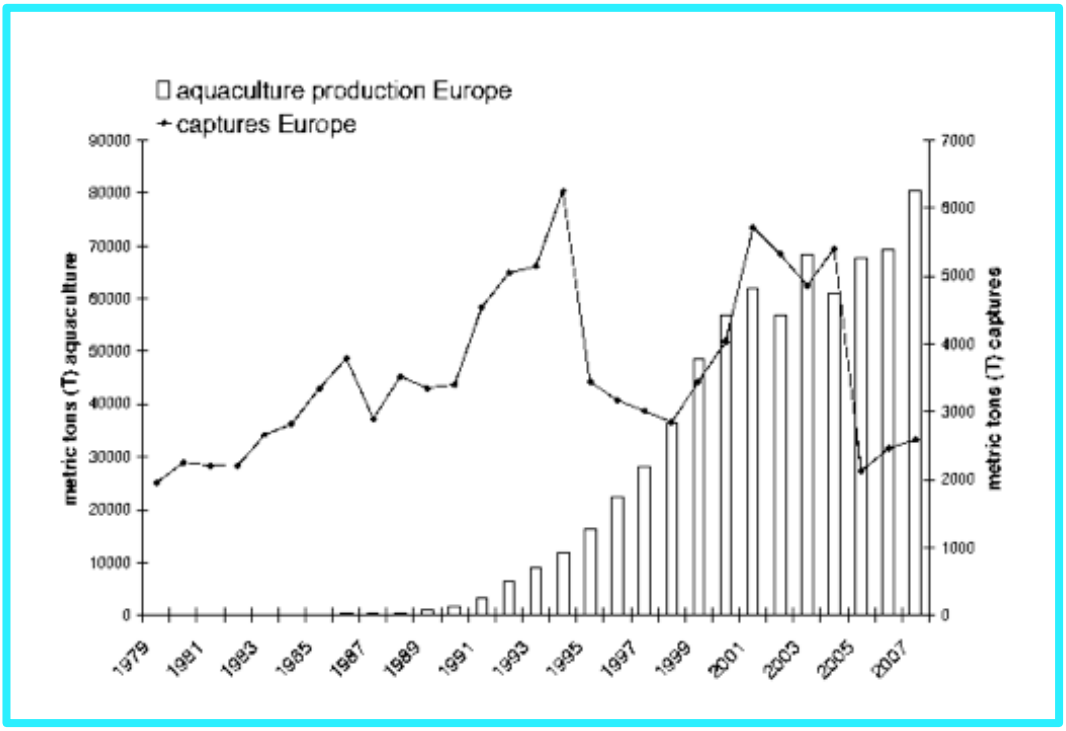


Figure 1.1 Aquaculture production *versus* captures of gilthead sea bream in Europe from 1979 to 2007 (data elaborated from FAO FISHSTAT Plus).

Lipids are a group of organic compounds that are insoluble in water, but soluble in organic solvents. Many lipids, namely those saponifiable, contain fatty acids, hydrocarbon chains with a carboxylic group at one end and a terminal methyl group at the other (n or ω carbon). The acyl chain may be saturated or unsaturated. The fatty acids commonly found in biological tissues possess an even-numbered carbon chain of between 12 and 24 atoms and 0 to 6 methylene-interrupted double bonds or unsaturations with double bonds. Being a heterogeneous and wide group of compounds, different classifications of lipids are currently used. For instance, according to their degree of polarity, they are known as polar or neutral lipids. The latter include free fatty acids and their derivative glycerolipids, sterols, waxes and tocopherols, among others. Polar lipids include glycerophospholipids (currently named as phospholipids), glycoglycerolipids, sphingomyelin and glycosphingolipids.

Dietary lipids provide a rich source of energy and phospholipids that are essential components of biomembranes. Dietary lipids also serve as carriers for the absorption of other nutrients, including the fat-soluble vitamins A, D, E, and K, and natural or synthetic pigments. Moreover, dietary lipids are recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe *et al.*, 1983), since they are the source of essential materials for the normal formation of cell and tissue membranes and organ development (Izquierdo *et al.*, 1989b, 2003; Pousão-Ferreira *et al.*, 2003). In recent years there has been an increasing interest in all these aspects of lipid nutrition in fish larvae, due to the importance of dietary lipid utilization for optimal larval growth and survival (Izquierdo *et al.*, 2000).

1. 2 Essential fatty acids and their role in neural function

Marine organisms, especially algae, can contain a plethora of polyunsaturated fatty acids (PUFA) of chain length C16 (with two to four double bonds), C18 (with two to five double bonds), C20 (with two to five double bonds), and C22 (with two to six double bonds) (Sargent *et al.,* 1995). These PUFA are predominantly of the *n*-3 series, although representatives of the *n*-6 and, in the case of C16 PUFA, the *n*-1 series also occur. However, in fresh water fish PUFA from *n*-6 family are also very important, including 20:4*n*-6 (arachidonic acid; ARA) and its metabolic precursor 18:2*n*-6 (linoleic acid; LA), together with 20:5*n*-3 (eicosapentaenoic acid; EPA) and

22:6*n*-3 (docosahexaenoic acid; DHA) and their metabolic precursor 18:3*n*-3 (linolenic acid; LNA). Freshwater fish posses Δ6 and Δ5 desaturase and elongase activities to produce ARA, EPA and DHA if their precursors are present in the diet (Figure 1.2). Such enzymatic activity is very limited in marine fish and, as a consequence, those long chain fatty acids have to be included in the diet and are considered essential (Sargent *et al.*, 1995; Izquierdo, 1996). Thus, PUFA are obtained by fish through the diet and incorporated selectively in the body tissues. However, not only optimum levels of fatty acids must be provided, but also the different fatty acids at the required ratios, which are species specific, as well as, dependent on the life stage and metabolic conditions (i.e. smoltification) (Sargent and Tacon, 1999). Recently (Izquierdo *et al.*, 2008), it has been shown that dietary lipids are able to regulate Δ6-desaturase expression in gilthead sea bream (*Sparus aurata*), although the ability of this fish to synthesise DHA was not enough to fulfil sea bream requirements for this fatty acid. Another term in common usage in the aquaculture field is LCPUFA, the abbreviation for long chain polyunsaturated fatty acids. LCPUFA, such as DHA, EPA and ARA, are those PUFA of 20 and 22 carbon atoms in their aliphatic chain with 3 or more unsaturations, which are essential since they play very important functions in marine fish species (Izquierdo, 2005), although they are poorly synthetised by these species. In general, the main roles of fatty acids in fish larvae are consistent with those in juveniles and adults. Thus, they function as (1) a source of metabolic energy, (2) structural components in the phospholipids of cellular membranes and (3) precursors of bioactive molecules (Sargent *et al.*, 1999a; Tocher *et al.*, 2003).

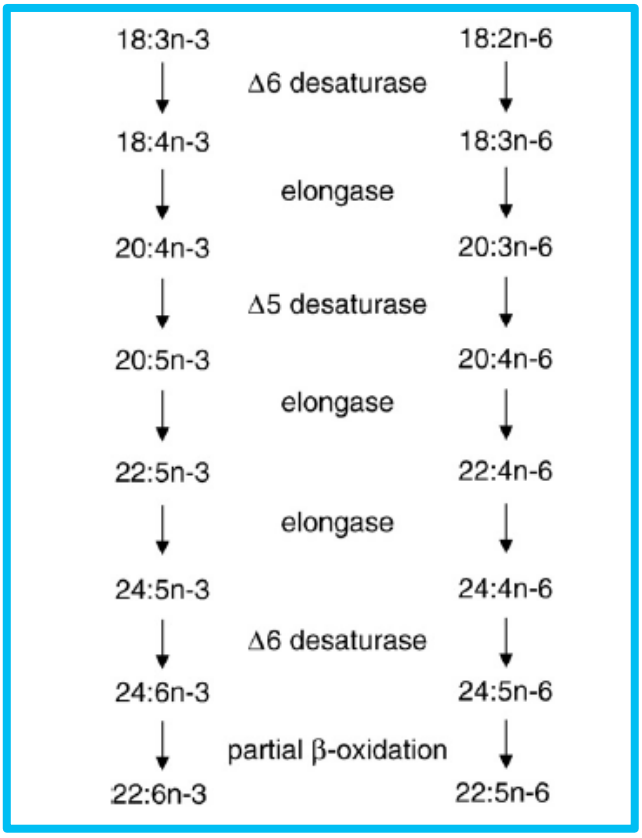


Figure 1.2 Biosynthesis pathways of long-chain polyunsaturated fatty acids from C18 precursors, 18:3n-3 and 18:2n-6.

Fatty acids, as cell membrane constituents, are present in all life forms. Since cell membranes are responsible for receiving information and anticipating a response to any factor that perturbs the intracellular organization, changes in their fatty acid composition will directly affect cell function. Thus, the role of a fatty acid is not just structural or energetic, but also functional. Fatty acids are involved in several processes such as the control of membrane permeability, membrane plasticity, cell division, hormone formation and immune response. Inadequate contents of those dietary essential fatty acids (EFA) give rise to several alterations such as poor feeding and swimming activities, poor growth and dropping mortality, fatty livers, abnormal pigmentation, disaggregation of gill epithelia, immune-deficiency and raised basal cortisol levels (Izquierdo, 1996).

Marine fish larvae appear to possess higher specific requirements for DHA than juveniles and pre-adults. This fatty acid is incorporated in the developing visual and neural tissues (Mourente, 2003), which, at this stage, account for a higher proportion of body weight relatively to other fully developed fish. DHA is necessary for the normal development of nervous system and sensory organs, larval eye and brain fatty acid composition clearly reflecting that of the diet (Navarro *et al.*, 1995; Benítez-Santana *et al.*, 2007). Despite variations in the dietary level of such fatty acids would markedly affect behaviour, few studies have been conducted to elucidate the effect of EFA on larval behaviour. DHA deficiency in the diet is related to impaired ability of herring larvae to capture prey at natural light intensities (Bell *et al.*, 1995), retarded development of normal behaviour of yellowtail (Masuda *et al.*, 1998) and gilthead sea bream (Benítez-Santana *et al.*, 2007), increasing incidence of skeletal deformities (Cahu *et al.*, 2003; Izquierdo and Koven, 2010), immunosuppression (Montero *et al.*, 2004), malpigmentation and impaired eye migration in flatfish (Mc Evoy *et al.*, 1998; Bell *et al.*, 2003). DHA and EPA are competitively incorporated into cell membranes; however, the former is superior as an essential fatty acid for growth performance and stress resistance in red sea bream among other species (Watanabe *et al.*, 1989; Watanabe and Kiron, 1994).

Dietary *n*-3 LCPUFAs have a great relevance in brain health. In mammals, it has been established that they are critical for proper infant growth and neurodevelopment (Marszalek and Lodish, 2005). DHA is highly enriched in neural membranes, constituting approximately 30–40% of the phospholipids of the gray matter of cerebral cortex and photoreceptor cells in the retina (Innis, 1991; Lauritzen *et al.*, 2001). Thus DHA constitutes >17% by weight of the total fatty acids in the brain of adult rats and >33% of the total fatty acids in the retina (Hamano *et al.*, 1996). Among subcellular fractions of brain tissue, the highest levels of DHA are found in synaptosomal membranes, synaptic vesicles, and growth cones (Scott and Bazan, 1989). The amount of DHA in the brain increases dramatically before birth and during the brain growth spurt, due to both of the growth in brain size and the increase in the relative DHA contents (Lauritzen *et al.*, 2001).

Throughout its growth, the brain tissue utilizes large amounts of DHA for the biosynthesis of rapidly expanding neural cell plasma membranes. Because neurons lack the enzymatic activity necessary for de novo DHA synthesis (Martin *et al.*, 2003), these fatty acids are derived either directly from the diet or are mainly synthesized from the dietary n-3 precursors in liver from where they are transported to the brain tissue.

Among the *n*-3 LCPUFAs, DHA is the most important fatty acid with physiological significance for brain function (Bourre, 2004; Marszalek and Lodish, 2005), especially during prenatal brain development when it is incorporated into nerve growth cones during synaptogenesis (Martin and Bazan, 1992) (Figure 1.3). The human brain growth spurt that takes place from the third trimester of pregnancy until 18 months post birth correlates with DHA accretion into brain phospholipids (Lauritzen *et al.*, 2001). Insufficient dietary supply of *n*-3 LCPUFAs during pre- and postnatal development decreases the levels of DHA in neural tissue with a reciprocal increase of docosapentanoic acid (DPA, 22:5n6) (Schiefermeier and Yavin, 2002), leading to behavioural deficits in animal models (Moriguchi *et al.*, 2000; Lim *et al.*, 2005). Accordingly, dietary DHA supplementation in breastfeeding has been shown to improve mental development in human children (Willatts *et al.*, 1998; Birch *et al.*, 2000; Hibbeln *et al.*, 2007).

The membrane phospholipids of neural cells naturally contain large amounts of PUFAs of the n-6 and n-3 families, principally AA and DHA. Experimental studies on rats have shown that a lack of dietary n-3 PUFA severely disrupts the PUFA composition in the nerve membrane phospholipids by dramatically reducing the amount of DHA while increasing that of n-6 fatty acids (Aïd *et al.*, 2003). Such reduction in brain tissues DHA is associated with impaired attention and learning abilities (Reisbick and Neuringer 1997; Moriguchi *et al.*, 2000; Salem *et al.*, 2001), in relation to disruptions of serotoninergic and dopaminergic neurotransmission (Zimmer *et al.*, 1998, 2002; Takeuchi *et al.*, 2002; Kodas *et al.*, 2004). More recently, Aïd *et al.* (2003) demonstrated that a chronic lack of dietary n-3 PUFA leads to changes in the release of acetylcholine in the rat hippocampus. Together,

these data demonstrated that the brain is highly sensitive to a lack of PUFAs during maturation and that a chronically unbalanced early environment produced by deficient nutrition could lead to durable changes in cerebral function (Lauritzen *et al.*, 2001).

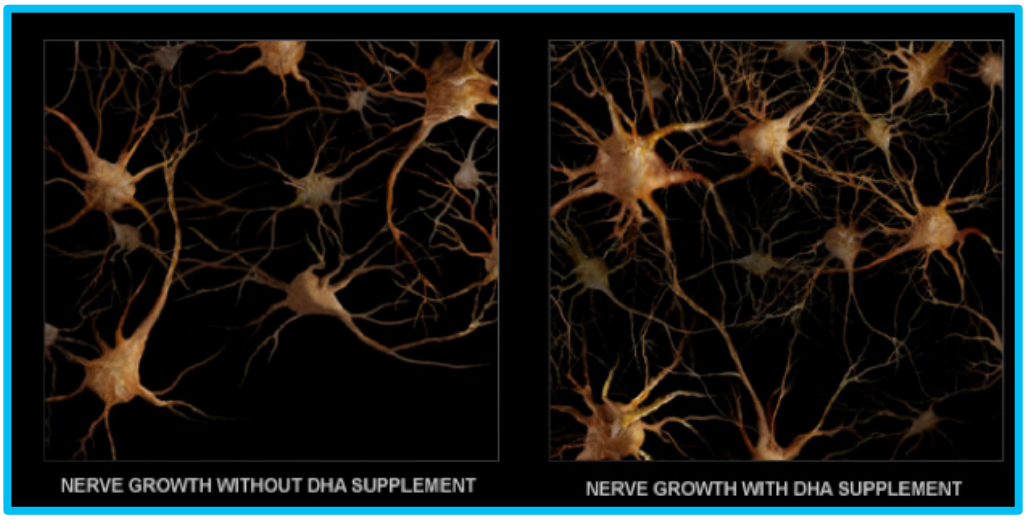


Figure 1.3 Nerve growth without and with DHA supplementation (http://www.thevisualmd.com/health_centers/child_health/infant_nutrition/dha_ara).

Other reports also implicate PUFAs in a number of seemingly unrelated neuropathologic conditions including depression, schizophrenia, hyperactivity disorder, and autism. In these patients, the plasma and erythrocytes membranes contain subnormal amounts of *n*-3 and *n*-6 PUFAs (Glen *et al.*, 1994; Edwards *et al.*, 1998; Maes *et al.*, 1999; Burgess *et al.*, 2000; Yao *et al.*, 2000; Vancassel *et al.*, 2001). Furthermore, intervention trials suggest that DHA, as well as its direct metabolic precursor, EPA, can be beneficial for treating some of these psychiatric diseases (Mellor *et al.*, 1996; Emsley *et al.*, 2002; Nemets *et al.*, 2002; Peet and Horrobin, 2002; Richardson and Puri, 2002).

The brain is functionally and anatomically lateralized, with the hemispheres being specialized differently for cognitive and motor control. Deviations from the normal patterns of asymmetry appear to be related to cognitive behavioural deficits,

and anomalies in brain asymmetry have been widely documented in psychiatric disorders, such as schizophrenia, autism, and depression (Cowell *et al.*, 1999; Petty, 1999). Studies in rodents indicate that behavioural lateralization reflects inter hemispheric asymmetries involving complex genetic and environmental factors that are established during the prenatal and postnatal development of the brain (Glick and Cox, 1978; Carlson and Glick, 1996; Nielsen *et al.*, 1997). Early experiences and variables, such as prenatal stress and maternal ingestion of specific nutrients, cause long-lasting changes in the behavioural lateralization of the offspring (Rodríguez *et al.*, 1994; Alonso et al., 1997). In addition, differences in brain laterality appear to determine susceptibility to depression-like behaviours in rats exposed to stressors that cannot be controlled (Carlson and Glick, 1991; Alonso *et al.*, 1997). These data suggest that behavioural laterality is a labile process that can be modified by environmental stimuli, and these studies on n-3 PUFA deficiency and behavioural impairments led us to assume that a change in dietary n-3 PUFA supply from conception throughout life can lead to changes in specific lateralized behaviour by affecting structural or neuro chemical patterns of asymmetry in motor related brain structures, with potential consequences for cognition (Vancassel *et al.*, 2005).

In fish, inadequate dietary EFA levels also result in poor feeding and swimming activities (Izquierdo, 1996) and altered fish larvae behaviour (Benítez-Santana *et al.*, 2007). Moreover, EFA deficient feeds delay the appearance of response to visual stimulus, in agreement with the reduction in DHA content in eyes and brains of these larvae and suggesting a delay in the functional development of brain and vision (Benítez-Santana *et al.*, 2007). In agreement with these results, reduction in DHA and EPA contents, reduces gilthead sea bream larvae eye diameter (Roo *et al.*, 1999; Izquierdo *et al.*, 2000). This fact, along with the higher density of cone photoreceptors, implies a significant improvement in larval visual potential (Roo *et al.*, submitted). Lower swimming and feeding activities in EFA-deficient larvae (Izquierdo *et al.*, 1989a; Rodríguez *et al.*, 1993; 1994) are frequently recognized by the larvae floating on the water surface denoting as well alterations in the functioning of swim bladder (Koven, 1991). Effects have also been reported on

swimming performance of Atlantic salmon (*Salmo salar*) in seawater when it is fed diets containing different supplemental oils (anchovy oil and poultry fat) depending on the different n-3 HUFA/saturated fatty acids ratios (Wagner *et al.*, 2004). Thus, n-3 HUFA have been recognised as a major limiting factor defining the nutritional value of larval diets for marine species, being indispensable through early stages of life (Watanabe and Kiron, 1994; Izquierdo, 1996; Sargent *et al.*, 1999b). Since very few studies have been aimed to determine the ontogeny of fish behaviour, the effect of dietary fatty acids on behaviour of this larva is also unknown.

1.3 Behaviour

Exploration is a key animal behaviour in response to novelty (Kim *et al.*, 2005; Kliethermes and Crabbe, 2006; Kalueff and Zimbardo, 2007). Fishes are by far the most species-rich vertebrate taxon, and it is also the vertebrate group with the most strikingly diverse repertoire of behaviours and behavioural adaptations. As such, they provide us with many opportunities to explore the fascinating complexities of animal behaviour. Concern for the welfare of fish has increased during the last years (Huntingford *et al.*, 2006; Ashley, 2007; Arlinghaus *et al.*, 2007) to a large extent because of the possibility that fish can feel pain (Rose, 2002; Chandroo *et al.*, 2004). Stressful situations have been shown to affect the behaviour of fish (Sneddon *et al.*, 2003; Ashley, 2007) and recent studies suggests that their central processing of potentially painful stimuli leads to prioritization of

motivational drivers and modifies normal behaviour patterns (Ashley *et al.*, 2009).

The physiological and behavioural mechanisms that result in pain and stress are evolved adaptations that enable animals to cope with natural challenges, such as the need to avoid unfavourable habitats, to find food or to avoid being killed by predators. Pain (or nociception as it is more properly called) allows animals to detect and avoid localised harmful stimuli. The stress response can be considered to be part of an adaptive strategy for coping with a perceived threat to homeostasis. Concern for the welfare of the animals that humans keep in captive conditions (for example, on farms or as pets) arises from the fact that these adaptive systems may be activated in contexts where they cannot bring about their natural outcome of either removing the stressor or removing the animal from the source of pain or stress. Much welfare research involves identifying signs that this is the case and finding ways of ameliorating the conditions responsible.

From a physiological viewpoint, good welfare can be recognized by objective measurement of a range of biochemical and physiological indicators (Arlinghaus *et al.*, 2007). Exposure to some stressors and the resultant physiological response are not equivalent to suffering and may be beneficial, at least in the short term (Pickering, 1998; Huntingford *et al.*, 2006). Fish are able to adapt to stress for a period of time; they may look and act normal. However, energy reserves are eventually depleted and hormone imbalance occurs, suppressing their immune system and increasing their susceptibility to infectious diseases. Nevertheless, repeated exposure to acute stressors or prolonged exposure to poor conditions results in maladaptive responses (Wendelaar Bonga, 1997; Pickering, 1998), and monitoring of multiple components of the stress responses provides valuable insights into the welfare of fish (Turnbull *et al.*, 2005; Adams *et al.*, 2007). Fish have the same stress response and powers of nociception as mammals. Their behavioural responses to a variety of situations suggest a considerable ability for a higher level of neural processing, a level of consciousness equivalent perhaps to that attributed to mammals. Fish showing low blood concentrations of cortisol and normal values for parameters such as plasma osmolality and plasma concentrations

of glucose and lactate, as well as adequate growth and condition index are generally considered to be experiencing good health and welfare (Adams *et al.*, 1993; Turnbull *et al.*, 2005). Persistent disruption of homeostasis, results in a reduced appetite, poor growth and condition, and decreased immunocompetence (Adams *et al.*, 1993; Balm, 1997). In this sense, fish have the same stress response and powers of nociception as mammals and good welfare preserves or enhances fish health and well being (Arlinghaus *et al.*, 2007).

Animals often express consistent individual differences in behaviour. Part of the individual variation in behaviour may be caused by random noise around an adaptive average and small variations in state or context, but in a variety of animal species individual behavioural differences are consistent across different social and environmental contexts and independent of sex, age and size (Sih *et al.*, 2004; Carere & Eens 2005; Bell, 2007; Reale *et al.*, 2007). For example, some individuals may be more aggressive or bolder than others. Such consistent individual differences are commonly termed personalities or temperament (Reale *et al.*, 2007). The fitness benefits associated to a specific behaviour in a particular context (e.g. aggression during a contest) may become costs in another (aggression during courtship). Therefore, different personalities may have different context-dependent fitness and the inherent trade-offs may, in general, hinder individuals from attaining theoretical fitness optima. There is little available information about the ecological and evolutionary implications of personalities, but there are indications that personalities may affect dispersal, antipredator behaviour, foraging and reproductive strategies and, therefore, play a part in population and community ecology (Sih *et al.*, 2004; Dingemanse & Reale, 2005; Reale *et al.*, 2007). Personalities have been found in various types of behaviours, mostly having to do with aggressiveness, boldness and reaction to novelty. Some of these include exchange of signals as basic components, but there are no direct investigations of the implications of personalities for communication. Reale *et al.* (2007) have suggested five behavioural categories for personalities: shyness–boldness; exploration–avoidance; activity; aggressiveness; sociability. Only the last two of these require the use of signals at least to some

degree, but the frequency of signal use and reception per se have not been investigated as specific personality traits. Yet the frequency of visual threat display has been used as an index of aggressiveness in fish, and the rate, frequency or timing of specific vocalizations could be used as an index of aggression or eagerness to mate as in birds (Matessi *et al.*, 2010).

The spectacular array of behaviours exhibited by animals has been generated through the processes of evolution. Behavioural modifications are ultimately produced by differences in neural circuits. Probing the causes and consequences of nervous system evolution requires a tractable neural circuit and a group of animals that exhibit variation in this circuit (Tierney, 1996; Nishikawa, 1997; Katz and Harris-Warrick, 1999; Wright, 2000; Carr *et al.*, 2001; Rose, 2004). The circuitry that generates escape behaviour in teleost fishes presents such a system. The escape response, also known as the fast-start or startle response, is initiated by a pair of large, identifiable neurons in the hindbrain: the Mauthner cells (M-cells) (Figure 1.4) (Zottoli and Faber, 2000; Eaton *et al.*, 2001; Korn and Faber, 2005).

Fast-starts in most teleost fish consist of a C-type fast-start (C-start), the first stage of which is characterized by a rapid unilateral contraction of trunk musculature leading to head and tail movement, which causes the fish to bend into a C-shape (Figure 1.5). Stage 1 is typically followed by subsequent movements, including a tail stroke that results in a forward propulsion of the centre of mass (stage 2), and either gliding or a burst swim (stage 3) (Eaton *et al.*, 1981; Foreman and Eaton, 1993; Domenici and Blake, 1997). M-cell activity precedes the C-start, and electrical stimulation of Mauthner axons can elicit a C-start (Zottoli, 1977; Nissanov *et al.*, 1990).

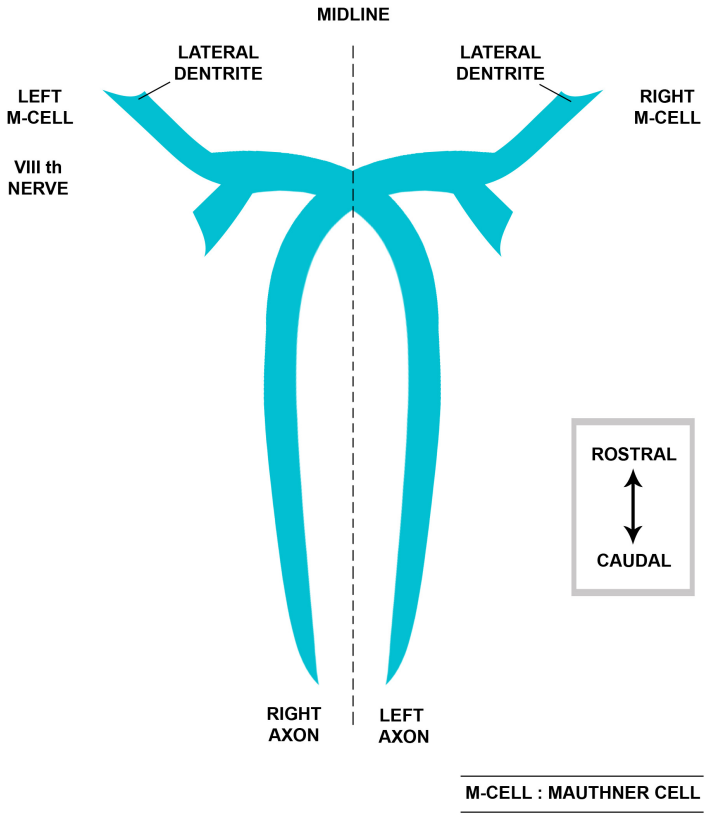


Figure 1.4 Diagram of M-cells.

Figure 1.5 Sequential body orientations of a fish engaging in a tail-flip escape behaviour, what time progressing from left to right (Figure adapted from Eaton *et al.*, 2001).

1.4 Mauthner cells

Fish can elude predatory attacks by producing a stereotyped escape behaviour, which is characterized by a rapid and powerful unilateral bending of the body and tail that involves most of its somatic musculature (Korn and Faber, 2005). This behaviour has a characteristic short latency when triggered by abrupt acoustic stimuli, and it is initiated by the activation of the M-cells. The M-cells are a pair of reticulospinal neurons located in the medulla of teleost fish (Beccari, 1907). These uncommonly large cells are anatomically and physiologically identifiable and have historically constituted a valuable preparation for the study of the cellular correlates of behaviour (Faber *et al.*, 1989; Korn and Faber, 2005). Their characteristic large myelinated axons, first noticed by Mauthner (1859), cross the midline to descend the length of the spinal cord (Figure 1.6), issuing axon collaterals that massively activate cranial and spinal motor systems via reliable (with high safety factor) chemical synapses (Faber *et al.*, 1989). Such an anatomical arrangement allows a single action potential in this cell to initiate an escape response by producing a tail-flip.

Reticulospinal neurons form one of the most important descending pathways in the vertebrate central nervous system. These neurons function as integration centers for sensory inputs and higher motor command in the brain and ultimately regulate motor functions in the spinal cord (Rovainen, 1967; Rovainen, 1982). Certain reticulospinal neurons, the Müller and M-cells, can be identified from animal to animal in anamniotic vertebrates. Because they are large cells and can be easily identified, these neurons have provided convenient experimental models to study neuronal properties and the neuronal basis of motor behaviours.

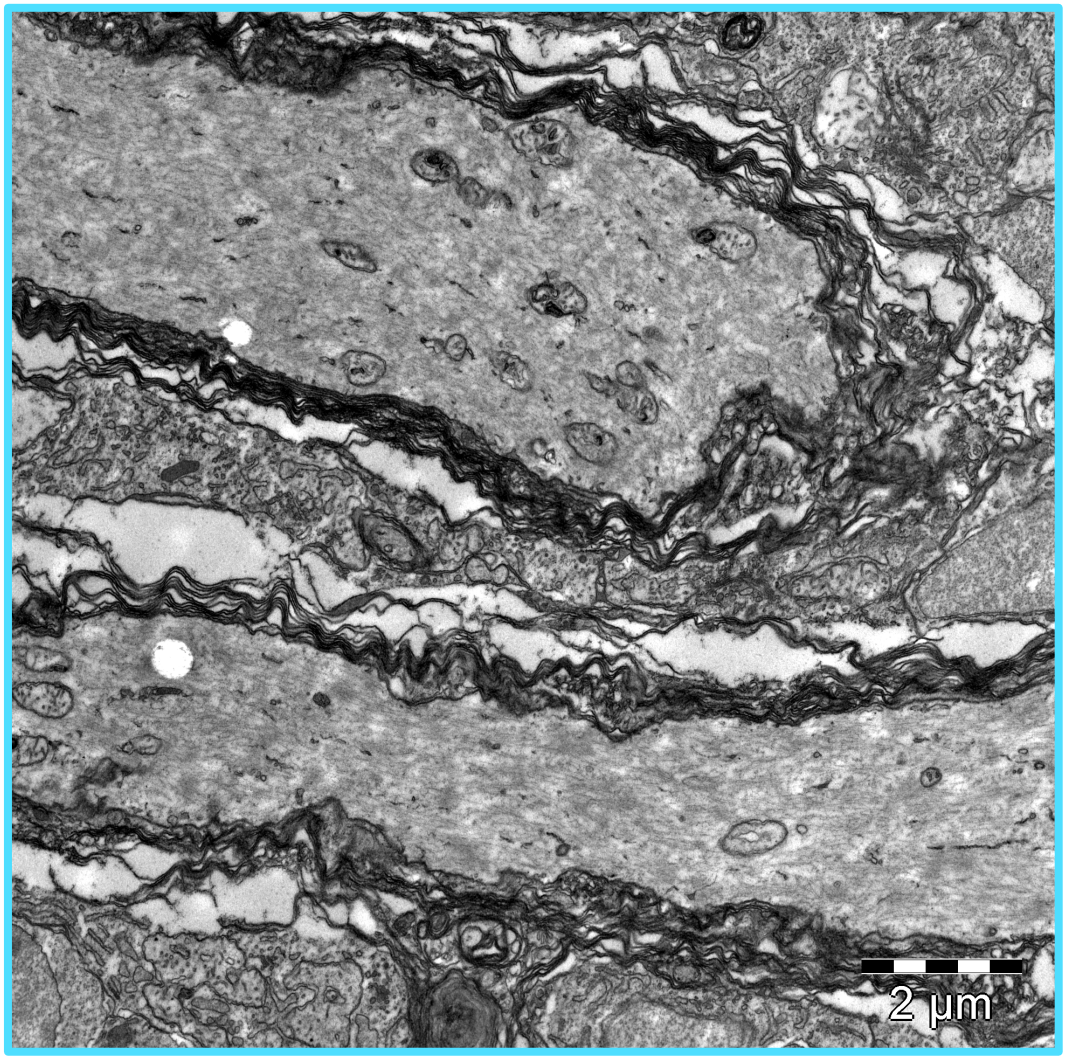


Figure 1.6 Myelinated axons from M-cells cross the midline to descend the length of the spinal cord.

The M-cells are a pair of identifiable neurons found in the brain of teleost fishes and certain amphibians (Stefanelli, 1951). They have two major dendrites, both at least 500 μm in length; one extends laterally and the other either ventrally or medially. One of the more impressive features of the M-cells is the size of its dendrites, which allows repeated microelectrode penetrations along their entire length. The soma is located in the rostroventral medulla and the large lateral dendrite of each M-cell receives inputs mainly from the sensory neurons of the statoacoustic system. The club endings of the ipsilateral VIIIth nerve have been shown to make gap junctions with the lateral dendrite of the M-cells (Robertson *et*

al., 1963), a finding consistent with an electrically transmitting synaptic mechanism (Furshpan, 1964). The axon from the M-cells first traverses a group of interlacing nerve fibres, the so-called 'axon cap' (Bartelmez, 1915). It then crosses the midline, enters the spinal cord on the contralateral side as a myelinated giant nerve fibre and tapers towards its caudal end. Short collaterals project from the axon at regular intervals and contact interneurons and motoneurons. The medullary and spinals outputs of the M-cell have been well characterized electrophysiologically and morphologically (Hackett and Faber, 1983; Fetcho, 1991).

Studies on Mauthner neurons have provided fundamental information on neuronal biochemistry, development, synaptic morphology and physiology, and control of behaviour than can be generalized to many central neurons throughout vertebrates (Faber and Korn, 1978; Nissanov and Eaton, 1989; Korn *et al.*, 1990). In fact, these identifiable pair of cells are the most studied in the central nervous system of vertebrates (Bullock, 1978). In fish, the shortest pathway for an escape in response to an acoustic input involves only four neurons the hair cells in the ear that connect to eighth nerve fibbers which in turn connect to the M-cell, which directly connects to motor neurons in spinal cord to activate the startle response. The speed of the pathways is further optimized by the presence of electrical synapses, which allow the direct current flow between one neuron and the next and reduce the delays associated with chemical neurotransmission. Finally, the startle circuits contain large, relatively fast conducting neurons in the pathways. These so-called giant neurons have relatively very large axons and, often, large cell bodies as well.

Alberto Stefanelli's publications on M-cells have helped establish these neurons as model system used to study the role of reticulospinal system in vertebrate motor control (Eaton *et al.*, 1981, Ward and Azizi, 2004) and the response of vertebrate central neurons to spinal cord injury (Zhang *et al.*, 2005). Stefanelli made many important contributions to neuroscience that include descriptions of the determination and differentiation of the M-cells in amphibian (Stefanelli, 1946; Stefanelli, 1951), axonal path finding of the Mauthner axon

(Stefanelli, 1951; Kimmel and Model, 1978; Stefanelli, 1979), afferent inputs to amphibian M-cells (Cioni and De Palma, 1989; Cioni and De Palma, 1992). His description of the differences in axon cap structure among many fishes and amphibians and his use of the "morpho-ecological" approach to determine M-cell function has provided the basis for future studies on the neuronal basis of behaviour and its evolution.

Since M-cells are determinant of startle reaction and EFA seem to affect escaping response in fish, but there are no studies about the effect of EFA in M-cells, the general aim of this thesis included to study the potential implication of these neurons in the regulation of escaping behaviour in fish by EFA, to contribute to understand the importance of dietary nutrients in behavioural responses.

2. Objectives

Objectives

Considering that long chain polyunsaturated fatty acids (LCPUFA) are indispensable for the normal development of the central nervous system and taking into account the welfare of all farm animals, including fish, the study fish larvae behaviour in function to their diet has become an interesting field of study in recent years. However information is still available. Thus, the main objective of this thesis was to determine the effect of n-3 LCPUFA in the escape behaviour of different fish species and its relation to neuronal activity.

To achieve this goal the specific objectives were grouped in five complementary and successive phases: The first part of this thesis aimed to show the effect of essential fatty acids contents in live preys on behaviour of gilthead sea bream (*Sparus aurata*) during early larval development; in the second part we further investigated the importance of LCPUFA on behaviour on the later stage of sea bream larval development using specific microdiets and newly developed techniques to study the implication of Mauthner cells (M-cells) activity; a third part meant to identify the specific effect of each n-3 LCPUFA, comparing the efficacy of different dietary DHA and EPA contents in escaping behaviour and neuron activity of gilthead sea bream larvae; a fourth part aimed to determine the effect of dietary n-3 LCPUFA levels on M-cells functioning in relation to escaping behaviour on a different species, the zebrafish (*Danio rerio*); finally the objective of the last part of the thesis was to further investigate on M-cells, conducting a histological study of their ontogeny in sea bass larvae (*Dicentrarchus labrax*).

Therefore five different studies were included:

- Study 1. The goal of this study was to determine the effect of distinct feeding regimes differing in their content in LCPUFA on the behavioural

responses to different stimulus during early larval development in gilthead sea bream.

- Study 2. The aim of this study was to better understand the effect of dietary LCPUFA on gilthead sea bream during later larval development on fish escape and avoidance behaviour, as well as M-cell activity.

- Study 3. In view of the enhanced escaping response in marine fish larvae fed increased n-3 LCPUFA in our previous research, the purpose of the present study was to compare the efficacy of different dietary DHA and EPA contents in escaping behaviour and neuron activity of gilthead sea bream larvae.

- Study 4. The aim of this study was to determine the effect of dietary n-3 LCPUFA levels on M-cells functioning in zebrafish in relation to escaping behaviour and neural function.

- Study 5. This study goal was to better understand ontogeny of the M-cells and the presence of calretinin and parvabumin using immunohistochemical and immunofluorescence tecniques in sea bass, adapting the method used previously in freshwater fish to marine fish studies.

3. Material and Methods

3.1 Species used

3.1.1 Sea bream

Sea bream (*Sparus aurata*) larvae (Figure 3.1a) were obtained from natural spawning from broodstocks at the Grupo de Investigación en Acuicultura (GIA) facilities where the experiments were carried out. Broodstocks were fed with a commercial diet called Vitalis Repro (Skretting, France), supplemented once a week with pieces of fish, molluscs such as cuttlefish, squid and mussels.

Eggs were collected using a collecting net of 500 mm, arranged on the drain tank top, resting on a cement tank of 200 liters capacity to avoid drying. Positively buoyant eggs fell by overflow in the collector. After harvesting, the start was incubated 24 hours in a cylindrical tank with mild aeration open circuit. Afterwards, the separation of eggs was performed by decanting, to distinguish viable and nonviable by the negative buoyancy of the latter. In parallel, tests were performed to calculate the rates of hatching, viable and death, eggs performed in 6 cups of 2 l for 72 h.

3.1.2 Sea bass

Sea bass (*Dicentrarchus labrax*) larvae (Figure 3.1b) were obtained from a natural spawning from a french hatchery (Ecloserie Marine de Gravelines, Gravelines, France). During the first days of acclimation of the larvae, water temperature was monitored through coolers (16° C) and water renewal rate was progressing increased until temperature reached Canary Islands values (20.2° C). The experiments were carried out at GIA (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain) facilities.

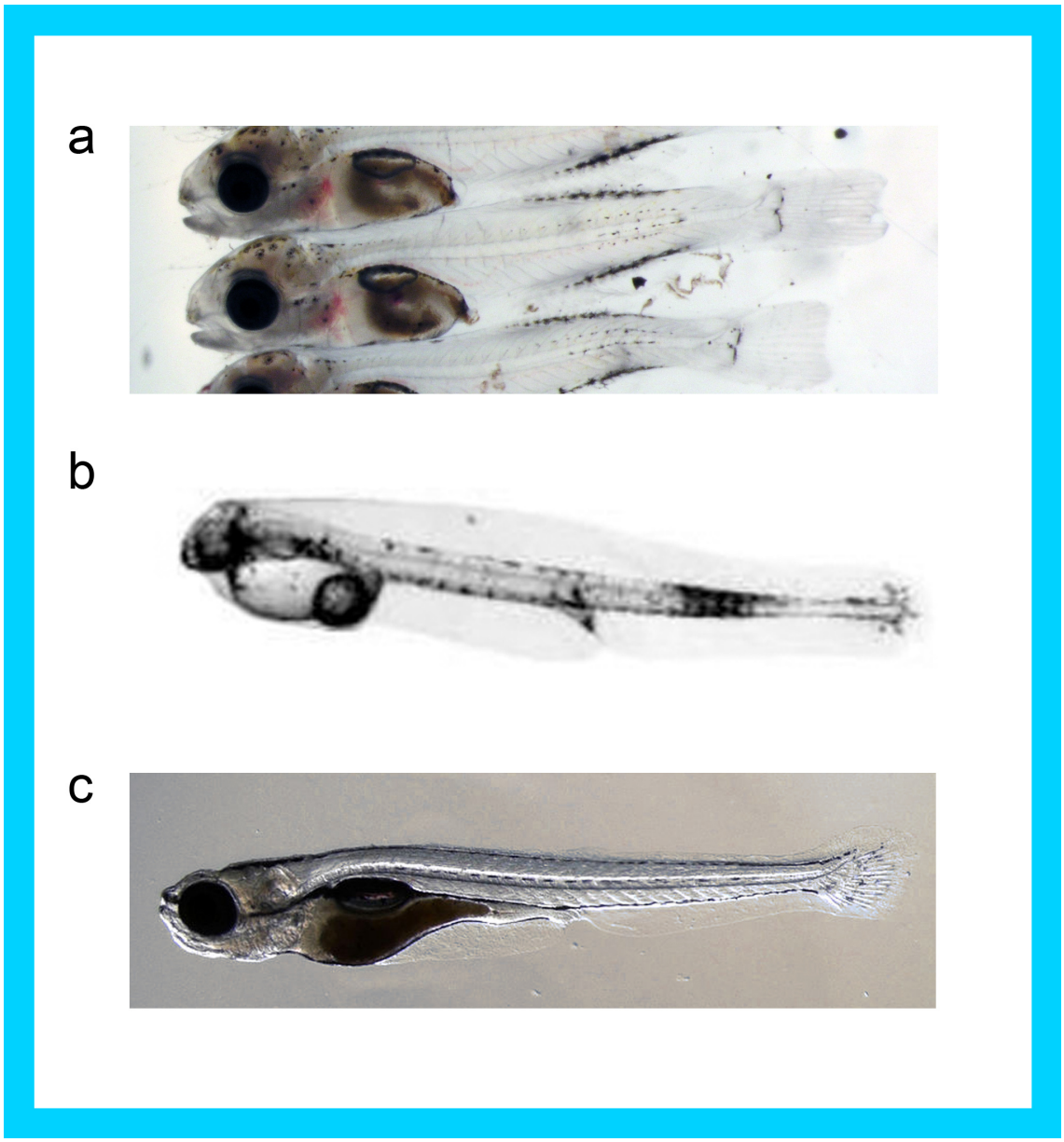


Figure 3.1 a) Sea bream larva. b) Sea bass larva. c) Zebra fish larva.

3.1.3 Zebrafish

Zebrafish (*Danio rerio*) larvae type Nacre (without pigments) (Figure 3.1c) were obtained from natural spawning from Department of Neurobiology and Behavior at Cornell University (Ithaca, USA) where these experients were carried out.

3.2.1 Experimental conditions

All tanks were supplied with sea water (about 37 ppm salinity) filtered by 50 µm mesh. Light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Temperature and oxygen were daily measured by using an Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, USA). Tanks were daily cleaned by hand between 18:00 and 20:00 pm with a hose by a siphon system. All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water previously stored in a 500 l tank for degasification. Tanks were subjected to an open circulation at different water flowing rates which were increasingly adjusted along the feeding trials. Water quality was daily tested and no deterioration was observed. Water was continuously aerated (125ml/min). Water temperature and dissolved oxygen were daily recorded at 15:00 (attaining 5-8 ppm) and saturation ranged between 60 and 80% in all experimental tanks. Photoperiod was kept at 12h light: 12h dark by fluorescent lights.

3.2.2 Growth determination

Growth was determined by measuring dry body weight and total length of starved larvae. Whole body weight was determined by 4-3 replicates of 5-10 starved larvae washed with distilled water and dried in a glass slide at an oven at 110° C until constant weight, for approximately 24 h, followed by 1 h periods. Total or standard length of 20-30 anesthetised larvae from each tank were measured in a Profile Projector (V-12A Nikon, Nikon Co., Tokyo, Japan).

3.3.1 Live food

3.3.1.1 Rotifers cultive

The rotifer *Brachionus plicatilis* was used in larval rearing experiences; adult individuals had an average total length of 240µm, being classified as L morphotype. Rotifers production was carried out on cylinder conical fibre glass tanks with a total capacity of 1700 l filled with a mixture of fresh and seawater to attain a salinity of 25 ppt. Production cycles lasted 8 days. Initial rotifers density was 265 indv · ml^{-1}; from day 4^{th} of culture, harvest of 400 l volume was performed in alternative days, harvested volume being replaced by a mix of seawater and fresh water to reach a 25 ‰ salinity. The 8^{th} day, the total volume was harvested and a new production cycle started. Routinely, after rotifers harvesting, a 1 min freshwater bath was applied to kill potential contaminants such as ciliates. Average total density and percentage of ovigerous females were daily calculated after individual counts (n=3) of 0.5 ml collected with a micropipette from a sample randomly collected from the culture tank. Similarly, oxygen and temperature were registered twice a day at 9:00 and 15:00 hours by means of a portable probe (Mod. Handy Polaris, OxyGuard; Birkerød, Denmark). Rotifers feeding consisted in lyophilized baker yeast (*Saccharomyces cerevisiae*) supplied at a dose of 0.4 g /10^6 rotifers. The first day of culture yeast was supplemented with lyophilized microalgae 0.1g /10^6 rotifers. Food was added manually at 09:00 and 15:00 hours, and automatically at 21h00 and 3h00.

3.3.1.2 Rotifers enrichment

Rotifer *Brachionus plicatilis* reared under the, previously described protocols are deficient in n-3 LCPUFA and other nutrients, needing to be enriched previously to be fed to the larvae. Enrichment was performed in cylinder-conical fibre glass tanks 500 l capacity. Rotifers concentration was generally high (over 400 rotifers · ml^{-1}), and an air diffuser was settled 15 cm from the tank bottom centre, to prevent sedimentation and to maintain the

oxygen levels. Along the different trials, rotifers enrichment with commercial or experimental products was performed. Generally, different enrichment products were used following manufacturer instructions, including 6 h enrichment time before harvesting. The enrichment product was spread in two separate doses (0h and 3 h). Thus, two tanks were daily used for rotifers enrichment; the first's one was harvested at 8h30 and fed to the larvae. In this case the enrichment product was automatically added at 2h00 and 5h00. The second enrichment tank was harvested at 14h00, and the enrichment product was manually supplied at 8h00 and 11h00. After rotifers enrichment, they were harvested and cleaned to eliminate oil emulsions particles, concentrated in a 20 l basket and kept with air supply until larval feeding. A 5 ml sample was taken, diluted in 250 ml, homogenized and counted. Once calculated the needs for each larval tank, rotifer were fed to the larvae and the remaining rotifers discarded. Along the whole experimental period, different samples of the products and rotifers used were stored at -80ºC to study the biochemical composition and fatty acid profile.

3.3.1.3 Enrichment

Emulsions were prepared with 2 g of oil, 5 g of soybean lecithin and 400 ml fresh water mixed in a blender for 2 min. This emulsion was added to the 30 l of rotifer culture at a concentration of 250 rotifers/ml during 12 h. After rotifers enrichment, they were harvested and cleaned to eliminate oil emulsions particles, concentrated in a 10 l basket and kept with air supply until larval feeding. A 5 ml sample was taken, diluted in 250 ml, homogenized and counted. Once calculated the needs for each larval tank, rotifer were fed to the larvae and the remaining rotifers discarded. Along the whole experimental period, different samples of the products and rotifers used were stored at -80ºC to study the biochemical composition and fatty acid profile.

3.3.1.4 Brine shrimp cultive

Nauplii of the brine shrimp *Artemia* constitute one of the basic foods items for marine larval rearing. For all the larval rearing experiences *Artemia* nauplii from two different origin were used, *Artemia franciscana* (AF Type;

INVE; Dendermode, Belgium) and *Artemia salina* (EG Type; INVE, Dendermode, Belgium). The decapsulation process to eliminate the external corion and to reduce the risk of external pathogens introduction was performed for all the *Artemia* batches.

3.3.1.5 Enrichment

To improve nutritional quality of *Artemia*, an enrichment procedure was necessary. For this purpose, cylinder-conical fibre glass tanks of 1700 l total volume were employed. Strong aeration from the tank bottom and 24 h photoperiod were applied. Besides, an internal heater was used to maintain a culture temperature of 25-26° C. Just hatched out *Artemia nauplii* were introduced in the enrichment tank at a concentration of 250,000-300,000 nauplii $\cdot l^{-1}$. In most of the experimental trials the Easy DHA Selco (Inve, Dendermonde, Belgium) enrichment product was used. The enrichment last for 18-24 h with a concentration 0.6 gr $\cdot l^{-1}$, added in a single dose at the beginning of the enrichment process (h=0). Enriched *nauplii* were harvested in a sock net of 125 µm mesh size, washed with seawater to eliminate residual lipid particles and concentrated in a 20 l beaker to be counted and check the enrichment success. Enriched nauplii were added to the larval tank manually.

3.3.2 Microdiets

Several isonitrogenous and isolipidic experimental microdiets (pellet size < 250 µm) having differing ratios of DHA, EPA were formulated in the different experiments using: EPA50 oil and DHA50 (CRODA, East Yorkshire, England, UK) oil in a triglycerides form as an sources of EPA and DHA. Vegetable oils were used in one of the experiments: fish oil (FO), soybean oil (SBO), lindseed oil (LSO) and rapeseed oil (RSO) (vegetal oils used were commercial food grade oil, Agroalimentary Commercial Oils; and FO was sardine oil, Croda, East Yorkshire, UK). The desired lipid content was completed with a non-essential fatty acid source, oleic acid (Merck, Darmstadt, Germany). The protein source used was squid meal (Riber & Son, Bergen, Norway) which was defatted (3 consecutive times with a chloroform:meal ratio of 3:1) to allow a better control of the microdiet fatty acid profile, except in one specific experiment. The

defatted squid meal (2.6% lipid content), DHA50 and EPA50 fatty acids profiles are shown in Table 3.1.

3.3.2.1 Microdiets preparation

The microdiet was prepared in the following manner: the squid powder was carefully mixed with the other hidrosoluble ingredients (attractants, minerals and hidrosoluble vitamins, Sigma-Aldrich, Madrid, Spain) in a mortar. In a separated mixture, oils and fat-soluble vitamins were combined to obtain a homogeneous mix which was afterwards put together with the powder mixture. Then, gelatin was dissolved in warm water and when its temperature was lower than 35° C, it was added to the rest of the previously mixed ingredients. The paste was then compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38° C for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain the desired particle size. Diets were analyzed for proximote and fatty acid composition and each diet was tested in triplicate.

Table 3.1 Main fatty acids of the major dietary lipid sources and deffated squid meal used in this thesis.

Fames	DHA50	EPA50	Defatted squid
14:0	0.03	0.04	1.68
14:1n-5	0.20	0.24	0.04
14:1n-7	n.d.	0.01	0.04
15:0	n.d.	0.01	n.d.
15:1n-5	n.d.	n.d.	0.04
16:0iso	0.01	0.02	0.11
16:0	1.21	0.36	23.80
16:1n-9	n.d.	n.d.	0.02
16:1n-7	0.47	0.43	0.52
Me 16:0	0.01	0.01	0.10
16:1n-5	0.02	n.d.	0.16
16:2n-6	n.d.	0.05	0.06
16:2n-4	0.16	0.49	0.48
17:0	0.12	n.d.	0.06
16:3n-4	0.10	0.21	0.07
16:3n-1	0.06	0.14	0.12
16:4n-3	n.d.	n.d.	0.38
16:4n-1	n.d.	0.33	0.03
18:0	2.32	0.27	3.64
18:1n-9	4.92	1.81	1.73
18:1n-7	0.87	0.55	0.94
18:1n-5	0.04	n.d.	0.24
18:2n-9	n.d.	0.44	n.d.
18:2n-6	0.59	2.16	0.24
18:2n-4	0.07	0.68	n.d.
18:3n-6	0.34	0.73	n.d.
18:3n-4	0.04	0.44	n.d.
18:3n-3	0.20	1.67	n.d.
18:3n-1	n.d.	0.32	n.d.
18:4n-3	0.31	10.44	0.07
18:4n-1	n.d.	1.01	n.d.
20:0	0.73	0.42	n.d.
20:1n9+n7	3.98	0.39	2.80
20:1n-5	n.d.	n.d.	n.d.
20:2n-9	0.07	0.41	n.d.
20:2n-6	0.54	0.13	0.12
20:3n-9 +7	0.21	0.72	n.d.
20:3n-6	n.d.	n.d.	n.d.
20:4n-6	2.46	3.49	0.49
20:3n-3	0.44	0.11	0.34
20:4n-3	0.92	2.49	0.06
20:5n-3	12.66	46.48	4.31
22:1n-11	2.29	0.31	0.04
22:1n-9	1.21	0.74	0.11
22:4n-6	2.84	0.49	0.13
22:5n-6	0.19	n.d	n.d.
22:5n-3	5.53	3.21	n.d.
22:6n-3	53.80	17.72	9.40

n.d.≤0.005

3.4.1 Material

The preparation is very similar in both stimuli. We used a 500 ml glass beaker (beaker experiment) with a seawater depth of 4 cm. Under the glass a chart paper was placed. The answers to each type of stimulus individual larvae were tested, using 10 larvae of each tank. Each larva was transferred from the feeding tanks to the experimental beaker and then video-recorded using a Sony digital video camera DCR-TRV27. The acclimation time for each larva in the glass beaker was 2 minutes. Different tests were done to determine this time. After recording for 90 s without disturbance, larva was scared by sound and visual stimuli to introduce a startle response. Stimuli were provided three times at 10 s intervals for each larva.

To determinate the effects of diet on larval behaviour, different trials were performed throughout the experiments. In tests on day 1 after hatching (dah) and up to 6 dah larvae could scarcely been distinguish clearly in the digital camera and visual analysis had to be applied. In experiment 1, the escape behaviour was quantifed at 6, 10, 16 and 19 dah; in experiment 2 at 23, 27 and 34 dah; and in experiment 3 at 22, 25, 29 and 32 dah. These data were used for monitoring throughout the period of larval development and to acquire fluency in handling such sensitive animals with these techniques. Each larva used in the experiments was measured by a profile projector (V - 12ª Nikon, Tokyo, Japan).

3.4.2 Stimuli

3.4.2.1 Sonorous stimuli

Consistent sound stimuli were produced using a steal nut (≈10 g) hung by a string (26 cm) that was released from a distance of 18 cm from the beaker wall (Figure 3.2). This beaker was covered with a black vinyl sheet only when sound stimuli was applied. The pendulum was located at a distance of 8 cm from beaker glass. Using a tape measure, the nut was carried at a distance

of 10 cm from the same pendulum, (the nut would be at a distance of 18 cm from the experimental beaker) and was launched without using force, just dropping it. The distance had to be very precise since an error of one centimeter may affect the performance parameters evaluation.

Figure 3.2 Application of the sonorous stimuli.

3.4.2.2 Visual stimuli

A flash of white light to simulate natural conditions was used as visual stimulus. The flash was located at 10 cm from the experimental beaker (Figure 3.3).

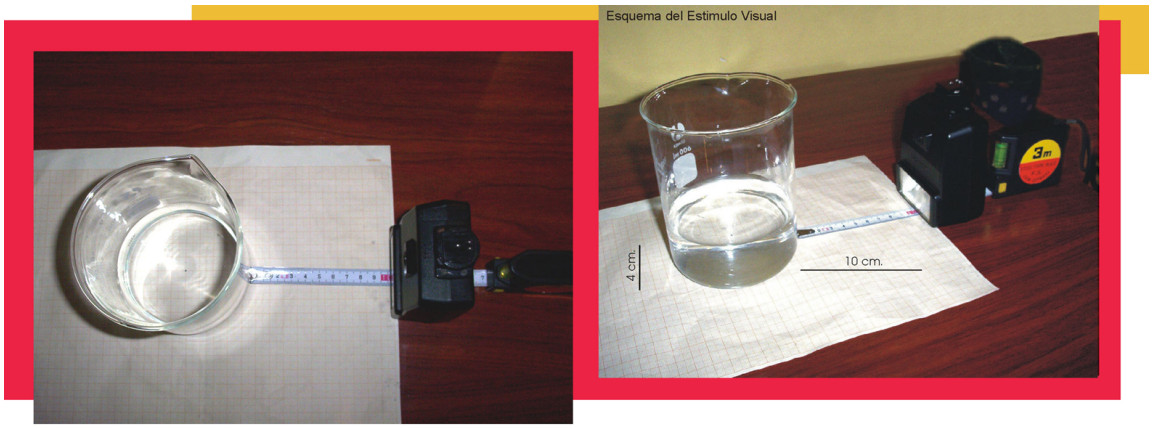


Figure 3.3 Application of the visual stimuli.

3.4.3 Parameters

As mentioned above, each larva was supplied three times the same stimulus with intervals of 10 s. In order to know if the larvae responds or not, that is to say, if the response is positive or negative, we had to observe with patience and dertermination because in the earliest days of sampling, the larvae could barely be clearly seen. The analysis began with the viewing of all the images to get an idea of which day the larvae will begin to react in front of the stumulus.

3.4.3.1 Cruise Swimming Speed

Cruise swimming speed is defined as the natural larvae movement just before application of the stimulus (Masuda *et al.*, 2002). Video analysis was conducted frame by frame to calculate cruise swimming speed and burst swimming speed. Cruise swimming speed estimation was based on the 10 s video recording starting 30 s after the recording began. The movement of the fish was traced on an overhead projector transparency sheet, and this distance was divided by the time taken (10 s), obtaining the cruise swimming speed. The units used were mm/s and SL/s (SL, standard length), it cruise swimming speed was divided by the SL of each individual (Masuda *et al.*, 2002). This analysis was conducted only in larvae that had movement for both, the sound and the visual stimulus.

3.4.3.2 Burst Swimming Speed

Burst swimming speed was calculated only when fish showed an obvious startle response. This obvious response could be a change of direction for visual and sonorus stimuli, and/or panic type for visual stimuli. After each stimulus, larval movement was traced for four consecutive frames, and the distance was divided by the time taken (4/30 s). Preliminary observations revealed that the faster movement appeared in any of the first four frames after providing a stimulus. Burst swimming speed was calculated as the average of the movement of four frames and defines it as the burst swimming speed.

The units used were mm/s, SL/s. Burst swimming speed was divided by the SL of each individual (Masuda *et al.*, 2002).

3.4.3.3 Burst Swimming Rate

To observe the response development, burst swimming rate was calculated by dividing the number of responses by the number of trials.

3.5 Zebrafish behaviour

The study of behaviour response in zebrafish was carried out in the Department of Neurobiology and Behavior facilities at Cornell University (USA). Response to a vibrational stimulus was studied determining the peak angle of escape bend, the peak angular speed of the escape turn and time to respond in challenged zebra fish larvae. The trials were recorded by a high-speed camera that digitally captures images at 1000 frames/s (EG&G Reticon, Sunnyvale, CA, USA) using the software called Photron Fastcam Viewer (San Diego, CA, USA). Data were analyzed using a custom written image analysis program focusing on the performance of the initial turn in the fish escape, since the Mauthner array is essential for generating the high-performance movements during this turn.

3.5.1 Electroporation

To view and study "in vivo" Mauthner cells (M-cells), we proceeded to fluorescent labelling by electroporesis. Electroporation or electropermeabilization is a significant increase in the electrical conductivity and permeability of the cell's plasma membrane caused by an externally applied electric field. When the voltage across a plasma membrane exceeds its dielectric, pores are formed. If the applied electric field strength and duration of exposure were chosen properly, the pores formed by the electrical pulse were sealed after a short period, during which extracellular compounds have

the opportunity to enter the cell. However, excessive exposure of live cells to electrical fields can cause processes leading to cell death, apoptosis or necrosis.

To carry out the electroporation of one M-cell, 4 dah nacre type larvae (not pigmented) were used. Older 4 dah larvae were never used because of the difficulty to perform this operation as larva is more developed. At this age, nacre zebrafish larvae are transparent with sparse pigmentation that allows the identification and picturing of neurons in the brain and spinal cord of the intact animal. Firstly larvae were anesthetized using 50 ml of Hank's solution and 3.2 ml of 0.03% tricaine methanesulfonate (MS-222, Sandoz, Basel, Switzerland) and positioned straight in 1.2% agarose in 0.3' phosphate buffered saline (PBS) on a glass slide. Then, a few drops of anaesthetic Hank' solution were supplied on the plate. Some specific glass pipettes for electroporation were prepared with fluorescein. Electroporation was carried out in an electroporator, an instrument that creates an electromagnetic field through the agar. Using a microscope M-cells were located. Once the right cell were found (always the same neuron electroporated), we proceeded to enter the pipette with fluorescein into the cell. A 10% solution of rhodamine (3000 molecular weight or MW; Molecular Probes, Eugene, OR) in 10% Hanks solution was electroporated into the M-cell (Bhatt et al., 2004). Applying an electric field, fluorescein was ntroduced into the neuron. Once satisfied that the operation was correct, larvae were released of the agar and placed in a petri dish with 50 ml of Hank's solution and 50 microns of Pen Strep (penicillin) and microdiet. The animals were given 24 h to allow for recovery. After this time larvae were again placed in experimental tanks to continue the study.

At the end of the experiment all the fish larvae were briefly anesthetized, embedded on their backs in soft agar on a cover glass in a petri dish (Eaton *et al.*, 1984) and then rinsed with 10% Hanks solution to allow recovery from the anesthetic. Confocal images were obtained by looking into the head of the intact fish using a Zeiss LSM 510 confocal microscope (Fetcho and O'Malley, 1995; O'Malley *et al.*, 1996). The fish transparency allowed not only to clearly visualize neurons inside the living animal but also to monitor fish

viability by observing the heart beat and blood flow. To confirm the identity of the cells studied physiologically, stacks of images showing the morphology in successive confocal sections were acquired. Signal averaging was used when acquiring this morphological data. Maximum projections were made from stacks of these sections. The image stacks were also reconstructed in three-dimensions using the Imaris software (Bitplane scientific software, South Windsor, CT, USA) allowing us to examine the details of the dendritic morphology and axonal projections of each cell.

3.6 Biochemical analysis

The biochemical analyses were made in the laboratory of Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA; University of Las Palmas de Gran Canaria, ULPGC). During the course of the different experiences, larval samples of different ages from each rearing tank and treatments were collected. In adition, dry feed samples and live preys before and after their enrichment were also taken. Samples were frozen (-80° C) in hermetic bags under nitrogen atmosphere, for its later analysis. Determinations of dry matter, ash, protein and lipid content as well as fatty acids were performed. All the analyses were performed at least in triplicate. At the end of the experiment number 2, one hundred larvae were separated for brain and eyes dissection for biochemical analysis.

3.6.1 Dry matter content

It was determined following the Official methodology of the American Chemical Analysts Association of (AOAC, 1995). Dry matter content was determined after drying the fresh known sample quantity (*Pi*) in an oven at 105° C until constant weight was obtained (*Pf*). Before being weighted, the

samples were introduced in a desecator for 30 min for ambient temperature adaptation, and, finally, they were weighted. The dry matter content was calculated by the following expression:

$$Pl = ((Pl + L) - (Pc))/n$$

3.6.2 Total lipids content

Total lipids were extracted according to Folch *et al.* (1957). A 50-200 mg sample was homogenised in an Ultra Turrax (IKA-Werke, T25 BASIC, Germany, Staufen) at 11,000 rpm during 5 min in a solution of 5 ml of Chloroform: Methanol (2:1) with 0,01% of BHT. The resulting solution was filtered at an ambient pressure through glass wool and adding KCl at 0.88%, to increase the water phase polarity. After decantation and centrifugation at 2000 rpm during 5min the watery and organic phases were separated. After discarding the water soluble phase, a N_2 current was used to evaporate the chloroform until completed dryness. Finally total lipid content was gravimetrically determined.

3.6.3 Separation of polar lipids

Neutral and polar fractions of the larval total lipids were separated by adsorption chromatography on silica cartridges (Sep-pak; Waters S.A., Massachussets, USA) using 30 ml chloroform and 20 ml chloroform/methanol (49:1, v/v) as solvent for neutral lipid, followed by a 30 ml methanol wash to obtain the polar fractions according to Juaneda and Rocquelin (1985).

3.6.4 Fatty acid esters preparation and quantification

Total or polar lipids were dissolved in toluene and fatty acid methyl esters obtained by transmethylation with 1% sulphuric acid in methanol (Christie, 1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16h at 50° C. Afterwards, fatty acid methyl esters were extracted with hexane: diethyl ether (1:1 v/v) and purified by adsorption chromatography on NH2 Sep-pack cartridges (Waters S.A., Massachussets,

USA) as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supelcolwax-10-fused silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180° C for the first 10 min, increasing to 215 °C at a rate of 2.5 °C/min and then held at 215 °C for 10 min, following the conditions described in Izquierdo *et al.* (1990). Fatty acid methyl esters were quantified by FID and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd Tokyo; Japan).

3.7 Histological studies

The histological analyses were made in the laboratory of IUSA. For histological studies 60 individuals from each larval tank were randomly sampled, anesthetized with MS-222 (1:20,000) and fixed in 10% buffered formalin (Annexe I). The basis of fixing is the preservation of the different tissues while maintaining the highest fidelity gives histological tissue *in vivo*, through the interaction of active radicals with formalin radicals blocks of proteins and lipids. The time of formalin get into tissues is about 3 mm per hour.

3.7.1 Sample collection

At least 24h after fixation, samples were embedded with three different inclusions methods. The first procedure consisted of paraffin inclusion technique, routinely used in histology with a highly standardized and automated protocol. In the second, samples were prepared to be cutted in the cryostat (Leica Jung CM 3000, Nussloch, Germany). Third, larvae were included

in a resin that allows thinne preparations for electron microscopy studies. The protocols used in each case are described below.

3.7.2 Processing and paraffin embedding

Fixed larvae from different ages and treatments were set in individual microcapsules previously identified, to be washed and dehydrated in a series of graded alcohol in a tissues processor (Mod. Histokinette 2000; Leica, Nussloch, Germany) (Table 3.2). Once paraffin infiltration has finalized, larvae were removed from the microcapsules and embedded in paraffin using a paraffinic dispenser (Mod. Jung Histoembedder; Leica, Nussloch, Germany). Larvae were decapitated to study the brain.

Table 3.2 Histological samples manipulation.

Alcohol 70°	3 h	Dehydration
Alcohol 96°	2.5 h	
Alcohol 96°	2.5 h	
Alcohol 96°	2.5 h	
Alcohol 100°	2 h	
Alcohol 100°	2 h	
Alcohol 100°	2 h	
Xylene	30 min	Clarification
Xylene	30 min	
Xylene	30 min	
Paraffin	3 h	Paraffin infiltration
Paraffin	6 h	
Paraffin vacuum	1 h	

3.7.2.1 Preparation of cuts

Paraffin blocks were cut in the microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany). Initially, gross cuts of 20-25 µm were performed until larvae tissues were reached and later definitive cuts of 3 µm were conducted. The cut series were introduced in a distilled water bath at 45-50° C. Brain tissue sections slides for immunohistochemistry studies were prepared with glue to increase the fixation of the tissues, preventing them off Poly-L (Sigma ®) (Annexe I) was applied in the slide.

3.7.2.2 Histological stains

3.7.2.2.1 Hematoxylin and eosin

It is one of the most common techniques in histology laboratories. The aim of this technique was created to obtain a contrast between different parts of the cell for a proper assessment of different tissues under the microscope, using the different affinity of the tissues to absorb dyes. In this technique the dyes used were:

- Hematoxylin: blue-purple coloration. Stains basic structures.
- Eosin: Red-pink coloration. Stains acid structures.

The samples were first dried in an oven (1h at 60° C). Then, samples were introduced in a xylol bath and re-hydrated in alcohol series of different gradation, finalizing with water:

Xylene	2 min
Xylene	2 min
Alcohol 100°	2 min
Alcohol 100°	2 min
Alcohol 70°	2 min
Distilled water	2 min
Distilled water	2 min
Distilled water	2 min

Then, the staining battery starts following the protocol modified from Matorja and Matorja-Pierson (1970) and Garcia del Moral (1993):

Harris hematoxylin	15 min
Rinse quickly in water	
Alcohol hydrochloric acid	4 quick passes
Rinse quickly in water	
Ammonia water	15 passes
Water	15 min
Eosin	4 min

Once staining was end, samples were dehydrated and clarified:

Alcohol 96°	2 min	Dehydration
Alcohol 96°	2 min	
Alcohol 100°	2 min	
Alcohol 100°	2 min	
Xylene	2 min	Clarification
Xylene	2 min	
Xylene	2 min	

The preparations were mounted using DPX medium.

3.7.2.2.2 Nissl staining

This method is used for the detection of Nissl body in the cytoplasm of neurons on paraformaldehyde or formalin-fixed, paraffin embedded tissue sections (Martoja and Martoja-Pierson, 1970). The Nissl body will be stained in purple-blue. This stain is commonly used for indentification of the basic neuronal structure in brain and spinal cord tissue.

Solutions

a) Acetate buffer

a. 2.71% sodium acetate.

b. 1.20% acetic acetate.

b) Cresyl extra solution

a. Violet or thionin extra cresyl 0.25 g.

b. Acetate buffer at pH = 3.8-4 100 ml.

Procedure

1. Dehydrate and hydrate the samples.

2. Stain in cresyl violet solution for 10 min.

3. Rinse in acetate buffer.

4. Dehydrate, clear in xylene (no time) and mount with permanet mounting medium.

3.7.2.3 Immunohistochemical techniques

To demonstrate the presence of different markers in the M-cells of fish larvae, we applied the avidin-biotin-peroxidase (ABC) immunohistochemical and immunofluorescence in method for simple markings. Immunohistochemistry is based on the application of a specific primary antibody in the tissues, usually created in rabbits (polyclonal) or hybridoma established in mouse spleen (monoclonal). The antibody is specific for the protein molecule studied. The method followed is described in the next paragraphs:

1. A blockade of endogenous peroxidase to avoid false positives.
2. An antigen unmasking process, used only when necessary to break the bonds created by formalin fixation.
3. Normal serum blocking. Normal serum should be long to the same species of which the secondary antibody is raised.

Subsequently, a secondary serum is applied after the primary, serving as a

bridge between this and the following reaction amplifier complex called avidin-biotin-peroxidase (ABC). The development process can be carried out using two different chromogens, commonly called "red" (AEC, 3-amino-9 ethylcarbazole) or "brown " (3-3-diaminobenzidine) as the positive staining.

Protocol

Each antibody was tested for versioning operation and it is subject to variations to determine the concentrations and types of unmasking epitopes that maximize the positive.

The antibodies tested were parvalbumin (PV) and calretinin (CR), both polyclonal (Table 3.3).

Table 3.3 Antibodies tested in immunohistochemistry technique.

Antibody	Dilution	Time to detection	Company
PV	1:700	10 min	Swant
CR	1:700	10 min	Swant

The steps of the ABC method protocol are as follows:

1. The samples were dried in an oven for at least 24 h at 37° C.
2. Wash 10 min and 2x5 min with xylene.
3. Wash 5 min in alcohol at 100°.
4. Inhibition of endogenous peroxidase by a solution of hydrogen peroxide 3% in methanol during 30 min with agitation.
5. Wash 5 min with alcohol at 100°.
6. Wash 5 min with alcohol at 96°.
7. Wash 5 with alcohol at 70°.
8. Wash 2x5 min with distilled water.

9. Wash 5 min with PBS (phosphate bufferd saline) in agitation.

10. Unmasking antigen epitopes using the enzyme treatment. This treatment involves applying a solution of the enzyme pronase in a proportion of 0.1 g per 100 ml of PBS buffer at room temperature for 7 min.

11. Wash 3x5 min with PBS buffer.

12. Application of normal goat serum for the following antibodies: parvalbumin and calretinin. Incubation in a humid chamber at room temperature with 10% normal goat serum (Vector ®) in PBS for 30 min.

13. Primary antibody: Incubate sections with primary antibodies. The antibodies were diluted in 1% normal goat serum in PBS. This process was conducted in a moist chamber at 4° C during 18 h. Tests were conducted with different concentrations of antibody to obtain the optimal dilution.

14. Remove the moist chamber of the refrigerator an hour before preceding the next steps.

15. Wash 3x5 min with PBS.

16. Secondary antibody: Incubation with secondary antibody diluted created in pig and normal rabbit serum 1% in PBS (concentration 1:250) for parvalbumin and rabbit anti-mouse calretinin (1:250). This process is conducted in a moist chamber at room temperature for 30 min. Note: Longer incubation time may produce higher background staining.

17. Wash 3x5 min with PBS.

Note: Slides should be protected from light starting from this step until the end by covering slides with aluminum foil or a black box.

18. Incubation with streptavidin-biotin-peroxidase (ABC) (Dako ®) in a humid chamber in darkness room during 1h at room temperature.

19. Wash 2x5 min with PBS.

20. Wash 1x10 min with Tris (1 part Tris mother /9 parts 0.85% saline).

21. Reaction revealed by immersion in diaminobenzidine (DAB) prepared by dissolving with a stirrer 0.07 g DAB in 200 ml of Tris buffer. Dual role is filtered in a darkroom and 200 ul of hydrogen peroxide at 30% was added. Is revealed during approximately 2 min observing the positive control.

22. Wash 1x10 min with water.

23. Counterstain with Harris hematoxylin during 30 s.

24. Wash 1x10 min with water.

25. Dehydrated in alcohols and xylene (2 min in each one).

26. Coverslip with DPX mounting medium.

3.7.3 Preparation of samples for cryostat

These techniques were learned at the Institute of Neurosciences of Castilla y León (INCYL) in Salamanca (Spain). Larvae were deeply anesthetized using an aqueous solution MS-222 and fixed in 1% (w./v.) paraformaldehyde and 15% (w./v.) picric acid in 0.1 M PBS, pH 7.4 during 4-6 hours (depending on the size of the sample) at room temperature. Some tests were performed using larvae fixed in 10% buffered formalin to improve the cut quality. Then, samples were washed 3 times with PBS during 5 min. Larvae heads were keeped in a freezer solution (Annexe I) at -20° C. After rinsing in PBS, tissue was cryoprotected with 30% (w./v.) sucrose in PBS during 12 hours at room temperature and embedded in a medium consisting of agar to 1.5% and 5% sucrose (w./v.). In each block was placed a single fish head and was oriented to have a horizontal cutting angle. Once cryoprotected, blocks were included in OCT 4583 (Middle of inclusion, Tissue-Tek, Miles), frozen with liquid nitrogen

and cut in cryostat at a temperature of -24° C. Gelatin-embedded larva blocks (Annexe I) were serially cut on Slee Mainz cryostat at 10 □m.

For immunofluorescence studies 30 larvae were collected and fixed in 10% buffered formalin. Each larva was included in a gelatin block horizontally orientated to obtain a better visualization of neuronal structures. Hematoxylin-eosin stains were tested to verify that sections right before the relevant evidence of study.

Hematoxylin-eosin

1. Wash 3x15 min with PBS.
2. Wash 3x15 min with distilled water.
3. Hematoxylin: 1 min and 30 sec.
4. Wash in water during 30 min.
5. Wash in distilled water.
6. Dehydrated in alcohols and xylene (three steps of 5 min each).
7. Coverslip with DPX mounting medium.

3.7.3.1 Immunofluorescence

This technique was setup in collaboration with the INCYL, which hosted a research visit. Subsequently, the samples of this thesis were processed in the IUSA. The sections previously cut in cryostat, were defrosted at room temperature during 1 h and washed in PBS to rehydrate the tissue, 3x10 min.

This was followed by a preincubation with non-immune rabbit serum 5% (Sigma) and Triton X-100 (Probus SA) 0.2% in PB for 1 h at room temperature (species same as secondary antibody). With this procedure we get a better penetration of antibodies and also reduce nonspecific binding of the same tissue. Then sections were incubated at an appropiate dilution in primary antibody anti-Choline acetyltransferase (ChAT) (Table 3.4) in a humid chamber for 2 days at 4° C. After incubation sections were rinsed with PBS.

Table 3.4 Primary antibody used in inmunofluorescence.

Antibody	Dilution	Company
ChAt	1:500	Millipore

After that, sections were incubated with the corresponding fluorescent secondary antibody anti-goat IgG (whole-molecule)_FITC (antibody made in rabbit, Sigma ®) diluted 1:250 for 1 h at room temperature in darkness. To mark the nuclei of all cells, incubation for 10 min in the dark with propidium iodide (PI, Sigma ®) (1:2000) in PB was performed. At the end, sections were washed with PB and mounted in a commercial medium Fluoromont ® (Sigma ®). To obtain and quantify the intensity of fluorescence, a Zeiss confocal microscope model LSM 510 of Department of Biochemistry (ULPGC) was used. To quantify the intensity of the sample a LSM 510 program was used by the next parameters: detector gain, amplifier offset and amplifier gain.

3.7.4 Processing and inclusion in resin

This section describes the procedures for cutting and staining of semithin and ultrathin sections of resin-embedded samples. Larvae were anesthetized with MS-222 and fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH = 7.2) (Annexe I) for 1 h and stored in cacodylate buffer at 4ºC (Bancroft and Stevens, 1996).

Samples were analyzed in the histological laboratory in IUSA. For this, samples were washed with washing liquid (Annexe I) for 24 h at 4º C. After this period, tissues were postfixed in 2% osmium tetroxide diluted in 1.5% ferrocyanide buffer (Annexe I) for 2 h. Then samples were dehydrated with acetone as follows:

30% acetone 10 min

50% acetone 10 min

70% acetone 10 min

80% acetone 10 min

90% acetone 10 min

Absolute acetone 10 min

Then samples using were incubated in Durcupan ACM Fluka resin (Analitikal Fluka, Buchs, Switzerland):

Resin/acetone; ½ (45 min or overnight) at 4° C

Resin/acetone; 1/1 (2 h or overnight) at 4° C

Resin/acetone; 2/1 (8 h or overnight) at 4° C

Pure resin overnight at 4° C

Afterward, samples were placed and oriented in the blocks with pure resin and kept overnight in an oven at 60° C.

3.7.4.1 Semithin sections

The inclusion of samples in resin allows the production of sections much thinner than with paraffin. Although semithin sections were used as to select regions for further for transmission electron microscopy to obtained a higher resolution.

Sections cut at 1 µm were floated in a water trough attached to the glass knife, and picked up with a shaved applicator stick. The sections were transferred to a drop of water on a clean microscope slide. The slide with floating sections was then placed on a hot plate prewarmed at 60° C. After few minutes, as the liquid warms, the sections spread and adhered to the slide. Without removing the slide from a hot plate, filtered toluidine blue in 1% sodium tetraborate is dropped on the sections. The slide was then heated on a hot plate at 60° C for 30 sec. After staining, rinse slide carefully with distilled water from wash bottle. The drain trained slide was blotted dry and mounted in DPX. The semi-thin section was then examined at the light microscope.

Procedure

a. Block face was trimmed to approximately 1 mm^2 and cutted off one corner so that orientation can be determined for later re-trimming.

b. Semithin sections (approximately 0.5 µm thick) were cutted with a glass knife. A drop of distilled water was approximately placed at 1 cm in diameter on a clean glass slide. The sections were picked up with a wire loop. The loop was then invert to place 2-4 sections into the drop of water on the slide.

c. The slide was label with the block number.

d. The slide was put with the water droplet containing sections on a hot plate adjusted to approximately 60° C and wait until all the water evaporates.

e. The sections were located on the slide and circle the bottom of the slide with a felt-tip marker to indicate their position.

f. Few drops of 1% toluidine blue O in 1% sodium tetraborate were placed into the sections and the slide located on the hot plate. After the edges of the drop of stain began to turn golden (approximately 20-30 sec), the sections were quickly rinsed with a stream of distilled water to wash off the excess stain. Even if the stain dried down completely, the sections were useable. When washing, the stream was directed slightly above the sections with the slide tilted at about a 45° angle above a catch basin for the excess stain. When the stream was pointed directly at the sections, they may come off the slide from the water pressure. If the sections came off the slide too easily when washing, they were too thick or the hot plate temperature is too low. The washed slide was then replaced on the hot plate after gently drying the bottom of the slide with a piece of paper towel and wait for the residual water to evaporate from the slide surface.

g. The slide was removed from the hot plate and add 1-2 drops of polymount

mounting medium to the top of the sections adhering to the slide. coverslip (#1.5) was gently lowered a 22 mm X 22 mm over the droplet of polymount over the sections.

h. The stained semithin sections were examined by compound light microscope.

i. A cartoon of the block face was drawed with the cut-off corner on a block sheet and the area of interest that should appear in the ultrathin sections sketched. The block face for ultrathin sectioning was approximately 0.5 mm^2. The block could be longer than 0.5 mm, but was not wider than 0.5 mm, to allow diamond knife edge sectioning.

3.7.4.2 Ultra thin sections

It is well known that, semithin section is useful and give a general idea of the orientation of the tissue, and for pinpointing areas of interest in the block face prior to further trimming of the block for ultrathin sectioning.

Ultrathin sections were cutted (approximately 50 nm) using an ultramicrotome (model LKB Ultratome Nova Leica, Germany) and diamond knife. Once prepared, the ultra-thin sections were mounted on copper grids with a diameter of about 3.05 mm and can be of different shapes and material. The grids with sections were contrasted with lead citrate. The sections were studied and photographed using an electron microscope of Electron Microscopy Service of ULPGC.

Procedure

a. The block was mounted and examine it with the dissecting microscope and compare the block face to the cartoon drawn previously from the semithin sections. The final block face was approximately 0.5 mm^2. It is also permissable to trim the block face so that it is 0.5mm x 1 mm, as long as the 0.5 mm wide side was the side presented to the diamond knife edge. If there was the potential for splitting of the sample surface from the resin block, orient the

block so that the splittable surface was perpendicular to the direction of cutting.

b. Ultra-thin sections were cutted approximately 50 nm thick for standard work (clearly gold in color by diffuse light examination while floating in the knife boat).

c. Around 5-6 sections were picked up for standard work (formvar-coated slot grids, Annexe I). The sections were picked up by orienting them into a block with an eye-brow lash tool and then submerging a clean grid and bringing the shiny side up underneath the floating sections. Try to get them into the center of the grid.

d. Grid with sections was blotted from the bottom with a clean piece of whatman filter paper to remove water on the grid. A clean piece of the filter paper was runned down between the forcep jaws to absorb the water drawn between the jaws by capillary action. The grid was placed down on a clean piece of filter paper in a plastic petri dish, pushing it out of the forcep jaws with the filter paper piece used to remove the capillary water.

e. The grid was stained during approximately 5 min in Reynolds' Lead Citrate (Annexe I). The lead citrate stain was removed from the 4° C refrigerator just before use. A clean pasteur pipet was used to obtain a droplet of stain solution from below the surface of the staining solution in the 50 ml volumetric flask. The droplet of the stain was placed onto a piece of parafilm tacked to the bottom of a plastic petri dish. The dish was covered and well away from your breath, since CO_2 was caused formation of lead carbonate that was seen on sections as a black, granular precipitate. When the petri dish lid was opened, the dry grid section-side (shiny side) down on the droplet of stain was touched and then the grid was inverted. Immediately the petri dish was covered to decrease exposure to CO_2. While the grid was staining, rinse all three rinsing beakers with deionized water and re-filled them for the next rinsing step.

f. After about 5 min, the grid was quickly removed from the stain and inserted it into a beaker of fresh deionized water. This was very important, since the stain was attracted CO_2 from the air and produced lead carbonate dirt on the sections.

g. The grid was jog up and down in the water about 10 times and cleaned in the next four beaker with water. The grid was remove from the beaker, inserted a piece of clean whatman filter paper between the forcep jaws to absorb the stain and water drawn up by capillary action.

h. The stained grid was putted into a grid box and filled out the block sheet so that it was relocated and filled out the small file sheet associated with the grid box as well. After staining the grid, a check mark was putted after the grid number on the small file sheet associated with the grid box as a reminder that it had been post-stained.

i. The filter paper and parafilm that has stain on it was putted in the waste box at the staining station, the rinse beakers rinsed out with deionized water and left upside down to dry at the deionized water sink, and all pipets were putted into the waste glass containers.

j. The lead citrate stain is kept in the refrigerator at approximately 4° C.

k. Used caution when working with these stains (heavy metals and toxic to humans).

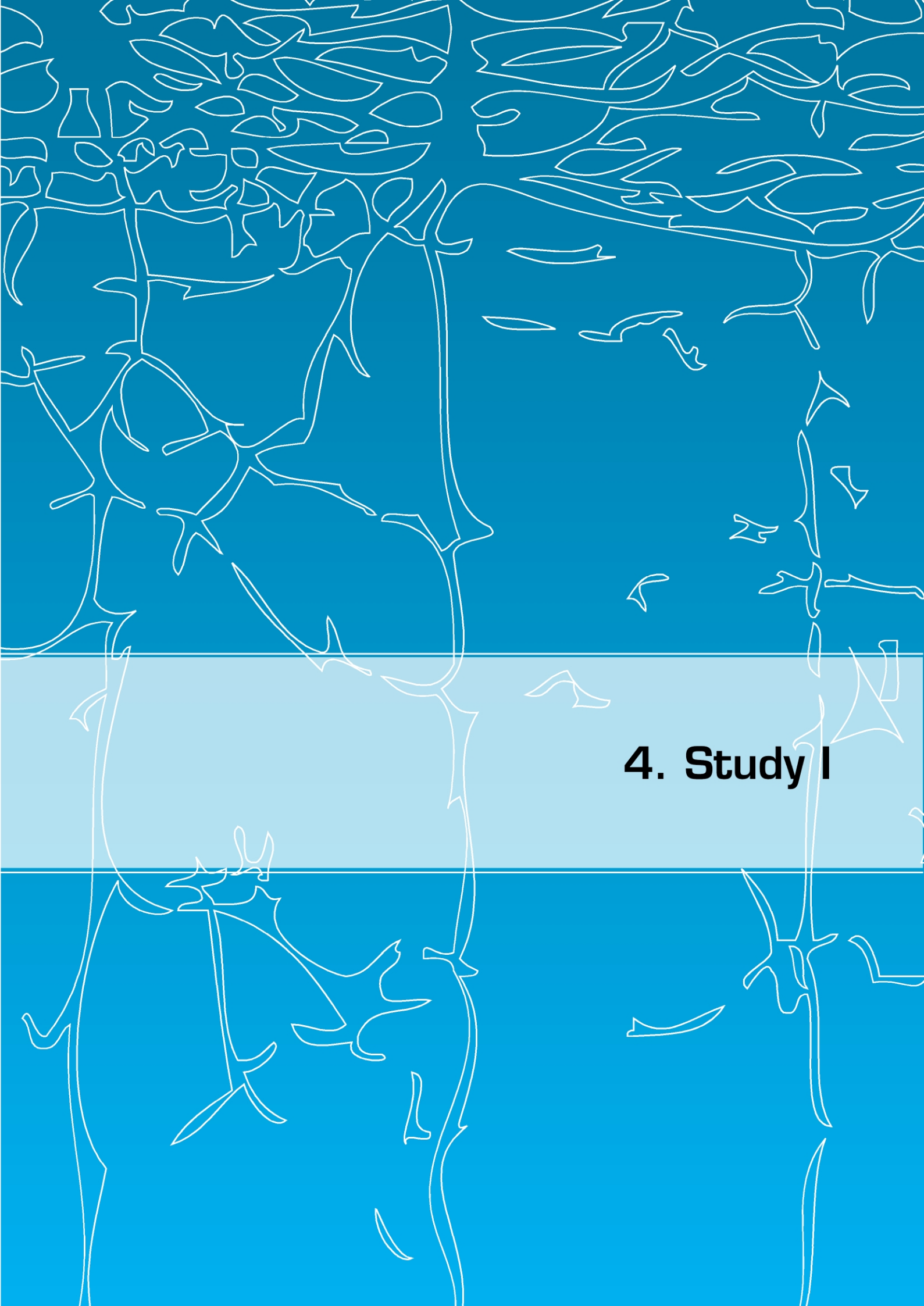
3.8 Statistical analysis

The mean and standard deviations were calculated for each parameter measured. Results were expressed as mean ± standard deviation and analyzed with the software using SPSS (SPSS 11.5 for Windows, SPSS Inc, Chicago, IL, USA). Differences between groups were determined by one-way analysis of variance (ANOVA) following general linear model:

$$Yijk = \mu^* + Fi + Hj + (FH)\ ij + \varepsilon ijk$$

Where is the average Yijk tank, μ is the mean of the population, Fi is the fixed effect of a factor, Hj is the fixed effect of another factor (eg PUFA) (FH) ij the interaction between the fixed effects and εijk is the residual error.

The normality of the variable distribution was verified using the Levene's test. Logarithmic transformation was used to normalize variables when necessary and Duncan's test or Student's t-test was employed to compare means of fatty acids and behavioural studies results ($P<0.05$).



4. Study I

Study I

Dietary DHA deficiency induce a reduced response in gilthead seabream (*Spaurs aurata*) larvae

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Abstract

Developmental changes of swimming speed were analysed in the seabream (*Sparus aurata*). Four feeding regimes using live preys (rotifer *Brachionus plicatilis*) enriched with fish oil, soybean oil, linseed oil and rapseed oil, differing in fatty acid profile were tested during the first weeks of larval life. There was an increase in burst swimming speed and cruise swimming speed during the visual stimulus experiment at day 16th of life in the present study in agreement with the better eye development in larvae of this age. Swimming activity before stimulus was significantly reduced rotifers enriched with vegetable oils. Larvae fed with rotifers enriched with fish oil reacted with a higher burst speed after a visual stimulus than after the sound stimulus (159.5 SL/s vs. 18.30 SL/s) denoting the importance of the vision during the period of the development not only for the predation but also for the burst. The reduction in dietary essential fatty acid contents, by the enrichment with vegetable oils, delays the appearance of response to visual stimulus, an agreement with the minor DHA content in eyes and brains of these larvae and suggesting a delay in the functional development of brain and vision. The results suggest that DHA-deficient fish show relatively slow burst swimming speed when visual stimuli were provided compared to those stimulated by sound stimuli.

Keywords: Seabream larvae, essential fatty acids, behaviour, visual stimulus, sound stimulus, DHA, EPA, cruise swimming speed, burst swimming speed.

Introduction

Sparus aurata is one of the most important products of the European aquaculture. Despite the good knowledge on the biology of this species, there is a lack of studies in areas like behaviour in intensive rearing. Description of the normal pattern of behaviour in fish larvae constitutes a powerful tool to study larval development since delays in the appearance of those patterns or deviations from the typical conduct in certain individuals or patches of larvae may constitute an effective non-invasive indicator of health, development and maturity of these fish. For instance, schooling behaviour is crucial to understand the great fluctuations from year to year which occur in wild fish stocks and hence numerous studies have focused the schooling behaviour of juvenile and adult fish (Pincher and Hart, 1982). However, only few studies focus on developmental aspects of behaviour in larvae. Despite numerous factors affect behavioural development, certain types of behaviour, such as schooling, seem to rely more on the proper development of central nervous system than in other causes such as alterations of sensorial organs or swimming capacity (Masuda and Tsukamoto, 1998). In turn deficiencies in certain nutrients, such as essentials fatty acids, markedly affect the normal development of the brain (Masuda *et al.*, 1999).

Three very long chain polyunsaturated fatty acids, namely docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have a variety of very important functions in fish species, as in most vertebrates. Inadequate contents of those dietary essential fatty acids (EFA) give rise to several alterations such as poor feeding and swimming activities, poor growth and dropping mortality, fatty livers, abnormal pigmentation, disgregation of gill epithelia, immune-deficiency and raised basal cortisol levels (Izquierdo, 1996). Besides, an inappropriate dietary content of such fatty acids in diets for broodstock reduces fecundity and fertilization rates, originate embryo deformities and damage larval quality. In gilthead seabream larvae, DHA, EPA and in some extend ARA, have been also shown to be determinant of growth and survival performance (Izquierdo *et al.*, 2000, 2001). Thus, increasing dietary EFA either in live food or in microdiets

improves larval growth, survival and stress resistance (Koven *et al.*, 1990; Rodríguez *et al.*, 1994; Watanabe and Kiron, 1994; Izquierdo, 1996; Bessonart, 1997; Salhi, 1997; Sargent *et al.*, 1999). EFA, particularly DHA, are also necessary for the normal development of nervous system and sensory organs, larval eye and brain fatty acid composition clearly reflecting that of the diet (Navarro *et al.*, 1995). Despite variations in the dietary level of such fatty acids would markedly affect behaviour, few studies have been conducted to elucidate the effect of EFA on larval behaviour. For instance, in yellowtail larvae (*Seriola quinqueradiata*) dietary DHA has been shown to affect ontogeny of schooling behaviour as well as brain development (Ishizaki *et al.*, 2001). Since very few studies have been aimed to determine the ontogeny of behaviour in gilthead seabream, the effect of dietary fatty acids on behaviour of this larva is also unknown.

At present, a stable high quality juveniles production is required to satisfy the constant increase in the production of gilthead seabream, a major species in Mediterranean aquaculture. Hence, finding of early noninvasive quality indicators such as behavioural patterns is of primary importance for commercial hatcheries. However, ontogeny of behaviour and its dependence on feeding is poorly understood in gilthead seabream. This study aimed to determine the parallelism between behavioural responses to different stimulus along the larval gilthead seabream development and the effect of distinct feeding regimes.

Materials and Methods

S. aurata eggs were obtained from natural spawning from broodstock of the ICCM (Instituto Canario de Ciencias Marinas, Las Palmas de Gran Canaria) and were distributed into sixteen 170 L fibreglass cylindrical tanks (100 eggs/L) filled with 50 µm filtered sea water at 21.28 °C±0.44 provided with constant aeration and water flow (0.5 L/min). From first feeding to day 4, water was stagnant, adding new water just to keep the water quality. Photoperiod of 12 h artificial light was kept constant during the experimental

period and no microalgae were added to the rearing tanks to obtain a better control of the EFA consumed by the larvae. From day 4th after hatching, larvae were fed with rotifers (*Brachionus plicatilis*) twice a day (at 900 h and 1500 h) for the following 20 days. In order to see the effect of different dietary fatty acids profiles, four types of rotifers were fed: "FO rotifers" enriched with fish oil, "SBO rotifers" enriched with soybean oil, "LSO rotifers" enriched with linseed oil and "RSO rotifers" enriched with rapeseed oil. Each type of rotifers was tested in four larval rearing tanks. Emulsions were prepared with 2 g of oil, 5 g of soybean lecithin and 400 ml fresh water mixed in a blender for 2 min. This emulsion was added to the 30 L of rotifer culture at a concentration of 250 rotifers/ml during 12 h. Rotifers concentrations in the larval tanks were kept at 5, 7, and 10 rotifers/ml until days 8, 15 and afterwards, respectively. Samples of rotifers were taken three times along the experimental period and stored at –80 °C until lipid analysis.

Larval growth was assessed by determination of larval standard length at 1, 6, 10, 16 and 19 days after hatching by a profile projector (Nikon V-12A, Nikon, Tokyo, Japan). At day 10, larvae from one tank of each diet were sacrificed for analysis of their total lipid content and fatty acid composition. At the end of the experimental period, one hundred larvae were separated for eyes and brain dissection, whereas the remaining alive larvae were stored at –80 °C until lipid analysis.

Methyl esters of fatty acids were obtained by transesterification with 1% sulfuric acid and methanol using heneicosanoic acid (10% of total lipids) as an internal standard. The fatty acid methyl esters obtained were separated by gas chromatography (ShimadzuGC-14 a, Kyoto, Japan) run at the operating conditions described previously by Izquierdo and Gil (1998), quantified by flame ionisation detectors (FID) and identified by comparison to well characterized external standards.

Swimming speed of larvae from all groups was determined at 6, 10, 16 and 19 days after hatching, respectively, in a 1 L glass beaker (10 cm in diameter) with a water depth of 4 cm. This beaker was covered with a black vinyl sheet only when sound stimuli was applied. Each larva was transferred

from the feeding tanks to the experimental beaker and then video-recorded using a Sony digital video camera DCR-TRV27. After recording for 90 s without disturbance in order to determine cruise swimming speed, larvae was scared by sound and visual stimuli to introduce a startle response and determine cruise swimming speed. Consistent sound stimuli were produced using a steal nut (≈10 g) hung by a string (26 cm) that was released from a distance of 18 cm from the beaker wall. Sound stimuli were provided three times at 10 s intervals for each larva. Visual stimuli were produced using a flash from a distance of 10 cm from the beaker wall. After this operation, larvae standard length (SL) was measured by a profile projector (Nikon V-12A, Nikon, Tokyo, Japan). This procedure was repeated using 5 individuals of each rearing tank (Masuda *et al.*, 2002) for each stimulus.

Video analysis was conducted frame by frame to calculate cruise swimming speed recording and burst swimming speed. Cruise swimming speed estimation was based on the 10 s video recording from 30 s after the recording was started. The movement of the fish was traced on an overhead projector transparency sheet, and this distance was divided by the time taken (10 s) obtaining the cruise swimming speed. To observe the response development to both stimuli, burst swimming rate was calculated by dividing the number of responses by the number of trials. Burst swimming speed was calculated only when fish showed an obvious startle response. After each stimulus, larval movement was traced for four consecutive frames, and the distance was divided by the time taken (4/30 s). Preliminary observations revealed that the faster movement appeared in any of the first four frames after providing a stimulus. Burst swimming speed was calculated as the average of the movement of four frames and defines it as the burst swimming speed. Both cruise swimming and burst swimming speeds were divided by the SL of each individual (Masuda *et al.*, 2002).

Data were statistically analyzed with the software STATGRAPHICS PLUS for Windows 3.1 (Statistical Graphics Corp., Englewood Cliffs, NJ, USA) using one way analysis of variance (ANOVA) and Duncan test ($P<0.05$) for multiple comparison of means was applied.

Results

Fatty acid analysis of prey, fish whole body, and the brain and eyes of experimental fish

Analysis of the fatty acid composition of oils and rotifers showed that the fish oil used contained 17.11% n-3 HUFA as % of total fatty acids, with an EPA (20:5n-3)/ DHA (22:n-3) ratio of 1.03/1 (Table 4.1). This oil also showed the highest content of saturated fatty acids and was rich in 22:1n-11. Soybean oil was rich in fatty acids of the n-6 series, particularly 18:2n-6 and in a lesser extent oleic acid (18:1n-9), whereas linseed oil showed the greatest proportion of n-3 fatty acids, mainly linolenic acid (18:3n-3). Finally, rapeseed oil was characterized by a high proportion of n-9 fatty acids due to the high content in oleic acid. As expected, rotifers enriched with these oils reflected their particular enrichment oil fatty acid composition (Table 4.1). Hence, rotifers fed with vegetable oils showed a lower n-3 HUFA content than fish oil enriched ones. Although the highest DHA content was found in FO rotifers, EPA/DHA ratios were highest (1.2) for rotifers fed fish oil and rapeseed oil, and only 0.4 and 0.5 for rotifers fed soybean or linseed oils. Linoleic acid was high in all enriched rotifers, reflecting the use of soybean lecithin as an emulsifier, but it was about 50% higher in rotifers enriched with soybean and rapeseed oils.

Regarding larval composition (Table 4.2), at the beginning the exogenous phase (3 day-old initial larvae), the main fatty acids of total lipids from larvae were 22:6n-3>16:0>18:1n-9>18:2n-6>16:1n-7>18:0>20:5n-3. Besides, EPA/DHA ratio in larval total lipids was very low (1/5.04). Larvae fed rotifers enriched with vegetable oils progressively reduced the n-3 HUFA proportion (both EPA and DHA in the same proportion), except for larvae fed FO rotifers. Hence, in FO larvae a slight reduction in n-3 HUFA proportion was found at day 10, followed by an increase in 20 d larvae, particularly due to the EPA increase. Nevertheless, the similar DHA contents between larvae fed rotifers enriched FO and the initial ones suggested the adequate level of this fatty acid in such rotifers to cover EFA requirements of seabream larvae. Compared to FO larvae, larvae fed rotifers enriched with vegetable oils showed lower proportion 22:1n-11, a fatty acid particularly rich in FO. Besides, higher

contents of certain Δ6 desaturase products such as 18:3n-9 in SBO larvae and 18:3n-6 in SBO and LSO were found, despite they were not detected in the rotifers.

Table 4.3 shows the results of the lipid analyses for the determination of total lipids of brain and eye fatty acids in larvae of 20 days fed with the different diets. In FO larvae EPA/DHA ratio in eye samples was lower than in larval total lipids. Saturated fatty acids proportion was also higher in brain and eyes than in larval total lipids, although in eyes of SBO larvae the proportion was lower than in the rest of larvae. Both tissues fatty acids composition reflected to some extend that of the fed rotifers. Hence, linoleic acid was highly incorporated into brain and, in a higher extend, eye lipids of larvae fed rotifers enriched with rapeseed and, particularly, soybean oils. Linolenic acid was only slightly higher in brain and eyes of LSO larvae. Docosahexaenoic and, particularly, eicosapentaenoic acids were markedly reduced in larvae fed rotifers enriched with vegetable oils. Compared to FO larvae higher 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3 contents in eyes of larvae fed with rotifers enriched with linseed oil, rich in linolenic acid (18:3n-3) but low in those very long chain fatty acids was found.

Table 4.1 Some fatty acids contents in total lipids from oils and enriched rotifers used to feed gilthead seabream larvae (% total determined fatty acids, n=3).

Fatty acids	FO oil	SBO oil	LSO oil	RSO oil	FO rotifers	SBO rotifers	LSO rotifers	RSO rotifers
14:0	0.28	0.08	0.07	0.02	6.98	1.45	0.06	1.38
14:1	0.34	n.d.	n.d.	n.d.	1.10	0.99	0.01	0.53
15:0	0.08	0.02	n.d.	n.d.	0.74	0.28	0.03	0.31
16:0	13.23	11.18	6.95	4.88	18.99	8.99	5.39	10.89
16:1n-7	7.61	0.01	n.d.	0.46	30.62	9.57	0.12	15.49
16:1n-5	0.12	0.08	n.d.	n.d.	0.79	0.36	0.01	0.03
17:0	0.48	0.07	n.d.	n.d.	1.44	0.67	0.04	0.15
16:4n-4	0.45	0.05	n.d.	n.d.	0.33	0.69	0.01	0.57
18:0	1.46	3.39	0.97	1.31	0.98	0.86	2.85	0.71
18:1 (n-9+ n-7)	12.53	27.13	3.21	63.37	31.10	24.23	19.64	71.86
18:2n-6	1.56	51.39	14.80	20.72	2030	35.42	14.89	33.52
18:3n-3	0.88	5.04	53.67	n.d.	3.52	3.76	56.42	9.51
18:4n-3	2.29	n.d.	n.d.	8.03	3.33	0.13	n.d.	0.04
18:4n-1	0.15	n.d.	n.d.	n.d.	0.26	0.11	n.d.	n.d.
20:0	0.13	0.36	n.d.	n.d.	0.18	0.13	0.05	0.28
20:1n-9	12.92	n.d.	0.15	1.17	10.25	1.70	0.14	3.68
20:1n-7	n.d.	0.25	0.23	n.d.	n,d,	0.43	n.d.	n.d.
20:2n-9	0.20	n.d.	n.d.	n.d.	0.65	0.25	n.d.	0.11
20:2n-6	n.d.	n.d.	n.d.	n.d.	0.52	0.74	n.d.	0.36
20:3n-6	0.09	0.03	n.d.	n.d.	0.13	0.15	n.d.	0.07
20:4n-6	0.02	0.02	0.01	n.d.	0.69	0.61	0.01	0.11
20:3n-3	0.15	0.02	n.d.	n.d.	0.15	0.09	0.02	0.13
20:4n-3	0.43	0.02	n.d.	n.d.	1.16	0.15	0.02	0.19
20:5n-3	8.12	0.48	n.d.	n.d.	13.47	0.90	0.02	0.36
22:1n-11	16.63	0.03	n.d.	0.01	11.25	0.70	0.02	1.62
22:3n-6	0.28	n.d.	n.d.	n.d.	0.45	0.35	0.01	0.07
22:4n-6	0.02	n.d.	n.d.	n.d.	0.07	0.29	n.d.	n.d.
22:5n-6	0.11	0.05	n.d.	n.d.	0.16	0.01	n.d.	n.d.
22:4n-3	0.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.10
22:5n-3	0.45	n.d.	n.d.	n.d.	0.86	0.29	0.02	0.08
22:6n-3	7.91	0.18	n.d.	n.d.	11.19	2.16	0.04	0.31
Saturated	21.79	15.10	8.20	6.20	21.02	12.49	8.44	13.76
Monounsaturated	53.98	27.59	3.59	65.01	59.15	37.78	20.05	93.18
n-3	20.28	5.73	53.67	8.03	17.81	10.17	56.53	13.77
n-6	2.34	51.47	14.81	20.72	0.48	37.63	14.91	34.14
n-9	25.74	25.72	23.10	64.54	29.84	26.49	19.89	76.27
n-3HUFA	17.11	0.69	n.d.	n.d.	17.81	3.59	0.11	1.17
AA/EPA	n.d.	0.04	n.d.	n.d.	0.05	0.68	0.55	0.32
EPA/DHA	1.03	0.04	n.d.	n.d.	1.20	0.42	0.48	1.13
n-3 HUFA % dry wt					15.77	3.18	0.10	1.04

n.d. ≤ 0.005

Table 4.2 Fatty acids composition of initial 3 day-old larvae and 10 and 20 day-old larvae for each experimental group (n.d. ≤ 0.005).

Fatty acid	3 d old	FO 10d	SBO 10d	LSO 10d	RSO 10d	FO 20d	SBO 20d	LSO 20d	RSO 20d
14:0	2.56	1.41	1.09	1.17	0.91	1.82	0.86	0.48	0.52
14:1	0.08	0.86	0.35	0.38	0.27	0.48	0.49	0.02	0.33
15:0	0.30	0.15	0.40	0.44	0.51	0.81	0.60	0.09	0.13
16:0iso	0.43	0.46	0.38	0.41	0.34	0.36	0.26	0.11	0.19
16:0	20.50	10.32	13.44	14.45	16.27	17.72	15.15	7.09	10.40
16:1n-7	6.54	9.39	5.84	6.28	5.72	11.27	1.20	4.91	5.90
16:1n-5	0.08	0.46	0.36	0.39	0.31	0.69	4.32	0.48	0.17
16:2	0.48	0.95	0.57	0.61	0.70	1.36	0.78	0.52	0.72
17:0	0.38	0.53	0.65	0.67	0.66	1.21	0.32	0.17	0.74
16:4n4	n.d.	0.06	n.d.	0.18	0.42	0.89	0.33	0.20	0.31
16:4n-3	0.18	4.49	0.04	7.74	0.22	12.20	0.51	4.40	0.06
18:0	4.91	0.87	7.20	0.92	9.70	0.84	0.56	0.47	5.28
18:1(n-9+ n-7)	19.57	14.23	7.41	14.98	32.18	3.53	20.17	28.21	39.87
18:1n-5	0.29	0.99	n.d.	0.70	n.d.	0.32	0.33	0.07	n.d.
18:2n-9	0.20	n.d.	0.65	n.d.	n.d.	0.32	0.78	0.19	0.13
18:2n-6	7.88	8.56	20.37	0.10	14.66	0.15	28.73	14.40	16.56
18:3n-9	0.07	0.10	1.26	n.d.	n.d.	0,17	0.42	n.d.	n.d.
18:3n-6	0.19	0.12	1.01	1.08	n.d.	1.13	0.51	0.31	n.d.
18:4n-6	n.d.	1.39	0,51	n.d.	n.d.	0.21	n.d.	n.d.	n.d.
18:3n-3	0.13	0.06	n.d.	21.90	1.80	0.66	2.14	23.85	2.93
18:4n-3	1.39	0.51	0.09	0.07	n.d.	0.12	0.24	0.35	0.12
18:4n-1	0.43	0.05	0.12	n.d.	n.d.	0.14	0.28	n.d.	n.d.
20:0	0.15	0.10	0.06	0.21	0.09	0.26	0.37	0.13	0.35
20:1n-9	1.24	2.64	0.20	2.01	2.71	3.07	1.05	1.09	1.68
20:2n-9	0.06	0.43	n.d.	0.26	0.27	0.41	n.d.	0.10	0.29
20:2n-6	n.d.	0.63	0.24	1.01	0.28	0.85	1.11	0.56	1.18
20:3n-6	n.d.	0.19	0.93	0.24	0.16	0.21	0.56	0.11	0.43
20:4n-6	1.10	1.80	0.12	1.40	0.09	1.49	1.18	0.49	1.40
20:3n-3	0.10	0.12	1.31	0.14	1.14	0.11	0.28	1.36	0.33
20:4n-3	0.43	0.71	0.13	0.24	0.19	1.08	0.23	0.34	0.07
20:5n-3	4.59	6.73	0.22	2.20	2.25	9.12	1.67	1.51	1.91
22:1n-11	0.29	1.48	0.71	0.76	0.12	0.81	0.37	0.22	0.47
22:1n-9	0.11	n.d.	0.30	0.32	n.d.	0.66	0.20	0.34	n.d.
22:1n-7	n.d.	n.d.	0.24	0.26	n.d.	0.44	n.d.	0.22	n.d.
22:3n-6	0.13	0.18	0.05	0.05	0.21	0.72	0.38	0.02	0.06
22:4n-6	0.08	0.13	0.15	n.d.	n.d.	0.15	0.22	n.d.	n.d.
22:5n-6	0.19	0.30	0.21	0.23	n.d.	0.29	0.44	0.14	n.d.
22:4n-3	0.04	n.d.	0.03	n.d.	0.22	0.13	0.21	0.22	0.24
22:5n-3	1.57	1.26	0.47	0.51	0.10	1.88	0.37	0.19	0.23
22:6n-3	23.15	18.38	9.88	10.63	7.50	21.92	6.99	6.66	7.06
Saturated	29.23	13.85	17.02	18.28	28.49	23.02	18.78	8.53	17.60
Monounsat.	28.30	38.54	16.51	32.74	41.0	20.58	28.54	35.08	48.24
n-3	31.58	32.27	20.19	21.62	13.42	47.23	12.65	38.88	12.93
n-6	9.57	13.30	23.64	25.93	15.40	5.19	33.13	16.03	19.62
n-9	21.25	17.41	30.32	17.57	35.16	6.05	22.62	23.56	41.97
n-3HUFA	29.88	27.21	12.78	13.71	11.39	34.25	9.76	10.28	9.83
AA/EPA	0.24	0.27	0.64	0.64	0.04	0.16	0.70	0.32	0.73
EPA/DHA	0.20	0.37	0.21	0.21	0.30	0.42	0.24	0.23	0.27
Total lipids % dry wt	14	17	15.30	16.5	12.2	19.17	16.42	20.75	17.96

Table 4.3 Some fatty acids contents in total lipids from brain and eyes in larvae after 20 days of feeding (% total determined fatty acids, n=3).

Fatty acids	Brain FO	Brain SBO	Brain LSO	Brain RSO	Eyes FO	Eyes SBO	Eyes LSO	Eyes RSO
12:0	5.37	9.12	6.41	9.01	4.29	3.95	6.08	7.13
14:0	3.47	6.25	4.24	4.75	3.21	3.42	3.47	2.71
14:01	1.57	2.42	0.95	n.d.	1.67	0.60	1.73	2.71
15:0	0.74	1.17	0.88	1.68	1.61	1.06	0.50	0.89
16:0iso	2.02	3.97	2.56	2.56	2.68	0.47	0.30	2.59
16:0	17.07	18.10	17.24	20.06	19.50	2.03	16.72	18.40
16:1 n-7	7.01	4.09	3.14	5.94	5.93	19.43	3.45	3.76
16:1n-5	n.d.	2.25	2.33	2.42	2.53	0.13	1.71	0.57
16:02	n.d.	0.54	n.d.	n.d	n.d.	0.26	n.d.	n.d.
17:0	n.d	0.49	0.40	n.d	n.d.	0.19	0.46	0.30
16:4n4	n.d	0.86	n.d	n.d	n.d.	0.71	n.d.	1.49
16:4 n-3	0.66	n.d	0.35	n.d	n.d.	0.72	0.30	n.d.
18:0	10.35	8.30	13.66	9.84	9.27	10.52	10.63	9.99
18:1n-9	13.73	15.54	21.18	17.24	15.81	27.59	19.94	15.98
18:1n-7	3.79	2.05	2.62	2.35	2.47	1.55	2.46	2.48
18:1 n-5	n.d	n.d	0.35	n.d	n.d.	0.17	11.70	0.91
18:2n-9	0.84	n.d	0.42	n.d	n.d.	n.d.	n.d.	0.61
18:2 n-6	7.34	10.94	7.56	13.95	12.29	20.16	3.27	10.90
18:3 n-9	n.d	0.47	n.d	n.d	n.d.	0.94	n.d.	2.32
18: 3n-6	n.d	n.d	0.43	n.d	n.d.	0.23	n.d.	0.47
18: 4 n-6	1.47	1.45	n.d	1.26	1.91	0.13	n.d.	1.67
18:3 n-3	n.d	0.70	3.48	n.d	n.d.	0.47	0.85	0.56
18:4n-3	0.66	n.d	1.04	1.37	1.38	0.98	1.67	2.23
18:4n-1	n.d	1.27	n.d	n.d	n.d.	n.d.	1.33	n.d.
20:0	n.d	n.d	0.84	n.d	0.91	0.69	0.94	0.82
20:1n-9	1.58	0.63	0.83	1.26	0.95	0.67	0.52	1.46
20:1n-7	n.d	0.81	0.41	n.d	n.d.	0.24	0.37	0.30
20:2n-9	n.d	n.d	n.d	n.d	n.d.	0.27	n.d.	n.d.
20:2n-6	n.d	n.d	0.36	n.d	n.d.	0.16	n.d.	1.28
20:3n-6	n.d	n.d	0.62	n.d	n.d.	0.45	n.d.	0.17
20:4n-6	0.99	0.70	0.27	1.08	n.d.	0.17	0.63	0.82
20:3n-3	n.d	n.d	n.d	n.d	n.d.	0.29	0.84	n.d.
20:4n-3	0.87	0.74	1.24	n.d	n.d.	0.49	0.84	1.00
20:5n-3	5.95	1.45	0.49	1.28	2.78	0.34	1.23	0.89
22:1n-11	n.d	n.d	0.42	n.d	n.d.	n.d.	0.54	1.04
22:5n-6	0.66	n.d	n.d	n.d	1.38	n.d.	n.d.	n.d.
22:4n-3	n.d	n.d	n.d	n.d	n.d.	n.d.	1.03	n.d.
22:5n-3	1.65	n.d	0.61	n.d	n.d.	0.20	1.05	0.51
22:6n-3	12.21	5.69	4.66	3.97	9.42	0.34	4.81	3.04
Saturated	39.02	47.39	46.24	47.89	41.49	22.32	39.10	42.82
Monounsat.	27.68	25.55	29.91	26.79	26.83	50.25	41.37	28.66
n-3	22.01	8.58	11.87	6.62	13.58	3.82	12.60	8.23
n-6	10.46	13.09	9.23	16.29	15.57	21.30	3.90	15.30
n-9	16.14	16.64	22.43	18.50	16.76	29.47	21.12	20.38
n-3HUFA	20.68	7.88	6.99	5.25	12.20	1.65	9.79	5.44
EPA/DHA	0.49	0.25	0.10	0.32	0.30	1.00	0.25	0.29

n.d. ≤ 0.005

Growth and behavioural performance

Development of larval growth along the feeding experiment expressed in terms of standard length is recorded in Figure 4.1. After 16 d of feeding a significantly higher growth was found in FO larvae, fed with the highest HUFA level.

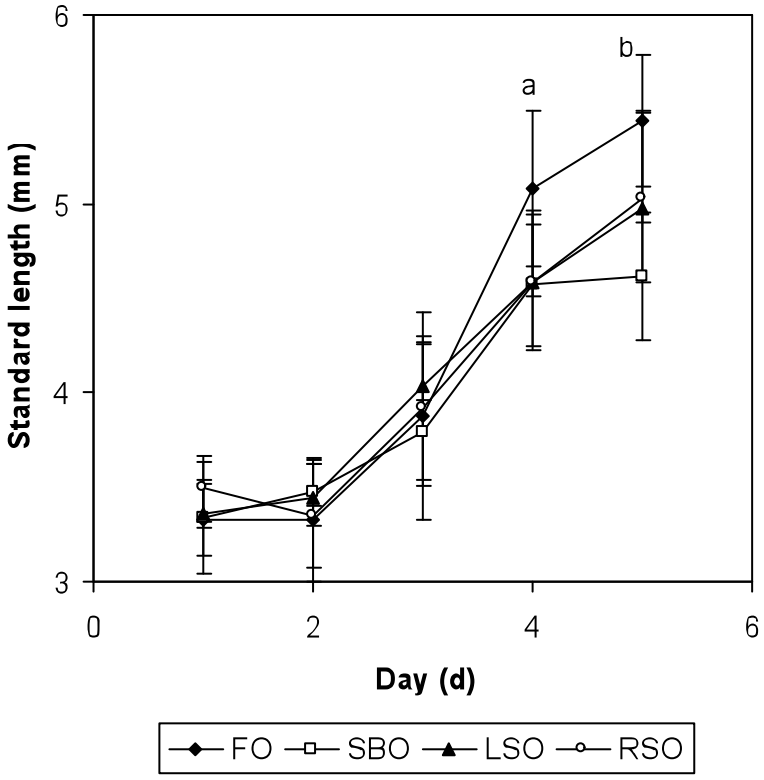

Figure 4.1 Development of growth in seabream larvae fed rotifers enriched with different types of lipids (n = 30, P<0.05).

Cruise swimming speed under conditions of the sound stimuli experiment (dark beaker walls) gradually increased in FO larvae during the first 16 days, dramatically increasing on day 19th (Figure 4.2a). However cruise speed decreased in larvae fed rotifers enriched with vegetable oils from day 10th. Hence, at the end of the experiment, cruise swimming speed in FO larvae was significantly higher than those of fish fed rotifers enriched with vegetable oils. Number of reacting larvae after the sound stimuli (Figure 4.2b) increased with age. Burst swimming speed was not affected by larval growth or dietary fatty acids during this stage of development, although at day 10th, a slightly higher

burst swimming speed was found in FO larvae, in comparison with larvae fed rotifers enriched with soybean oil (Figure 4.2c).

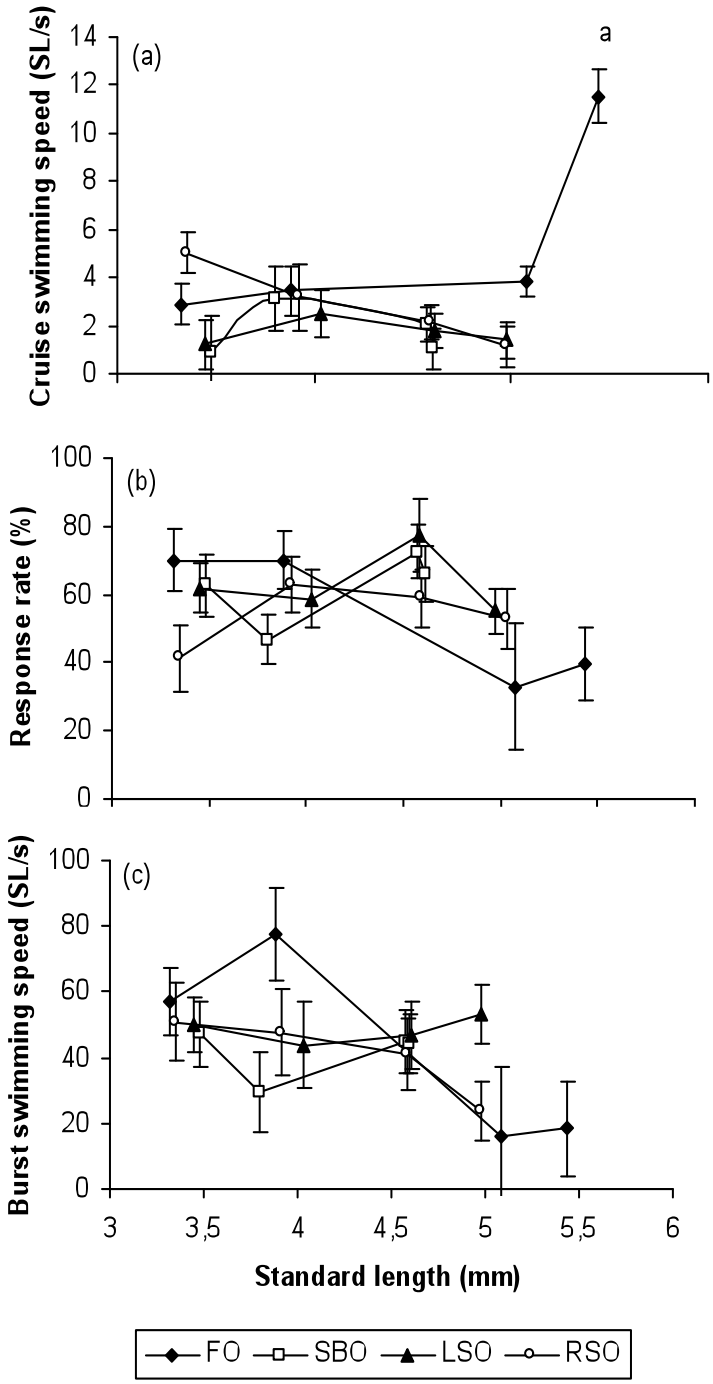

Figure 4.2 (a) Development of cruise swimming speed (mm/s) under conditions of sound stimuli experiment (dark walls) in seabream larvae fed rotifers enriched with different types of lipids (n = 10, P<0.05). (b) Number of reacting seabream larvae for each experimental group after the sound stimuli. (c) Development of burst swimming speed (mm/s) after sound stimuli in seabream larvae fed rotifers enriched with different types of lipids (n = 10, P<0.05).

Cruise swimming speed under the conditions of visual stimuli experiments (clear walls) increased with age along larval development, particularly in FO larvae which showed a significantly higher speed than larvae fed rotifers enriched with vegetable oils from day 16th (Figure 4.3a). However, almost no larvae reacted to visual stimulus during the first day of experiment, whereas from day 10th an increasingly higher number of larvae reacted after the stimuli (Figure 4.3b). Burst swimming speed, after the visual stimuli, increased also along larval development. FO larvae showed a significantly higher burst swimming speed than larvae fed vegetable oils enriched rotifers from day 16th. However, burst swimming speed of larvae fed LSO rotifers increased at the end of the experiment and was not significantly different than that of FO larvae (Figure 4.3c).

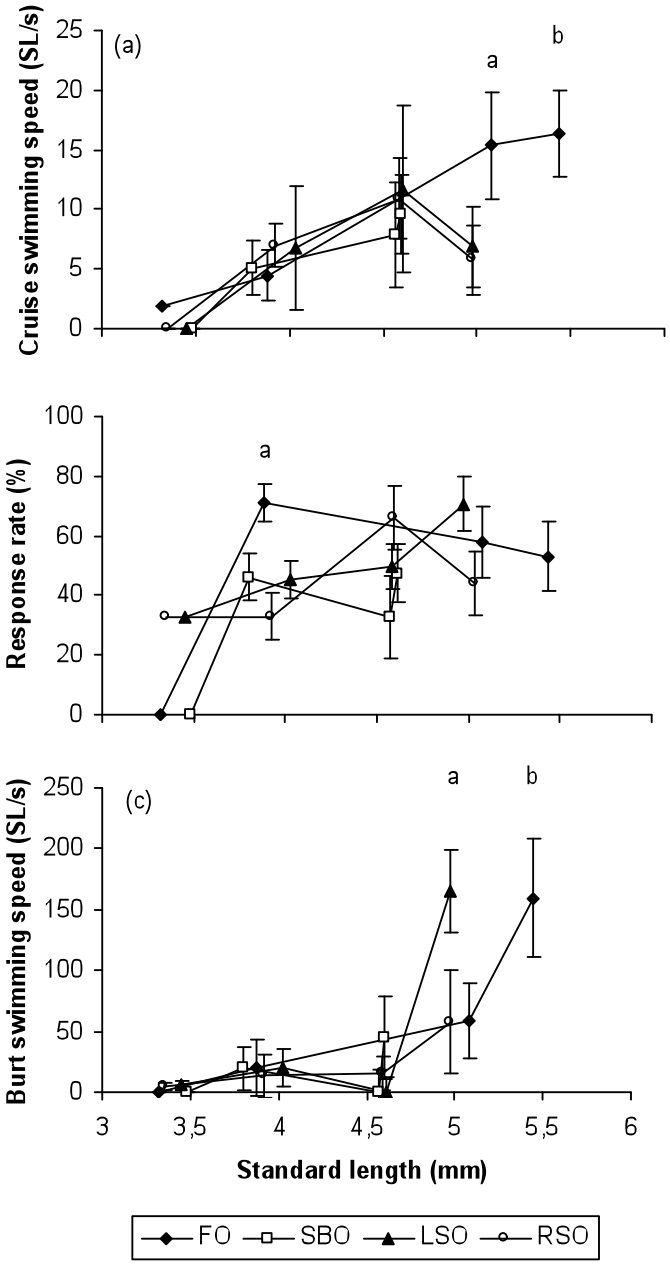

Figure 4.3 (a) Development of cruise swimming speed (mm/s) under conditions of visual stimuli experiment (clear walls) in seabream larvae fed rotifers enriched with different types of lipids (n = 10, P<0.05). (b) Number of reacting seabream larvae for each experimental group after the sound stimuli. (c) Development of burst swimming speed (mm/s) after visual stimuli (clear walls) in seabream larvae fed rotifers enriched with different types of lipids (n = 10, P<0.05).

Discussion

Biochemical aspect of DHA deficiency

Growth results showed that fish oil replacement by vegetable oils negatively affected gilthead seabream larval growth. Hence, larvae fed fish oil enriched rotifers showed a significant ($P<0.05$) increase in growth in comparison with larvae from the other treatments, which seemed to be related with the higher n-3 HUFA and DHA levels found in fish oil enriched rotifers. This is in agreement with other author's results (Rodríguez *et al.*, 1994; Izquierdo, 1996). Throughout larval development, several authors indicate that the essential fatty acid requirements in gilthead seabream larvae are around 1.5% n-3 HUFA in dry matter, when larvae are fed with any type of prey (Rodríguez *et al.*, 1998) or microdiets, independently of the dietary lipid content (Salhi *et al.*, 1994, 1999). Results of the present study showed that the n-3 HUFA contents in rotifers enriched with vegetable oils were lower than 0.19, being below the minimum level necessary to cover the fatty acid requirements of this species. Regarding the DHA, minimum requirements in gilthead seabream larvae are around 0.8% dry matter (Rodríguez *et al.*, 1998; Salhi *et al.,* 1999), whereas deleterious effects of excess of this fatty acid in diets for larvae have not been found yet (Izquierdo, 2005). Thus, high levels (5% dry base) of dietary DHA in microdiets have not caused any problem skipjack tuna (*Katsuwonus pelamis*). Bell *et al.* (1995), feeding larval herring (*Clupea harengus* L.) on a DHAfree diet reduce efficiency of prey capture in conditions of low light intensities, probably affecting rods receptors development in the retina. In the present study the retention of DHA and n-3 HUFA in larvae fed rotifers enriched with fish oil (10 and 20 days after hatching) confirmed their importance in the larval development. Besides, fatty acids composition in both brain and eyes, revealed a retention of n-3 HUFA, particularly DHA, even in larvae fed rotifers low in these fatty acids.

Development of response against sound and visual stimuli

Fish larval are visual predators, the larvae trofic behaviour is intimately related with the development of visual ability (Izquierdo, 2005). In sparides,

like gilthead seabream and red porgy (*Pagrus pagrus*) the most important changes in ocular structure occur during the lecitotrofic phase (Roo *et al.*, 1999). The rod photoreceptors for a correct vision in low intensities of light appear in gilthead larvae at the 18th of day. This agrees with the noticeable increase in burst swimming speed and cruise swimming speed during the visual stimulus experiment at day 16th of life in the present study. Both n-3 HUFA, but particularly DHA, play a critical role in functions of neural and retinal tissue. Moreover, elevation of dietary DHA and EPA content, increase gilthead seabream larvae eye diameter (Roo *et al.*, 1999; Izquierdo *et al.*, 2000). This fact, along with the higher density of cone photoreceptors, implies a significant improvement in larval visual potential (Roo *et al.*, 1999). This would explain the greatest burst swimming speed in response to the visual stimuli of larvae fed with rotifers enriched with fish oil in the present experiment and agree with the visual found in incapacity found in yellowtail larvae fed DHA-deficient diets (Masuda *et al.*, 1999).

Along larval development, cruise swimming speed appeared earlier in dark wall beaker (sound stimulus trial) than clear wall ones (visual stimulus trial). Besides, burst swimming activity after sound stimulus appeared earlier than after a visual stimulus. Masuda *et al.* (2002), reported cruise swimming speed for club mackerel *Scomber japonicus* (mm/s and SL/s) in the same conditions of sonorous stimuli than the present study. At day 14 and 17 chub mackerel has a 1.58 and 4.28 SL/s of cruise swimming speed, *S. aurata* fed with rotifers enriched fish oil were at day 19, three times faster. Larvae fed with rotifers enriched with vegetable oils had slower cruise swimming than mackerel. Also in a comparison between SL of both species, mackerel has a bigger size (around 12 mm).

Regarding behaviour of larvae fed rotifers enriched with fish oil, swimming activity before stimulus (cruise swimming) was low during the first 10 days and markedly increased from day 16, denoting the higher development of nervous system along the previous days. Reaction after a sonorous stimulus (burst swimming speed) appeared as early as day 6 after hatching and was about 10 times that of cruise swimming before the stimulus at the same day.

Reaction after a visual stimulus appeared later, at day 10 after hatching, being about 10 times that of cruise speed, but increased from day 16 being from there always higher than that after sonorous stimulus, in agreement with the better eye development at this age (Roo, 1999). The appearance of the cruise swimming speed obtained in the sonorous stimulus by larvae fed with rotifers rich in fish oil presents similarities with other species, such as club mackerel (Masuda *et al.*, 2002) but with inferior absolute values. The same happens with the burst swimming speed. The moment which it begins to detect answer before the stimulus is very similar but the results are totally opposed, since in mackerel they are increased throughout all the experiment.

Swimming activity before stimulus was significantly reduced by feeding rotifers enriched with vegetable oils. Despite reaction against sonorous stimulus was not affected by feeding vegetable oils, appearance of reaction after visual stimulus was delayed to day 19th in larvae fed LSO rotifers and it was also delayed and significantly reduced by feeding the other vegetable oils. Higher burst swimming speed in larvae fed LSO is in agreement with the higher response to acute stress found in juveniles of the same species fed with linseed oil compared to fish oil (Montero *et al.*, 2003). Besides, larvae fed with rotifers enriched with fish oil reacted with a higher burst speed after a visual stimulus than after the sound stimulus, denoting the importance of the vision during this period of the development not only for the predation but also for the burst.

However, the reduction in dietary essential fatty acid contents, by the enrichment with vegetable oils, delays the appearance of response to visual stimulus, in agreement with the minor DHA content in eyes and brains of these larvae and suggesting a delay in the functional development of brain and vision. In addition, larvae fed with rotifers enriched with fish oil, with levels of n-3 HUFAs sufficient to cover their requirements denoted a greater cruise swimming speed than larvae fed rotifers enriched with vegetable oils with low essential fatty acid content. Lower cruising speed may also be related with lower prey capture efficiency.

Regarding the burst swimming speed, the greatest movement of the burst swimming speed took place in the four first sequences after the performance of the stimulus, in agreement with findings in other fish species (Masuda *et al.*, 2002). Burst swimming speed after visual stimulus was much higher than that obtained after sonorous stimulus. These results indicate once more the importance of visual stimulus and visual sharpness during this period of larval development, which is faster transmitted through seawater and is essential for prey finding and flee from predators. Besides, dietary changes did not affected larval reaction to the sound stimuli, whereas it significantly affected reaction to the visual stimuli. Indeed, higher burst and cruise swimming speed in the larvae fed rotifers enriched with fish oil and linseed oil, would be related to the higher levels of DHA in larval eyes and brain, since this fatty acid is involved in several neural tissue related functions such as neurocytes myelination and synapse construction, both functions being sensitive to nutritional deficiencies (Krigman and Hogan, 1976). A histological study comparison of DHA, EPA, and oleic acid enriched juveniles striped jack (*Longirostris delicatissimus*) demonstrated the DHA enriched juveniles have a more developed superficial white and gray zones on their optic tectum than to the other two groups (Masuda, 1995).

Conclusion

Reduction in the rotifer EFA content by enrichment with vegetable oils, affects larval normal behaviour, reducing cruise swimming speed, and particularly delaying the appearance of the response to visual stimulus, suggesting a delay in the functional development of brain and vision, in agreement with the minor EPA and DHA found in eyes and brains of these larvae.

Acknowledgements

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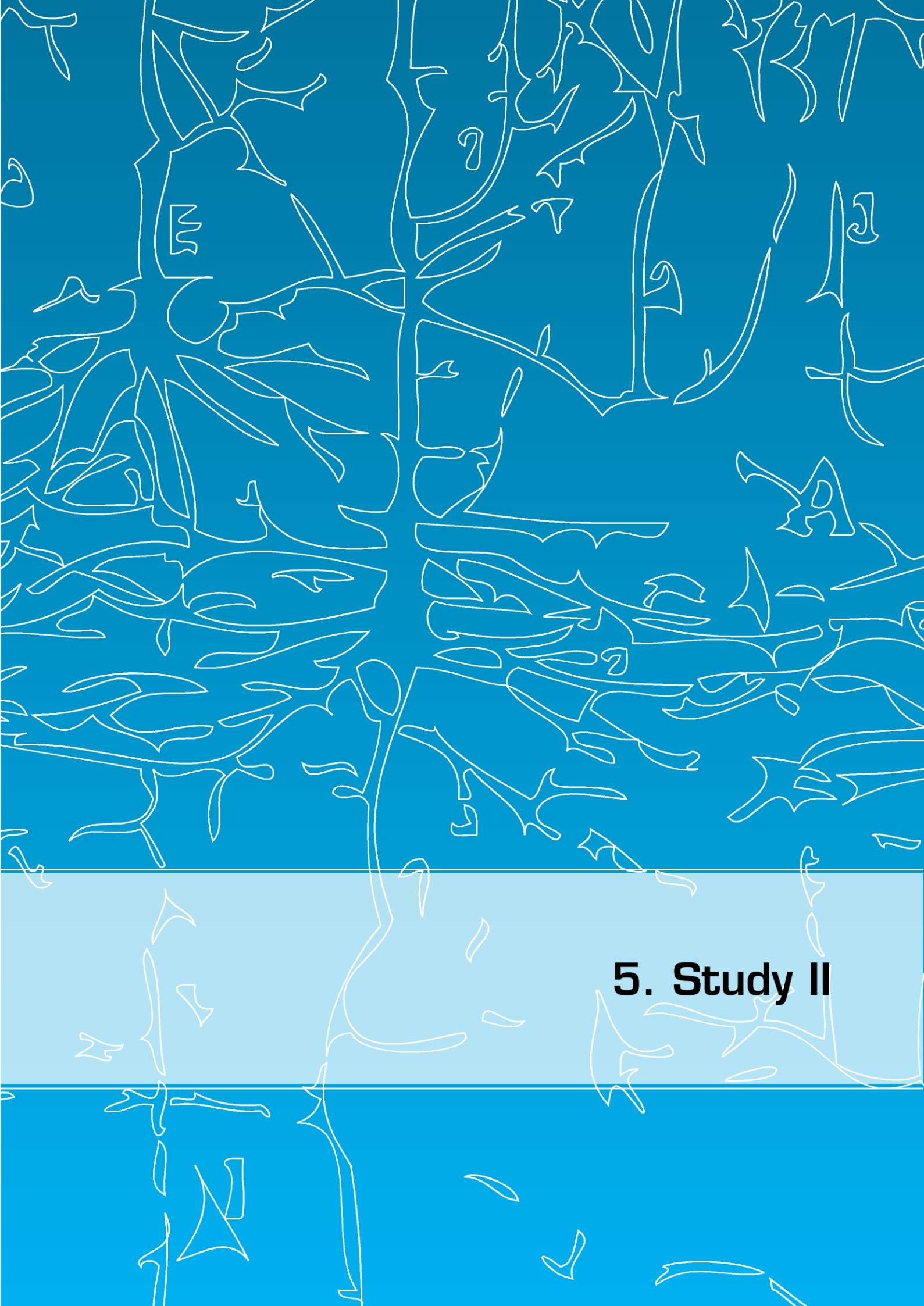

5. Study II

Study II

Increased Mauthner cells activity and escaping behaviour in sea bream (*Spaurs aurata*) fed long chain polyunsaturated fatty acids

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Abstract

There is limited information on the specific effects of long chain polyunsaturated fatty acids (LCPUFA) on neuron development and functioning. Deficiency of those essential fatty acids impairs escape and avoidance behaviour in fish, where Mauthner cells play a particularly important role to initiate this response. Gilthead seabream larvae fed two different LCPUFA profiles were challenged with a sonorous stimulus. Feeding Omega-3 (n-3) LCPUFA increased the content of these fatty acids in fish tissues and caused a higher number of larvae to react to the stimulus and a faster burst swimming speed response. This faster startle response in fish fed n-3 LCPUFA was also associated to an increased immunepositive neural response, particularly in M-cells, denoting a higher production of acetylcholine. The present study shows the first evidence of the effect of n-3 LCPUFA on functioning of particular neurons in fish, the Mauthner cells, and the behaviour response that they modulate to escape from a sound stimulus.

Keywords: Burst swimming behaviour, DHA, fish larvae behaviour, Mauthner cells.

Introduction

Despite Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) have been long recognized as being important for brain development and function, little is known on their specific effects on neuron activity in relation to behaviour. N-3 LCPUFA play important roles in neural growth, development of synaptic processing of neural cell interaction, and expression of genes regulating cell differentiation and growth (Uauy and Dangour, 2006). Essential fatty acid metabolism can influence many aspects of brain development, including neuronal migration, axonal and dendritic growth, and the creation, remodelling and pruning of synaptic connections (Guesnet and Alessandri, 2010; Robson *et al.*, 2010). Animal studies have shown that both neural integrity and function can be permanently disrupted by deficits of n-6 and n-3 fatty acids during foetal and neonatal development (Yamamoto *et al.*, 1988; Bourre *et al.*, 1989). While both n-6 and n-3 fatty acids are required, the n-3 fatty acids such as docosahexaenoic acid (DHA, 22:6n-3) appear to play a special role in highly active sites such as synapses and photoreceptors, and deficiencies have particularly been linked to visual and cognitive deficits (Neuringer *et al.*, 1986; Neuringer *et al.*, 1994).

In marine fish, n-3 LCPUFA are essential and play very important physiological roles (Izquierdo *et al.*, 1989; Izquierdo, 1996). Specifically, DHA and eicosapentaenoic (EPA, 20:5n-3) acids must be supplied in the diet and function as critical structural and physiological components of the cell membranes of most tissues, being necessary for fish growth, welfare, survival and development (Watanabe and Kiron, 1994; Izquierdo *et al.*, 2000). In particular, DHA has been found to be required for normal development of the nervous system and sensory organs, such that larval brain and eye fatty acid compositions reflect the diet (Masuda *et al.*, 1999; Benítez-Santana *et al.*, 2007). Moreover, DHA deficiency impairs vision in juvenile herring (*Clupea harengus*) (Bell *et al.*, 1995). Therefore, dietary fatty acid contents could potentially affect behavior, stress reactions or pain and comfort, despite the lack of studies on this subject in fish. Recently, dietary fatty acids have been

found to affect escape and avoidance behavior in fish larvae after a sound or visual stimulus (Masuda *et al.*, 1999; Benítez-Santana *et al.*, 2007). Fish can elude predatory attacks by producing a stereotyped escape behaviour, which is characterized by a rapid and powerful unilateral bending of the body and caudal fin that involves most of its somatic musculature (Korn and Faber, 2005). This behaviour is initiated by the activation of the Mauthner cells (M-cells).

M-cells integrate diverse sensory inputs (Faber *et al.*, 1989; Eaton *et al.,* 2001) and are able to reset swimming rhythms in the course of its initiation of escape behaviours (Svoboda and Fetcho, 1996). In chronic recordings of freely swimming intact fish, the M-cells have been shown to fire an action potential at the initiation of C-start responses (Zottoli, 1977; Eaton *et al.*, 1988; Canfield and Rose, 1996; Weiss *et al.*, 2004). Because the axons of each M-cell decussate, the firing of one leads to a contraction of trunk musculature that is contralateral to the cell soma (Zottoli, 1977; Foreman and Eaton, 1993). To date there is no evidence of an effect of essential fatty acids on M-cells activity. Therefore, the aim of this study was to better understand the effect of dietary n-3 LCPUFA on fish escape and avoidance behaviour and neural function. For that purpose the effect of two different lipid sources, with different n-3 LCPUFA content, fed to gilthead sea bream (*Sparus aurata*) during early larval and brain development on the fish reaction to a sonorous stimulus and M-cells activity was investigated. M-cells activity was determined by choline acetytransferase (ChAt) distribution by immuno-labelling as a marker of cholinergic neuron density, since the cholinergic neurotransmission system has been found to be sensitive to dietary n-3 PUFA in rats (Aïd *et al.*, 2003).

Material and Methods

All animal studies complied with the guidelines for animal experimentation of our laboratories and were approved by institutional review boards.

Experimental conditions

Six thousand six hundred (6,600) gilthead sea bream larvae of 22 days of age (post-hatch) (5.06±0.59 mm in standard length (SL)) were randomly distributed into six 170 L fiberglass cylindrical tanks and fed in triplicate with 2 experimental diets. Diets differed only in the lipid source to obtain different fatty acid profiles. Lipid sources were fish oil rich in n-3 LCPUFA and soybean oil rich in linoleic acid (18:2n-6). Diets were analyzed for crude lipid content and fatty acid composition (Table 5.1). Microdiets were prepared according to Atalah *et al.* (2010). No significant differences were found in dry lipid content (20.09). All tanks were supplied with filtered seawater (34 ppm salinity) at 19-20º C, constant aeration (125 ml/min), seawater flow (0.4 L/min at the beginning to 1.0 L/min at the end) and artificial light (12 h photoperiod). Feeds (2.5 g/tank/d) were manually supplied fourteen times per day every 45 min during the light period.

Swimming Speed Behaviour

Swimming speed of larvae from each tank was determined at 23, 27 and 34 days of age in a 1 L black wall glass beaker (10 cm diameter) keeping a water depth of 4 cm. Each larva was transferred from the feeding tanks to the glass beaker and then video-recorded using a Sony digital video camera (DCR-TRV27, Sony, Tokyo, Japan). After recording for 30 s, the larva was scared by a sound stimulus to induce a startle response and determine burst-swimming speed. Consistent sound stimuli were produced using a steel nut (Benítez-Santana *et al.*, 2007). Sound stimuli were provided three times at 10 s intervals for each larva. Afterwards, larval standard length (SL) was measured by a profile projector (Nikon V-12A, Nikon, Tokyo, Japan).

Table 5.1 Fatty acid profile of the experimental diets (% total identified fatty acids) used to feed gilthead sea bream larvae.

Fatty acids	FO diet	SBO diet
14:0	0.53	0.85
14:1n-7	0.02	0.04
14:1n-5	0.16	0.05
15:00	0.54	0.22
15:1n-5	n.d.	0.03
16:0ISO	0.06	0.03
16:0	23.37	17.94
16:1n-7	6.83	0.48
16:1n-5	0.38	0.06
16:2n-4	1.09	0.08
17:0	1.22	0.26
16:3n-4	0.29	0.09
16:3n-3	0.03	0.02
16:3n-1	0.07	0.04
16:4n-3	0.58	0.11
16:4n-1	0.16	0.08
18:0	2.38	1.92
18:1n-9+n-7	12.11	19.65
18:1n-5	0.18	0.46
18:2n-6	9.35	34.11
18:2n-4	0.17	n.d.
18:3n-6	0.14	n.d.
18:3n-4	n.d.	2.40
18:3n-3	n.d.	1.47
18:4n-3	1.26	0.07
18:4n-1	0.14	0.02
20:0	0.07	0.17
20:1n-9+n-7	2.75	1.84
20:1n-5	0.22	0.06
20:2n-9	0.07	0.03
20:2n-6	0.22	0.14
20:3n-6	0.12	n.d.
20:4n-6	1.06	0.45
20:3n-3	0.31	0.22
20:4n-3	0.61	0.06
20:5n-3	12.73	4.97
22:1n-11	0.58	0.28
22:1n-9	0.36	0.13
22:4n-6	0.09	0.09
22:5n-6	0.24	0.11
22:5n-3	0.49	0.07
22:6n-3	13.89	10.91

*n.d.=not detected, n< 0.005

This procedure was repeated using 10 individuals from each rearing tank. Frame by frame video image analysis was conducted to calculate burst-swimming speed (Masuda *et al.*, 2002; Benítez-Santana *et al.*, 2007). Burst swimming speed was analyzed only when the larva showed an obvious startle response. The fastest movement appeared always on the first frames after providing a stimulus as it occurs in younger larvae (Benítez-Santana *et al.*, 2007). The speed was presented as a function of the SL of each individual to avoid larval size interference (Masuda *et al.*, 2002; Benítez-Santana *et al.*, 2007).

Biochemical analyses

At the end of the study larvae were sampled for lipid and fatty acid composition of total lipids. Lipids were extracted by chloroform:methanol (Folch *et al.*, 1957). Methyl esters of fatty acids were prepared by transesterification with 1% sulfuric acid and methanol using heneicosanoic acid (21:0; 10% of total lipids) as an internal standard (Christie, 1982). The fatty acid methyl esters obtained were separated by gas chromatography (ShimadzuGC-14a, Kyoto, Japan) run using the operating conditions described previously (Izquierdo *et al.*, 1990), quantified by flame ionisation detection (FID) and identified by comparison to well characterized external standards.

Immunofluorescence study

Thirty larvae per tank (n=90) were collected and fixed in 10% buffered formalin at the end of the experiment. Each larva head was mounted in a gelatin block in horizontal orientation to obtain better visualization of neuronal structures. Gelatin-embedded larva head blocks were cryoprotected in 30% sucrose and serially cut on a Slee Mainz cryostat at 10 □m. Each gelatin section for immunofluorescence slides was collected on gelatin-coated slides. The antibody was tested to determine the optimal working concentration and quality of the signal and sea bream larvae were processed for the demonstration of immunoreactivity. The slides were covered with 5% rabbit serum and 0.2% Triton in phosphate-buffered saline (PBS) for 1 h.

Incubations with anti-choline acetyltransferase primary antibody (1:250, Millipore, Billerica, USA) was carried out for 48 h at 4°C in a 0.1 M (PBS) solution with 0.1% Triton X-100 and 2% goat serum (Vector). As secondary antibody we used anti-goat IgG FITC conjugate (1:250, antibody developed in rabbit affinity isolated antigen specific antibody; Sigma) diluted in PBS and applied for 1 hour in a dark room at room temperature. A propidium iodide complex at concentration of $1:10^4$ was applied for 5-10 min at room temperature and was used for nuclei contrast staining. In order to see the M-cells green immunofluorescence a Zeiss LSM 510 confocal system (Zeiss, Thornwood, NY) was used. In order to prevent fluorescence data were not compromised, in each batch of samples processed always all treatmenst were included. Negative controls were run by replacing each primary antibody by PBS to test for the specificity of an antibody involved.

Statistic analysis

Data were statistically analyzed with the software SPSS (SPSS 11.5 for Windows, SPSS Inc, Chicago, IL, USA). The normality of the variable distribution was verified using the Levene's test, not requiring any transformation. Values of fatty acid levels were expressed as means±S.D., and Student's t-test were employed to compare fatty acids and behavioural studies results.

Results

Feeding trial

The larvae fed the fish oil diet showed the highest DHA and EPA ($P<0.05$) content in whole body (Table 5.2). The fatty acid composition of larval fish (Table 5.2) at 36 days after hatching showed that larvae fed with soybean oil microdiets lost n-3 LCPUFA content (both EPA and DHA in the same proportion) in comparison with larvae fed with fish oil microdiet where the content of DHA and EPA ($P<0.05$) is higher in whole body compared to fish fed soybean oil. Larvae fed the fish oil microdiet showed a reduction in monoenoics and unsaturated fatty acids, while showing the highest content

of n-3 fatty acids. Larvae fed with soybean oil microdiets were rich in fatty acids of the n-6 series, particularly 18:2n-6 showed a significant reduction compared to levels found in fish fed the fish oil diet. Soybean oil larvae showed a higher content of monoenoics and saturated fatty acids which may reduce membrane fluidity. These fatty acid incorporation results demonstrated the good assimilation of the diet by the larvae.

Behavioural performance

Under the stimulus challenges conducted during the study, the number of larvae (n=20 larvae/diet/challenge) that reacted to the sound stimulus was related to larval age and diet. Thus, at 23 d of age a low percentage of larvae reacted to the stimulus, whereas at 27 d this number significantly ($P<0.05$) increased (Figure 5.1a). At 32 d, a similar proportion of larvae reacted when they were fed fish oil, whereas the percentage of reacting larvae was lower in those fed soybean oil (Figure 5.1a).

In larvae fed fish oil, burst swimming speed after sound stimuli increased with age being highest in larvae of 34 d ($P<0.05$) (Figure 5.1b). However in larvae fed soybean oil burst swimming speed only slightly increased at 27 d in comparison to 23 d and it was even reduced in 34 d-old larvae when they were fed soybean oil (Figure 5.1b). Therefore, at day 34 burst swimming speed after sound stimulus of larvae fed fish oil was significantly higher ($P<0.05$) than that of fish fed soybean oil (Figure 5.1b).

Table 5.2 Fatty acids content (% total determined fatty acids, n=3) of 35 day-old sea bream larvae fed with fish oil microdiet and soybean oil microdiet (Mean values with their standard desviation).

Fatty acid	FO larvae	SBO larvae
14:0	1.08±0.21	0.69±0.08
14:1n-7	0.18±0.02	0.10±0.02
14:1n-5	0.11±0.03	0.02±0.02
15:0	0.32±0.03	0.27±0.02
15:1n-5	0.03±0.00	0.02±0.01
16:0ISO	0.08±0.01	0.06±0.02
16:0	19.33±1.69	22.66±1.98
16:1n-7	3.64±0.15	1.66±0.19
16:1n-5	0.21±0.02	0.16±0.03
16:2n-6	0.13±0.06	0.18±0.02
16:2n-4	0.50±0.10	0.26±0.03
17:00	0.42±0.01	0.22±0.06
16:3n-4	0.06±0.02	0.02±0.00
16:3n-3	0.08±0.05	n.d.
16:3n-1	0.16±0.01	n.d.
16:4n-3	0.65±0.11	0.67±0.05
16:4n-1	0.08±0.03	0.14±0.11
18:0	9.74±1.24	11.73±0.32
18:1n-9	9.57±0.77	12.56±2.24
18:1n-7	3.84±0.63	2.35±0.90
18:1n-5	0.33±0.05	0.35±0.08
18:2n-9	0.23±0.03	0.12±0.15
18:2n-6	4.77±0.37[b]	15.37±0.63[a]
18:2n-4	0.09±0.00	0.06±0.06
18:3n-6	0.10±0.03	0.11±0.01
18:3n-4	0.09±0.12	0.09±0.03
18:3n-3	1.19±0.37	0.98±0.07
18:3n-1	0.06±0.00	n.d.
18:4n-3	0.32±0.02	0.09±0.04
18:4n-1	0.03±0.00	0.01±0.00
20:00	0.17±0.06	0.26±0.06
20:1n-9+n-7	1.41±0.02	1.28±0.18
20:1n-5	0.29±0.01	0.22±0.06
20:2n-9	0.13±0.02	0.11±0.02
20:2n-6	0.47±0.02	0.93±0.14
20:3n-6	0.16±0.01	0.12±0.03
20:4n-6	2.43±0.13	1.72±0.13
20:3n-3	0.20±0.04	0.21±0.01
20:4n-3	0.32±0.02	0.07±0.00
20:5n-3	7.44±0.07[a]	3.53±0.39[b]
22:1n-11	0.29±0.20	0.29±0.05
22:1n-9	0.11±0.04	0.10±0.02
22:4n-6	0.16±0.02	0.04±0.01
22:5n-6	0.75±0.11	0.51±0.04
22:5n-3	2.00±0.01	0.67±0.01
22:6n-3	26.26±0.85[a]	18.86±2.03[b]
Saturated	30.40±3.19	35.35±2.24
Unsaturated	13.40±1.19	15.48±2.33
Monoenoics	15.56±1.19	16.93±3.41
Polyunsaturated	48.84±1.82	44.87±1.53
n-3	38.45±1.42	25.08±2.51
n-6	8.97±0.50	18.99±0.89
n-9	11.45±0.65	14.17±2.59
n-3 LCUFA	40.35±0.87	39.83±2.42
AA/EPA	0.33±0.01	0.49±0.10
EPA/DHA	0.28±0.01	0.19±0.00

n.d.≤0.005

a,b Mean values across the row with unlike superscript letters were significantly different as determined by Student's t-test honestly significant difference (n=3, P<0.05).

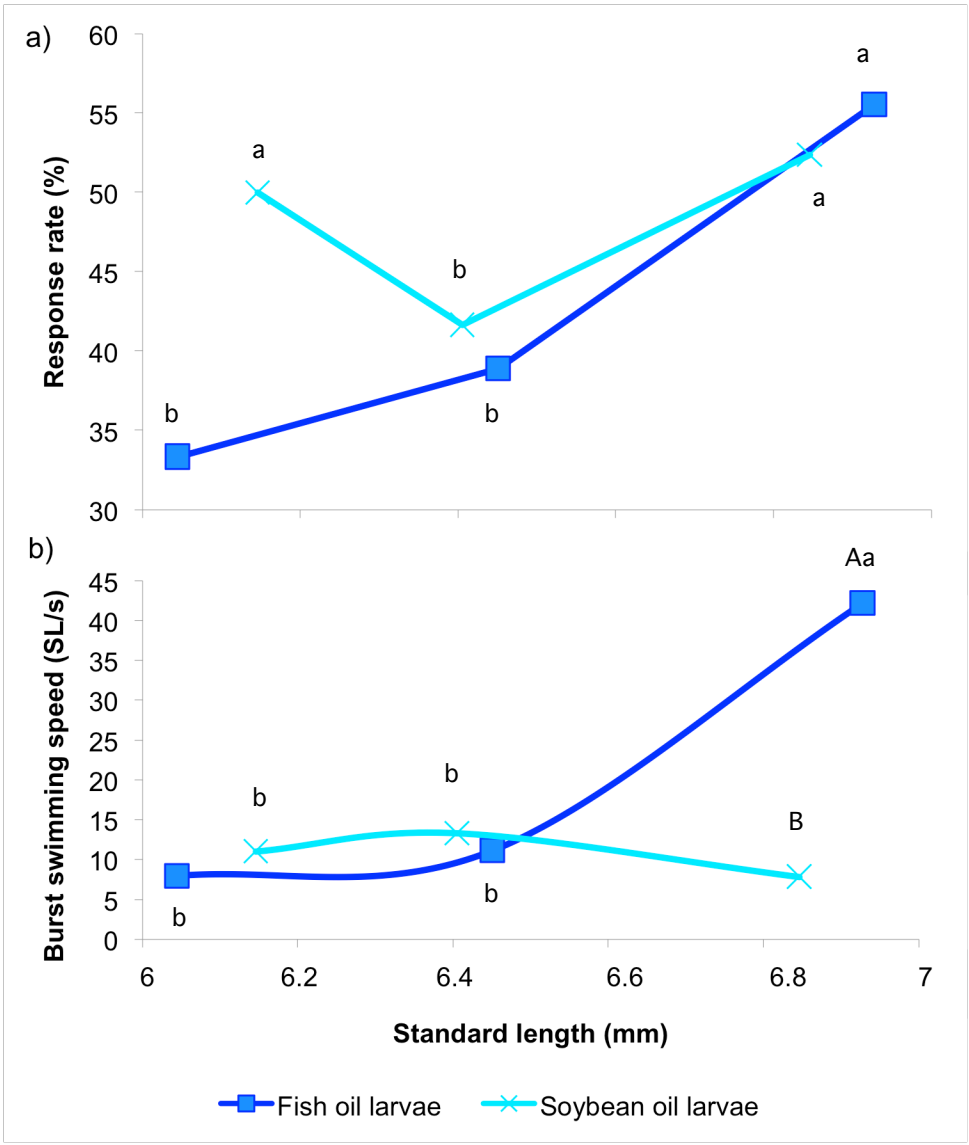

Figure 5.1 Larval reaction after the sound stimuli along larval development (a) Number (%) of reacting sea bream larvae for each experimental group after the sound stimuli fed diets containing fish oil and soybean oil. (b) Burst swimming speed (SL/s) in sea bream larvae fed microdiets enriched with different types of lipids: fish oil and soybean oil. a,b Mean values with unlike letters were significantly different between animals of same treatment ($P<0.05$), n=30. A,B Mean values with unlike letters were significantly different between animals of different treatment (n=30, $P<0.05$).

Mauthner cells activity

Confocal microscopy analysis showed a greater acetylcholine immunopositive response (green fluorescence) in larvae fed the fish oil microdiet than in those fed the soybean oil microdiet (Figure 5.2). Fluorescence quantification showed a significantly higher immunopositive response in larvae fed fish oil than in larvae fed the soybean oil microdiet (Figure 5.3).

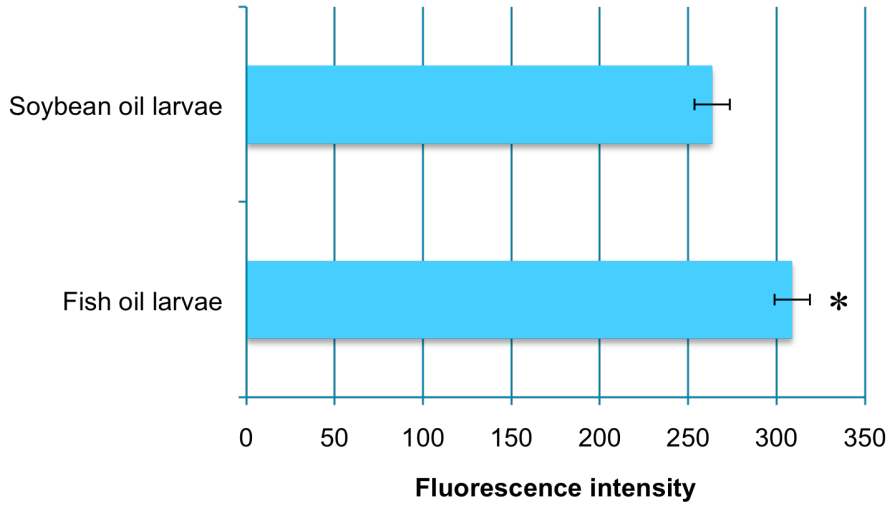

Figure 5.2 Quantification of green fluorescent intensity by confocal microscopy according to the immunopositive response of M-cells. * Mean values were significantly different between animals of different treatment (n=30, P<0.05).

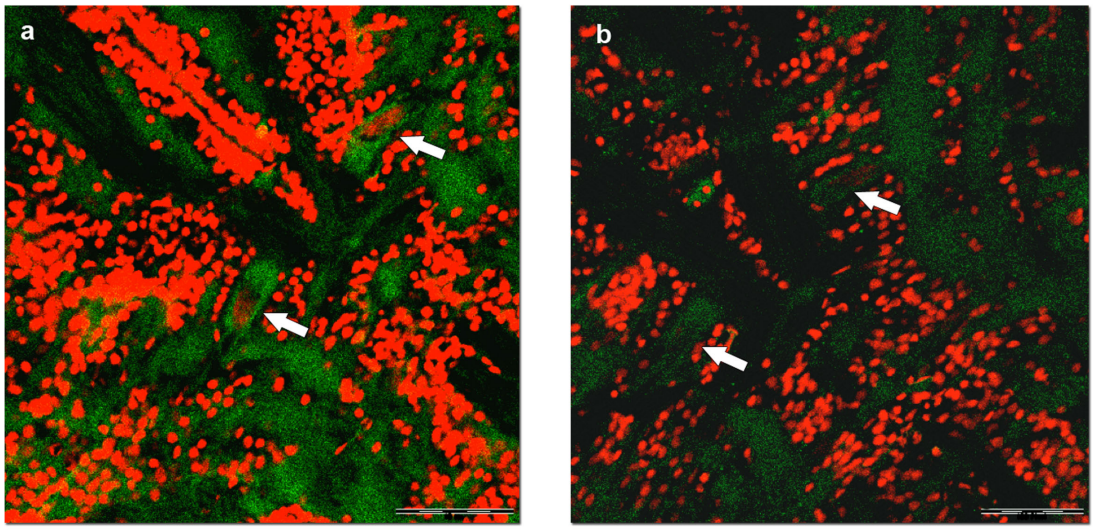

Figure 5.3. Confocal microscopy image of the acetylcholine immunopositive response of M-cells longitudinal sections from larvae fed with fish oil microdiet (A) and soybean oil microdiet (B). Scale bar 10 μm.

Discussion

In the present study, the reduction in dietary n-3 LCPUFA caused a significant reduction in the contents of these fatty acids in the fish. In gilthead Sea bream, brain fatty acid composition is also modified by the n-3 LCPUFA content of the diet (Benítez-Santana *et al.*, 2007), and therefore, being necessary for the normal development of nervous system and sensory organs, these fatty acids could affect physiological functions in the brain. In fact, a diet unbalanced in n-3 PUFA may cause changes in cell permeability and synaptic membrane fluidity (Yehuda *et al.*, 2005), or modifications in the number and affinity of receptors, in the function of ion channels and on the activity of neurotransmitters (Jump, 2002). Thus, alterations in brain fatty acid composition could potentially affect behaviour.

An important behaviour to escape from predation is the startle response, which is initiated by a sudden stimulus and results in a rapid reaction. In the present study, substitution of soybean oil by fish oil in microdiets for larval gilthead Sea bream markedly increased n-3 LCPUFA content in fish tissues and affected fish behaviour in terms of the startle response to a sonorous stimulus. Thus, during fish development, a higher but not statistically different number of larvae reacted to the stimulus when they were fed fish oil, rich in n-3 LC-PUFA. Moreover, a n-3 LC-PUFA increase in the diet also led to a faster swimming speed burst in those larvae that reacted to the sonorous stimulus at 32 dph. Therefore, dietary reduction in n-3 LC-PUFA impaired fish larval response to the stressor, reducing the escape behaviour in larvae with a lower content of n-3 LC-PUFA in their body tissues. These results highlight the important role of these fatty acids in the response to a sonorous stimulus, in agreement with their importance for sensory organs functioning (Izquierdo, 2005). In contrast with the present study, younger and less developed gilthead sea bream burst swimming response to a sound stimulus was not affected by dietary n-3 LCPUFA, whereas they had a high burst swimming speed after a light stimulus (Benítez-Santana *et al.*, 2007). This suggests that despite the neural and

muscular responses were well developed in those young larvae since they were able to react to a visual stimulus, the reception to the sound stimulus was not sufficiently developed to produce a consistent response according to dietary differences. The mechanoreceptive neuromast cells associated with the lateral line system and the inner ear (auditory nerve) are the major receptors for external vibrational and gravitational stimuli in fish. The lateral line system of teleost fish typically consists of a row of pores along the tail, body and head, leading into an underlying fluid-filled lateral line canal. The neural impulses from these receptors are transmitted along the anterior and posterior lateral line nerves to the octavolateralis area of the medulla (Bleckmann *et al.*, 1987). In the fish used in the present study the lateral line was better developed, whereas in the former trial, conducted with younger larvae, the lateral line had not started to appear until the end of the experiment (15-20d). A further development of the sensorial organs in the larvae of the present study would allow a different perception of the stimulus by larvae fed different n-3 LC-PUFA suggesting the importance of these fatty acids for the normal functioning of the lateral line.

The Mauthner neurons are known to receive sensory information not only from the auditory nerve (Faber *et al.*, 1991; Zottoli *et al.*, 1995), but also from the optic tectum (Canfield, 2003), from the lateral line mechanosensory system (Zottoli and Danielson, 1989), from somatosensory channels (Chang *et al.*, 1987), and via the electrosensory system in weakly electric fish (Zottoli *et al.*, 1995). Thus a variety of sensory modalities could modulate the relative excitation or inhibition of the M-cells prior to a startling stimulus driving it beyond threshold levels. Escaping behaviour in fish has been particularly related to M-cells. This pair of reticulospinal neurons initiates fast startle responses in fishes and amphibians and constitute an important model system in studies of vertebrate neurons and their control of behaviour (Korn and Faber, 2005). In the present study, faster startle response in fish fed n-3 LCPUFA was also associated with an increased immunopositive neural response, particularly in M-cells, denoting a higher production of acetylcholine. Acetylcholine release in the hippocampus has been found to be

reduced under neuronal activation in rats receiving a chronically n-3 PUFA deficient diet (Aïd *et al.*, 2003; Kodas *et al.*, 2004). N-3 LC-PUFA, and particularly DHA, markedly affect membrane fluidity and functioning and have been found to be important for neurocyte myelination and synapse construction, with both functions being sensitive to nutritional deficiencies (Krigman and Hogan, 1976). Moreover, these fatty acids are nutritional antioxidants that prevent the formation of cerebral lipid peroxides (Choin-Kwon *et al.,* 2004), stabilizing the oxidant/antioxidant status of membrane structures in brain (Sarsilmaz *et al.*, 2003), and protecting from several neurological and neuropsychiatric disorders (Black *et al.*, 2004). Most of DHA accumulation occurs during late prenatal and early postnatal development, coinciding with the formation of synapses (Green *et al.*, 1999). Similarly, DHA accumulates in brain and sensorial organs of fish during larval development and it is retained in neural tissues even during periods of starvation (Izquierdo *et al.*, 2001; Izquierdo, 2005). Adequate dietary availability of DHA during this period is essential for optimal central nervous system development and functioning. Inadequate intake of DHA is thus associated with impaired attention and learning performance as well as modifications in emotional status including elevated behavioural indices of anxiety, aggression and depression (Fedorova and Salem, 2006).

The present study shows the first evidence of the importance of n-3 LCPUFA for the adequate functioning of particular neurons, the M-cells, and, subsequently for the behaviour response that they modulate to escape from a sound stimulus. Further studies are being conducted to understand the role of these essential fatty acids on neural development and functioning.

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6. Study III

Study III

DHA but not EPA, enhances sound induced escape behaviour and Mauthner cells activity in *Sparus aurata*

This work has been submitted to Physiology and Behavior.

Abstract

Dietary omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have a marked effect on fish behaviour. There is limited information on the mechanisms involved in this effect and its relation to neuron development and functioning. Deficiency of n-3 LCPUFA reduces fish escape behaviour. Mauthner cells (M-cells) are neurons responsible for escape behaviour. The aim was to compare the efficacy of dietary DHA and EPA contents in escape behaviour and neuron activity of sea bream larvae. Behaviour was determined by burst swimming speed. M-cell activity was studied by ChAT immuno-fluorescence. Feeding the lowest n-3 LCPUFA levels lead to a lower escape response. Increase in dietary EPA did not significantly improve escape response. Elevation of dietary DHA was correlated with a higher burst speed denoting the greater importance in escaping behaviour. Incorporation of DHA into larval tissues was proportional to DHA dietary levels and significantly correlated with burst speed. In addition, a marked immunoreactivity was found in M-cells. These results show first evidence of n-3 LCPUFA on fish neuronal activity and their implications in behaviour, denoting that DHA boosts escaping behaviour and this effect is at least partly mediated by the increase in neural activity of M-cells.

Keywords: Burst swimming speed, DHA, EPA, Mauthner cells, n-3 LCPUFA.

Introduction

Dietary omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), play critical roles in optimum larval growth, development and survival of marine fish larvae (Watanabe, 1993; Izquierdo, 1996; Rainuzzo *et al.*, 1997; Sargent, 2000; Izquierdo *et al.*, 2001; Atalah *et al.*, 2007; Atalah *et al.*, 2010), since they are essential components for the normal formation of cell and tissue membranes and organs development (Izquierdo, 1988; Izquierdo *et al.*, 2003). Inadequate dietary n-3 LCPUFA levels also resulted in reduced feeding and swimming activities (Izquierdo, 1996) and abnormal behaviour (Benítez-Santana *et al.*, 2007) in marine fish larvae. Moreover, n-3 LCPUFA deficient feed reduces cruise swimming speed and delays the response to a visual stimulus, postponing the appearance of behaviour patterns which suggests a delay in the functional development of brain and vision (Benítez-Santana *et al.*, 2007).

Among the different n-3 LCPUFAs, docosahexaenoic acid (DHA) has been recognized to be particularly important for growth and survival (Watanabe *et al.*, 1989; Izquierdo, 1996; Sargent *et al.*, 1997), regulation of stress response (Watanabe, 1993; Izquierdo, 2005; Ganga *et al.*, 2006) and normal behaviour (Masuda *et al.*, 1999). Indeed, DHA is found in high quantities in neural and sensorial tissues (Mourente *et al.*, 1992; Bell *et al.*, 1995; Ushio *et al.*, 1996; Benítez-Santana *et al.*, 2007). In mammals, DHA constitutes approximately 30–40% of the phospholipids of the gray matter of cerebral cortex (Lauritzen *et al.*, 2001), being the most important n-3 fatty acid with physiological significance for brain function (Bourre, 2004; Marszalek and Lodish, 2005). A decrease in the levels of DHA in neural tissue leads to behavioural deficits (Lim *et al.*, 2005). Accordingly, dietary DHA supplementation in breastfeeding children has been shown to improve mental development (Hibbeln *et al.*, 2007). However, in fish the specific effect of DHA on neural activity and its relation to abnormal behaviour has not yet been investigated.

Other LCPUFA may also play essential roles in neural tissues. For instance, eicosapentaenoic acid (EPA) present in live prey fed to fish larvae enhances development of the brain (Furuita *et al.*, 1998) and eye (Izquierdo *et al.*, 2001). In marine fish larvae, EPA improves larval performance (Watanabe *et al.*, 1989; Furuita *et al.*, 1996; Furuita *et al.*, 1998; Liu *et al.*, 2002). Moreover, dietary EPA has been

found to increase red sea bream (*Pagrus major*) stress resistance to handling (Watanabe *et al.*, 1989) and gilthead sea bream (*Sparus aurata*) resistance to air exposure and temperature shock (Liu *et al.*, 2002) and regulates cortisol production by modulating adrenocorticotropic hormone-stimulated interrenal cells in sea bream (Ganga *et al.*, 2005). Whereas in mammals, EPA has been shown to have a neuro-protective role important for brain development, aging and behavior, in fish the function of EPA on behavior patterns, such as escaping response, or on neural activity, has not yet been studied.

Escape behaviour in fish, also known as the fast-start or startle response, is initiated by a pair of neurons in the hindbrain, the Mauthner cells (M-cell) (Korn and Faber, 2005). The M-cells are easily identifiable morphologically by the large size of their axons, soma and dendrites. The M-cells integrate diverse sensory inputs (Zottoli and Faber, 2000; Eaton *et al.*, 2001), and are able to reset swimming rhythms in the course of its initiation of escape behaviours (Svoboda and Fetcho, 1996). In most teleost fish, the escape response consists of a C-type fast-start (C-start) — the first stage of which is characterized by a rapid unilateral contraction of trunk musculature leading to head and tail movement, which causes the fish to bend into a C-shape. M-cell activity precedes the C-start, and electrical stimulation of Mauthner axons can elicit a C-start (Nissanov *et al.*, 1990). Although, dietary n-3 LCPUFAs have been found to affect escape behaviour in fish, their potential effect on M-cells has not yet been investigated.

Besides the importance of absolute DHA and EPA contents in diets for marine fish, the ratio between these two fatty acids seems to be a determinant of the requirements for each of those fatty acids. For instance, studies in turbot larvae have suggested the need for a suitable DHA/EPA balance for normal pigmentation of developing flatfish (Reitan *et al.*, 1994). Indeed, increased dietary EPA reduces dietary DHA incorporation into larval phospholipids, and increased dietary DHA reduces incorporation of EPA in phosphatidyl ethanolamine (Izquierdo *et al.*, 2000), a main phospholipid in neural tissues. Thus, optimum dietary DHA/EPA ratios described in the literature range from around 1.2 for gilthead sea bream (Rodríguez *et al.*, 1997; Rodríguez *et al.*, 1998) or 2 for red drum (*Sciaenops ocellatus*) (Brinkmeyer and Holt, 1998) to 8 for yellowtail flounder, *Limanda ferruginea*

(Copeman *et al.*, 2002).

Thus, in view of the enhanced escaping response in marine fish larvae fed increased n-3 LCPUFA in our previous research (Benítez-Santana *et al.*, 2007), the aim of the present study was to compare the efficacy of different dietary DHA and EPA contents in escaping behaviour and neuron activity of gilthead sea bream larvae. For that purpose, sea bream larvae were fed different weaning diets with several EPA and DHA contents and their effect on survival, growth, sound stimuli response behaviour and M-cell activity were studied. M-cells activity was studied by ChAT immuno-fluorescence considering acetylcholine as a major neuro-transmitter in the nervous system.

Material and Method

All animal studies complied with the guidelines for animal experimentation of our laboratories and were approved by institutional review boards. All compounds studied were sourced from recognized suppliers, and commercially available reagents were used according to manufacturers' instructions unless otherwise indicated.

Experimental conditions

Gilthead Sea Bream larvae of 18 days after hatching (DAH) with standard length (SL) 4.98±0.899 (mean±SD), were obtained from a natural spawning from genetically characterized broodstock of the GIA (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain), and were randomly distributed into twelve 170 L fibreglass cylinder tanks (conical bottom and painted a light grey colour) at a density of 1200 larvae/ tank. Fish larvae, which had been previously fed enriched rotifers (Selco, DHA Protein Selco, INVE, Dendermonde, Belgium) until they reached 18 DAH, were fed one of the experimental diets (4 microdiets in total) tested in triplicates for 14 days. All tanks were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4 L/min – 1.0 L/min along the feeding trials to assure good water quality during the entire trial. Water flowed in from the top of the tank and out from the bottom using a siphon system. Water was continuously aerated (125 ml·min-1) attaining 5-8 g·L-1 dissolved O_2 87 and 60-80% saturation in all tanks. Water temperature ranged between 19.6 and 20.9° C during

the trial. A photoperiod regime of 12h light: 12h dark was obtained with fluorescent lights and was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Larval growth was determined by measuring the total length of 30 larvae at 18, 22 and 29 DAH using a profile projector (Mitutoyo Profile Projector PJ-A3000). At the end of the experiment, survival rate was determined by manually counting the remaining live larvae.

Four isonitrogenous and isolipidic (theoretically 70.1/16.80 protein/lipid) experimental microdiets (pellet size < 250 µm) with different proportions of DHA (0.6-9%) and EPA (0.3-4%) contents, were formulated using EPA50 and DHA50 (CRODA, East Yorkshire, England, UK) as sources of EPA and DHA, and using oleic acid oil (Oleic acid vegetable, Merck, Darmstadt, Germany) as complement lipid source (Table 6.1). Two control diets were used: a negative control with deficient contents of n-3 LCPUFA (0.3/0.6 diet) and a positive control that included n-3 LCPUFA in sufficient amount to cover sea bream nutritional requirements (4/1 diet). The other two diets had different EPA/DHA ratios to compare relative contents of these fatty acids. Microdiets were prepared according to Atalah *et al.* (2010). Diets were analyzed for crude lipid content and fatty acid composition (Table 6.2).

Table 6.1 Lipid sources (% total ingredients) and crude lipid (% dry basis) content in the experimental microdiets.

EPA/DHA	0.3/0.6	1.5/9	4/1	4/3
EPA50[1]	0.00	12.00	6.00	5.30
DHA50[1]	1.30	1.00	0.00	3.30
Oleic acid[2]	11.70	0.00	7.00	4.40
Crude lipids	16.04	17.16	16.95	16.99

[1]CRODA, East Yorkshire, England, UK. [2] Oleic acid vegetable, Merck, Darmstadt, Germany.

Table 6.2 Fatty acid profile of the experimental diets (% total identified fatty acids) used to feed gilthead sea bream larvae.

EPA/DHA	0.3/0.6	1.5/9	4/1	4/3
14:0	0.32	0.06	0.22	0.16
14:1n-5	0.08	n.d.	0.05	0.03
14:1n-7	0.03	n.d.	0.02	0.01
15:0	0.05	0.02	0.04	0.03
15:1n-5	0.03	n.d.	0.02	0.01
16:0	1.21	0.92	1.01	0.90
16:1n-9	0.05	n.d.	0.03	0.02
16:1n-7	0.70	0.07	0.45	0.32
16:1n-5	0.03	n.d.	0.02	0.01
16:2n-6	0.11	n.d.	0.07	0.05
16:2n-4	0.04	0.03	0.04	0.03
16:3n-4	0.22	0.02	0.15	0.10
18:0	0.22	0.40	0.18	0.21
18:1n-9	10.15	0.96	6.38	4.51
18:1n-7	0.05	0.15	0.42	0.37
18:1n-5	n.d.	0.01	0.03	0.02
18:2n-9	0.05	n.d.	0.02	0.02
18:2n-6	1.34	1.21	1.27	1.22
18:3n-3	0.13	0.17	0.19	0.20
18:4n-3	n.d.	0.04	0.36	0.35
18:3n-1	n.d.	n.d.	0.03	0.03
18:4n-1	n.d.	0.08	0.00	n.d.
20:1n-9	0.12	0.05	0.00	n.d.
20:1n-7	n.d.	0.47	0.06	0.13
20:3n-6	n.d.	0.07	0.00	0.02
20:4n-6	0.04	0.29	0.22	0.27
20:3n-3	n.d.	0.06	0.02	n.d.
20:4n-3	n.d.	0.11	0.17	0.18
20:5n-3	0.28	1.62	4.09	4.14
22:1n-11	n.d.	0.28	0.02	0.08
22:1n-9	n.d.	0.14	0.00	0.03
22:4n-6	0.03	0.34	0.02	0.10
22:5n-6	n.d.	0.02	0.00	n.d.
22:5n-3	0.06	0.66	0.13	0.27
22:6n-3	0.62	8.86	1.12	3.09
Saturated	1.8	1.4	1.45	1.3
Monoensaturated	11.24	2.13	7.5	5.54
n-3	1.09	11.52	6.08	8.23
n-6	1.52	1.93	1.58	1.66
n-9	10.37	1.15	6.43	4.58
n-3 LCPUFA	0.96	11.31	5.53	7.68
ARA/EPA	0.14	0.18	0.05	0.07
DHA/EPA	2.21	5.47	0.27	0.75

*n.d.=not detected, n<0.005

All larvae of each tank were washed with distilled water and kept at -80° C for biochemical composition after 16 h of starvation at the end of the trials. Moisture (A.O.A.C., 1995) and crude lipid (Folch *et al.*, 1957) contents of larvae and diets were analyzed. To determine fatty acid profiles, methyl esters of fatty acids were obtained by transesterification with 1% sulfuric acid and methanol using heneicosanoic acid (10% of total lipids) as an internal standard. The fatty acid methyl esters obtained were separated by gas chromatography (ShimadzuGC-14a, Kyoto, Japan) run at the operating conditions described previously (Izquierdo *et al.*, 1990), quantified by flame ionisation detectors (FID) and identified by comparison to well characterized external standards.

Swimming speed behaviour

Swimming speed of larvae from all dietary groups was determined at 22 and 29 DAH, respectively, in a 1 L cylindrical black glass container (10 cm in diameter) with a water depth of 4 cm. Each larva was transferred from the feeding tanks to the experimental beaker and then video-recorded using a Sony digital video camera DCR-TRV27. After recording for 30 s, the larva was auditorially stimulated to induce a startle response and determine burst swimming speed. Consistent sound stimuli were produced using a steel nut (≈10 g) hung by a string (26 cm) that was released from a distance of 18 cm from the beaker wall. The sound was produced by swinging the nut like a pendulum to tap the side wall of the tank. Sound stimuli were provided three times at 10 s intervals for each larva. After this experiment, larva standard length (SL) was measured by a profile projector (Nikon V-12A, Nikon, Tokyo, Japan). This procedure was repeated using 10 individuals of each rearing tank following the procedure described in (Masuda *et al.*, 2002; Benítez-Santana *et al.*, 2007). Frame by frame video analysis was conducted to calculate burst swimming speed. To observe the response development to sound stimuli, burst swimming speed rate was calculated by dividing the number of responses by number of trials. Burst swimming speed was analyzed only when the larva showed an obvious startle response. The fish movement was traced for four consecutive

frames, and the distance was divided by the time taken (4/30 s). Preliminary observations in very young larvae (Benítez-Santana *et al.*, 2007) revealed that the fastest movement appeared in any of the first frames after providing a stimulus. In the present research, later larval stages provided the same results, where the fastest movement appeared in the first frames. Burst swimming speed was calculated as the average of the movement of four frames (Masuda *et al.*, 2002; Benítez-Santana *et al.*, 2007).

Immunofluorescence study

Thirty larvae per tank (n=60) were collected and fixed in 10% buffered formalin at the end of the experiment. Each larva was mounted in a gelatin block in horizontal orientation to obtain better visualization of neuronal structures. Gelatin-embedded larva blocks were serially cut on a Slee Mainz cryostat at 10 □m. The antibody was tested to determine the optimal working concentration and quality of the signal, and sea bream larvae were processed for the demonstration of immunoreactivity. Each section for immunofluorescence slides was collected on gelatin-coated slides. The slides were covered with 5% rabbit serum and 0.2% Triton in PBS for 1 h prior to incubation with the primary antibody for 48 h at 4° C. When the primary polyclonal antibody was used, anti-choline acetyltransferase (Millipore, Billerica, USA) diluted 1:250 in PBS was applied for 1 hour as secondary (anti-goat IgG FITC conjugate; antibody developed in rabbit affinity isolated antigen specific antibody; Sigma) reagent in a dark room at room temperature. A propidium iodide complex diluted 1 mg in 250 ml PBS was applied for 5-10 min at room temperature to detect the different substrates. A Zeiss LSM 510 confocal system (Zeiss, Thornwood, NY, USA) was used to visualize M-cell green immunofluorescence. To quantify the intensity of the sample, a LSM 510 program was used, which measured the laser ray intensity required to excite the neurons to the same threshold level. Confocal images were collected at x1000.

Statistic analysis

Statistical analysis was performed using the software SPSS (SPSS 11.5 for Windows, SPSS Inc, Chicago, IL, USA) using one-way analysis of variance (ANOVA) following the general linear model:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

Means of fatty acid levels and behavioural studies were compared by Duncan's test ($P<0.05$).

Results

Feeding trial

All experimental microdiets were well accepted by the larvae. Lowest survival rate (14.6%) was obtained in larvae fed the lowest dietary LCPUFA content (diet 0.3/0.6), whereas there were no significant differences among larvae fed the other diets (1.5/9 = 18.9%; 4/1 = 25.67%; 4/3 = 27.59%). Since only the biggest larvae survived in treatment with the lowest dietary LCPUFA content (diet 0.3/0.6), no significant differences were found in larval growth. Average lipid content of whole larval body did not differ significantly among larvae fed the different diets. By contrast, fatty acid composition of larval whole body lipids reflected the dietary fatty acid profile (Table 6.3). Thus, the elevation of dietary oleic acid from 0.92 to 10.15 (g/100g diet) induced a significantly correlated (r^2=0.94) increase in larval oleic acid content. In addition, dietary elevation of either EPA (r^2= 0.82) or DHA markedly increased the incorporation of these fatty acids into larval tissues. Therefore, larvae fed with 0.3/0.6 microdiet showed the lowest content of DHA and EPA. Despite low levels of dietary saturated fatty acids, particularly for palmitic acid (16:0), they were high in larval lipids, and particularly in larvae fed the 0.3/0.6 diet which had the lowest n-3 LCPUFA contents. In these larvae, oleic acid (18:1 n-9) and linoleic acid (18:2n-6) were also very high. Thus, in general, the larvae content of linoleic acid was not correlated with that of the diet, but with the dietary n-

6/ARA (r^2=0.92), denoting a higher incorporation of linoleic acid into larval tissues when dietary ARA was lower.

Behavioural performance

For the startle response assay, there was no significant difference in percentage of larvae that reacted to the stimulus (n=30 larvae/diet/challenge). The results of the behavioural trial showed that after only one week of feeding (22 days after hatching, DAH) larvae fed the highest DHA (1.5/9 diet) level tended to show a higher burst swimming speed as an escape response to the auditory stimulus. After two weeks of feeding, 29 DAH larvae fed the highest level of DHA (1.5/9 diet) showed significantly ($P<0.05$) the highest burst swimming speed followed by larvae fed the second highest dietary DHA level (4/3 diet) (Figure 1). The lowest burst swimming speed was found in larvae fed the lowest DHA and EPA content (0.3/0.6 diet), whereas elevation of dietary EPA (4/1) did not significantly increase burst swimming speed. Thus, burst swimming speed was significantly correlated to DHA dietary levels (r^2=0.89), but not with dietary EPA. Similarly, the increase in burst swimming speed was significantly correlated to whole body DHA contents (r^2=0.98), but not to EPA content in whole body lipids. About the percentage of larvae not shown a startle response 29 DAH larvae fed the lowest level of DHA (0.3/0.6 diet) significantly ($P<0.05$) showed the higher percentage of not startle response. Larvae fed the lowest level of DHA (1.5/9 diet) showed significantly ($P<0.05$) the lowest percentage of response.

Table 6.3 Fatty acids content (% total determined fatty acids, n=3) of 32 day-old sea bream larvae fed with different EPA/DHA microdiets.

Diet EPA/DHA	0.3/0.6	1.5/9	4/1	4/3
Lipid w.b.	2.22±0.20[a]	2.26±0.28[a]	2.86±0.59[a]	2.5±0.30[a]
14:0	0.70	0.57	0.77	1.08
14:1n-5	0.02	0.02	0.01	n.d.
14:1n-7	0.10	0.23	0.12	0.21
15:0	0.42	0.44	0.40	0.43
15:1n-5	0.16	0.16	0.13	0.16
16:0iso	0.44	0.37	0.44	0.33
16:0	21.62	16.10	18.54	15.76
16:1n-9	n.d.	0.66	0.80	0.78
16:1n-7	1.37	2.89	1.80	3.58
Me16:0	0.18	0.07	0.17	0.13
16:1n-5	0.32	0.59	0.32	0.51
16:2n-6	0.52	0.21	0.42	0.29
16:2n-4	0.81	0.96	0.83	0.83
16:3n-4	1.06	0.72	0.81	0.65
16:3n-3	n.d.	0.04	n.d.	0.06
16:3n-1	n.d.	n.d.	n.d.	0.06
16:4n-3	0.95	1.06	0.93	0.99
16:4n-1	0.88	0.50	1.00	0.69
18:0	9.23	9.80	8.50	8.48
18:1n-9	30.37	11.84	22.76	17.17
18:1n-7	4.74	3.47	4.12	4.25
18:1n-5	0.22	0.18	0.19	0.28
18:2n-9	0.15	0.68	0.26	0.73
18:2n-6	6.59	3.07	3.96	3.28
18:2n-4	n.d.	0.11	n.d.	0.06
18:3n-6	0.14	0.26	n.d.	0.09
18:3n-4	0.10	n.d.	0.12	0.06
18:3n-3	n.d.	0.23	0.29	0.31
18:3n-1	n.d.	0.09	n.d.	0.05
18:4n-3	n.d.	n.d.	0.32	0.36
18:4n-1	0.28	0.40	0.28	0.27
20:1n-9	n.d.	n.d.	n.d.	0.09
20:1n-7	0.67	1.00	0.54	0.84
20:2n-9	0.06	0.33	n.d.	0.24
20:2n-6	n.d.	0.19	n.d.	0.14
20:3n-9	0.19	0.17	n.d.	0.14
20:3n-6	0.16	0.09	0.15	0.12
20:3n-3	n.d.	0.13	n.d.	0.04
20:4n-6	2.52	2.75	3.18	2.73
20:4n-3	n.d.	0.09	0.34	0.36
20:5n3	3.27	3.98	10.67	8.68
22:1n-11	n.d.	0.34	0.11	0.46
22:1n-9	n.d.	0.09	0.08	0.10
22:4n-6	0.80	1.34	0.42	0.70
22:5n-3	1.05	1.44	2.09	1.86
22:6n-3	9.92	32.33	14.16	21.56
Saturated	31.99	26.84	28.25	25.65
Unsaturated	33.03	15.08	26.23	20.39
Monoenoics	36	16.92	27.80	23.19
Polyunsaturated	29.26	51	40.23	45.21
n-3	14.14	37.86	26.71	32.36
n-6	10.73	7.91	8.13	7.35
n-9	31.25	13.94	23.64	19.17
n-3 LCUFA	13.19	36.53	25.17	30.64
AA/EPA	0.77	0.69	0.30	0.31
EPA/DHA	3.03	8.12	1.33	2.48

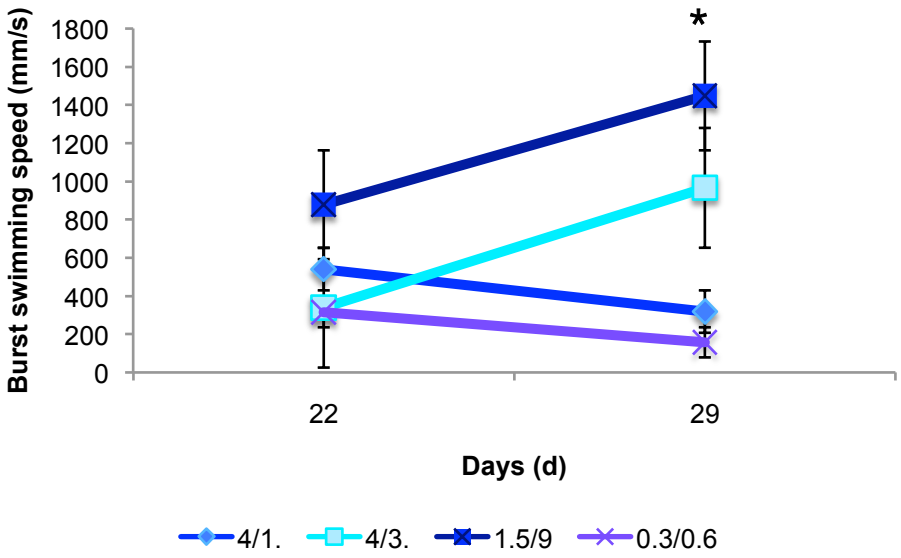

Figure 6.1. Larval reaction after the sound stimuli along larval development. Burst swimming speed (mm/s) in sea bream larvae fed with EPA/DHA microdiets. * Mean values were significantly different between animals of different treatment (n=30, P<0.05).

Mauthner cells activity

Observation of M-cell activity by confocal microscopy showed higher choline acetyltransferase activity in larvae fed the highest dietary DHA levels (1.5/9 diet) and lowest in larvae fed lowest dietary LCPUFA (0.3/0.6 diet) (Figure 2). Quantification of the amount of green fluorescence produced by the antibody reaction to choline acetyltransferase showed a significantly (P<0.05) higher immunopositive response in larvae fed the highest DHA levels (1.5/9 diet) than in larvae fed the lowest content of these essential fatty acids (Figure 6.3).

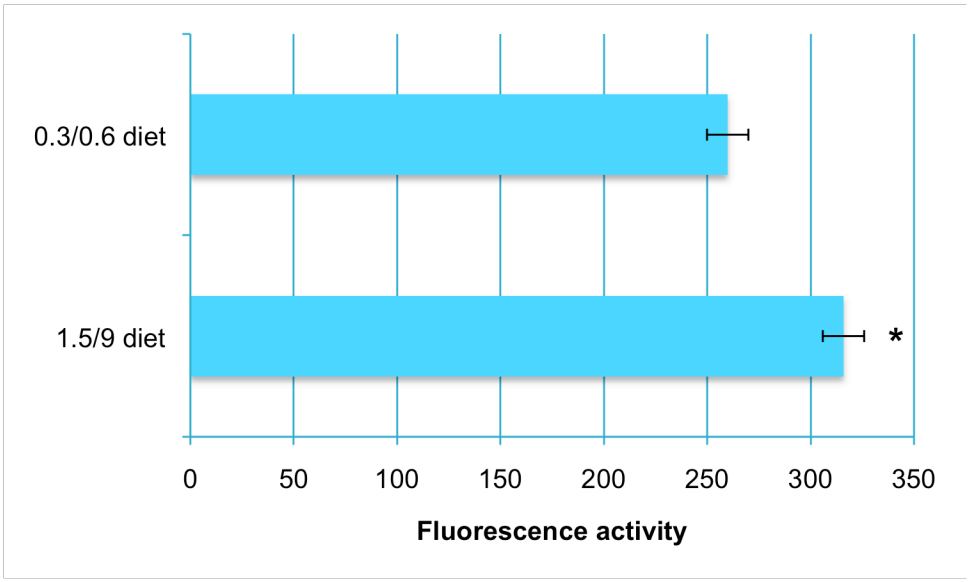

Figure 6.2 Quantification of green fluorescent intensity by confocal microscopy according to the anti-choline acetyltransferase immunopositive response of M-cells. * Mean values were significantly different between animals of different treatment (n=30, P<0.05).

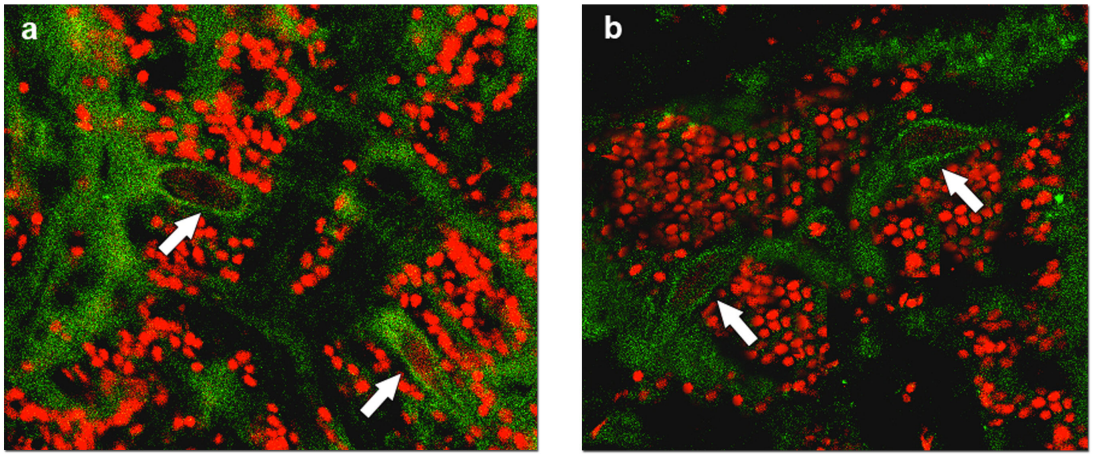

Figure 6.3 Confocal microscopy image of the acetylcholine immunopositive response of M-cells longitudinal sections from larvae fed with 1.5/9 microdiet (A) and 0.3/0.6 microdiet (B) (x1000).

Discussion

Feeding the lowest n-3 LCPUFA levels lead to a lower escape response after an auditory stimuli in gilthead sea bream larvae, in agreement with the reduced response behaviour to visual stimulus found in our previous studies in younger larvae of this species (Benítez-Santana *et al.*, 2007). These results are in agreement with the great relevance of dietary n-3 LCPUFA for behaviour and brain health known in mammals (Marszalek and Lodish, 2005).

Elevation of dietary DHA correlated with a higher burst swimming speed in response to a sound stimulus, denoting the higher importance of this n-3 LCPUFA in escaping behaviour in comparison to EPA. In agreement, dietary DHA has been found to improve development of normal behaviour in yellowtail (Masuda and Tsukamoto, 1998). Incorporation of DHA into gilthead sea bream larval tissues was proportional to dietary levels of this fatty acid and correlated well with the burst swimming activity in response to sound stimuli. In this species, a previous study to determine brain and eyes fatty acid profile composition showed that DHA is the main LCPUFA in tissues of these organs in quantities dependant on its dietary levels (Benítez-Santana *et al.*, 2007). In turbot, DHA is selectively assimilated in phosphatidyl ethanolamine, the largest phospholipid class in neural tissue (Mourente and Tocher, 1992). In other fish species, DHA has been found to accumulate in olfactory nerve and photoreceptors (Bell *et al.*, 1995), being associated with sensory organ function (Izquierdo, 2005). However, in the present study, increase in dietary EPA did not significantly improve escaping response of fish, as will be discussed later on.

Neural regulation of escaping behaviour in fish is based on the activation of M-cells to initiate the startle response. Startle response is a fast primary sensorimotor reaction to avoid predators initiated by simple and fast neural circuits, the large Mauthner neurons. In the present study, increased M-cell activity, denoted by the higher production of acetylcholine, was found in larvae fed and containing in their tissues the highest levels of DHA and showing the fastest startle response. Several pathways may be involved in the effect of DHA on neural activity. On one hand, in mammals, DHA promotes outgrowth of neurites, through the enhancement of phospholipids synthesis, particularly phosphatidyl ethanolamine, in the

membranes needed for neurite elongation (Ikemoto *et al.*, 1997). On the other hand, DHA content in neural membrane affects Na^+ and K^+ channels as well as neurotransmitter receptors (Bowen and Clandinin, 2002; Farkas *et al.*, 2002; Levant *et al.*, 2004), interfering with eicosanoid production and transcription factors (Samadi *et al.*, 2006). Particularly, acetylcholine levels are restored in hippocampus when dietary DHA increases, improving learning performance (Horrocks and Yeo, 1999). In agreement, dietary DHA apparently ameliorates the learning performance failure caused by cholinergic dysfunction (Minami *et al.*, 1997). Finally, another mechanism for the neuronal protective role of DHA could be the inhibition of apoptosis induced by sphingosine (Horrocks and Yeo, 1999). In fish, EPA also plays an important role in modulation of eicosanoid synthesis and transcription factors (Ganga *et al.*, 2005), but this fatty acid is much less efficient than DHA in regulation of membrane fluidity and incorporation into phosphatidyl ethanolamine (the main phospholipid in neural tissues), what could explain its lower ability to initiate a startle response. Moreover, increased dietary EPA reduces incorporation of dietary DHA into marine fish larvae phospholipids (Izquierdo *et al.*, 2000).

The results of the present study have shown that DHA, rather than EPA, boosts escape behaviour in gilthead sea bream, and this effect may be at least partly mediated by the increase in neural activity in M-cells. Nevertheless, this reaction could also be related to a faster response against a stressor (auditory stimuli) since it has been recently shown that DHA has the ability to regulate cortisol release by ACTH induced interrenal cells of gilthead sea bream (Ganga *et al.*, 2006). Reduced dietary supply of DHA during early development may have long-lasting effects on brain function and cytoarchitecture of the developing brain (Koletzko *et al.*, 2008). Early detection of insufficient DHA contents in neural tissues of marine fish larvae by simple behaviour observations may constitute a non-invasive tool to allow the prevention of posterior neural disorders and improve marine fry production by reducing mortality.

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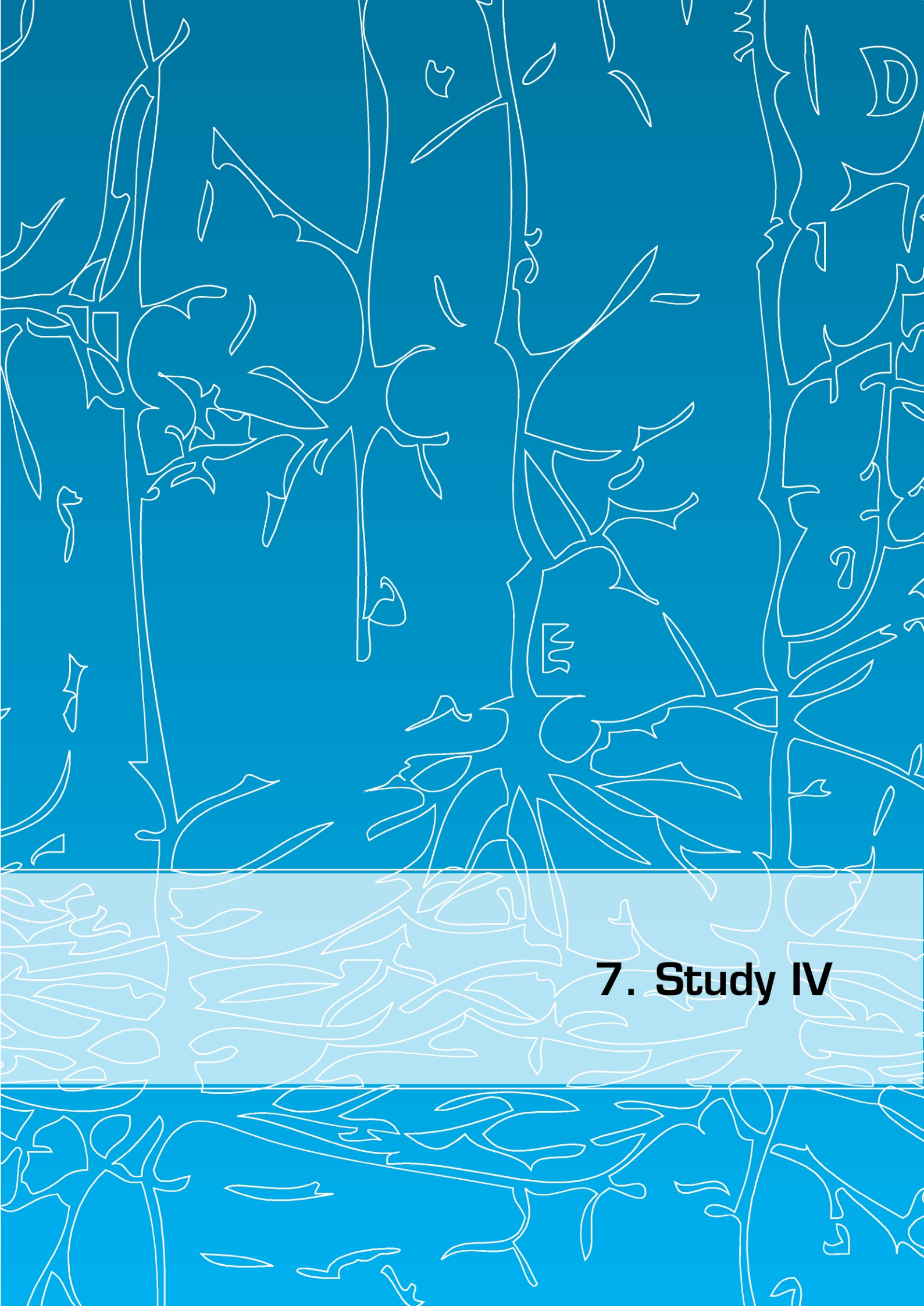

7. Study IV

Study IV

Dietary polyunsaturaed fatty acids affect zebrafish (*Danio rerio*) behaviour and Mauthner cells

This study, which remains un-published, has been done in collaboration with the Department of Neurobiology and Behaviour, Cornell University, Ithaca, USA.

Abstract

Behaviours like the escape response are initiated by a pair of large identifiable neurons in the hindbrain: the Mauthner cells (M-cells). Several factors seem to affect M-cell performance but little is known about the effect of nutritional factors. N-3 long chain fatty acids (n-3 LCPUFA), and particularly docosahexaenoic acid (DHA), are essential components of cellular membranes and they are particularly accumulated in neural tissues and sensorial organs. Inadequate contents of n-3 LCPUFA in fish diets give rise to several alterations such as poor feeding and swimming activities. The aim of this study was to determine the effect of dietary n-3 LCPUFA levels on M-cells functioning in zebrafish (*Danio rerio*) in relation to escape behaviour and neural function. Behaviour was determined by eliciting an escape to a vibratory stimulus and measuring the peak angle speed of the turn and the time to the peak of the initial bend. M-cells activity was studied by ChAT immuno-fluorescence. Feeding the lowest n-3 LCPUFA levels led to a lower frequency of escape response. Elevation of dietary DHA was correlated with a higher speed angle and peak time denoting its importance on escaping behaviour. In addition, a marked immunoreactivity was found in M-cells. The present study denotes the relevance of dietary DHA for normal growth and enhanced startle response and neural activity of zebrafish, pointing out the importance of optimum nutritional conditions when zebrafish is used as a model in behaviour and neural studies.

Keywords: DHA, fish larvae behaviour, long chain fatty acids, Mauthner cells.

Introduction

Over the past twenty years, the zebrafish (*Danio rerio*) has emerged as a great vertebrate model system for screening of therapeutic drugs (Penberthy *et al.*, 2002; Sumanasa and Lin, 2004) and to better understand the neural basis of behaviour (Zottoli and Faber, 2000; Eaton *et al.*, 2001; Kohashi and Oda, 2008; Fetcho and MacLean, 2010; MacLean and Fetcho, 2010). Behaviours like the escape response, also known as the fast-start or startle response, are initiated by a pair of large, identifiable neurons in the hindbrain: the Mauthner or M-cells (Zottoli and Faber, 2000; Eaton *et al.*, 2001; Korn and Faber, 2005). The M-cells are a pair of reticulospinal neurons located in the medulla teleost fish (Beccari, 1907). They are characterised by the large size of their somata and their myelinated axons that cross the body midline to descend the length of the spinal cord on the opposite side of the body from its soma, issuing axon collaterals that massively activate cranial and spinal motor systems (Faber *et al.*, 1989). The presence of two distinct dendrites, the association of the lateral dendrite with the VIIIth cranial nerves, and the presence of a unique structure, the so-called axon cap (Triller and Korn, 1980), around the cell's initial segment, convey a morphological identifiably to this pair of cells in different animals like the otophysan fish (Zotoli, 1978, Bierman *et al.*, 2009). During sudden stimuli, fish perform an escape that starts with a rapid bend of the body to one side. M-cells play an important role in initiating this escape response: a single spike in one of the M-cells triggers an escape toward the contralateral side (Eaton et al., 2001; Korn and Faber, 2005). During Mauthner-triggered escapes, motoneurons on the side of the escape bend are activated, while those on the opposite side are almost simultaneously inhibited (Yasargil and Diamond, 1968). Fast-starts in most teleost fish consist of a C-type fast-start (C-start), the first stage of which is characterized by a rapid unilateral contraction of trunk musculature leading to head and tail movement, which causes the fish to bend into a C-shape. Subsequent movements typically follow stage 1, including a tail stroke that results in a forward propulsion of the centre of mass (stage 2), leading to gliding or a burst swim (stage 3) (Foreman and Eaton, 1993; Domenici and Blake, 1997). M-cells activity precedes the C-start and electrical stimulation of Mauthner axons can elicit a C-start (Nissanov

et al., 1990).

Several studies have used zebrafish as a model to study M-cells functioning (Eaton and Nissanov, 1985; Fetcho and Liu, 1999; Lorent *et al.*, 2001; Hale, 2002). Several factors seem to affect M-cells performance (Chang *et al.*, 1987; Zottoli and Danielson, 1989; Canfield and Rose, 1993; Zottoli *et al.*, 1995) but little is known on the effect of nutritional factors. N-3 long chain fatty acids (LCPUFA), and particularly docosahexaenoic acid (DHA, 22:6n-3), are essential components of cellular membranes and they are particularly accumulated in neural tissues and sensorial organs (Bell and Tocher, 1989; Bell and Dick, 1991; Mourente *et al.*, 1991; Lauritzen *et al.*, 2001; Benítez-Santana *et al.*, 2007). LCPUFA are essential compounds that play key roles in numerous metabolic and physiological processes ensuring normal cellular function. In fish, DHA and eicosapentaenoic (EPA, 20:5n-3) acids, or their precursor, linolenic acid (LNA, 18:3n-3), must be supplied in the diet, and function as critical structural and physiological components of the cell membranes, being necessary for fish growth, welfare, survival and development (Watanabe and Kiron, 1994; Izquierdo, 1996; Sargent *et al.*, 1999; Izquierdo *et al.*, 2000). Inadequate contents of those dietary essential fatty acids (EFA) in fish diets give rise to several alterations such as poor feeding and swimming activities (Masuda *et al.*, 2002; Benitez-Santana *et al.*, 2007), poor growth and dropping mortality (Hernández-Cruz *et al.*, 1999), fatty livers, abnormal pigmentation, disgregation of gill epithelia, immune-deficiency (Izquierdo, 1996) and raised basal cortisol levels (Montero *et al.*, 2003). Our previous studies (Benítez-Santana *et al.*, in press) showed the first evidences that DHA, rather than EPA, boosts escaping behaviour in gilthead sea bream (*Sparus aurata*) and suggest that this effect is at least partly mediated by the increase in neural activity, in particular, of M-cells. Dietary fatty acids have been found to affect escape behaviour in fish larvae after a sound or visual stimulus (Masuda *et al.*, 2002; Benítez-Santana *et al.*, 2007). As in mammals, inadequate intake of DHA is associated with impaired attention and learning performance as well as modifications in emotional status including elevated behavioural indices of anxiety, aggression and depression (Fedorova and Salem, 2006).

Therefore, the aim of this study was to determine the effect of dietary n-3 LCPUFA levels on M-cells functioning in zebrafish in relation to escape behaviour and neural function.

Material and Methods

All experiments conformed to NIH guidelines regarding animal care and were approved by Cornell University's Institutional Animal Care and Use Committee.

Feeding conditions and growth

The nacre type zebrafish larvae used were spawned at the Department of Neurobiology and Behaviour in Cornell University (Mudd Hall, Ithaca, USA). Twelve larvae were individually distributed into 12 aquariums filled with filtered water at 28.5 °C and submitted to a 14 h/10 h light /dark photoperiod. From 4-12 days after hatching (dah) larvae were fed Shrimp Larva Diet (Miami Aqua-culture, Inc. Florida). From 12 dah larvae were fed *Artemia* twice a day (at 9:00 h and 15:00 h) for 25 days. Three different lots of *Artemia* were fed with three levels of DHA: "Diet L" (low content of n-3 LCPUFA), "Diet M" (medium content of n-3 LCPUFA), supplemented with 1 capsule of MorDHA (241 mg DHA and 33 mg EPA, Minami Nutrition, Belgium) and "Diet H" (high content of n-3 LCPUFA) enriched with 2 capsules of MorDHA. Larval growth was evaluated by determining larval total length at 5 and 30 dah under the light microscope.

Biochemical analyses

Diets were analyzed for crude lipid content and fatty acid composition (Table 7.1). Diets were sampled for lipid and fatty acid composition of total lipids. Lipids were extracted by chloroform:methanol (Folch *et al.*, 1957). Methyl esters of fatty acids were prepared by transesterification with 1% sulfuric acid and methanol using hexaenoicosanoic acid (10% of total lipids) as an internal standard (Christie, 1982). The fatty acid methyl esters obtained were separated by gas chromatography (ShimadzuGC-14a, Kyoto, Japan) run at the operating conditions previously

described (Izquierdo *et al.*, 1992), quantified by flame ionisation detectors (FID) and identified by comparison to well characterized external standards.

Behavioural studies

Response to a vibrational stimulus was studied determining the peak angle of escape bend, the peak angular speed of the escape turn and time to respond in challenged zebra fish larvae (6 larvae per diet, 10 stimulus per larva) at 19 dah, after one week of feeding *Artemia* containing different n-3 fatty acid levels. The trials were recorded by a high-speed camera that digitally captures images at 1000 frames/s (EG&G Reticon, Sunnyvale, CA) using the software called Photron Fastcam Viewer (San Diego, CA, USA). Data were analyzed using a custom written image analysis program focusing on the performance of the initial turn in the fish escape, since the Mauthner array is essential for generating the high-performance movements during this turn.

Mauthner cells studies

In order to study Mauthner cells development and morphology these neurons were labelled via electroporation in 6 larvae per treatment at 4 dah prior to the feeding trial. At this age, nacre zebrafish larvae are transparent with sparse pigmentation, that allows the identification and picturing of neurons in the brain and spinal cord of the intact animal. For electroporation, larval zebrafish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222, Sigma Aldrich, St. Louis, MO, USA) and positioned straight in 1.2% agarose in 0.3' phosphate buffered saline (PBS) on a glass slide. A 10% solution of rhodamine (3000 molecular weight or MW; Molecular Probes, Eugene, OR) in 10% Hanks solution was electroporated into the Mauthner cell (Bhatt *et al.*, 2004). After injection, the animals were given 24 h to allow for recovery.

Table 7.1 Some fatty acids contents in total lipids from *Artemia* fed different levels of DHA and used to feed zebrafish larvae (% d.w.).

Fatty acids	Diet L	Diet M	Diet H
14:00	0.08	0.22	0.31
14:1n-7	0.10	0.27	0.41
14:1n-5	0.05	0.13	0.21
15:00	0.02	0.04	0.07
15:1n-5	0.01	0.05	0.06
16:0ISO	0.05	0.14	0.21
16:00	1.11	3.35	4.51
16:1n-7	0.23	0.63	0.95
16:1n-5	0.07	0.18	0.25
16:2n-6	n.d.	0.03	0.05
16:2n-4	0.08	0.22	0.28
17:00	0.06	0.17	0.25
16:3n-4	0.05	0.17	0.24
16:3n-3	0.04	0.17	0.25
16:3n-1	0.01	0.04	0.06
16:4n-3	n.d.	0.01	0.02
18:00	0.60	1.71	2.14
18:1n-9	1.98	6.13	8.00
18:1n-7	0.64	1.62	2.51
18:2n-9	0.04	0.12	0.18
18:2n-6	0.61	3.15	3.49
18:2n-4	n.d.	0.02	0.01
18:3n-6	0.03	0.08	0.13
18:3n-3	2.40	7.23	9.90
18:4n-3	0.40	1.03	1.47
18:4n-1	n.d.	n.d.	n.d.
20:00	0.01	0.06	0.08
20:1n-9+n-7	0.05	0.27	0.37
20:1n-5	0.01	0.05	0.06
20:2n-9	0.01	0.01	0.02
20:2n-6	0.03	0.09	0.11
20:3n-6	n.d.	0.02	0.03
20:4n-6	0.05	0.15	0.33
20:3n-3	0.08	0.29	0.38
20:4n-3	0.13	0.41	0.46
20:5n-3	0.17	2.19	3.05
22:1n-11	0.08	0.18	0.23
22:1n-9	0.07	0.45	0.62
22:4n-6	0.01	0.01	0.02
22:5n-6	n.d.	0.10	0.11
22:5n-3	0.03	0.71	0.96
22:6n-3	0.06	4.71	6.39
Saturated	1.85	5.49	7.26
Monoenoics	2.85	8.74	11.86
Polyunsaturated	4.23	21.02	27.97
n-3	3.30	16.73	22.86
n-6	0.72	3.63	4.26
n-3 LCPUFA	0.46	8.30	11.25
AA/EPA	0.28	0.07	0.11
EPA/DHA	2.86	0.46	0.48

n.d.≤0.005

At the end of the experiment all the fish larvae were briefly anesthetized, embedded on their backs in soft agar on a cover glass in a petri dish (Eaton *et al.*, 1984) and then rinsed with 10% Hanks solution to allow recovery from the anesthetic. Confocal images were obtained by looking into the head of the intact fish using a Zeiss LSM 510 confocal microscope (Fetcho and O'Malley, 1995; O'Malley *et al.*, 1996). The fish transparency allowed not only to clearly visualize neurons inside the living animal but also to monitor fish viability by observing the heartbeat and blood flow. To confirm the identity of the cells studied physiologically, stacks of images showing the morphology in successive confocal sections were acquired. Signal averaging was used when acquiring this morphological data. Maximum projections were made from stacks of these sections. The image stacks were also reconstructed in three-dimensions using the Zeiss software or Imaris, allowing us to examine the details of the dendritic morphology and axonal projections of each cell.

After getting the images, larvae were collected and fixed in 10% buffered formalin. Each larva head was mounted in a gelatin block in horizontal orientation to obtain better visualization of neuronal structures. Gelatin-embedded larva head blocks were cryoprotected in 30% sucrose and serially cut on a Slee Mainz cryostat at 10 □m. Each gelatin section for immunofluorescence slides was collected on gelatin-coated slides. The antibody was tested to determine the optimal working concentration and quality of the signal and zebrafish larvae were processed for the demonstration of immunoreactivity. The slides were covered with 5% rabbit serum and 0.2% Triton in phosphate-buffered saline (PBS) for 1 h. Incubations with anti-choline acetyltransferase primary antibody (1:250, Millipore, Billerica, USA) was carried out for 48 h at 4° C in a 0.1 M (PBS) solution with 0.1% Triton X-100 and 2% goat serum (Vector). As secondary antibody we used anti-goat IgG FITC conjugate (1:250, antibody developed in rabbit affinity isolated antigen specific antibody; Sigma) diluted in PBS and applied for 1 hour in a dark room at room temperature. A propidium iodide complex at concentration of $1:10^4$ was applied for 5-10 min at room temperature and was used for nuclei contrast staining. In order to see the M-cells green immunofluorescence a Zeiss LSM 510 confocal system (Zeiss, Thornwood, NY) was used. In order to prevent fluorescence data were not

compromised, in each batch of samples processed always all treatments were included. Negative controls were run by replacing each primary antibody by PBS to test for the specificity of an antibody involved.

Statistical treatment of data

Data were statistically analyzed with the software SPSS (SPSS 11.5 for Windows, SPSS Inc, Chicago, IL, USA) using one-way analysis of variance (ANOVA) following the general linear model:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

Means of fatty acid levels and behavioural studies were compared by Duncan's test ($P<0.05$).

Results

Biochemical analysis of non-enriched *Artemia* (Diet L) and *Artemia* enriched with one (Diet M) or two (Diet H) capsules of DHA showed a progressive increase in total lipids content (9.5, 36.8 and 49.3% lipids in dry basis of *Artemia*, respectively for diets L, M and H). As expected, enrichment with DHA capsules markedly changed the fatty acid profile of *Artemia*. Thus, major fatty acids in Diet L (non enriched *Artemia*) were 18:3n-3, 18:1n-9 and 16:0, whereas in diets M and H (*Artemia* enriched with DHA) were 18:3n-3, 18:1n-9 and DHA, followed by 18:2n-6 and EPA. Enrichment with DHA capsules progressively increased DHA in larvae, together with other fatty acids such as 18:2n-6, 18:3n-3 and 20:5n-3.

After 17 days of feeding, zebra fish fed *Artemia* not enriched with DHA capsules, containing the lowest polyunsaturated fatty acid contents (Diet L), showed significantly lower growth than fish fed with *Artemia* enriched with one capsule of DHA (Diet M) (Figure 7.1). However, further enrichment with two DHA capsules (Diet H) significantly reduced final fish total length.

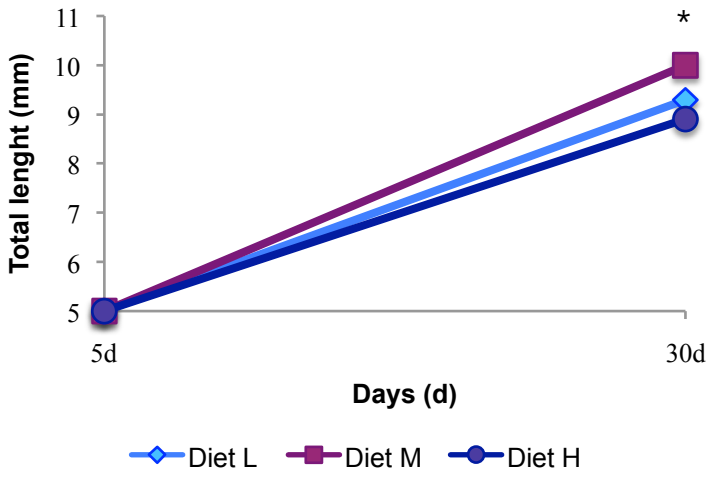

Figure 7.1 Evolution of growth in zebrafish larvae fed *Artemia* enriched with different levels of DHA (n=12, P<0.05).

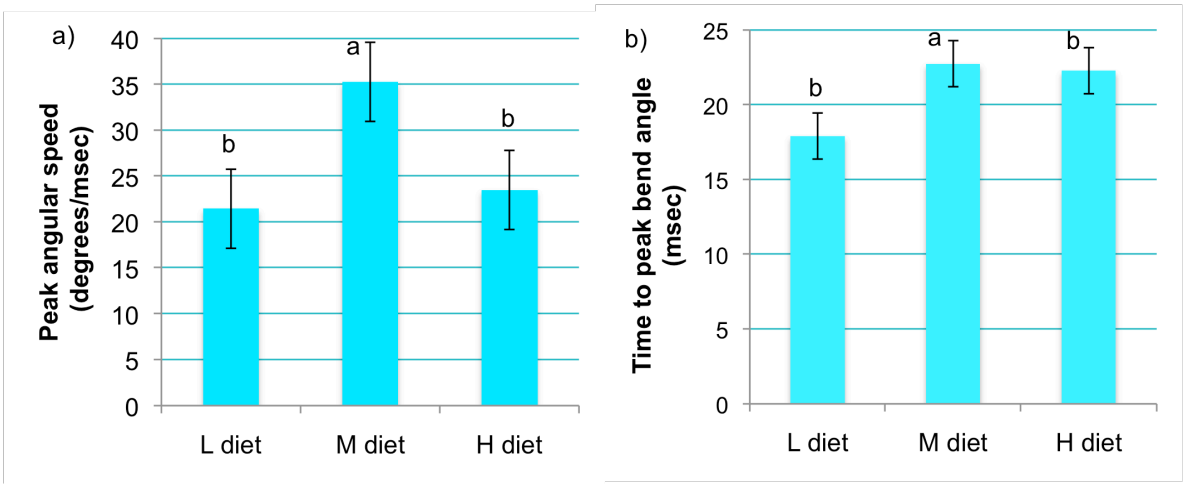

Figure 7.2 (a) Peak angular speed (Degrees/msec) (b) Time to peak bend angle (msec) in zebrafish larvae fed with different levels of DHA. a, b Mean values with unlike letters were significantly different between animals of different treatment (n=6, P<0.05).

Behavioural performance

At the beginning of the study there was a good feeding activity in the fish larvae, regardless the type of feed. After one week of feeding *Artemia* the different diets, zebrafish fed non-enriched *Artemia* (Diet L) containing the lowest EPA and DHA (0.17 & 0.06, respectively) showed a lower response to the vibration stimulus, whereas a significantly highest response was found in fish fed Diet M. Thus, fish fed Diet M showed a significantly higher peak angle and speed angle after sound stimuli (Figure 7.2a, b). However, fish fed with *Artemia* enriched with two DHA capsules (Diet L) showed lower values of peak angle and speed angle than fish fed Diet M, and only slightly higher than those of fish fed Diet L.

Mauthner cells activity

In agreement with the lower response after the vibration stimulus in fish fed Diet L, confocal microscopy analysis showed a low anti-choline acetyltransferase signal (green fluorescence) in M-cells from this fish, whereas enrichment of *Artemia* with one DHA capsule (Diet M) increased fluorescence suggesting a perhaps a healthier neuron (Figure 7.3). On the contrary, further enrichment with two DHA capsules (Diet H) reduced the immunopositive response (Figure 7.3). Analysing the amount of green fluorescence by confocal microscopy we found a significantly highest signal in fish fed Diet M, denoting a higher neuron activity than fish fed Diets L or H (Figure 7.4).

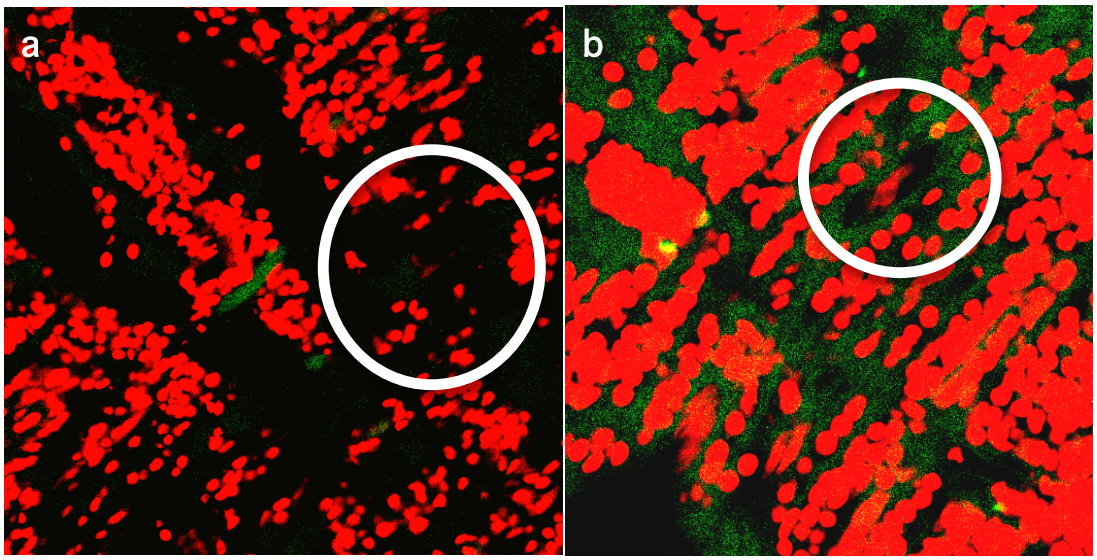

Figure 7.3 Immunopositive response (green fluorescence) observed by confocal microscopy in Mauthner cells from zebrafish fed different diets. (a) Zebrafish fed Diet L (0.06 DHA content). (b) Zebrafish fed Diet M (4.71 DHA content) (x1000).

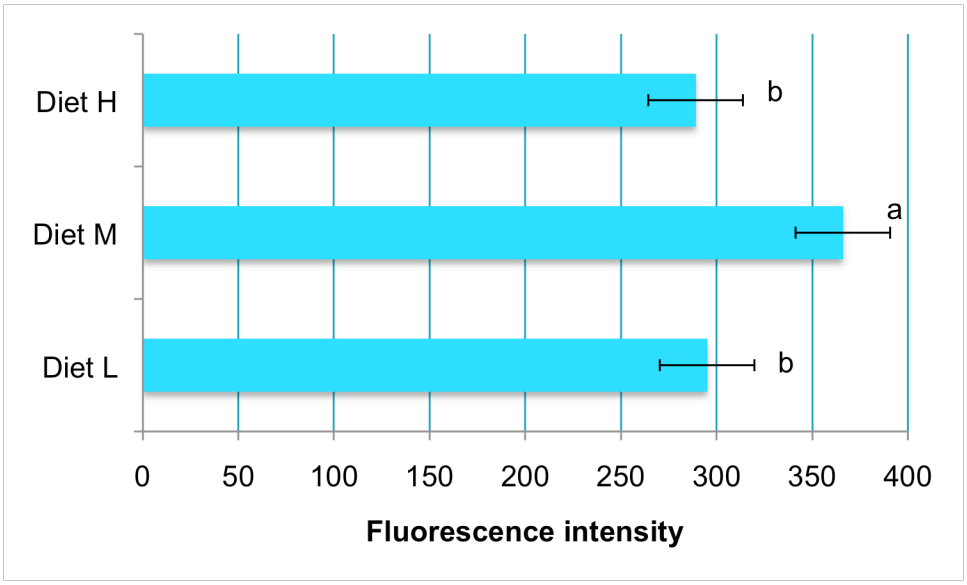

Figure 7.4 Quantification of green fluorescent intensity by confocal microscopy according to the anti-choline acetyltransferase immunopositive response of M-cells in zebrafish fed diets with different DHA levels. a, b Mean values with unlike letters were significantly different between animals of different treatment (n=30, $P<0.05$).

Discussion

Zebrafish fed non enriched *Artemia*, particularly low in DHA (0.06%) as well as in other polyunsaturated fatty acids such as EPA (0.17%), ARA (0.05%) or 18:2n-6 (0.61%), showed poor growth and reduced escaping response to sonorous stimulus, together with lower M-cells immunostaining for anti-choline acetyltransferase, denoting a deficiency in essential fatty acids in this fish. Despite its importance as a model for physiological and neurological research, few studies have focus the essential fatty acid requirements for zebrafish along its life cycle. In the present study DHA, EPA, ARA and LA in Diet L were, respectively, 13, 6, 2 and 13 times lower than the values obtained by Jaya-Ram *et al.* (2008) as optimum for zebrafish broodstock, suggesting insufficient amounts of these essential fatty acids in Diet L. In fish, dietary PUFA have been shown to be necessary for optimum growth (Izquierdo, 1996; Sargent *et al.*, 1999), stress resistance (Koven *et al.*, 2001; Montero *et al.*, 2003; Izquierdo, 2005) and, more recently, normal behaviour (Benítez-Santana *et al.*, 2007). In agreement, in the present study, elevation of dietary DHA to 4.71% significantly improved zebrafish growth, escaping response and M-cells immunostaining for anti-choline acetyltransferase, covering the DHA requirements found as optimum for zebrafish broodstock (1.28%, Jaya-Ram *et al.*, 2008).

Despite early studies in fish suggested the importance of dietary DHA for adequate feeding and swimming activities (Izquierdo, 1996; Wagner *et al.*, 2004), very few studies have been aimed to determine the effect of this fatty acid on particular aspects of fish behaviour and the potential mechanisms implied. Dietary DHA has been found to increase escape response to visual and sonorous stimulus (Benítez-Santana *et al.*, 2007; in press) promoting the early the appearance of response to visual stimulus and suggesting the importance of this fatty acid for the functional development of brain and vision (Benítez-Santana *et al.*, 2007) in marine fish larvae. Zebrafish can constitute an excellent model to study behaviour in relation to dietary DHA and the involvement of specific neurons. In the present experiment, larvae fed with Diet M, containing 4.71% DHA, showed the highest angular speed

and peak time to the initial bend in response to a sonorous stimulus. These results are in agreement with the better response to different types of stressors in marine fish fed increased DHA dietary levels (Izquierdo *et al.*, 2000, Benítez-Santana *et al.*, 2007) and point out the importance of this fatty acid also for behaviour of freshwater fish species such as zebrafish.

All the necessary biosynthetic enzymes for DHA synthesis from its precursor 18:3n-3 (linolenic acid) has been characterized in the zebrafish genome (Lykidis, 2007). However, in the present study, although Diet L contained enough 18:3n-3 to cover the essential fatty acid requirements for this fatty acid, zebrafish fed this diet showed a significantly lower response than fish fed with increased amounts of DHA, denoting a specific requirement for this fatty acid at least in very young stages of zebrafish. These results are in agreement with the specific requirements for DHA and EPA found during reproduction of this species (Jaya-Ram *et al.*, 2008). DHA is particularly important for brain development and it is accumulated in neural tissues (Bell and Dick, 1993), being associated with sensory organs functioning (Izquierdo, 2005). In higher vertebrates, DHA is associated with impaired attention and learning abilities (Moriguchi *et al.*, 2000; Reisbick and Neuringer 1997; Salem *et al.*, 2001), in relation to disruptions of serotoninergic and dopaminergic neurotransmission (Kodas *et al.*, 2004; Takeuchi *et al.*, 2002; Zimmer *et al.*, 2000, 2002). More recently, the synthesis of very long-chain fatty acids derived from LC-PUFA has been characterized in zebrafish brain (Monroig *et al.*, 2010). The importance of VLC-PUFA for neural function remains unknown and deserves further studies. However, DHA does not seem to be a good precursor of VLC-PUFA and is only marginally elongated, remaining essentially unmodified and directly accumulated in the zebrafish brain for the normal development functions for the neural activity.

The startle response is a fundamental sensorimotor response in animals that is a fast, protective response often used to avoid predators. The plasticity of the startle response and its disruption in disease states with broad neuropsychiatric consequences have made it a model system for studies of the neuronal generation of behaviour and its disruption and treatment during disease. In the present study, the higher immuno-fluorescence to anti-choline acetyltransferase in zebrafish fed

Diet M, containing 4.71% DHA denotes an enhancement in acetylcholine production and, perhaps, neuronal activity, which was identified in M-cells. Other mechanisms could be also involved in the effect of DHA on neural activity such as regulation of Na^+ and K^+ channels, neurotransmitter receptors, or eicosanoid production and E receptors which regulate neurotransmitter release in mammals.

Despite the beneficial effects of *Artemia* enrichment with one DHA capsule, further elevation of DHA levels in Diet H negatively affected growth, escaping response to sonorous stimulus and M-cells immunostaining. Negative effects of elevated dietary DHA contents in fish have been associated with the high sensitivity of this polyunsaturated fatty acid to peroxidation (Betancor *et al.*, 2010). Under normal physiological conditions, reactive oxygen species are rapidly eliminated in fish by antioxidant enzymes such as superoxidate dismutase, catalase, and glutathione peroxidase (Zhang *et al.*, 2009). However, augmentation of dietary DHA markedly increases peroxidation risks, raising TBARs contents and enhancing expression of superoxide dismutase and catalase genes, and creating an imbalance in reactive oxygen species that can outright oxidative damage in different fish tissues (Betancor *et al.*, 2010, in press; Izquierdo, unpublished data). Indeed, in zebrafish, oxidative stress has been found not only to promote expression of antioxidant enzymes and apoptosis related genes, but also to cause a heavy damage of DNA (Jin *et al.*, 2011).

In summary, the present study denotes the importance of dietary DHA for normal growth and enhanced startle response and neural activity of zebrafish, pointing out the importance of optimum nutritional conditions when zebrafish is used as a model in behaviour and neural studies.

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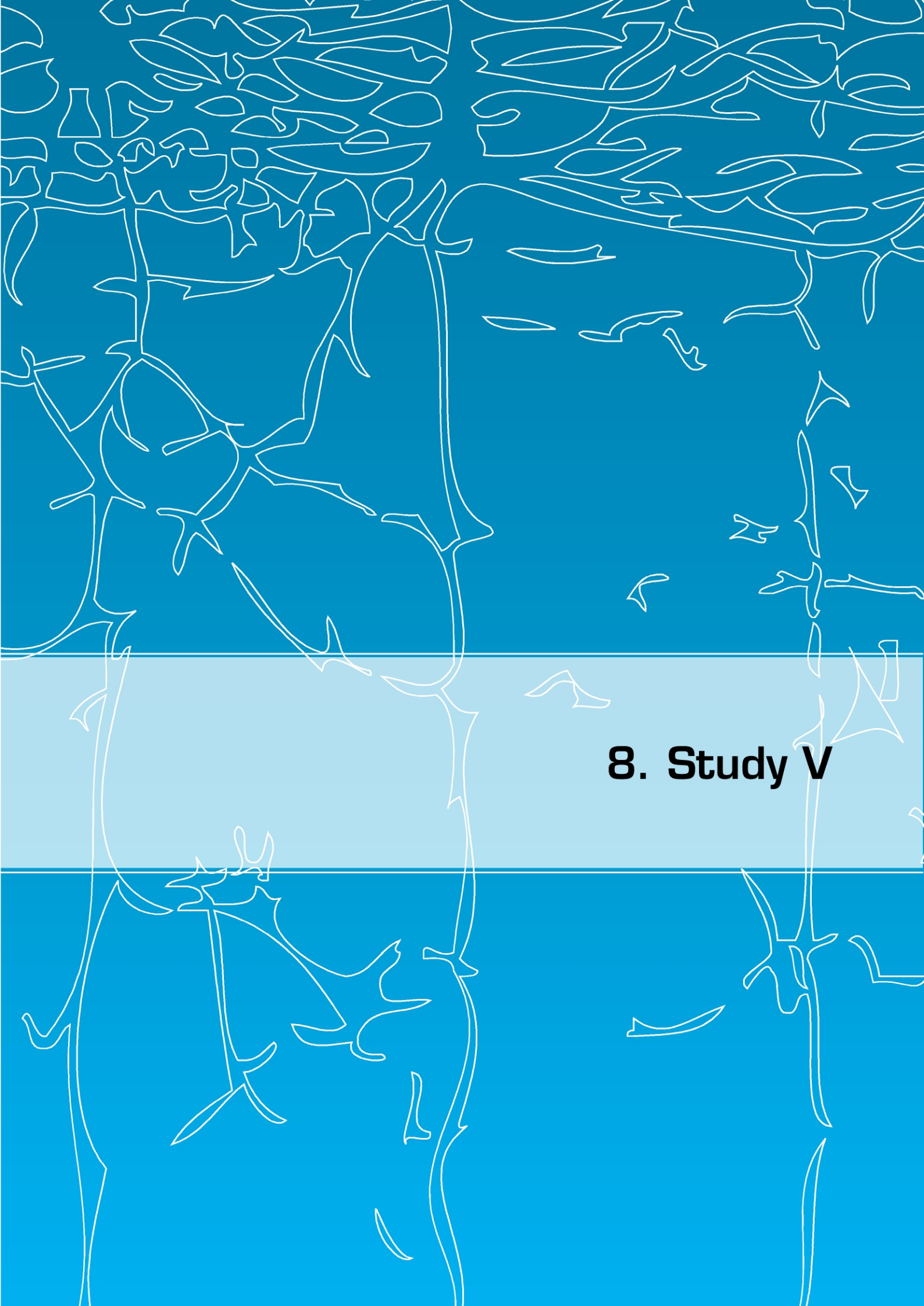

8. Study V

Study V

Use of calretinin (CR) and parvalbumin (PV) as Mauthner cells markers in sea bass (*Dicentrarchus labrax*)

This work has been done in collaboration with the Instituto de Nuerociencias de Castilla y León (INCYL), Salamanca (Spain) and has been submitted to International Journal of Developmental Neuroscience.

Abstract

Monitoring larval behaviour can be a powerful tool for early detection of stress or diseases with negative implications in larval rearing success. Behaviour is markedly affected by brain development. Mauthner cells, located in the hindbrain, are easily identifiable morphologically by the large size of its soma, dentrites and axons that cross the midline to descend the length of the spinal cord on the opposite side of the body from its soma, issuing axon collaterals that massively activate cranial and spinal motor systems. This pair of reticulospinal neurons initiate fast startle responses in fishes and amphibians and have been an important model system in studies of vertebrate neurons and their control of behaviour. The present study applied and developed histological techniques to specifically study this cell type, allowing their study along larval development in European sea bass (*Dicentrarchus labrax*) using two different calcium-binding proteins: calretinin and parvalbumin.

Keywords: Calretinin, immunohistochemistry, Mauthner cells, parvalbumin.

Introduction

Teleost fish are the most abundant and diverse group of vertebrates (Nelson, 2006). There are a wide variety of brain morphological types in teleosts, which vary both in external form and internal structure. Studies have focused on brain anatomy as well as complete cytoarchitectonic studies on gilthead sea bream (*Sparus aurata*) (Muñoz-Cueto *et al.*, 2001), tilapia (*Oreochromis mossambicus*) (Peppels *et al.*, 2002), medaka (*Oryzias latipes*) (Anken and Bourrat, 1998), platyfish (*Xhiphopho rushelleri*) (Anken and Rahmann, 1994) and sea bass (*Dicentrarchus labrax*) (Cerdá-Reverter *et al.*, 2008) including the Mauthner cells (M-cells). However, there is a high variability among fish species and it would be interesting to study these cells in European sea bass (*Dicentrarchus labrax*) because it may have different characteristics depending on the species. One of the most typical characteristics of the central nervous system (CNS) of teleosts and certain amphibians is the presence in the hindbrain of two large nerve cells whose thick axons extend all the way along the spinal cord (Zottoli, 1978; Stefanelli, 1980). This bilateral pair of large reticulospinal neurons located in the hindbrain was discovered by Mauthner in 1859, function as integration centers for sensory inputs and higher motor commands in the brain and ultimately regulate motor functions in the spinal cord (Rovainen, 1978; Rovainen, 1982). The M-cells are easily identifiable morphologically by the large size of its soma, dentrites and axons that cross the midline to descend the length of the spinal cord on the opposite side of the body from its soma, issuing axon collaterals that massively activate cranial and spinal motor systems (Faber *et al.*, 1989). The presence of two distinct dentrites, the association of the lateral dentrite with the VIIIth nerve, and the presence of a unique structure, the so-called axon cap, around the cell´s initial segment convey a morphological identifiably to this pair of cells in different species like otophysan fish (Zotoli, 1978).

The M-cells have served as particular models in neuroscience as they play key roles in escape behaviours (Eaton *et al.*, 1977; Kimmel *et al.*, 1980; Metcalfe *et al.*, 1986). Thus, the escape response, also known as the fast-start or startle response, is initiated by M-cells (Zottoli and Faber, 2000;

Eaton *et al.*, 2001; Korn and Faber, 2005). In this sense, studies on M-cells have provided primary information on neural biochemistry, development, synaptic morphology and physiology, and control of behaviour such as fast-escape motor response after vibrational and/or visual stimuli (Eaton and Bombardieri, 1978), that can be generalized to many central neurons throughout vertebrates (Faber and Korn, 1978a; Nissanov and Eaton, 1989; Korn *et al.*, 1990). They receive acoustic and other sensory inputs and connect directly to spinal motoneurons. Together with other reticulospinal cells, the M-cells trigger the C-start, a characteristic escape movement of fishes and also mediate tail-flip escape responses in fish (Eaton *et al.*, 1977; Webb, 1978; Will, 1991; Domenici and Batty, 1997; Domenici and Blake, 1997; Eaton *et al.*, 2001; Azizi and Landberg, 2002; Ward and Azizi, 2004; Korn and Faber, 2005), challenging this perception as their synapses undergo activity-dependent potentiation (Yang *et al.*, 1990; Pereda *et al.*, 2004). Stimulation of these afferents with high frequency trains evokes a long-term potentiation of both components of their mixed, electrical (gap-junction mediated) and chemical, synaptic response (Yang *et al.*, 1990). The plastic properties of these synapses likely represent a mechanism for input sensitization (Yang *et al.*, 1990) and therefore an unusual specialization for a primary auditory afferent, which in this case provides a decision-making neuron (Eaton *et al.*, 2001) with relevant sensory information that could be directly translated into a behavioural response essential for the survival of the fish. This behaviour occurs when the animal is confronted to a sudden aversive stimulus, such a predator attack. Morphology and physiology of the neural circuit that drives the C bend have been studied in depth (Furukawa and Furshpan, 1963; Hackett and Faber, 1983; Fetcho and Faber, 1988; Faber *et al.*, 1989; Fetcho, 1991). Numerous reports have classified the different morphological and neurochemical types of afferences establishing synaptic contacts with M-cells (Nakajima, 1974; Korn *et al.*, 1990).

Calretinin (CR) and parvalbumin (PV) are members of a large family of cytoplasmic calcium-binding proteins that are involved in the regulation of intracellular calcium levels and could buffer calcium transients resulting from different cellular processes (Van Brederode *et al.*, 1991). These proteins

occur in certain subpopulations of nerve cells in the central and peripherals nervous systems (García-Segura *et al.*, 1984; Celio, 1990; Résibois and Rogers, 1992). Besides, Anken *et al.* (1996) suggested that the M-cells of teleosts contain nitric oxide synthase. Neurons producing nitric oxide require a high-sensitivity buffer system of the intracellular calcium levels, because neuronal nitric oxide synthase is dependent on calcium and calmodulin (Vincent, 1995). Immunohistochemistry studies have described the positive staining of M-cells to CR and PV in tench (*Tinca tinca*), their presence indicating that these neurons need complex calcium-buffering system (Crespo *et al.*, 1998). In the present study, we analyze the presence of CR, PV in the M-cells of sea bass larvae ontogeny using immunohistochemical and immunofluorescence tecniques and adapting the method used by Crespo *et al.* (1998) to marine fish.

Material and Methods

Animals

European Sea bass larvae were obtained from natural spawnings from France (Ecloserie Marine de Gravelines, Nord-Pas-de-Calais) where the experiment were carried out. For each species, five hundred thousand 2-days-after-hatching (dah) larvae were randomly distributed into 2 stock tanks ($2m^3$), and fed rotifers enriched with EFA (Selco, DHA Protein Selco, INVE, Dendermonde, Belgium), until they reached 15 dah. At 7 dah, larvae were co-fed the crustacean *Artemia* nauplii, to continue with metanauplii enriched with EFA (Selco, INVE, Dendermonde, Belgium) until they reached 22 dah. From that day larvae were fed a commercial microdiet (GM, Gemma Micron, Skretting, Stavanger, Norway) to 50 dah (Table 8.1). All tanks were supplied with filtered seawater (34 g L^{-1} salinity). Water temperature and dissolved oxygen during the experimental period ranged between 16.5-21°C and 5.04 - 8.32 ppm, respectively. Water quality was daily tested by pH quantification and no deterioration was observed.

Table 8.1 Feeding protocol during the experimental trials.

Dah	Rotifers	Artemia	Fitoplancton	Comercial diet
2			40 l	
3			20 l	
4			20 l	
5	2.5 rotifers/ml		15 l	
6	5-10 rotifers/ml		-	
7	5-10 rotifers/ml	0.25 nauplii/ml	-	
8	5-10 rotifers/ml	0.5 nauplii/ml	-	
...	↓	↑	-	
22	-	0.5 metanauplii/ml	-	50% GM 150 50% GM 300
...	-	-	-	↑

Histological, immunohistochemical and immunofluorescence studies

Thirty sea bass larvae per day were collected and fixed in 10% buffered formalin, dehydrated through graded alcohols followed by xylene, and, finally, embedded in paraffin wax for histological and immunohistochemical studies. Each larva head was included in an individual paraffin block orientated horizontally to obtain a better visualization of neuronal structures. Paraffin-embedded larva blocks were serially cut on a Leica microtome at 3 □m and stained for Hematoxilin and Eosin (H&E) (Martoja and Martoja-Pearson, 1970). When the M-cells were visualized through H&E sections, the next two sections were used to detect M-cells reactions with Nissl staining and immunohistochemistry.

For immunofluorescence studies thirty larvae were collected and fixed in 10% buffered formalin. Each larva head was mounted in a gelatin block in horizontal orientation to obtain better visualization of neuronal structures.

Gelatin-embedded larva head blocks were cryoprotected in 30% sucrose and serially cut on a Slee Mainz cryostat at 10 □m.

a) Nissl staining technique

This method was used for the detection of Nissl body in the cytoplasm of neurons (García del Moral, 1993) on paraformaldehyde or formalin-fixed, paraffin embedded tissue sections. Sections were dewaxed through xylene, 100%, 96% and 70% ethanol, and rinsed in tap water and then in distilled water. Afterwards, sections were stained in 0.1% cresyl violet solution for 10 minutes, washed quickly in acetate buffer, dehydrated in alcohol and xylene, and then mounted with a permanent mounting medium.

b) Immunohistochemical technique

All the antibodies were firstly tested to determine the optimal working concentration and quality of the signal. Sea bass larvae were processed for the demonstration of calretinin (CR) and parvalbumin (PV) immunoreactivity. Sections for immunohistochemistry were mounted on Poly-L coated slides (Sigma P1524). These sections were dewaxed in xylene, rehydrated in a graded alcohols series and incubated with 3% hydrogen peroxidase in methanol for 30 minutes to block endogenous peroxidase activity on a moving platform. Enzymatic treatment was applied according to the used primary antibody. 0.1% pronase (Sigma P5147) in PBS was employed in both primary polyclonal antibodies (Swant, Bellinzona, Switzerland; CR, 1:700 or PV, 1:700) for 3 min at room temperature. Next, the slides were covered with 10% goat serum in PBS for 30 minutes before incubation with the primary antibody for 18 hours at 4ºC. When the primary polyclonal antibody was used, a biotinylated pig anti-rabbit immunoglobulin G diluted 1 in 250 in PBS was applied for 30 min as secondary reagent. An avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) diluted 1 in 50 in PBS was applied for 1 h at room temperature to detect the different substrates. Sections were then incubated with DAB (3,3´-diaminobenzidine tetrahydrochloride), diluted in 0.1 M Tris containing 3% hydrogen peroxide, and checked microscopically for adequate chromogen development. Finally,

sections were rinsed in tap water, counterstained with Harris' Hematoxylin, dehydrated and mounted. Negative controls were run by replacing each primary antibody by PBS. The positive staining (brown staining) was scored as follows: ++, intensive positive staining +, positive staining -, no positive staining.

c) Immunofluorescence study

For immunofluorescence slides, each gelatin section was collected on gelatin-coated slides. The slides were covered with 5% goat serum and 0.2% Triton in PBS for 1 hour before incubation with the primary antibody for 48 hours at 4°C. When the primary polyclonal antibodies were used, a CR or PV diluted 1 in 700 in PBS medium was applied for 1 h as secondary (anti-rabbit IgG FITC conjugate, an antibody developed in goat affinity isolated antigen specific antibody; Sigma) reagent in a dark room at room temperature. A propidium iodide complex diluted 1 mg in 250 ml PBS was applied for 5-10 min at room temperature and was used for nuclei contrast staining. In order to see the M-cells green immunofluorescence, a confocal microscope was used, positive staining scored as indicated in the previous section. For this study, larvae from 17 dah were used as younger larvae could not be cut on the cryostat due to their small size.

Results

Histological detection

The presence of Nissl body-like positive lumps in the most external part of the cytoplasm of M-cells, including the surrounding synaptic endings (Figure 8.1a) was observed throughout the development of sea bass larvae, since as early as 10 dah.

M-cells were firstly identified on H&E staining paraffin sections prior to the employment of Nissl staining and immunohistochemical technique. With this staining all the related structures of M-cells could be appreciated. M-cells axon presented a basophilic staining, whereas nucleous staining was eosinophilic (Figure 8.1b, 8.1c, 8.1d, 8.1e). All the soma appeared surrounded by a zone clearly differentiated from the surrounding tissue and

was identified as the synaptic bed. The initial segment of the axons appeared surrounded by the axon cap and continued with the myelinated axon that crossed the brain midline and extended down to the spinal cord.

Immunohistochemical and immunofluorescence detection

Positive staining of calcium-binding proteins CR and PV was studied on M-cells soma, dendirites and axons (Table 8.2). Calcium-binding protein immunohistochemistry provided a good visualization of Mauthner structures (soma, dendrites and axon) in sea bass larvae.

Table 8.2 Scores of the staining of M-cells in soma, dendrites and axon using PV and CR antibodies.

	Days-old post-hatch							
PV Immunostaining	**6**	**10**	**13**	**17**	**19**	**24**	**28**	**47**
Soma	-	-	-	+	+	++	++	++
Dendrites	-	-	+	+	++	++	++	++
Axon	-	-	+	+	++	++	++	++
PV Immunofluorescence								
Soma	n.d.	n.d.	+	+	+	++	++	++
Dendrites	n.d.	n.d.	+	+	++	++	++	++
Axon	n.d.	n.d.	+	+	++	++	++	++
CR Immunostaining								
Soma	-	-	-	-	-	-	-	-
Dendrites	-	-	-	-	-	-	-	-
Axon*	-	-	-	-	-	-	++	++
CR Immunofluorescence								
Soma	n.d.	n.d.	-	-	-	-	-	-
Dendrites	n.d.	n.d.	-	-	-	-	-	-
Axon*	n.d.	n.d.	-	-	-	-	++	++

* Mauthner axon cap
- negative staining
+ positive staining
++ Intense positive staining
n.d. = not determined

Larvae of 6 and 10 dah showed a PV negative staining in the Mauthner soma, dendrites and axon (Figure 8.2a), whereas in 13 dah larvae dendrites and axon of the M-cells stained positive for PV (Figure 8.2b). In 17 dah larvae light PV-like protein immunoreactive positive neurons were clearly distinguishable in all structures including dendrites, soma and axon (Figure 8.2c). From 19 dah, Mauthner soma, dendrites and axon showed an intensive positive PV immunoreaction (Figure 8.2d), nevertheless, the Mauthner soma membrane was still markedly PV immunonegative (Figure 8.2d). In the PV immunofluorescence studies, the Mauthner nucleus showed negative staining through the whole study, whereas the Mauthner cytoplasm was PV immunopositive together with dendrites and axon showed (Figure 8.3a, 8.3b). The specificity of PV antibody was reduced throughout the development of M-cells.

Despite the high immune-reaction found to PV, in the present study, CR immune-staining or immune-fluorescence was negative in M-cells soma and dendrites along the whole study, and only an intensive positive reaction was detected in M-cells axon cap at 28 and 47 dah (Figure 8.4).

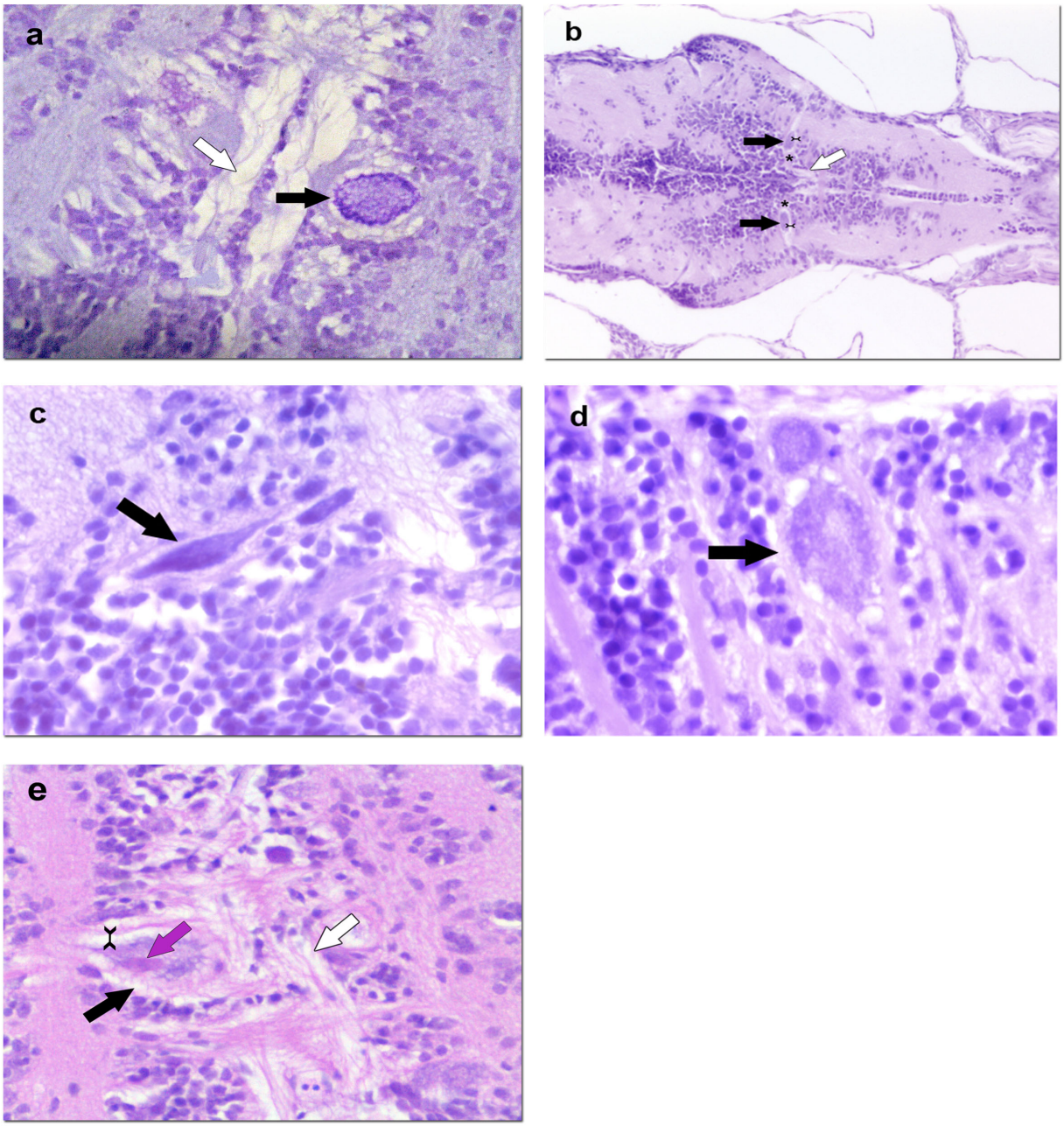

Figure 8.1 (a) Nissl body-like positive lump in the most external part of the cytoplasm of M-cells (black arrow). White arrow shows axon (x1000). (b) Basophilic staining present in soma (black arrow), dendrites, axon (white arrow) and axon cap (*) M-cells present in larvae of 10 days old. (›-‹) shows synaptic bed (x400). (c) Acidophilic stain in dendrites and axon and basophilic stain in soma (black arrow) in larvae of 13 days old (x1000). (d) Acidophilic stain in dendrites and axon and basophilic stain in soma (black arrow) in larvae of 17 days old (x1000). (e) Acidophilic stain in dendrites, axon (white arrow) and nucleus (pink arrow). Basophilic stain in soma (black arrow) in larvae of 28 days old. (›-‹) shows synaptic bed (x1000).

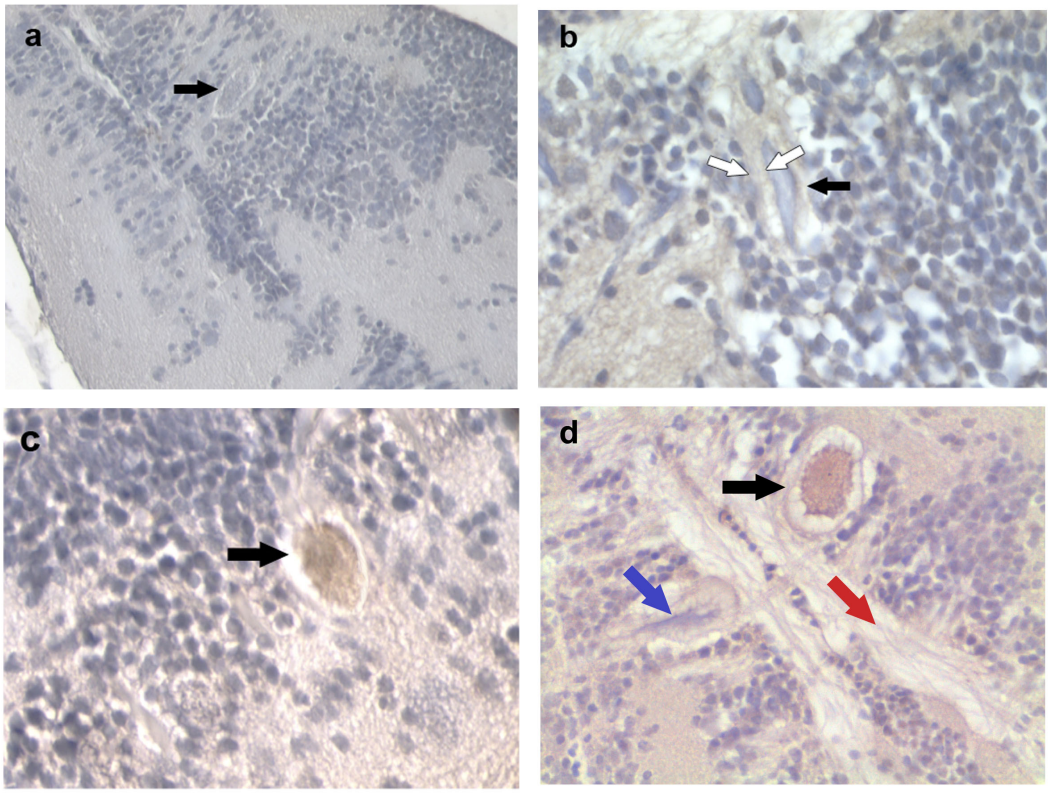

Figure 8.2. (a) The soma, dendrites and axon of M-cells (black arrow) are PV immunonegative (x400). (b) PV immunostained in axon and dendrites (white arrows) of M-cells (black arrow) (x1000). (c) PV-containing soma, dendrites and axon of a M-cell (black arrow) (x1000). (d) PV-immunopositive Mauthner soma (black arrow), dendrites and axon (red arrow). PV-immunonegative Mauthner soma membrane (blue arrow) (x1000).

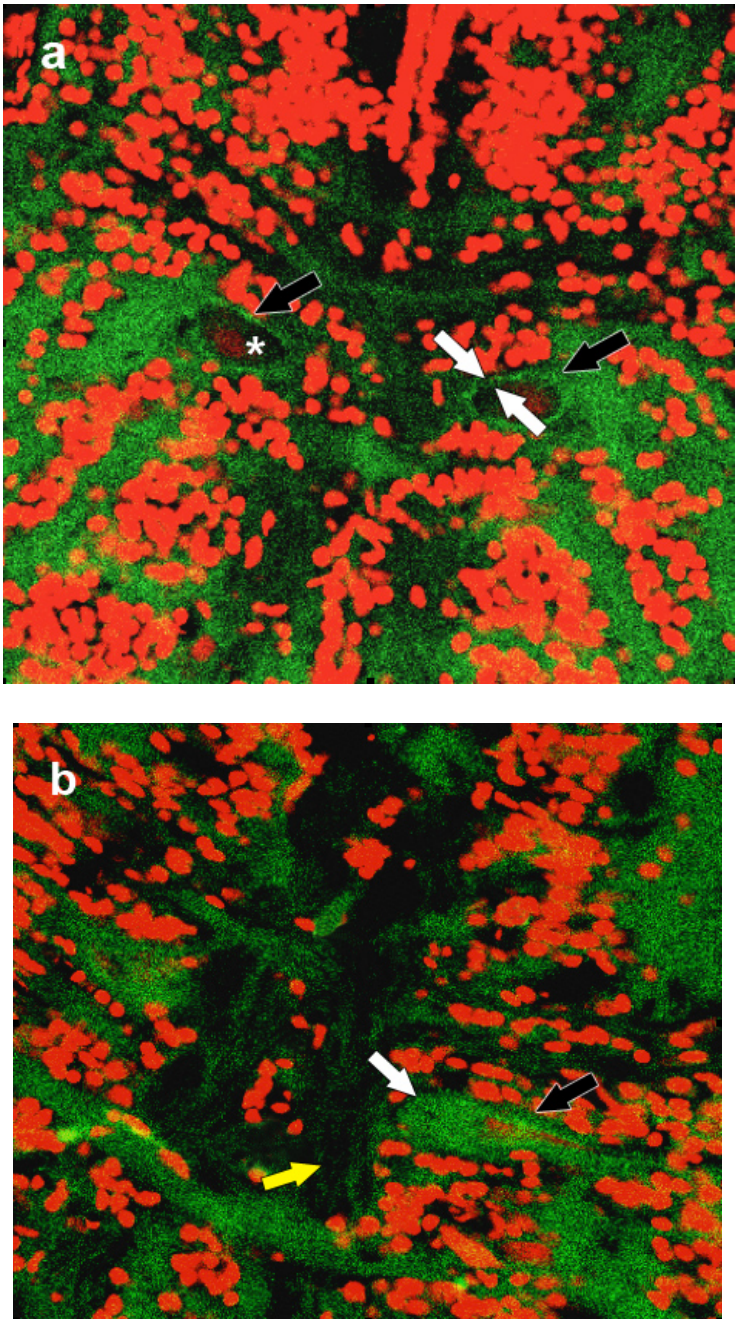

Figure 8.3 (a) PV immunonegative nucleus (*) surrounded by PV immunostained cytoplasm of M-cells (black arrow) in sea bass larvae of 17 days old (x1000). (b) PV-immunopositive soma (white arrow) and axon (yellow arrow) of M-cells and PV-immunonegative nucleus (black arrow) in larvae of 24 days old (x1000).

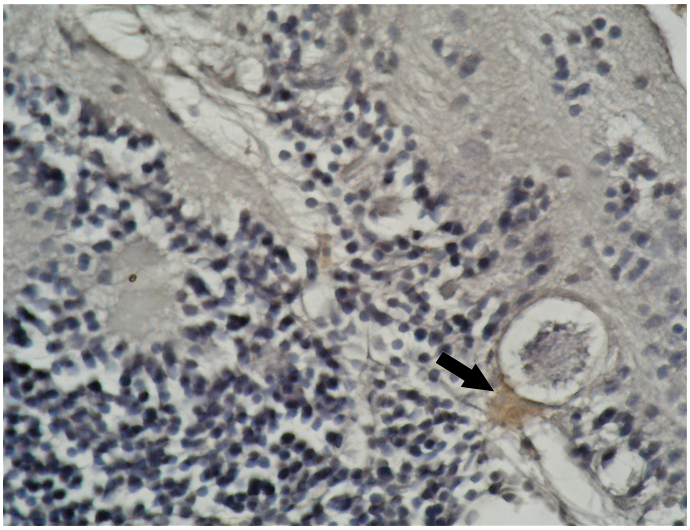

Figure 8.4 CR immunopositive in Mauthner axon cap (black arrow) (x1000).

Discussion

The M-cells appeared dorsal to the medial reticular nucleus and lateral to the nucleus of the medial longitudinal fascicle, coinciding with the emergence of the magnocellular nucleus of the *Area octavolateralis* in sea bass, in agreement with previous studies (Cerdá-Reverter *et al.*, 2008). The present study describes for the first time the presence of buffer calcium-binding proteins, such as CR and PV, in the M-cells of sea bass, an important species in the Mediterranean and Atlantic aquaculture. Moreover, it constitutes the first ontogenetic study describing the presence of these proteins in M-cells along larval development in any fish species. Antibodies are popular tools used not only for neuronal identification but also to reveal their cellular anatomical features. M-cells CR and PV activities have been only studied in very few fish species including swordtail fish (*Xiphophorus helleri*) (Anken *et al.*, 1996) and tench (Crespo *et al.*, 1998). The presence of these proteins suggests an important role of nitric oxide in the circuitries of fast escape responses. Nitric oxide is a gaseous intercellular messenger that has been suggested to play a major role in several different in *vivo* models of learning processes (Bohme *et al.*, 1993; Hawkins *et al.*, 1994). Neurons constitutively synthesizing nitric oxide denote a high-sensitivity calcium-buffer system in M-cells, since the synthesis of this neurotransmitter is

calcium-dependent (Vicent, 1995). The presence of nitric oxide synthase in M-cells could indicate the involvement of nitric oxide, as retrograde messenger, in long-term potentiation events taking place in these neurons (Anken *et al.*, 1996). Nicotinamide adenine dinucleotide phosphate diaphorase produces nitric oxide that could be implicated in long-term potentiation events taking place in the M-cells at the level of both excitatory and inhibitory synaptic junctions (Anken *et al.*, 1996). The suggestion of a high-sensitivity calcium-buffer system in M-cells, is also supported by the high postsynaptic increase in calcium concentrations required by cellular coupling through gap-junctions (Pereda *et al.*, 1996).

In goldfish brain NADPH-diaphorase histochemical staining showed that the nitric oxide synthase (NOS) in M-cells is uniformly distributed along its axon, soma, and ventral and lateral dendrites (Bell *et al.*, 1997). Nevertheless, in the present study, the different moments of appearance of CR and PV along larval development and their diverse distribution in the different subcellular regions of M-cells suggest independent roles for both proteins. These results are in agreement with the different subcellular distributions in the adult tench M-cells (Crespo *et al.*, 1998) and heterogeneous distributions in rat brain (Tan *et al.*, 1997). The M-cells are fast-firing neurons involved in the motor startle reflex (Korn *et al.*, 1990), and PV may shorten the refractory phase of action potentials in the fast-firing events, protecting the cells from calcium overload. The axons of the M-cells have been reported to contain the basic components of the protein synthesis (Weiner *et al.*, 1996). This fact may account for the specific presence of CR immunoreactivity in the Mauthner axons but not in the somata or dendrites of these neurons. The specific distribution of CR in the axons suggests an involvement of CR-mediated calcium buffering mechanisms in functional aspects of the axonal physiology such as neurotransmitter release, or nervous signal transduction (Crespo *et al.*, 1998). It has been demonstrated that the ultrastructural localization of calcium ions in the M-cells changes under normal conditions and after prolonged stimulation (Moshkov *et al.*, 1995).

Evolution of immunostaining and immunofluorescence to PV and CR along larval development also provide interesting information about functionality of M-cells along marine fish ontogeny. Mauthner neurons, mediate fast-escape motor responses, important in predator avoidance, after the reception of unexpected vibrational and/or visual stimuli (Eaton and Bombardieri, 1978). Marine fish larvae are subjected to an intense predator activity and accordingly very low survival rates. At 6 dah (3.32 mm of standard length) escape response is very low or completely lacking (20% population) in marine fish larvae (Benítez-Santana *et al.*, 2007), whereas around 16 dah (5.08 mm) slightly increases in speed and number of responding fish, further increasing around 19 dah (5.44 mm). Moreover, escaping response to sonorous stimulus significantly increases in larvae around 30 dah (6.87 mm) (Benítez-Santana *et al.*, in press). In the present study, the lack of PV or CR reactivity at 6 and 10 dah denote the poor functionality of M-cells in agreement with the low escaping response found in previous studies. Moreover, PV seems to play an important role in the initial development of M-cell functionality and marine fish larvae startling response, since its detection in immunostainning or immunofluorescence at 17 dah and its increase in intensity at 19 dah coincides with the improved escaping response found in previous studies (Benitez Santana *et al.*, 2007). Besides, CR does not seem to play an essential role in M-cell functioning since despite the lack of CR immunostaining and immunofluorescence in 6-24 dah larvae, at this age larvae show a significant startling response (Benitez *et al.*, 2007, Benitez *et al.*, in press) as well as M-cell activity denoted by the reaction to acetil-choline antibodies (Benitez *et al.*, in press 2). However, CR may be important to further booster M-cell activity in older larvae, since its appearance at 28 and 47 dah coincides with the higher startling response found in previous studies (Benítez-Santana *et al.*, in press).

Interestingly, reduction in long chain polyunsaturated fatty acids (LC-PUFA), and more specifically docosahexaenoic acid (DHA) resulted in a lower escaping response and M-cells activity in marine fish (Benítez-Santana *et al.*, in press). This fatty acid is involved in several neural tissue related functions such as neurocytes myelination and synapse construction, both functions

being sensitive to nutritional deficiencies (Krigman and Hoga, 1976). Moreover, DHA has been found to affect calcium channels and cell calcium mobilization (Horrocks and Yeo, 1999) what could be related to the efficacy of PV and CR for adequate functioning of M-cells. Indeed dietary DHA not only reduced startle response and M-cell activity (Atalah *et al.*, 2010; Izquierdo *et al.*, 2010), but also reduced inmunohistaining (PV o CR) in marine fish larvae (Benítez-Santana and Izquierdo, unpublished data). Further studies are being conducted to understand the role of DHA on functioning of M-cells applying the immunestaining techniques developed in the present study.

In summary, the results of this study provide some interesting techniques to further understand the role of M-cells in very young marine larvae, the potential implications in escaping response and the effect of dietary or environmental factors. In addition, the morphological studies of the sea bass brain have provided fine detail specific to the brain of a perciform species that has been used as nomen- clatural references in some other perciform species (Ahrens and Wullimann, 2002; Giusi *et al.*, 2005; Pandolfi *et al.*, 2005). In general, variation in brain morphology is more clearly correlated to behaviour and niches than in other vertebrates (Ito *et al.*, 2007).

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9. Conclusions

Conclusions

The results of this thesis can be summarize in the following conclusions for each chapter:

- Study 1. Dietary n-3 long chain polyunsaturated fatty acids deficiency induces a reduced visual response in early larvae of gilthead sea bream *Sparus aurata*.

Fish oil replacement in rotifers enrichment by vegetable oils negatively affected gilthead sea bream larval growth. Reduction in the rotifer essential fatty acids content by enrichment with vegetable oils, affects larval normal behaviour, reducing cruise swimming speed, and, particularly, delaying the appearance of the response to visual stimulus, suggesting a delay in the functional development of brain and vision, in agreement with the minor eicosapentaenoic acid and docosahexaenoic acid found in eyes and brains of these larvae.

- Study 2. Increased Mauthner cells activity and escaping behaviour in gilthead sea bream larvae (*Sparus aurata*) fed long chain polyunsaturated fatty acids.

The present study showed the first evidence of the importance of n-3 long chain polyunsaturated fatty acids for the adequate functioning of particular neurons, the M-cells, and, subsequently for the behaviour response that they modulate to escape from a sound stimulus.

- Study 3. Docosahexaenoic acid, but not eicosapentaenoic acid, enhanced sound stimuli induced escaping behaviour and Mauthner cells activity in gilthead sea bream larvae.

The results of this research study have shown that docosahexaenoic acid, rather than eicosapentaenoic acid, boosts escaping behaviour in gilthead sea bream and that this effect is at least partly mediated by the increase in neural activity, in particular of Mauthner cells.

- Study 4. Dietary polyunsaturated fatty acids affect zebrafish (*Danio rerio*) behaviour and Mauthner cells activity.

This study denotes the importance of dietary docosahexaenoic acid for normal growth and enhanced startle response and neural activity of zebrafish, pointing out the importance of optimum nutritional conditions when zebrafish is used as a model in behaviour and neural studies.

- Study 5. Use of calretinin (CR) and parvabumin (PV) as Mauthner cells markers in European sea bass (*Dicentrarchus labrax*).

The results of this last study provide some interesting techniques to further understand the role of Mauthner cells in very young marine larvae, the potential implications in escaping response and the effect of dietary or environmental factors. The lack of PV or CR reactivity at 6 and 10 dah denote the poor functionality of M-cells in agreement with the low escaping response found in previous studies. PV seems to play an important role in the initial development of M-cell functionality and marine fish larvae startling response, since its detection in immunostainning or immunofluorescence at 17 dah and its increase in intensity at 19 dah coincides with the improved escaping response found in our previous studies. However, CR may be important to further booster M-cell activity in older larvae, since its appearance at 28 and 47 dah coincides with the higher startling response found our studies.

10. Spanish Summary

Resumen

Hoy en día la captura de larvas de los stocks naturales no es una estrategia sostenible para el desarrollo de la acuicultura intensiva de peces marinos, estando restringida a otros organismos marinos como son los moluscos. En este sentido, uno de los principales cuellos de botella para el desarrollo de la acuicultura de peces marinos es la producción de larvas de buena calidad en cantidades adecuadas. Las larvas de la mayoría de las especies de peces marinos no están completamente desarrolladas cuando eclosionan y ciertas estructuras visuales, esqueléticas, digestivas, o su sistema nervioso no están del todo formados en comparación con un adulto. Por lo tanto, las larvas de peces marinos son muy sensibles al estrés y a las enfermedades, alcanzando tasas de mortalidad superiores al 80% de la población durante este periodo. Por ello, el conocimiento del bienestar de peces es determinante para el éxito de la cría larvaria. El comportamiento de peces representa la reacción de los mismos con el medio ambiente y por consiguiente es un elemento clave del bienestar de los peces. El estudio del comportamiento a lo largo del desarrollo larvario permitiría establecer los momentos de aparición de determinadas pautas típicas en los animales sanos. Posibles desviaciones en el tipo de comportamiento o retrasos en la aparición de dichas pautas, constituirían indicadores no invasivos del estado de desarrollo, madurez y salud del animal. Sin embargo, muy pocos estudios se habían centrado en aspectos del comportamiento a lo largo del desarrollo larvario en peces y a los factores que afectan al mismo antes de la realización de esta tesis. Determinados tipos de comportamiento son esenciales para la supervivencia de las larvas, como por ejemplo la respuesta de escape, que depende en gran medida del correcto desarrollo del sistema nervioso central. A su vez, el desarrollo normal del cerebro podría estar afectado por deficiencias en la dieta de ciertos nutrientes, tales como los ácidos grasos poliinsaturados omega tres de cadena larga (n-3

LCPUFA), ya que estos ácidos grasos, en particular el ácido docosahexaenoico (DHA), se acumulan en las neuronas y juegan un papel importante en el funcionamiento de membranas y la regulación de su fluidez. Un par de grandes neuronas, fácilmente identificables en el cerebro posterior, denominadas células de Mauthner, inician la respuesta de escape también conocida como "fast-start" o respuesta de alarma. Por lo tanto, esta tesis prueba por primera vez en la ciencia la hipótesis de que las deficiencias en n-3 LCPUFA pueden afectar el comportamiento de larvas de peces, al contenido de los ácidos grasos del sistema nervioso central y al funcionamiento neuronal.

Los resultados muestran las primeras evidencias del efecto de los LCPUFA de la dieta en el comportamiento de escape de los peces y su relación con el sistema nervioso central. Teniendo en cuenta que los LCPUFA son esenciales para el desarrollo normal del sistema nervioso central, la importancia del bienestar de todos los animales de granja (incluidos los peces), y la escasa información disponible en teleósteos, el objetivo principal de esta tesis fue determinar el efecto de la acción de los LCPUFA en la actividad de las células de Mauthner y sus implicaciones en el comportamiento en diferentes especies de peces. Para ello, dietas con diferentes perfiles de ácidos grasos fueron diseñadas y probadas. Con el fin de determinar la respuesta de las larvas a diferentes estímulos (visuales y sonoros), ciertos parámetros del comportamiento fueron estudiados como la velocidad de crucero de natación, la velocidad huída, la tasa de velocidad de huída, el ángulo y la velocidad en el ángulo en el pico de la respuesta de escape. Las propiedades nutricionales de los LCPUFA en las células de Mauthner se investigaron en en diferentes especies de peces: dorada (*Sparus aurata*), lubina (*Dicentrarchus labrax*) y pez cebra (*Danio rerio*).

La primera parte de esta tesis muestra el efecto específico de los ácidos grasos esenciales contenidos en presas vivas en el comportamiento larvario de dorada. El objetivo de este estudio fue determinar el paralelismo entre las respuestas del comportamiento utilizando diferentes estímulos a lo largo del desarrollo larvario de dorada y su efecto bajo diferentes regímenes de

alimentación (Estudio 1). Una vez que este efecto fue demostrado, se investigó la importancia de los LCPUFA utilizando microdietas específicas en el comportamiento de larvas de dorada en una etapa superior de desarrollo y, por otro lado, el desarrollo de técnicas para estudiar la implicación de la actividad de las células de Mauthner en el comportamiento de peces. Por lo tanto, el objetivo del estudio 2 consistió en entender mejor el efecto de los n-3 LCPUFA de la dieta en la respuesta de escape y en la función neuronal en larvas dorada. En vista de la mejora de la respuesta de escape en larvas de peces marinos por el incremento del contenido de n-3 LCPUFA en la dieta observado en los capítulos anteriores, el propósito del estudio 3 fue determinar el efecto específico de cada uno de los n-3 LCPUFA, comparando la eficacia de los diferentes ácidos grasos esenciales (ácido eicosapentaenoico y ácido docosahexaenoico), en el comportamiento de huída y de la actividad neuronal y en larvas de dorada. El siguiente paso consistió en determinar en una especie diferente, el pez cebra, el efecto de diferentes niveles n-3 LCPUFA de la dieta en el funcionamiento de las células de Mauthner en relación con el comportamiento de escape y la función neuronal (Estudio 4). La última parte se basó principalmente en el estudio histológico de la ontogenia de las células de Mauthner utilizando diferentes anticuerpos donde se describen aspectos específicos de la morfología de estas células (Estudio 5). El propósito de este ensayo fue describir la presencia de la calretinina y parvalbúmina en la células de Mauthner en la ontogenia de larvas de lubina utilizando técnicas inmunohistoquímicas y de inmunofluorescencia, adaptando a los peces marinos el método utilizado por otros autores en peces de agua dulce.

Los resultados obtenidos en esta tesis muestra la importancia de n-3 LCPUFA, especialmente el ácido docosahexaenoico, en la función normal de las células de Mauthner en relación con el comportamiento de huída en larvas de peces.

Objetivos

Considerando que los ácidos grasos poliinsaturados de cadena larga (LCPUFA) son indispensables para el desarrollo normal del sistema nervioso central y teniendo en cuenta el bienestar de todos los animales de granja (incluidos los peces), el efecto de la alimentación en el comportamiento de larvas de peces se ha convertido en un campo de estudio muy interesante a pesar de la escasa información disponible. Por lo que el objetivo principal de esta tesis consistió en estudiar las evidencias de la acción n-3 LCPUFA en la actividad neuronal y sus implicaciones en el comportamiento en diferentes especies de peces.

Para lograr este objetivo principal, el trabajo fue organizado en cinco fases complementarias y sucesivas con unos objetivos específicos: En la primera parte de la tesis se estudió el efecto de los ácidos grasos esenciales de presas vivas en el comportamiento de dorada (*Sparus aurata*) durante la primera parte del desarrollo larvario; en la segunda parte se investigó más a fondo la importancia de los LCPUFA en el comportamiento larvario de dorada utilizando microdietas específicas y aplicando nuevas técnicas desarrolladas para estudiar la actividad de las células de Mauthner. En la tercera fase se indentificó el efecto específico de los n-3 LCPUFA comparando la eficacia de diferentes niveles del ácido docosahexanoico (DHA) y ácido eicosapentaenoico (EPA) en el comportamiento de huída y en la actividad neuronal en larvas de dorada; la cuarta fase del estudio tuvo como objetivo determinar el efecto de los diferentes niveles de n-3 LCPUFA en el funcionamiento de las células de Mauthner en relación con el comportamiento de huída en una especie diferente, el pez cebra (*Danio rerio*). Finalmente, el objetivo específico de la última parte de la tesis se basó en estudiar la morfología y actividad de las células Mauthner

realizando un estudio histológico a lo largo de la ontogenia en larvas de lubina (*Dicentrarchus labrax*).

Por lo tanto para la realización de esta tesis y para cumplir los objetivos planteados, se llevaron a cabo cinco estudios:

- Estudio 1. El objetivo de este primer experimento fue determinar el efecto de diferentes regímenes de alimentación de LCPUFA en las respuestas de comportamiento a diferentes estímulos durante el desarrollo temprano de larvas de dorada.

- Estudio 2. El propósito de este estudio fue entender mejor el efecto de LCPUFA de la dieta durante el posterior desarrollo de larvas de dorada en su comportamiento de huída, así como en la actividad de las células de Mauthner.

- Estudio 3. En vista de los resultados obtenidos en el anterior capítulo donde la respuesta de huída en larvas de peces marinos incrementó debido a los niveles de n-3 LCPUFA, el objetivo del presente estudio fue comparar la eficacia de los diferentes contenidos de la dieta de DHA y EPA en la respuesta de huída así como en la actividad neuronal en larvas de dorada.

- Estudio 4. La finalidad de este ensayo fue determinar el efecto de los niveles de n-3 LCPUFA de la dieta en el funcionamiento de las células de Mauthner en larvas de pez cebra en relación con el comportamiento de huída y la función neuronal.

- Estudio 5. El objetivo de este último estudio se basó en comprender mejor la ontogenia de las células de Mauthner estudiando la presencia de calretinina y parvalbúmina mediante técnicas inmunohistoquímicas y de inmunofluorescencia en larvas de lubina. Este Se experimento esta adaptado de métodos utilizados previamente en peces de agua dulce a peces marinos.

10.1 Especies utilizadas

10.1.1 Dorada

Las larvas de dorada (*Sparus aurata*) (Figura 10.1a) fueron obtenidos de las puestas naturales de reproductores existentes en las instalaciones del Grupo de Investigación en Acuicultura (GIA) donde se llevaron a cabo los experimentos con esta especie. Los reproductores fueron alimentados con una dieta comercial Vitalis Repro (Skretting, Francia), suplementada una vez a la semana con trozos de pescado, moluscos como sepia, calamar y mejillón.

Los huevos fueron recogidos mediante una red recolectora de 500 µm, dispuesta en el desagüe superior del tanque, que descansaba sobre un tanque de fibrocemento de 200 l de capacidad con el fin de que no quedasen secos. Los huevos con flotabilidad positiva, cayeron por rebose en dicho colector. Una vez recogida la puesta, se incubó 24 horas en una malla cilíndrica dentro de un tanque con circuito abierto y aireación suave. Posteriormente se hizo la separación de los huevos por decantación, diferenciándose los viables y no viables por la flotabilidad negativa que presentaban éstos últimos. Después se procedió a la siembra por volumetría. Paralelamente, se realizaron pruebas para calcular los índices de eclosión, malformaciones y mortalidad, efectuadas en 6 vasos de 2 l durante 72 horas.

10.1.2 Lubina

Las larvas de lubina (*Dicentrarchus labrax)* (Figura 10.1b) utilizadas en este experimento procedían de la empresa francesa Ecloserie Marine de Gravelines. Durante los primeros días de aclimatación de las larvas se controló la Tª del agua gracias a enfriadores (16º C) y poco a poco se incrementó la tasa de renovación del agua hasta llegar a Tª ambiente (20,2º C) en las instalaciones del GIA (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, España) en donde se llevaron a cabo los experimentos de esta especie.

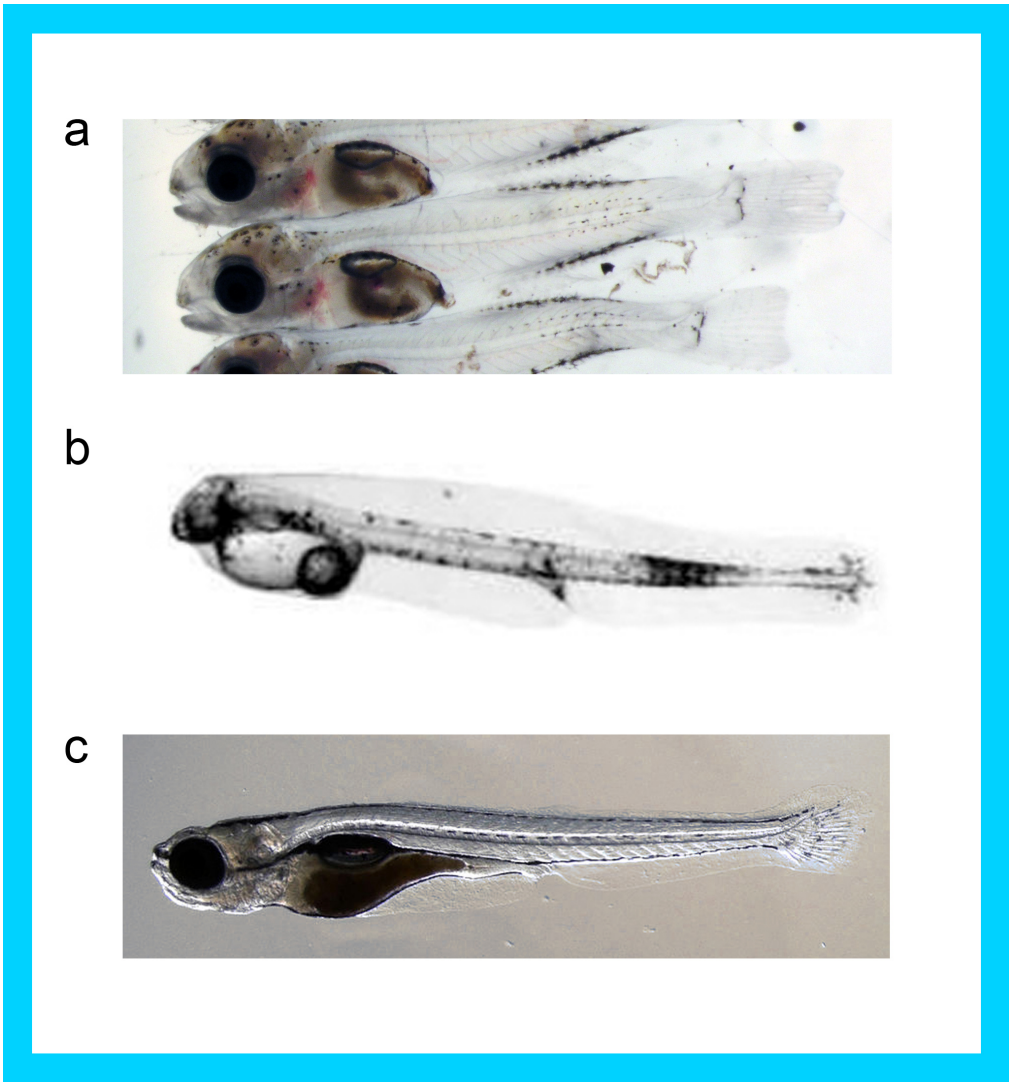

Figure 10.1 a) Larva de dorada. b) Larva de lubina. c) Larva de pez cebra.

10.1.3 Pez Cebra

Las larvas de pez cebra (*Danio rerio*) tipo Nacre (sin pigmentos) (Figura 10.1c) fueron obtenidas de la puesta natural de los reproductores existentes del Departamento de Neurobiología y Comportamiento de la Universidad de Cornell (Ithaca, Estados Unidos) donde se realizó dicha experiencia.

10.2 Experimentos

10.2.1 Condiciones experimentales

Los tanques de cultivo fueron provistos con agua de mar (alrededor de 37 ppm de salinidad) filtrada por malla de 50 µm de luz. La intensidad de la luz se mantuvo a 1700 lux (Lux digital Tester YF-1065, Powertech, Australia Occidental, Australia). La temperatura y el oxígeno se midieron diariamente utilizando el aparato Oxy Guardhandy beta instrument (Zeigler Bros, Gardners, EE.UU.). Los tanques se limpiaron diariamente de manera manual entre las 18:00 y las 20:00, con una manguera por un sistema de sifón. Los tanques (170 l, cilíndricos de fibra de vidrio, con color gris claro) fueron suministrados con agua de mar filtrada y previamente almacenada en un tanque de 500 l para eliminar el exceso de gases disueltos. Los tanques fueron sometidos a una circulación abierta del agua que fluyó a diferentes tasas según la edad de la larva y que fueron incrementadas a lo largo de los ensayos alimentarios. La calidad del agua fue comprobada diariamente y no se observó contaminación

alguna. El agua era continuamente aireada (125ml/min). La temperatura del agua y el oxígeno disuelto se midieron diariamente a las 15:00. Se mantuvo entre 5-8 ppm y la saturación osciló entre el 60 y el 80% en todos los tanques experimentales. Se utilizó un fotoperiodo artificial de 12h luz: 12h oscuridad mediante luces fluorescentes.

10.2.2 Determinación del crecimiento

El crecimiento fue determinado midiendo el peso seco corporal y la longitud de las larvas en inanición. El peso corporal se determinó por 4-3 repeticiones de 15 larvas en inanición lavadas previamente con agua destilada y secadas en un porta objetos de vidrio en una estufa a 110° C hasta peso constante, durante aproximadamente 24 h, seguido de períodos de 1 hora. La longitud estándar de 20-30 larvas anestesiadas de cada tanque fue medida en un proyector de perfil (V-12A Nikon, Nikon Co, Tokio, Japón).

10.3 Dietas y alimentación

10.3.1 Alimento vivo

10.3.1.1 Cultivo de rotíferos

El rotífero utilizado en las experiencias de cultivo larvario fue *Brachionus plicatilis* cepa tipo L con una longitud total media de los individuos adultos de

240 µm. En el proceso de producción masiva de rotíferos se utilizaron tanques cilindro-cónicos de fibra de vidrio, con una capacidad de 1700 l. Los ciclos de producción fueron de 8 días de cultivo, iniciándose el proceso con una densidad aproximada de 265 rotíferos $\cdot ml^{-1}$, a partir del 4º día de cultivo se cosechó en días alternos 400 l de volumen del tanque que fueron repuestos con agua previamente mezclada para alcanzar la salinidad establecida (25 ppt). Al 8º día se procedió a la cosecha del volumen total y reinicio de un nuevo ciclo. Como medida rutinaria, tras la cosecha, los rotíferos eran sumergidos durante 1 minuto en agua dulce para eliminar posibles contaminantes. Diariamente se determinó la densidad media y el porcentaje de hembras ovígeras en tres contajes individuales de 0,5 ml con una micropieta, tomados de una muestra recogida en la zona central del tanque de cultivo, para asegurar una buena homogeneización de la muestra en cada medida. De la misma manera, a diario se tomaron medidas de temperatura y oxígeno disuelto a las 9h00 y a las 15h00 con una sonda portátil (Mod. Handy Polaris, OxyGuard; Birkerød, Dinamarca). En cuanto a la alimentación, de manera general se utilizó levadura de panificación (*Saccharomyces cerevisiae*) añadiendo 0,4 g/10^6 rotíferos. Únicamente el día de inicio del ciclo se suplementó la levadura con fitoplancton liofilizado, en una concentración de 0,1 g/10^6 rotíferos. La alimentación se distribuyó manualmente a las 9h00 y a las 15h00 horas y mediante un distribuidor automático a las 21h00 y 3h00.

10.3.1.2 Enriquecimiento de rotíferos

Los rotíferos cultivados, en las condiciones anteriormente descritas, son

deficientes en ácidos grasos poli-insaturados y aminoácidos, por lo que es necesario enriquecerlos antes de suministrarlos a las larvas. El enriquecimiento se realizó en tanques de fibra de vidrio opacos de 500 l de capacidad. Estos tanques tienen un sistema de aireación central mediante un difusor de manguera porosa, la concentración de rotíferos que se mantuvo bastante elevada (>400 rotíferos $\cdot$ ml^{-1}). En este proceso se utilizaron productos comerciales o experimentales, de acuerdo a las necesidades de cada una de las experiencias planteadas. De manera general, los diferentes productos se utilizaron siguiendo las instrucciones del fabricante, que incluían un tiempo de enriquecimiento 6h, y un reparto en dos dosis (9h00 y 15h00) de la cantidad necesaria. Diariamente se utilizaron dos tanques de enriquecimiento, uno de ellos para la primera comida de la mañana, que era cosechado a las 8h30. En este caso el producto enriquecedor se suministró mediante un dosificador automático programado para dar el producto a las 2h00 y a las 5h00. El segundo tanque, era cosechado a las 14h00, se le suministró el enriquecedor de forma manual a 8h00 y a las 11h00. Tras el enriquecimiento, los rotíferos eran filtrados con una bolsa de malla de 63 µm de luz, enjuagados para eliminar los restos de la emulsión enriquecedora y concentrados en un cubo de 20 l donde se mantenían con aireación para proceder a su contaje. Para ello, se tomaba una muestra de 5 ml de la zona central del cubo y se diluía en un vaso con agua de mar en un volumen de 250 ml, una vez homogeneizada la mezcla se realizaban tres contajes individuales de 0,5 ml con una micropieta. A lo largo de todo el desarrollo experimental se tomaron muestras del producto utilizado para la alimentación de los rotíferos y para su enriquecimiento en las diferentes

experiencias.

10.3.1.3 Enriquecedores

Las cantidades necesarias para enriquecer 5 millones de rotíferos a una concentración de 200-300 individuos/ml, se muestran a continuación:

Emulsión enriquecedora:

1. Preparar 60 ml de lecitina de soja al 0,01% (Solución madre).

   (Solución madre = 5 g de lecitina de soja en 500 ml de agua dulce = 100 mg/l)
2. Mezclar la lecitina en 400 ml de agua dulce.
3. 2 g de aceite.
4. Mezclar el aceite con 400 ml de agua dulce y 60 ml de lecitina de soja.
5. Homogeneizado: 2 minutos.

La correcta realización de este proceso fue de vital importancia para proporcionar los niveles de estos ácidos grasos esenciales durante el desarrollo de la larva. Una vez transcurrido el tiempo de enriquecimiento (12 h), y tras retirar los restos de emulsión flotante, se cosechaba la cantidad de rotíferos necesarios en una malla de 62 µm, previo paso a través de otra malla de 315 µm, que se utilizaba para eliminar posibles restos de enriquecedor y copépodos presentes en el tanque, se lavaba bien con agua de mar y se suministraba a las larvas.

10.3.1.4 Cultivo de *Artemia*

La *Artemia* sigue siendo uno de los alimentos básicos sobre los que se sustenta la cría larvaria de peces marinos actualmente. En las experiencias de

cultivo se utilizaron cistes o huevos de *Artemia* de dos calidades, *Artemia franciscana* (Tipo AF; INVE; Dendermode, Bélgica) y *Artemia salina* (Tipo EG; INVE, Dendermode, Bélgica). Es aconsejable realizar la descapsulación del corion o cubierta protectora del huevo, con lo que se evita la introducción de posibles patógenos asociados a estas cubiertas de resistencia. Del mismo modo, el ciste sin descapsular puede ser ingerido por la larva y dada su baja digestibilidad puede causar una obstrucción del tubo digestivo de la misma. Por último, el exceso de materia orgánica que suponen estos cistes si son introducidos en el tanque de larvas, contribuye a la disminución de la calidad de agua.

10.3.1.5 Enriquecimiento de *Artemia*

Para mejorar el valor nutritivo de la *Artemia*, se procedió a su enriquecimiento, al igual que en proceso de eclosión se utilizaron tanques cilindro-cónicos de fibra de vidrio, de 1700 l de capacidad, con aireación central fuerte, iluminación durante 24 horas, con una intensidad de 2000 lux, equipado con un intercambiador de calor que permitía mantener la temperatura de cultivo en torno a 25-26º C. Los nauplios de *Artemia* recién cosechados, se introducían en el tanque de enriquecimiento, lleno con la cantidad necesaria de agua del mar filtrada y esterilizada para mantener una concentración de 250.000-300.000 nauplios $\cdot l^{-1}$. De modo general, el enriquecedor utilizado fue Easy DHA Selco (Inve, Dendermonde, Bélgica), producto que se presenta en forma de emulsión lipídica. El tiempo de enriquecimiento de los nauplios fue de 18-24 horas a una concentración de 0,6 g $\cdot l^{-1}$, y añadido en una única dosis al inicio del enriquecimiento. Una vez enriquecidos los metanauplios de 18-24

horas, se filtraron en una bolsa de 125 µm de luz de malla, se lavaron con agua de mar abundante para eliminar los posibles restos de emulsión y se concentraron en un cubo de 20 l procediendo a su contaje y comprobando visualmente su correcto enriquecimiento por la presencia de gotas lipídicas en el tracto de la *Artemia*.

10.3.2 Alimento inerte (microdietas)

En algunas experiencias se utilizaron microdietas experimentales (tamaño < 250 µm) que se formularon para ser isoproteicas e isolipídicas. Se utilizaron diferentes tasas de EPA y DHA formuladas usando los aceites EPA50 y DHA50 (CRODA, East Yorkshire, Inglaterra, Reino Unido), en forma de triglicéridos como fuente de EPA y DHA. También se utilizaron diferentes fuentes de aceites vegetales como el aceite de soja (SBO), aceite de lino (LSO) y aceite de colza (RSO), así como el aceite de pescado (FO) (aceites vegetales cedidos por la empresa Rafoa; el FO utilizado fue aceite de sardina). Como fuente complementaria de lípidos se utilizó el ácido oleico (Merck, Darmstadt, Alemania). Se utilizó harina de calamar (Riber & Son, Bergen, Noruega) como fuente proteica, la cual fue desengrasada (3 veces consecutivas con una tasa cloroformo: harina de 3:1) para permitir un mejor control del perfil de ácidos grasos de la misma, salvo en uno de los experimentos en el que se especifica. Los perfiles de los ácidos grasos de la harina de calamar desengrasada (2,6% del contenido en lípidos), DHA50, EPA50 se muestran en la Tabla 10.1.

Table 10.1 Principales ácidos grasos de las fuentes lipídicas utilizadas en los experimentos.

Fames	DHA50	EPA50	Harina de calamar desengrasada
14:0	0,03	0,04	1,68
14:1n-5	0,20	0,24	0,04
14:1n-7	n.d.	0,01	0,04
15:0	n.d.	0,01	n.d.
15:1n-5	n.d.	n.d.	0,04
16:0iso	0,01	0,02	0,11
16:0	1,21	0,36	23,80
16:1n-9	n.d.	n.d.	0,02
16:1n-7	0,47	0,43	0,52
Me 16:0	0,01	0,01	0,10
16:1n-5	0,02	n.d.	0,16
16:2n-6	n.d.	0,05	0,06
16:2n-4	0,16	0,49	0,48
17:0	0,12	n.d.	0,06
16:3n-4	0,10	0,21	0,07
16:3n-1	0,06	0,14	0,12
16:4n-3	n.d.	n.d.	0,38
16:4n-1	n.d.	0,33	0,03
18:0	2,32	0,27	3,64
18:1n-9	4,92	1,81	1,73
18:1n-7	0,87	0,55	0,94
18:1n-5	0,04	n.d.	0,24
18:2n-9	n.d.	0,44	n.d.
18:2n-6	0,59	2,16	0,24
18:2n-4	0,07	0,68	n.d.
18:3n-6	0,34	0,73	n.d.
18:3n-4	0,04	0,44	n.d.
18:3n-3	0,20	1,67	n.d.
18:3n-1	n.d.	0,32	n.d.
18:4n-3	0,31	10,44	0,07
18:4n-1	n.d.	1,01	n.d.
20:0	0,73	0,42	n.d.
20:1n9+n7	3,98	0,39	2,80
20:1n-5	n.d.	n.d.	n.d.
20:2n-9	0,07	0,41	n.d.
20:2n-6	0,54	0,13	0.12
20:3n-9 +7	0,21	0,72	n.d.
20:3n-6	n.d.	n.d.	n.d.
20:4n-6	2,46	3,49	0,49
20:3n-3	0,44	0,11	0,34
20:4n-3	0,92	2,49	0,06
20:5n-3	12,66	46,48	4,31
22:1n-11	2,29	0,31	0,04
22:1n-9	1,21	0,74	0,11
22:4n-6	2,84	0,49	0,13
22:5n-6	0,19	n.d	n.d.
22:5n-3	5,53	3,21	n.d.
22:6n-3	53.80	17.72	9.40

*n.d.=not detected, n<0.005

10.3.2.1 Preparación de las microdietas

Las microdietas fueron preparadas de la forma siguiente: la harina de calamar fue cuidadosamente mezclada con los otros ingredientes hidrosolubles (atractantes, minerales y vitaminas hidrosolubles, Sigma-Aldrich, Madrid, España), en un mortero. A parte, los aceites y las vitaminas liposolubles fueron mezclados para obtener una mezcla homogénea, que fue luego añadida a la mezcla de polvo. A continuación, se disolvió la gelatina en agua caliente y cuando su temperatura fue inferior a 35° C se añadió al resto de los ingredientes previamente mezclados. La pasta formada se moldea en cuerdas finas (Severin, Suderm, Alemania) y se seca en una estufa (Ako, Barcelona, España) a 38° C durante 24 h. Por último, se pulveriza (Braun, Kronberg, Alemania) y se tamiza (Filtra, Barcelona, España) para obtener el tamaño deseado de las partículas. Se realizó el análisis proximal y la composición en ácidos grasos de las dietas en peso seco y cada dieta se ensayó por triplicado.

10.4 Estudios del comportamiento larvario de dorada

10.4.1 Material

La preparación, material y acondicionamiento, es muy similar en ambos estímulos. Se utilizó un vaso precipitados de 500 ml (vaso de experimentación) con agua de mar, manteniendo una profundidad de 4 centímetros, forrado con un plástico negro. Debajo del vaso, se ubicó un papel milimetrado. Las

respuestas a cada tipo de estímulo se ensayaron con larvas individuales, utilizando unas 10 larvas de cada tanque. Las larvas fueron trasladadas desde los tanques de cultivo a los vasos de experimentación. El tiempo a esperar para que la larva se acondicionara al nuevo medio era de 2 minutos. Se realizaron varias pruebas para determinar este tiempo. Transcurrido este periodo, se grababa con una cámara de video digital Sony DCR-TRV27. Durante un minuto y medio de tiempo, se observaba el movimiento de que presentaba la larva sin proporcionar ningún tipo de estímulo. A continuación, se suministró el estímulo. Cada 10 s se proporcionaba un estímulo hasta completar un total de tres.

Para observar el efecto de la alimentación en el comportamiento larvario, se realizaron pruebas a lo largo de la experiencia. En las pruebas realizadas al día 1 de edad (1 dah) así como en los primeros días de vida de las larvas (hasta llegar a 6 dah), apenas se podían diferenciar con claridad en la cámara digital, por lo que se procedió a realizar el análisis visualmente. En el experimento 2 el comportamiento se midió a los días 6, 10, 16 y 19 ph. En el experimento 3 la pruebas se realizaron los días 23, 27 y 34 ph, mientras que en el experimento 4 se midió los días 22, 25, 29 y 32 ph. Estos datos sirvieron para obtener un seguimiento a lo largo del periodo de desarrollo larvario y para adquirir soltura en el manejo de estos animales tan delicados con estas técnicas. Todas las larvas utilizadas en las experiencias fueron medidas con ayuda de un proyector de perfiles (V – 12ª Nikon, Tokyo, Japan).

10.4.2 Estímulos

10.4.2.1 Estímulo sonoro

Para simular un estímulo sonoro, se utilizó un péndulo construido a partir de un tubo de PVC constituido por una cordón que sujetaba una tuerca (Figura 10.2). Más que un estímulo sonoro, se trata de un estímulo vibracional. Para que la larva no "viese" por donde se le suministraba los golpes, se cubrió el vaso de experimentación con una funda negra. El péndulo se situaba a una distancia 8 cm del vaso de experimentación. Con ayuda de una cinta métrica, se llevaba la tuerca a una distancia de 10 cm desde el mismo péndulo, (la tuerca estaría a una distancia de 18 cm del vaso de experimentación) y se lanzaba sin aplicar fuerza, simplemente se dejaba caer la tuerca. Las distancias tenían que ser muy precisas ya que un centímetro de más o de menos, podría marcar la diferencia a la hora de evaluar los parámetros de comportamiento.

Figura 10.2 Material utilizado en el estímulo sonoro.

10.4.2.2 Estímulo visual

Como estímulo visual se empleó un flash de luz blanca. Se utilizó este color para simular las condiciones naturales. El flash estaba situado a una distancia de 10 cm del vaso de experimentación (Figura 10.3).

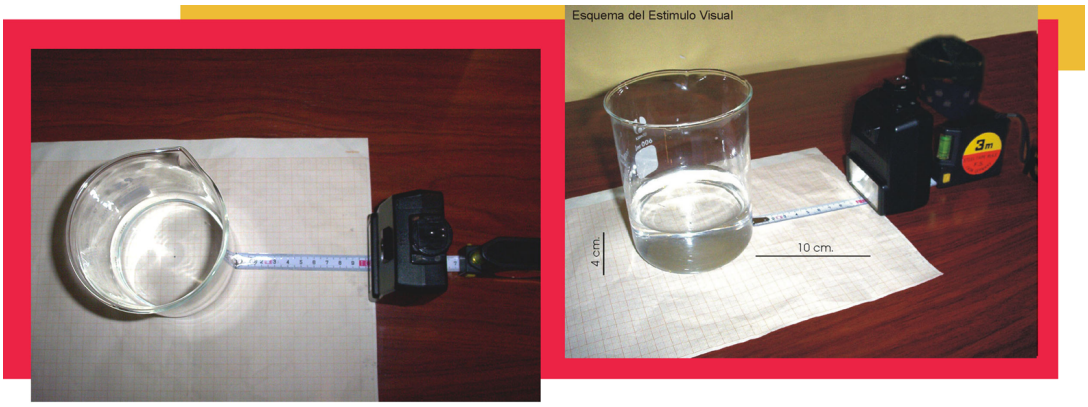

Figura 10.3 Material utilizado en el estímulo visual.

10.4.3 Parámetros determinados

Como ya se comentó anteriormente, a cada larva se le suministraba tres veces el mismo estímulo en intervalos de 10 segundos. Para saber sí la larva responde o no, es decir si la respuesta es positiva o negativa, hubo que observar con mucha paciencia y determinación, ya que en los primeros días de muestreo, las larvas apenas se podían apreciar con claridad. El análisis comenzó con la visualización de todas las imágenes para tener una idea de a partir de qué día comenzaban a reaccionar las larvas ante los estímulos.

10.4.3.1 Velocidad de crucero

La Velocidad de Crucero se define como el *movimiento natural* que presentan las larvas justo antes de suministrar el estímulo (Masuda *et al.*, 2002). Para poder determinar esta medida, se colocó un papel transparente sobre la pantalla del televisor fijado con cinta adhesiva. Para ello, se observó el movimiento *natural* de la larva durante 30 s. Durante los 10 s siguientes, se trazó el recorrido de la larva sobre el papel transparente. Hay dos formas de medir esta distancia. Una de ellas, con ayuda del papel milimetrado situado bajo el vaso de experimentación o bien, con ayuda de un hilo. El hilo se coloca sobre el recorrido realizado por la larva. Con una regla se mide el recorrido anotado en el hilo, de esta forma se obtiene la distancia recorrida por la larva. Como la velocidad de un objeto se define como distancia recorrida entre un tiempo determinado, en este caso 10 s, se obtiene la *Velocidad de Crucero*. Las unidades de esta velocidad se presentan mm/s y SL/s. Para obtener esta última expresión, se dividió entre la talla estándar. Este análisis se realizó sólo en aquellas larvas que presentaban movimiento, tanto para el estímulo sonoro como para el estímulo visual.

10.4.3.2 Velocidad de huída

La velocidad de huída fue medida en aquellas larvas que presentaron una respuesta obvia ante el estímulo. Este tipo de respuesta podía ser del tipo cambio de dirección, para el estímulo visual y sonoro, y/o del tipo de pánico, para el estímulo visual. El análisis de estas imágenes fue muy similar al realizado en la velocidad de huída. Desde el momento en que se suministró el estímulo se

analizaron las cuatro primeras secuencias o fotogramas. El movimiento de las larvas fue trazado para estas cuatro primeras secuencias, y la distancia fue dividida entre el tiempo tomado (4/30 s). Observaciones preliminares, revelan que el movimiento más rápido aparece justo en las primeras cuatro secuencias después de haber suministrado el estímulo. Para estandarizar la comparación entre la condición de la dieta y los estados del desarrollo, se realizó la media del movimiento en las cuatro secuencias y esto se definió como Velocidad de Huída. Al igual que en la Velocidad de Crucero, las unidades de esta velocidad se presentan mm/s y SL/s.

10.4.3.3 Tasa de la velocidad de huída

La tasa de velocidad de huída fue calculada dividiendo el número de respuestas registrado para cada una de las larvas entre el número de ensayos, es decir, el número de veces que se suministraba el estímulo.

10.5 Estudio del comportamiento larvario del pez cebra

El comportamiento del pez cebra se realizó en las instalaciones del Departamento de Neurobiología y Comportamiento de la Universidad de Cornell (USA), donde se realizó una estancia de investigación. La respuesta ante un estímulo vibracional fue estudiada determinando el ángulo en el pico del escape, la velocidad en el ángulo en el pico del escape y el tiempo de respuesta

en el pez cebra. Estas pruebas fueron realizadas utilizando una cámara de video de alta velocidad que capturaba imágenes digitalizadas a 1000 frames/s (EG&G Reticon, Sunnyvale, CA, EEUU) mediante el programa Photron Fastcam Viewer (San Diego, CA, EEUU). Los datos fueron analizados utilizando un programa de imágenes basado en la curva inicial de escape del pez, ya que la matriz de Mauthner es esencial para la generación de movimientos de alto rendimiento durante esta curva.

10.5.1 Electroporación

Con el fin de visualizar y estudiar "in vivo" las células Mauthner, se procedió a su marcaje con fluorescencia mediante electroporesis. La electroporación o electropermeabilización es el aumento de la conductividad eléctrica y la permeabilidad de la membrana plasmática celular causados por un campo eléctrico aplicado externamente. Cuando el voltaje que atraviesa una membrana plasmática excede su rigidez dieléctrica se forman poros. Si la fuerza del campo eléctrico aplicado o la duración de la exposición al mismo se eligen apropiadamente, los poros formados por el pulso eléctrico se sellan tras un corto período, durante el cual los compuestos extracelulares tienen la oportunidad de entrar a la célula. Sin embargo, una exposición excesiva de células vivas a campos eléctricos puede causar procesos que provocan la muerte celular, apoptosis o necrosis.

Para llevar a cabo la electroporación de una de las células Mauthner siempre se utilizaron larvas tipo nacre (sin pigmentos) de 4 días de edad. En

estas pruebas nunca se utilizaron larvas de mayor edad ya que hubiese sido muy difícil realizar esta operación debido a que la larva está más desarrollada. Primeramente se procedía anestesiar las larvas. Para ello se preparaban 50 ml de solución de Hank y 0.03% tricaine methanesulfonate (MS-222, Sandoz, Basel, Switzerland). Una vez que larvas fueron anestesiadas se preparó una solución de agar previamente calentada en el microondas. Cada larva fue colocada en una pequeña placa. Se retiraba el agua y se añadía un poco de agar. Antes de que el agar se solidificase se orientaba la larva en sentido horizontal. Una vez que el agar estuviese solidificado, se suministraban unas gotas de la solución de Hank con anestésico en la placa. Seguidamente, se preparaban una pipetas especiales de vidrio para electroporación. En el interior de las mismas se introducía la fluoresceína: 10% de rodamina (3000 peso molecular o MW; Molecular Probes, Eugene, OR) en una solución de Hanks al 10%. La electroporación se lleva a cabo en un electroporador, un aparato que crea un campo electromagnético a través del agar. Gracias a un microscopio se localizaron las células de Mauthner. Una vez encontrada la célula derecha (siempre se electroporó la misma neurona), se procedió a introducir la pipeta con la fluoresceína dentro de la neurona (Bhatt et al., 2004). Aplicando un campo eléctrico se consiguió introducir la fluoresceína dentro de la neurona. Una vez verificado este proceso, se procedió a liberar la larva del agar y fue colocada en una placa de petri con 50 ml de solución de Hank, 50 μm de Pen Strep (penicilina) y un poco de microdieta durante 24 horas. Transcurrido este tiempo las larvas fueron nuevamente colocadas en los tanques de experimentación para comenzar las experiencias de nutrición.

Finalizadas las experiencias de nutrición, las larvas fueron anestesiadas y colocadas en agar en una placa de petri (Eaton *et al.*, 1984). Las imágenes de las neuronas marcadas con fluoresceína fueron obtenidas utilizando el microscopio confocal Zeiss LSM 510 (Fetcho and O'Malley, 1995; O'Malley *et al.*, 1996). La transparencia de las larvas tipo nacre permitió visualizar las neuronas del animal vivo pero también la observación de los latidos del corazón y el flujo sanguíneo. Para confirmar la identidad de las células estudiadas fisiológicamente, se realizaron una serie de imágenes concecutivas a través del microscopio confocal que muestran la morfología de las mismas. Estas imágenes se reconstruyeron en tres dimensiones utilizando el programa Imaris (Bitplane scientific software, South Windsor, CT, USA) permitiendo examinar los detalles de la morfología dendrítica y proyecciones axonales de la célula.

10.6 Análisis bioquímicos

Los análisis bioquímicos de todas las experiencias de la presente tesis se realizaron en el laboratorio de nutrición en las instalaciones del Instituto Universitario de Sanidad Animal (IUSA). Todas las muestras de larvas y dietas fueron sacadas del congelador -80° C, descongeladas y homogeneizadas en el interior de las mismas bolsas de las muestras para evitar la evaporación de agua. En el experimento 2 se procedió también a la extracción del cerebro y

ojos para el análisis bioquímico. Para ello, con ayuda de dos agujas se realizó un corte limpio por la base del cráneo de las larvas bajo una lupa. A continuación, se retiraba la mandíbula y con sumo cuidado se procedía a la extracción de los ojos.

10.6.1 Peso seco

Se determinó el peso seco de larvas y dietas. Para ello, se colocaron 50 larvas (*n*) en un portaobjetos previamente marcado y seco del cual se conocía su peso (*Pc*). Se eliminó la mayor cantidad de agua con un papel secante. Se dejó 24 horas en una estufa a 100º C. Al sacar las muestras, se dejaron 30 minutos en un desecador para adaptarlas a temperatura ambiente. Posteriormente, se pesó el portaobjetos con las muestras. El proceso se repitió durante otra hora, hasta que el peso no varió o incrementó, dándose por adecuado el menor peso obtenido. El peso individual de cada huevo y larva (*Pl*) se obtiene de la expresión siguiente:

$$Pl = ((Pc+L)-(Pc))/n$$

10.6.2 Humedad

Se realizó siguiendo el Método Oficial de Análisis de la Asociación de Química Analítica de Estados Unidos (A.O.A.C., 1980). Con el fin de reducir el error analítico, la determinación del contenido de humedad de cada muestra se realizó por triplicado. El procedimiento consiste en secar en una estufa a 110º C una cantidad conocida de muestra (*Pi*). El proceso de secado se detiene

cuando el peso de la muestra se hace constante (*Pf*), calculándose el porcentaje de humedad con la siguiente expresión:

$$\%H = ((Pi-Pf)*100)/Pi$$

El porcentaje en peso seco (% P.s) de los distintos parámetros bioquímicos analizados y expresados como porcentaje del peso fresco de la muestra (% P.f) se obtuvo, en función del contenido de humedad de la muestra (% H), aplicando la siguiente fórmula:

$$\%PS = (\%Pf*100)/(100-\%H)$$

10.6.3 Lípidos totales

Los lípidos se extrajeron por el método de Folch *et al*. (1957). Se pesó una cantidad de muestra de las dietas y las larvas enteras (0,1-0,2 g), se homogenizó en 10 ml de una mezcla de cloroformo: metanol (2:1, v:v) en ultra turrax durante 5 min. Tanto los cerebros como los ojos, se iban colocando en un tubo de ensayo que contenía 4 ml de cloroformo-metanol (2:1) con 0,01% de BHT, de ésta manera, se comenzaba con la extracción de lípidos. Luego los lípidos fueron separados por centrifugación durante 5 min (2000 rpm), la fase inferior de cloroformo que contiene los lípidos fue cuidadosamente aspirado y evaporada para obtener el peso de los lípidos.

10.6.4 Separación de lípidos polares

Las fracciones de lípidos neutros y polares de los lípidos totales de las larvas fueron separados por cromatografía de adsorción en cartuchos de sílice

(Sep-pak; Water S.A., Massachusetts, EE.UU.), utilizando 30 ml de cloroformo y 20 ml de cloroformo/metanol (49:1, v/v) como disolvente de los lípidos neutros, seguido por un lavado de 30 ml de metanol para obtener las fracciones polares según el método de Juaneda y Rocquelin (1985).

10.6.5 Preparación y cuantificación de ésteres metílicos de ácidos grasos

Los lípidos polares o totales se disolvieron en tolueno y los ésteres metílicos de los ácidos grasos fueron obtenidos por transmetilación con 1% de ácido sulfúrico en metanol (Christie, 1982). La reacción se llevó a cabo en la oscuridad durante 16 h a 50° C, en atmósfera de nitrógeno. Después, los ésteres metílicos de los ácidos grasos se extrajeron con hexano: éter dietílico (1:1, v/v) y purificados por cromatografía de adsorción en cartuchos NH_2 Sep-pack (Water S.A., Massachusetts, EE.UU.) (Christie, 1982). Los ésteres metílicos de ácidos grasos fueron separados por GLC (GC-14A, Shimadzu, Tokio, Japón) en una columna capilar de sílice (Supercolvax-10) (longitud 30 m, diámetro interior 0,32 mm; Supelco, Bellefonte, EE.UU.) utilizando helio como gas portador. La temperatura de la columna fue de 180° C durante los primeros 10 minutos, aumentando a 215° C a razón de 2,5° C/min y luego se fijo en 215° C durante 10 min, según las condiciones descritas por Izquierdo *et al.* (1990). Los ésteres metílicos de ácidos grasos se han cuantificado con FID e identificados por comparación con estándares externos y aceites de pescado bien caracterizados (EPA 28, Nippai, Ltd Tokio, Japón).

10.7 Estudios histológicos

Los estudios histológicos se realizaron en los laboratorios del IUSA. Para el estudio histológico se utilizaron 60 larvas de cada tratamiento y ontogenia. Las larvas fueron anestesiadas en metasulfonato de tricoína MS-222 (1:20.000). De esta manera se aseguró que mantuvieran su conformación natural rectilínea evitando que se curvasen. Una vez anestesiadas, las larvas fueron fijadas en formalina tamponada al 10% (Anexo I). El fundamento de la fijación es la conservación de los diferentes tejidos manteniendo la máxima fidelidad histológica de los tejidos *in vivo*, mediante la interacción de los radicales activos del formol con los radicales básicos de las proteínas y lípidos. El tiempo de avance de la formalina hacia el interior de los tejidos es de unos 3 mm por hora.

10.7.1 Recogida de muestras

Transcurrido un mínimo de 24 h desde el momento de la fijación, se ensayaron tres inclusiones diferentes para el estudio morfológico. El primer procedimiento consistió en inclusión en parafina, técnica empleada rutinariamente en histología con un protocolo muy estandarizado y automatizado. En el segundo, se prepararon muestras para su tratamiento en criostato. En tercer lugar, se incluyeron larvas en resina la cual permite realizar preparaciones con mejor detalle viendo sus resultados al microscopio electrónico. Los protocolos empleados en cada caso se describen a continuación.

10.7.2 Procesamiento e inclusión en parafina

Las larvas fueron colocadas es unos cassettes de plástico que permitiesen la entrada de los reactivos sin la pérdida de las muestras, y lavadas en agua corriente durante 30 min. A continuación las muestras fueron colocadas en el procesador de tejidos Histokinette 2000 (Leica, Nussloch, Alemania) con una duración de 24 h. Este proceso se basa en la inclusión de los tejidos en un gradiente ascendente de alcoholes, hasta llegar al xilol, de naturaleza completamente volátil, para conseguir su total deshidratación. Seguidamente las muestras se incluyeron en parafina caliente para evitar la retracción del tejido (Tabla 10.2). Tras este último baño de parafina caliente y antes de que se confeccionaron los bloques utilizando una máquina dispensadora de parafina (Leica®, Alemania), las larvas fueron decapitadas ya que el cerebro de las misma fue el tejido de estudio. Al enfriarse la parafina, los bloques adquieren la dureza necesaria para poder ser cortados. En estos bloques, el tejido puede conservarse durante largos periodos de tiempo.

Tabla 10.2 Pasos en el proceso de parafinado.

Alcohol 70º	3 h	Deshidratación
Alcohol 96º	2.5 h	
Alcohol 96º	2.5 h	
Alcohol 96º	2,5 h	
Alcohol 100º	2 h	
Alcohol 100º	2 h	
Alcohol 100º	2 h	
Xilol	30 min	Clarificado
Xilol	30 min	
Xilol	30 min	
Parafina	3 h	Infiltración parafina
Parafina	6 h	
Bomba de vacío	1 h	

10.7.2.1 Preparación de los cortes

Para la obtención de los cortes se utilizó un microtomo de rotación Jung Autocut 2055 (Leica, Nussloch, Alemania). En primer lugar se procedió a orientar el bloque y a desbastar la parafina sobrante. Los bloques se cortaron a 3 μm. Los cortes se pusieron en suspensión en un baño de agua caliente que se encontraba a 45-50º C. Esta temperatura ayuda a distender el corte para conseguir el máximo estiramiento del tejido.

Los portaobjetos destinados a recoger las secciones tisulares para técnicas inmunohistoquímicas se prepararon con pegamento para aumentar la fijación de los tejidos, evitando así su despegado. Para ello se aplicó Poli-L (Sigma®) (Anexo I) en los portaobjetos.

10.7.2.2 Tinciones histológicas

10.7.2.2.1 Hematoxilina y eosina

Es una de las técnicas más comunes en un laboratorio de Histología. El objetivo es crear un contraste entre las diferentes partes del tejido para una correcta valoración al microscopio, utilizando la distinta afinidad de los tejidos para absorber los colorantes. En esta técnica los colorantes que se utilizan son los siguientes:

- Hematoxilina: Coloración azul púrpura. Tiñe estructuras básicas.
- Eosina: Coloración rosácea. Tiñe estructuras ácidas.

La técnica comienza secando los cristales en una estufa a 100° C durante 30 min. Las preparaciones fueron posteriormente inmersas en xilol e hidratadas en alcohol descendiente hasta llegar a agua como se indica a continuación:

Xilol	2 min
Xilol	2 min
Alcohol 100°	2 min
Alcohol 100°	2 min
Alcohol 70°	2 min
Agua destilada	2 min

Agua destilada	2 min
Agua destilada	2 min

Seguidamente comienza la batería de tinción según el siguiente protocolo modificado por Matorja y Matorja-Pierson (1970) y García del Moral (1993):

Hematoxilina Harris	15 min
Lavado rápido en agua	
Alcohol clorhídrico	4 pases rápidos
Lavado rápido en agua	
Agua amoniacal	15 pases rápidos
Agua corriente	15 min
Eosina	4 min

La técnica termina deshidratando el tejido siguiendo los pasos que se muestran a continuación:

Alcohol 96°	2 min	Deshidratación
Alcohol 96°	2 min	
Alcohol 100°	2 min	
Alcohol 100°	2 min	
Xilol	2 min	Clarificación
Xilol	2 min	
Xilol	2 min	

Se concluye montando las preparaciones con el medio DPX.

10.7.2.2.2 Tinción de Nissl

Los grumos de Nissl, o sustancias tigroide, son acúmulos de retículo endoplasmático rugoso que se encuentran en el citoplasmas de las neuronas, y están compuestos de RNA ribosómico y RNA mensajero. Para teñir los grumos de Nisll se utilizan, en general, colorantes básicos de anilina (como azul de metileno, azul de toluidina, tionina y violeta de cresilo y otros colorantes básicos) como la galocianina. Estos colorantes se unen a los ácidos nucleicos por un mecanismo físico-químico de atracción electrostática, y además de colorear los grumos de Nissl tiñen los núcleos, ya que éstos contienen DNA. Para que la coloración sea adecuada se precisa un pH ácido, a fin de que los colorantes mencionados tiñan por un mecanismo de coloración terminal, al igual que ocurre con la hematoxilina (Martoja y Martoja-Pierson, 1970).

Soluciones:

a) Tampón acetato
    a. Acetato sódico al 2,71%
    b. Acetato acético al 1,20%
b) Solución de cresilo extra
    a. Violeta de cresilo extra o tionina 0,25 g
    b. Tampón acetato a pH = 3,8-4 100 ml

Modo de operar:

1. Deshidratar e hidratar.

2. Solución de violeta de cresilo en tampón, 10 min.
3. Lavar en solución tampón de acetato.
4. Deshidratar, aclarar en xilol (poco tiempo) y montar.

10.7.2.3 Técnicas inmunohistoquímicas

Para poner de manifiesto la presencia de diferentes marcadores en las células de Mauthner en larvas de peces, se utilizó la técnica inmunohistoquímica de la avidina-biotina-peroxidasa (ABC) y un método de inmunofluorescencia para marcajes simples. La inmunohistoquímica está basada en la aplicación de un anticuerpo primario específico a los cortes del tejido, creado generalmente en conejo (policlonales), o en hibridoma creado en bazo de ratón (monoclonal). El anticuerpo es específico contra una molécula de naturaleza proteica que se quiere evidenciar. Previa aplicación del mismo se realiza:

1. Un bloqueo de la peroxidada endógena, para evitar falsos positivos, puesto que el posterior revelado se basa en esta enzima.
2. Un proceso de desenmascarado de antígenos, sólo en caso de ser necesario y para romper los enlaces creados por la fijación formólica.
3. Un neutralizado a base de suero inespecífico también denominado normal cuya función es evitar falsos positivos resultantes de la fijación del suero secundario, por eso debe ser creado en la misma especie que el suero normal.

Posteriormente, un suero secundario se aplica tras el primario y que sirve de puente entre éste y el complejo amplificador de reacción siguiente denominado

avidina-biotina-peroxidasa (ABC). El proceso de revelado se puede llevar a cabo utilizando dos cromógenos diferentes, comúnmente denominados “rojo” (AEC, 3-amino-9 etilcarbazol) o “marrón” (3-3-diaminobenzidina) según la coloración de la positividad. Para la realización de estos estudios, se utilizó el revelado en marrón.

Protocolo

Cada anticuerpo fue testado para versionar su funcionamiento, y sometido a variaciones para conocer las concentraciones y el tipo de desenmascarado de epítopos que maximizan su positividad.

Los anticuerpos probados fueron la parvalbúmina (PV) y la calretinina (CR), ambos policlonales (Tabla 10.3).

Tabla 10.3 Anticuerpos probados en las técnicas inmunohistoquímicas.

Anticuerpo	Dilución	Tiempo de revelado	Casa comercial
PV	1:700	10 min	Swant
CR	1:700	10 min	Swant

Para la realización de la técnica inmunohistoquímica se procedió a seguir los siguientes pasos:

1. Se introducen las cestillas en la estufa durante al menos 24 h a 37° C.

2. Un pase de 10 minutos y dos pases de 5 min en xilol.

3. Un pase de cinco minutos en alcohol de 100º.

4. Tratamiento de inhibición de la peroxidada endógena con una solución de peróxido de hidrógeno al 3% en metanol durante 30 minutos en agitación suave.

5. Un pase de cinco min en alcohol 100º.

6. Un pase de 5 min en alcohol de 96º.

7. Un pase de 5 min en alcohol de 70º.

8. Dos lavados de 5 min en agua destilada.

9. Un lavado de 5 min en PBS (Phosphate bufferd saline) en agitación suave.

10. Desenmascarado de epítopos para liberar los antígenos usando el tratamiento *enzimático* mediante el empleo de la pronasa. Este tratamiento consiste en aplicar una solución de enzima pronasa en proporción de 0,1 g cada 100 ml de buffer PBS a temperatura ambiente durante 7 min.

11. Tres lavados con buffer PBS.

12. Aplicación del suero normal de cabra para los siguientes anticuerpos: parvalbúmina y calretinina. Incubación en la cámara húmeda a temperatura ambiente con suero normal de cabra (Vector®) al 10% en PBS durante 30 min.

13. Incubación con los anticuerpos primarios. Al ser policlonales fueron diluidos en suero normal de cabra al 1% en PBS. Este proceso se llevó a cabo en una cámara húmeda a 4º C durante 18 h. Se realizaron pruebas con diferentes concentraciones del anticuerpo primario para obtener la idónea.

14. Retirar la cámara húmeda de la nevera una hora antes de proceder a reanudar la técnica para atemperar las muestras.

15. Tres lavado de 5 min en PBS con agitación suave.

16. Incubación con el anticuerpo secundario creado en cerdo y diluido en suero normal de conejo al 1% en PBS (concentración 1:250) para la parvalbúmina y conejo anti-ratón para la calretinina (1:250). Este proceso se llevó a cabo en una cámara húmeda a temperatura ambiente durante 30 min.

17. Tres lavados de 5 minutos en PBS en agitación suave.

18. Incubación con el complejo Estreptavidina-Biotina-Peroxidasa (ABC) (Dako®) (45 µl de reactivo A y 45 µl de reactivo B en 5000 µl de PBS), en una cámara húmeda y en oscuridad durante una hora temperatura ambiente.

19. Dos lavados de 5 min en PBS en agitación suave.

20. Un lavado de 10 min en Tris (1 parte de Tris madre/9 partes de suero salino al 0,85%).

21. Revelado de la reacción por inmersión en diaminobenzidina (DAB) que se prepara disolviendo con un agitador 0,07 g de DAB en 200 ml de tampón Tris. Se filtra con doble papel en cámara oscura y se añaden 200 µl de peróxido de hidrógeno al 30%. Se revela durante 2 min aproximadamente observando el control positivo.

22. Lavado en agua corriente durante 10 min.

23. Contra tinción con hematoxilina de Harris durante 30 s.

24. Lavado en agua corriente durante 10 min.

25. Deshidratar en alcoholes de graduación creciente en xilol, con pases de dos min cada uno.

26. Montaje con resina DPX.

10.7.3 Preparación de muestras para criostato

Estas técnicas fueron aprendidas en el Instituto de Neurociencias de Castilla y León (INCYL) en Salamanca. Las larvas fueron fijadas por inmersión en una mezcla constituida por paraformaldehído al 1% y ácido pícrico (15%) en tampón fosfato (TF) 0,1 M, pH 7,4 durante 4-6 horas (dependiendo del tamaño de la muestra) a temperatura ambiente. Se realizaron algunas pruebas fijando las larvas con formaldehído al 10% obteniendo resultados semejantes e incluso mejores ya que se obtuvo una mejor calidad del corte. Seguidamente se lavaron las muestras 3 veces con tampón fosfato o PBS durante 5 min. A continuación se procedió a conservar las cabezas de las larvas en mezcla congeladora (Anexo I) a -20° C hasta su posterior utilización. Tras sucesivos lavados en TF o PBS se preparó el tejido para su posterior corte en un criostato. Las muestras se crioprotegieron en una solución de sacarosa al 30% (p/v) en TF o PBS durante 12 horas a temperatura ambiente y se encastraron en un medio constituido por agar al 1.5% y sacarosa al 5% (p/v) en TF o PBS. En cada bloque se colocó una cabeza y fue orientada de tal manera que el ángulo de corte fuera horizontal. Una vez crioprotegidos, los bloques fueron posteriormente incluidos en OCT 4583 (Medio de inclusión, Tissue-tek, Miles) congelados con nitrógeno líquido y cortados en criostato (Leica Jung CM 3000, Nussloch, Alemania) a una temperatura de -24° C. Se realizaron secciones de 10 µm de espesor que fueron recogidas en portaobjetos doblemente gelatinizados (Anexo I).

Tinciones en hematoxilina-eosina fueron ensayadas para comprobar que los cortes salían correctamente antes de realizar las pruebas pertinentes objeto de estudio.

Hematoxilina-eosina

1. Lavado de las secciones en TF o PBS, tres veces durante 15 minutos.
2. Lavado de las secciones en agua destilada durante 10 minutos.
3. Tinción con hematoxilina durante 1 minuto y 30 segundos.
4. Eliminación del exceso de hematoxilina por lavado en agua corriente durante 30 minutos.
5. Lavado en agua destilada.
6. Deshidratación en alcoholes de graduación creciente, 5 minutos en cada uno hasta xileno (tres pasos de 5 minutos cada uno).
7. Montar secciones.

10.7.3.1 Inmunofluorescencia

Esta técnica fue puesta a punto en colaboración con el INCYL, donde se realizó una estancia de investigación. Posteriormente se montaron las técnicas y se procesaron las muestras de la tesis en el IUSA. Las secciones previamente cortadas en criostato, se descongelaron a temperatura ambiente durante una hora y se lavaron en TF o PBS para rehidratar el tejido, 3x10 minutos en agitación.

A continuación se realizó una preincubación con suero de conejo no inmune (Sigma) al 5 % y Triton X-100 (Probus S.A.) al 0,2 % en TF o PBS

durante 1 hora a temperatura ambiente. Se utilizó suero procedente de la especie en que se había obtenido el anticuerpo secundario. Con este procedimiento conseguimos facilitar la penetración de los anticuerpos y, además, reducir las uniones inespecíficas de los mismos al tejido. A continuación las secciones se incubaron con el medio indicado anteriormente al que añadimos el anticuerpo primario Anti-Choline acetyltransferase (ChAT) correspondiente (Tabla 10.4), en una cámara húmeda durante 2 días a 4° C. Una vez finalizada la incubación, los cortes se lavaron con TF o PBS.

Tabla 10.4. Anticuerpo primario utilizado en la inmunofluorescencia.

Anticuerpo	Dilución	Casa comercial
ChAt	1:500	Millipore

Tras este lavado, las secciones se incubaron con el correspondiente anticuerpo secundario fluorescente Anti-goat IgG (whole-molecule)_FITC (anticuerpo hecho en conejo, Sigma ®) diluido a 1:250 en el medio de incubación, durante 1 hora, temperatura ambiente y en oscuridad. Para marcar los núcleos de todas las células se realizó una incubación de 10 minutos en oscuridad con ioduro de propidio (IP; Sigma ®) a 1:2000, en TF o PBS. Por último, las secciones se lavaron con TF y se montaron en un medio comercial Fluoromont ® (Sigma ®). Para la obtención y cuantificación de la intensidad de la fluorescencia se utilizó un microscopio confocal modelo Zeiss LSM 510 del Departamento de

Bioquímica de la ULPGC. Para cuantificar la intensidad de la muestra se utilizó el programa LSM 510 mediante los siguientes parámetros: ganancia del detector, amplificador y ganancia del amplificador.

10.7.4 Procesamiento e inclusión en resina

Las larvas fueron anestesiadas con MS222 y seguidamente fueron fijaron en glutaraldehído al 2,5% en tampón fosfato al 0,2M (pH=7,2) (Anexo I) durante 1 hora y conservadas en tampón cacodilato hasta su procesamiento (Bancroft y Stevens, 1996).

Las muestras se procesaron en el IUSA. Para ello, las muestras fueron lavadas con líquido lavador (Anexo I) durante 24 horas a 4° C. Transcurrido este periodo, las larvas se postfijaron en tetróxido de osmio al 2% diluido en tampón ferrocianuro al 1,5% (Anexo I) durante 2 horas. Seguidamente se procedió a deshidratar las muestras con acetona como se muestra a continuación:

Acetona al 30% 10 min

Acetona al 50% 10 min

Acetona al 70% 10 min

Acetona al 80% 10 min

Acetona al 90% 10 min

Acetona absoluta 10 min

A continuación, se procedió a incluir las muestras en resina Durcupan ACM Fluka (Fluka Analitikal, Buchs, Suiza):

Resina/acetona; ½ 45 min (toda la noche) a 4° C

Resina/acetona; 1/1 2 h (toda la noche) a 4° C

Resina/acetona; 2/1 8 h (toda la noche) a 4° C

Resina pura toda la noche a 4° C

Seguidamente se procedió a colocar y orientar las muestras de las cabezas de las larvas en los bloque utilizando resina pura y conservando en una estufa a 60° C.

10.7.4.2 Cortes semifinos

La inclusión de las muestras en resinas permite la obtención de cortes de grosor mucho más delgado que en el caso de la parafina. Aunque inicialmente esta técnica de inclusión se utilizó como un método para seleccionar regiones para la obtención de cortes ultrafinos para la microscopía electrónica de transmisión, hoy en día se utiliza frecuentemente para la microscopía la óptica, ya que se obtiene una resolución mucho mayor.

Los tejidos embebidos en resina se cortaron a 1 μm de grosor con un ultramicrotomo (Leica LKB modelo Ultratome Nova, Alemania) y se tiñeron con Azul de Toluidina (Hoffman et al., 1983) durante 30 segundos. Este colorante tiñe estructura basófilas, tales como la cromatina. Las preparaciones se secaron utilizando un calefactor.

Procedimiento

a. Recortar la cara del bloque de corte alrededor de 1 mm^2 en forma de pirámide cuadrangular para sea más fácil de identificar sí se quiere seguir cortando en un futuro.

b. Cortar secciones (aproximadamente 0,5 µm de espesor) con una cuchilla de cristal. Colocar una gota de agua destilada en un portaobjetos de vidrio limpio. Pescar las secciones y colocar en la gota de agua del portaobjeto. Es fundamental que las secciones estén al revés de la forma en la que estaban flotando, de modo que cuando se vea con un microscopio de luz compuesto tendrá la misma orientación que el bloque de corte.

c. Identificar los portaobjetos con las muestras.

d. Colocar el portaobjetos con la gota de agua que contiene secciones sobre una placa caliente (60° C) y esperar hasta se evapore el agua.

e. Buscar las secciones e identificarlas con un marcador para indicar su posición.

f. Colocar 1-2 gotas de la solución de azul de toluidina al 1% y borato de sodio (1%) en las secciones sobre la placa calefactora. Esperar hasta que los bordes de la solución comienzan a dorarse (aproximadamente 20-30 segundos) y enjuagar rápidamente con agua destilada para eliminar el exceso de tinte. Si la

solución se secase totalmente, las secciones seguirían siendo utilizables. Al lavar, dirigir el flujo del agua destilada ligeramente por encima de las secciones formando un ángulo de 45°. Si el flujo apunta directamente sobre las secciones, las secciones se podrían separar del portaobjeto. Sí las secciones se separasen con demasiada facilidad podría ser debido a que la temperatura de la placa es muy baja o que los cortes son demasiado gruesos. Seguidamente, colocar el portaobjetos en la placa calefactora y esperar a que el agua residual se evapore de la superficie.

g. Retirar el portaobjetos de la placa caliente y añadir 1-2 gotas del medio de montaje (Polymount) en la parte superior de las secciones. Colocr suavemente un cubreobjetos.

h. Examinar los cortes semifinos teñidos con un microscopio de luz compuesto.

i. Marcar el área de interés para realizar los cortes ultrafinos. La cara del bloque de corte ultrafino debe ser de aproximadamente 0,5 mm^2.

10.7.4.3 Cortes ultra finos

Una vez obtenido el corte en semifino e identificada la zona objeto de estudio, se procedió a realizar cortes ultrafinos de aproximadamente 50 nm de espesor en un ultramicrotomo (Leica LKB modelo Ultratome Nova, Alemania). Para ello se utilizó una cuchilla de diamante. Una vez preparados, los ultrafinos se montaron sobre unas rejillas de cobre con un diámetro alrededor de 3,05

mm. Éstas pueden ser de diferentes formas y materiales. Las rejillas con los cortes se contrastaron con citrato de plomo. Los cortes se estudiaron y fotografiaron empleando un microscopio electrónico del Servicio de Microscopía Electrónica de la Universidad de Las Palmas de Gran Canaria.

Procedimiento

a. Examinar con un microscopio la cara de corte del bloque para comprobar la orientación correcta de corte. La cara de corte del bloque debe ser de aproximadamente 0,5 mm^2. Orientar el bloque de manera que la superficie de corte sea perpendicular a la dirección del corte.

b. Cortar la extremidad del bloque a 50 nm bajo la forma de una pirámide cuadrangular que contiene el tejido.

c. La tira formada por la sucesión de varios cortes fue colocada sobre una rejilla muy fina recubierta por una membrana (Formvar, Anexo I).

d. Con una trozo de papel de flitro se utilizó para secar la rejilla con las secciones. La rejilla fue colocada en una placa de petri de plástico.

e. Teñir las rejillas con citrato de plomo aproximadamente 5 minutos (Anexo I). El citrato de plomo fue sacado de la nevera (4° C) antes de su uso. Con una pipeta Pasteur limpia se tomó una gota y fue colocada sobre un trozo de parafina pegada en la parte inferior de la placa de Petri de plástico. Se cubrió

la placa para evitar la formación de un precipitado negro del carbonato de plomo por la exposición al CO_2. Las rejillas fueron enjuagadas en tres vasos con agua desionizada.

f. Después de 5 min, introducir la rejilla en la solución y seguidamente meterla en un segundo vaso de agua desionizada. Este paso es muy importante, para limpiar las secciones del carbonato de plomo.

g. Se le aplicó a la rejilla 10 pasos rápidos y se limpió en un cuarto vaso con agua. La rejilla fue secada con papel de flitro. Se retiró la rejilla del vaso y fue limpiada con un trazo de papel de filtro.

h. Guardar la rejilla en su cajita e identificarla.

i. El papel de filtro y parafina de la tinción fueron colocados en la caja de los residuos, los vasos fueron enjuagados con agua desionizada y todas las pipetas utilizadas se tiraron en los contenedores de residuos.

j. La solución de citrato de plomo fue guardada en la nevera a 4° C.

k. Mucha precaución usando estas soluciones, contienen maetales pesados y tóxicos para humanos.

10.8 Análisis estadísticos

La media y las desviaciones estándar se calcularon para cada parámetro medido. Los resultados fueron expresados como media ± desviación estándar y analizados mediante el programa SPSS (SPSS 11,5 para Windows; SPSS Inc, Chicago, IL, EE.UU.). Las diferencias entre grupos se determinaron mediante ANOVA de una vía siguiendo el modelo linear:

$$Y_{ijk} = \mu^* + F_i + H_j + (FH)_{ij} + \varepsilon_{ijk}$$

donde Y_{ijk} es el valor medio de tanque, μ es la media de la población, F_i es el efecto fijo de un factor, H_j es el efecto fijo de otro factor (por ejemplo PUFA), $(FH)_{ij}$ la interacción entre los efectos fijos, y ε_{ijk} es el error residual.

La normalidad de la distribución de las variables fue verificada mediante el test de Levene. La transformación logarítmica fue usada para normalizar las variables y el test de Ducan o t-Student fue empleado para comparar las medias de ácidos grasos y los resultados de los estudios de comportamiento ($P<0.05$).

Conclusiones

Las conclusiones obtenidas en cada capítulo de esta tesis se presentan a continuación:

- Estudio 1. Dietary n-3 long chain polyunsaturated fatty acids deficiency induces a reduced visual response in early larvae of gilthead sea bream *Sparus aurata*.

El reemplazamiento del aceite de pescado por aceites vegetales en enriquecedores de rotíferos, afecta negativamente la crecimiento en larvas de dorada. La disminución del contenido de grasos esenciales en rotíferos debido al enriquecimiento de aceites vegetales, afecta al comportamiento normal de las larvas, reduciendo de velocidad de crucero, y, en particular, retrasa la aparición de la respuesta ante el estímulo visual, lo que sugiere un retraso en el desarrollo funcional del cerebro y de la visión, en relación con el menor contenido de ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA) encontrado en los ojos y el cerebro de estas larvas.

- Estudio 2. Increased Mauthner cells activity and escaping behaviour in gilthead sea bream larvae (*Sparus aurata*) fed long chain polyunsaturated fatty acids.

El presente estudio muestra la primera evidencia de la importancia de los ácidos grasos poliinsaturados de cadena larga omega-3 para el adecuado funcionamiento de las neuronas, en especial las células de Mauthner, modulando la respuesta d el comportamiento de huída ante un estímulo sonoro.

- Estudio 3. Docosahexaenoic acid, but not eicosapentaenoic acid, enhanced sound stimuli induced escaping behaviour and Mauthner cells activity in gilthead sea bream larvae.

Los resultados de este capítulo demuestran que el DHA, y no el EPA, incrementa el comportamiento de huída en larvas de dorada y que este efecto es al menos en parte, mediado por el aumento de la actividad neuronal, en particular por las células de Mauthner.

- Estudio 4. Dietary polyunsaturated fatty acids affect zebrafish (*Danio rerio*) behaviour and Mauthner cells activity.

Este estudio demuestra la importancia del DHA de la dieta en el crecimiento, la respuesta de escape y la actividad neuronal en el pez cebra, indicando la importancia de la dieta cuando se utiliza el pez cebra como modelo en estudios de comportamiento y neurológicos.

- Estudio 5. Use of calretinin (CR) and parvabumin (PV) as Mauthner cells markers in European sea bass (*Dicentrarchus labrax*).

Los resultados de este último estudio facilitan las técnicas necesarias para entender mejor el papel de las células de Mauthner en larvas de peces marinos, las posibles implicaciones en la respuesta de huída y el efecto de los factores dietéticos o ambientales. La falta de reactividad de la calretinina (CR) y de la parvalbúmina (PV) a los 6 y 10 días denotan una pobre funcionalidad células de Mauthner coincidiendo con la baja respuesta de huída encontrada en los capítulos anteriores. La PV parece jugar un papel importante en el desarrollo inicial de la funcionalidad de las células de Mauthner y la respuesta de huída en larvas de peces marinos, detectándose su positividad mediante técnicas inmunohistoquímicas o de inmunofluorescencia a los 17 días e incrementando su intensidad a los 19 días, coincidiendo con una mejor respuesta de escape. Sin embargo, la CR podría ser importante para actividades de refuerzo de las células de Mauthner en larvas mayores, debido a que aparece durante los 28 y 47 días coincidiendo con la mayor respuesta de huída encontrado nuestros estudios.

11. References

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12. Annexes

Annex I

10% buffered formalin

100 ml 40% concentrated formaldehyde

900 ml distilled water

4 g $NaH_2PO_4 \cdot H_2O$

6 g Na_2HPO_4

Poli L

0.1 g Poli-L in 1000 ml milli-Q water

0.01 g Poli-L in 100 ml of water

a) Wash slides in this solution during 5 min
   - 2 water baths during 5 min
   - Dry and use

b) Wash slides in this solution during 5 min

c) Dry and use

Freezing mixture

This mixture serves to preserve the tissues in the freezer without destroying, and preventing the formation of crystals. It also has a controlled pH.

- Material

Sodium dihydrogen phosphate, 2-hydrate. NaH_2PO_4 $2H_2O$ p.m.= 156.01

di-Sodium hydrogen-Na_2HPO_4 p.m. = 141.96

Polyethylene glycol 400. HO (C_2H_4O) p.m. = 380-420

98% glycerol. CH_2OH CHOH $CH_2OH \cdot$ (use pure)

Distilled water

- Formula

30% v / v glycerol

30% v / v ethylene glycol

0.1M phosphate buffer pH = 7.3

- Preparation

0.5 M phosphate buffer:

7.8 g NaH_2PO_4 in 100 ml H_20 (A)

14.2 g Na_2HPO_4 in 200 ml H_20 (B)

Mix slowly (A on B) pH = 7.3-7.4.

For 1l of solution, mix 200 ml of 0.5 M phosphate buffer (to make it a final concentration of 0.1 M), 300 ml glycerin, 300 ml of ethylene glycol and adjusting the volume to 1l with H_2O. Mixing thoroughly.

Gelatinized slides

Mix 4.5 g of gelatin powder with 900 ml of water and dissolve by heating to 80° C. Add 0.44 g of chromium III and potassium sulphate. Insert the clean slides in the filtered solution at 70° C for 5 min and allow to dry in an oven overnight. The operation is repeated, and stored in a cool place.

Reactives Electro Microscopy

- Lead citrate
  - 1.33 g of lead nitrate
  - 1.77 g of sodium citrate
  - 30 ml H_2=
    - Shake vigorously for 10 min
    - Add 8 ml of 1N NaOH Vol
    - Turn slowly
- 4% glutaraldehyde
  - 84 ml of 0.1 M cacodylate buffer (or 42 ml of distilled water and 42 ml of 0.2 M cacodylate buffer)

    - 16 ml of 25% glutaraldehyde
- 0.1 M cacodylate buffer
    - sodium cacodylate (PM 214.02), 2.1402 g
    - 100 ml distilled water
    - cacodylate is dissolved in water adjusting pH (7.2-7.4) with 0.1 N hydrochloric acid
- 0.2 M cacodylate buffer
    - 4.28 g sodium cacodylate
    - 100 ml distilled water
    - Adjust pH (7.2-7.4) with 0.1 N hydrochloric acid
- Washer liquid (cacodylate/sucrose)
    - 0.2 M cacodylate buffer 50 ml
    - 50 ml distilled water
    - 5 g sucrose

Formvar-coated slot grids

Dissolve 1.1 g Formvar in 100 ml chloroform in a glass-stoppered erlenmeyer flask while stirring. Transfer the Formvar solution gently into a coplin jar.

Lead citrate

Working solution: 0.1 g lead citrate in 50 ml 0.1 N NaOH

Procedure:

- Lead citrate solution 1 min.
- 3 times in ultrapure water.
- Dry the grils with whatman filter paper.

Annex 2

DESARROLLO DE LAS TÉCNICAS DE PRODUCCIÓN DE CRÍAS DE BOCINEGRO (*PAGRUS PAGRUS*) EN CANARIAS

T. Benítez-Santana

R. Masuda

A. Valencia

M.S. Izquierdo

La incorporación de nuevas especies de peces marinos a la acuicultura, es un reto para el futuro desarrollo de esta actividad. Los avances realizados en el establecimiento de las técnicas de cultivo de bocinegro (pargo, *Pagrus pagrus*), permiten considerarlo como una especie con un fuerte potencial para la acuicultura siendo previsible que su cultivo experimente un impulso importante en el archipiélago canario. Sin embargo, las investigaciones han permitido identificar varios problemas que deben solventarse antes de que el bocinegro pueda cultivarse comercialmente, siendo una de las necesidades más acuciantes la de completar las técnicas de producción de larvas y alevines, y estudiar detenidamente la alimentación a suministrar durante todo el ciclo larvario. Por ello, el trabajo que se presenta tiene como objetivo principal tratar de optimizar las técnicas de producción de larvas y alevines de bocinegro en las condiciones ambientales del archipiélago canario. Esto se realizará a partir de una técnica japonesa muy novedosa, para establecer según el comportamiento larvario, las pautas de alimentación a seguir para completar el ciclo larvario del bocinegro. El comportamiento obtenido se comparará con el de la dorada, ya que es una de las especies más estudiadas debido a la importancia adquirida en la acuicultura europea de los últimos años. Con todo esto, se construirá un criterio de calidad de la dieta a partir de las pautas de comportamiento establecidas.

The incorporation of new marine species in aquaculture is essencial for the development of this activity. The recent advances on the establishment of techniques to produce red porgy (Pagrus pagrus), allow us to consider it a strong potencial to aquaculture, being predictable its implement in the Canary Archipel. Nevertheless, it has been not possible so far to produce it comercially, due to difficulties in assuring a continual supply of larvae and fry. Knowing this, it is crucial to continue studying the production techniques of larvae and the feeding system during this period. The aim of the work is then to optimize production techniques of larvae and fry of the red porgy according to the environmental conditions of the Canary Archipel. The work is based on a novel japanese technique, which intents to establish the feeding system in accordance with larvae behaviour, as to complete the larval cycle of Pagrus pagrus. The observed behaviour will be compared with the seabream one (Sparus aurata), one of the most studied species due to its importance in European aquaculture in the past years. In the light of these analyses a quality diet criteria based on the padrons of larval behaviour will be established.

PRESENTACIÓN

Los avances realizados en el establecimiento de las técnicas de cultivo de bocinegro (pargo, *Pagrus pagrus*), permiten considerarlo como una especie con un fuerte potencial para la Acuicultura, siendo previsible que su cultivo experimente un impulso importante tanto en áreas del Mediterráneo, como en zonas templadas del Atlántico, incluyendo el archipiélago canario, ya que esta especie constituye un plato fundamental en la gastronomía típica de las Islas. Sin embargo, las investigaciones han permitido identificar varios problemas que deben resolverse

antes de que el bocinegro pueda cultivarse comercialmente, siendo una de las necesidades más acuciantes la de completar las técnicas de producción de larvas y alevines.

INTRODUCCIÓN

Importancia del cultivo larvario en el desarrollo de la acuicultura

Hoy en día, la producción larvaria y la obtención de alevines de buena calidad, siguen representando el cuello de botella para el desarrollo de la acuicultura, sobre todo en ciertas especies marinas como el bocinegro. Esto es debido a que las larvas de peces marinos no están completamente desarrolladas cuando eclosionan. Sus tejidos y órganos, así como el sistema nervioso, no están del todo formados y experimentan importantes cambios de tipo morfológico, funcional y fisiológico durante la maduración. Por esta razón, durante estas primeras etapas de desarrollo de estos organismos, se necesitará un manejo más delicado. Una solución a este inconveniente, sería optimizar las condiciones de manejo de las diferentes especies marinas que actualmente se cultivan.

Dentro de este contexto, son muy importantes los estudios referentes a la nutrición y selección genética de reproductores, requerimientos nutricionales de las larvas, mejora de las técnicas de cultivo, estudios patológicos,... Pero todavía existen diferentes áreas de estudio que no han sido prácticamente abordadas, como el estudio del comportamiento de larvas y alevines. El estudio del comportamiento de las larvas a lo largo de su desarrollo permitiría establecer los momentos de aparición de determinadas pautas típicas en animales sanos. Posibles desviaciones en el tipo de comportamiento o retrasos en la aparición de esas pautas, constituirían indicadores del estado de desarrollo, madurez y salud del animal. Así, estos estudios se revelan como excelentes indicadores del estado de bienestar de las larvas, y por lo tanto, del éxito del cultivo larvario. La llave del desarrollo del comportamiento de escuelas (formación de cardúmenes) implica en mayor medida al sistema nervioso central que a los órganos sensoriales y de natación (Masuda y Tsukamoto, 1998). El DHA (Ácido docosahexaenoico) en la dieta influye en el desarrollo del comportamiento de escuelas y cerebro en larvas de seriola (*Seriola quinqueradiata*) (Ishizaki, 2001). Determinados autores, trabajando con larvas de dorada, como con otras especies de peces marinos, han demostrado que al aumentar el porcentaje de n-3 HUFA tanto en alimento vivo como en microdietas, se obtienen larvas con un mejor crecimiento y una supervivencia más alta (Salhi, 1997; Sargent *et al*., 1999). También se ha descrito que cuando se suministran rotíferos o microdietas deficientes en n-3 HUFA se produce además una mayor sensibilidad al estrés para algunas especies marinas (Izquierdo, 1996). La composición de ácidos grasos de los tejidos como ojo y cerebro se muestra como un claro reflejo de la dieta de la larva, así, Navarro *et al*. (1993) señalan que la cantidad de DHA en los ácidos grasos de la fosfatidiletanolamida de los ojos es particularmente susceptible a la deficiencia de los mismos en la dieta. La importancia de altos niveles de DHA para el correcto desarrollo neural en larvas ha sido demostrada.

El estudio del comportamiento de las larvas a lo largo de su desarrollo permitiría establecer los momentos de aparición de determinadas pautas típicas en animales sanos

Bocinegro (*Pagrus pagrus*)

El DHA en la dieta influye en el desarrollo del comportamiento de escuelas y cerebro en larvas

Este trabajo tiene como objetivo principal establecer el momento de aparición de ciertas respuestas del comportamiento a lo largo de los primeros días de vida del bocinegro, determinar el efecto de la alimentación con diferentes tipos de fuentes lipídicas y distintos niveles de DHA sobre dichas pautas y construir un criterio de calidad de la dieta a partir de las pautas de comportamiento establecidas. El comportamiento obtenido se compará con el de la dorada, que es una de las especies más estudiadas debido a la importancia en la acuicultura mediterránea de los últimos años.

MATERIAL Y MÉTODOS

El presente trabajo se llevó a cabo en el Instituto Canario de Ciencias Marinas (ICCM). Los huevos de dorada utilizados procedían del "stock" de reproductores existente en la propia planta de cultivos del ICCM, mientras que los huevos de bocinegro procedían de un "stock" de reproductores de la planta de cultivos del Centro de Maricultura de Calheta (Madeira). Estos fueron distribuidos en dieciséis tanques de fibra de vidrio de 170 l de capacidad (100 huevos/l). Las larvas de bocinegro y dorada fueron alimentadas con rotíferos enriquecidos con aceite de pescado (FO), aceite de soja (SBO), aceite de lino (LSO) y aceite de colza (RSO).

Al día 10 de experimentación, un tanque de cada dieta de dorada fue sacrificado para realizar análisis del contenido total de lípidos y de la composición de ácidos grasos. Al final del periodo experimental, 100 larvas de dorada fueron separadas para la extracción de cerebro y ojos, y el resto de larvas supervivientes se guardaron en bolsas de plástico a -80ºC para realizar análisis bioquímicos.

La velocidad de natación en larvas de bocinegro fue medida los días 9, 10, 12 y 13 de vida, y en dorada los días 6, 10, 16 y 19. Se utilizó un vaso precipitado de 500 ml con agua de mar cubierto por una funda negra. Las larvas fueron grabadas con una cámara de video digital Sony DCR-TRV27. Después de 90 s de grabación, se suministró un estímulo (sonoro o visual) (Masuda *et al*., 2002). Se utilizaron 5 larvas de cada replicado. Se realizó un análisis de imagen para calcular la velocidad de crucero y la velocidad de huída.

Los datos fueron analizados estadísticamente con el programa STATGRAPHICS PLUS para Windows 3.1 (Stadistical Graphics Corp., Englewood Cliffs, NJ, USA) usando un análisis de una sóla vía (ANOVA) y Duncan ($P < 0.05$) para una comparación de medias.

RESULTADOS

Composición de ácidos grasos obtenida en las muestras de dorada

En el presente apartado se hace una valoración comparada de la composición lipídica que presentan los rotíferos cuando son enriquecidos con aceites que contienen distintos porcentajes de n-3 HUFA. En la tabla I se presentan los resultados de los análisis lipídicos para la determinación de los lípidos totales y ácidos grasos de los aceites y presas utilizados en el experimento.

Examinando los ácidos grasos de los aceites enriquecedores cabe destacar algunas diferencias importantes. El aceite de pescado posee un 17.11% de n-3 HUFA, de los cuales el EPA (20:5n-3) tiene una representación ligeramente superior al DHA (22:6n-3), siendo la relación entre ambas de 1.04/1. El contenido en ácidos grasos saturados es elevado, sobre todo en el ácido palmítico (16:0), 13.22%. También es bastante alto el contenido en ácidos monoenoicos, principalmente en 22:1n-11 con un 16.63%. El aceite de soja se caracteriza por presentar una mayor cantidad en ácidos grasos de la familia n-6, 52.77%. También es rico en ácido oleico (18:1n-9) con un porcentaje de

Ácidos Grasos	ACEITE FO	ACEITE SBO	ACEITE LSO	ACEITE RSO	Rot. FO	Rot. SBO	Rot. LSO	Rot. RSO
14:0	0,28	0.51	0,07	0,02	6,98	1,45	0,06	1,38
14:1	0,34	0.01	n.d.	n.d.	1,10	0,99	0,01	0,53
15:0	0,08	0.18	n.d.	n.d.	0,74	0,28	0,03	0,31
16:0	13,23	14.86	6,95	4,88	18,99	8,99	5,39	10,89
16:1n-7	7,61	0.31	n.d.	0,46	30,62	9,57	0,12	15,49
16:1n-5	0,12	0.04	n.d.	n.d.	0,79	0,36	0,01	0,03
17:0	0,48	0.08	n.d.	n.d.	1,44	0,67	0,04	0,15
16:4n-4	0,45	0.11	n.d.	n.d.	0,33	0,69	0,01	0,57
18:0	1,46	2.96	0,97	1,31	0,98	0,86	2,85	0,71
18:1 (n-9+ n-7)	12,53	15.12	3,21	63,37	31,10	24,23	19,64	71,86
18:2n-6	1,56	27.43	14,80	20,72	20,30	35,42	14,89	33,52
18:3n-3	0,88	2.73	53,67	n.d.	3,52	3,76	56,42	9,51
18:4n-3	2,29	n.d.	n.d.	8,03	3,33	0,13	n.d.	0,04
18:4n-1	0,15	0.04	n.d.	n.d.	0,26	0,11	n.d.	n.d.
20:0	0,13	0.18	n.d.	n.d.	0,18	0,13	0,05	0,28
20:1n-9	12,92	1.55	0,15	1,17	10,25	1,70	0,14	3,68
20:1n-7	n.d.	0.05	0,23	n.d.	n,d,	0,43	n.d.	n.d.
20:2n-9	0,20	n.d.	n.d.	n.d.	0,65	0,25	n.d.	0,11
20:2n-6	n.d.	0.11	n.d.	n.d.	0,52	0,74	n.d.	0,36
20:3n-6	0,09	n.d.	n.d.	n.d.	0,13	0,15	n.d.	0,07
20:4n-6	0,02	0.31	0.01	n.d.	0,69	0,61	0,01	0,11
20:3n-3	0,15	0,2	n.d.	n.d.	0,15	0,09	0,02	0,13
20:4n-3	0,43	0.05	n.d.	n.d.	1,16	0,15	0,02	0,19
20:5n-3	8,12	4.42	n.d.	n.d.	13,47	0,90	0,02	0,36
22:1n-11	16,63	0.25	n.d.	0,01	11,25	0,70	0,02	1,62
22:3n-6	0,28	0.06	n.d.	n.d.	0,45	0,35	0,01	0,07
22:4n-6	0,02	0.06	n.d.	n.d.	0,07	0,29	n.d.	n.d.
22:5n-6	0,11	n.d.	n.d.	n.d.	0,16	0,01	n.d.	n.d.
22:4n-3	0,05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,10
22:5n-3	0,45	0.12	n.d.	n.d.	0,86	0,29	0,02	0,08
22:6n-3	7,91	0.12	n.d.	n.d.	11,19	2,16	0,04	0,31
Saturados	21,79	19.02	8,20	6,20	21,02	12,49	8,44	13,76
Monoinsaturados	53,98	17.90	3,59	65,01	59,15	37,78	20,05	93,18
n-3	20,28	34.66	53,67	8,03	17,81	10,17	56,53	13,77
n-6	2,34	27.97	14,81	20,72	0,48	37,63	14,91	34,14
n-9	25,74	16.70	23,10	64,54	29,84	26,49	19,89	76,27
n-3HUFA	17,11	16.81	n.d.	n.d.	17,81	3,59	0,11	1,17
AA/EPA	n.d.	14.09	n.d.	n.d.	0,05	0,68	0,55	0,32
EPA/DHA	1,03	2.72	n.d.	n.d.	1,20	0,42	0,48	1,13
n-3 HUFA % peso seco					15.77	3.18	0.10	1.04

n.d. ≤ 0.005

Tabla I. Principales ácidos grasos encontrados en las muestras

26.91. El aceite de lino posee una mayor proporción de ácidos grasos de la familia n-3, sobre todo linolénico (18:3n-3) con un 53.68%. Por otro lado, el aceite de colza se caracteriza por mostrar una proporción alta en ácidos grasos de la familia n-9, sobre todo en ácido oleico (63.37%), y también en monoenoicos. La cantidad de n-3 HUFA presente en los aceites vegetales es inapreciable.

Reconociendo las muestras de rotíferos enriquecidos con los distintos aceites, se puede comprobar que los ácidos grasos presentes en los aceites, comentados anteriormente, están presentes en éstos. Hay que señalar que en la muestra de rotíferos enriquecidos con aceite de pescado, la relación EPA/DHA es mayor a la del aceite de pescado. Esta relación es de 1.2/1. Para el

Ácidos Grasos	L3d	FO 10d	SBO 10d	LSO 10d	RSO 10d	FO 20d	SBO 20d	LSO 20d	RSO 20d
14:0	2,56	1,41	1,09	1,17	0,91	1,82	0,86	0,48	0,52
14:1	0,08	0,86	0,35	0,38	0,27	0,48	0,49	0,02	0,33
15:0	0,30	0,15	0,40	0,44	0,51	0,81	0,60	0,09	0,13
16:0iso	0,43	0,46	0,38	0,41	0,34	0,36	0,26	0,11	0,19
16:0	20,50	10,32	13,44	14,45	16,27	17,72	15,15	7,09	10,40
16:1n-7	6,54	9,39	5,84	6,28	5,72	11,27	1,20	4,91	5,90
16:1n-5	0,08	0,46	0,36	0,39	0,31	0,69	4,32	0,48	0,17
16:2	0,48	0,95	0,57	0,61	0,70	1,36	0,78	0,52	0,72
17:0	0,38	0,53	0,65	0,67	0,66	1,21	0,32	0,17	0,74
16:4n4	n.d.	0,06	n.d.	0,18	0,42	0,89	0,33	0,20	0,31
16:4n-3	0,18	4,49	0,04	7,74	0,22	12,20	0,51	4,40	0,06
18:0	4,91	0,87	7,20	0,92	9,70	0,84	0,56	0,47	5,28
18:1(n-9+ n-7)	19,57	14,23	7,41	14,98	32,18	3,53	20,17	28,21	39,87
18:1n-5	0,29	0,99	n.d.	0,70	n.d.	0,32	0,33	0,07	n.d.
18:2n-9	0,20	n.d.	0,65	n.d.	n.d.	0,32	0,78	0,19	0,13
18:2n-6	7,88	8,56	20,37	0,10	14,66	0,15	28,73	14,40	16,56
18:3n-9	0,07	0,10	20,37	n.d.	n.d.	0,17	0,42	n.d.	n.d.
18:3n-6	0,19	0,12	1,01	1,08	n.d.	1,13	0,51	0,31	n.d.
18:4n-6	n.d.	1,39	0,51	n.d.	n.d.	0,21	n.d.	n.d.	n.d.
18:3n-3	0,13	0,06	n.d.	21,90	1,80	0,66	2,14	23,85	2,93
18:4n-3	1,39	0,51	0,09	0,07	n.d.	0,12	0,24	0,35	0,12
18:4n-1	0,43	0,05	0,12	n.d.	n.d.	0,14	0,28	n.d.	n.d.
20:0	0,15	0,10	0,06	0,21	0,09	0,26	0,37	0,13	0,35
20:1n-9	1,24	2,64	0,20	2,01	2,71	3,07	1,05	1,09	1,68
20:2n-9	0,06	0,43	n.d.	0,26	0,27	0,41	n.d.	0,10	0,29
20:2n-6	n.d.	0,63	0,24	1,01	0,28	0,85	1,11	0,56	1,18
20:3n-6	n.d.	0,19	0,93	0,24	0,16	0,21	0,56	0,11	0,43
20:4n-6	1,10	1,80	0,12	1,40	0,09	1,49	1,18	0,49	1,40
20:3n-3	0,10	0,12	1,31	0,14	1,14	0,11	0,28	1,36	0,33
20:4n-3	0,43	0,71	0,13	0,24	0,19	1,08	0,23	0,34	0,07
20:5n-3	4,59	6,73	0,22	2,20	2,25	9,12	1,67	1,51	1,91
22:1n-11	0,29	1,48	0,71	0,76	0,12	0,81	0,37	0,22	0,47
22:1n-9	0,11	n.d.	0,30	0,32	n.d.	0,66	0,20	0,34	n.d.
22:1n-7	n.d.	n.d.	0,24	0,26	n.d.	0,44	n.d.	0,22	n.d.
22:3n-6	0,13	0,18	0,05	0,05	0,21	0,72	0,38	0,02	0,06
22:4n-6	0,08	0,13	0,15	n.d.	n.d.	0,15	0,22	n.d.	n.d.
22:5n-6	0,19	0,30	0,21	0,23	n.d.	0,29	0,44	0,14	n.d.
22:4n-3	0,04	n.d.	0,03	n.d.	0,22	0,13	0,21	0,22	0,24
22:5n-3	1,57	1,26	0,47	0,51	0,10	1,88	0,37	0,19	0,23
22:6n-3	23,15	18,38	9,88	10,63	7,50	21,92	6,99	6,66	7,06
Saturados	29,23	13,85	17,02	18,28	28,49	23,02	18,78	8,53	17,60
Monoinsaturados	28,30	38,54	16,51	32,74	41,00	20,58	28,54	35,08	48,24
n-3	31,58	32,27	20,19	21,62	13,42	47,23	12,65	38,88	12,93
n-6	9,57	13,30	23,64	25,93	15,40	5,19	33,13	16,03	19,62
n-9	21,25	17,41	30,32	17,57	35,16	6,05	22,62	23,56	41,97
n-3HUFA	29,88	27,21	12,78	13,71	11,39	34,25	9,76	10,28	9,83
AA/EPA	0,24	0,27	0.64	0,64	0,04	0,16	0,70	0,32	0,73
EPA/DHA	0,20	0,37	0.21	0,21	0,30	0,42	0,24	0,23	0,27
Lípidos totales % Peso seco	14±0.0a	17±0.0a	15.30±0.0a	16.58±0.0a	12.21±0.0a	19.17±0.0a	16.42±0.0a	20.75±0.0a	17.96±0.0a

n.d. ≤ 0.005

Tabla II. Ácidos grasos presentes en lípidos totales de larvas de 72 horas (Ld3), larvas de 10 y 20 días de dorada alimentadas con las diferentes dietas

resto de las muestras, esta relación es de 0.40/1 para los enriquecidos con soja, 0.5/1 para los enriquecidos con lino y 1.16/1 para los enriquecidos con soja.

La composición en ácidos grasos de los lípidos totales de las larvas de dorada de 3 días, así como las alimentadas durante 10 y 20 días con cuatro dietas diferentes, se muestra en la tabla II. Al comenzar la fase exógena, las larvas presentan aún alto contenido lipídico representado principalmente por los ácidos grasos 16:0, 18:1n-9, 22:6n-3 y 16:1n-7. Otros ácidos grasos importantes cuantitativamente son los saturados 18:0 y 14:0, el 18:2n-6 y el 20:5n-3. La relación EPA/DHA de los lípidos totales de las larvas de 72 horas es 1/5.04 es decir, mucho más favorable para el DHA. Comparando esta muestra inicial de larvas con el resto de las muestras, se observa una pérdida de lípidos totales que se manifiesta principalmente en los ácidos grasos saturados y, de forma más acusada, en el 16:0. Además, como era de esperar, en las larvas alimentadas con rotíferos enriquecidos con aceites vegetales se aprecia una pérdida importante de n-3 HUFA. Sin embargo, en las larvas alimentadas con rotíferos enriquecidos con aceite de pescado, se observa un ligero descenso en larvas de 10 días y un fuerte incremento en larvas de 20 días. En todas las larvas alimentadas con rotíferos enriquecidos con aceites vegetales, disminuyen drásticamente tanto el contenido de EPA como de DHA, de modo que la relación entre ambos sigue manteniéndose favorable para el DHA. Pese a todo, en las larvas de 10 días alimentadas con rotíferos ricos en aceite de pescado, el contenido de EPA aumenta ligeramente mientras que el DHA disminuye levemente. A pesar de esto, las larvas de 20 días aumentan la proporción EPA/DHA con valores de 1/2.4. El contenido de DHA en las larvas de 20 días es muy similar al inicial, lo cual indica un buen nivel de este ácido graso esencial en la dieta.

Además de los n-3 HUFA, otros ácidos grasos procedentes de la dieta como los monoenoicos y saturados, se ven reflejados en la composición final de las larvas.

Hay que destacar la gran incorporación del 18:2n-6 en larvas de 20 días alimentadas con rotíferos ricos en aceite de soja. Los valores registrados en 20:2n-6, para larvas alimentadas con rotíferos enriquecidos con aceite de soja, y 20:3n-3 para larvas mantenidas con rotíferos ricos en lino, indican elongación del 18:3n-3 y del 18:2n-6 respectivamente.

En la tabla III se muestran los resultados de los análisis lipídicos para la determinación de los lípidos totales y ácidos grasos de cerebro y ojo en larvas de 20 días de dorada alimentadas con las distintas dietas.

Como se puede observar, la mayoría de los ácidos grasos presentes en las dietas han sido incorporados en cerebro y ojo. Comparando la cantidad de DHA obtenido en larvas de 20 días, hay que decir que se aprecia una alta retención de DHA en cerebro y ojo, salvo en la muestra de ojo de larvas alimentadas con rotíferos enriquecidos con soja. Los ácidos grasos saturados son bastante ricos en las muestras de cerebro en comparación con los encontrados en larvas de 20 días, mientras que en las muestras de ojo se aprecian cantidades muy bajas. La relación EPA/DHA en las muestras de ojo es menor a 1. Este resultado es más bajo que el obtenido en las muestras de larvas de 20 días, lo que indica la importancia del DHA. Los valores obtenidos en 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 y 22:6n-3 en la muestra de ojo de las larvas alimentadas con rotíferos ricos en aceite de lino, sugieren cierta elongación y desaturación a partir del 18:3n-3.

Ácidos Grasos	CER FO	CER SBO	CER LSO	CER RSO	OJO FO	OJO SBO	OJO LSO	OJO RSO
12:0	5,37	9,12	6,41	9,01	4,29	3,95	6,08	7,13
14:0	3,47	6,25	4,24	4,75	3,21	3,42	3,47	2,71
14:01	1,57	2,42	0,95	n.d.	1,67	0,60	1,73	2,71
15:0	0,74	1,17	0,88	1,68	1,61	1,06	0,50	0,89
16:0iso	2,02	3,97	2,56	2,56	2,68	0,47	0,30	2,59
16:0	17,07	18,10	17,24	20,06	19,50	2,03	16,72	18,40
16:1 n-7	7,01	4,09	3,14	5,94	5,93	19,43	3,45	3,76
16:1n-5	n.d.	2,25	2,33	2,42	2,53	0,13	1,71	0,57
16:02	n.d.	0,54	n.d.	n.d	n.d.	0,26	n.d.	n.d.
17:0	n.d	0,49	0,40	n.d	n.d.	0,19	0,46	0,30
16:4n4	n.d	0,86	n.d	n.d	n.d.	0,71	n.d.	1,49
16:4 n-3	0,66	n.d	0,35	n.d	n.d.	0,72	0,30	0,00
18:0	10,35	8,30	13,66	9,84	9,27	10,52	10,63	9,99
18:1 n-9	13,73	15,54	21,18	17,24	15,81	27,59	19,94	15,98
18:1 n-7	3,79	2,05	2,62	2,35	2,47	1,55	2,46	2,48
18:1 n-5	n.d	n.d	0,35	n.d	n.d.	0,17	11,70	0,91
18:2n-9	0,84	n.d	0,42	n.d	n.d.	n.d.	n.d.	0,61
18:2 n-6	7,34	10,94	7,56	13,95	12,29	20,16	3,27	10,90
18:3 n-9	n.d	0,47	n.d	n.d	n.d.	0,94	n.d.	2,32
18: 3n-6	n.d	n.d	0,43	n.d	n.d.	0,23	n.d.	0,47
18: 4 n-6	1,47	1,45	n.d	1,26	1,91	0,13	n.d.	1,67
18:3 n-3	n.d	0,70	3,48	n.d	n.d.	0,47	0,85	0,56
18:4 n-3	0,66	n.d	1,04	1,37	1,38	0,98	1,67	2,23
18:4 n-1	n.d	1,27	n.d	n.d	n.d.	n.d.	1,33	n.d.
20:0	n.d	n.d	0,84	n.d	0,91	0,69	0,94	0,82
20:1 n-9	1,58	0,63	0,83	1,26	0,95	0,67	0,52	1,46
20: 1n-7	n.d	0,81	0,41	n.d	n.d.	0,24	0,37	0,30
20: 2n-9	n.d	n.d	n.d	n.d	n.d.	0,27	n.d.	n.d.
20:2 n-6	n.d	n.d	0,36	n.d	n.d.	0,16	n.d.	1,28
20:3 n-6	n.d	n.d	0,62	n.d	n.d.	0,45	n.d.	0,17
20:4 n-6	0,99	0,70	0,27	1,08	n.d.	0,17	0,63	0,82
20: 3n-3	n.d	n.d	n.d	n.d	n.d.	0,29	0,84	n.d.
20:4 n-3	0,87	0,74	1,24	n.d	n.d.	0,49	0,84	1,00
20:5 n-3	5,95	1,45	0,49	1.28	2,78	0,34	1,23	0,89
22:1 n-11	n.d	n.d	0,42	n.d	n.d.	n.d.	0,54	1,04
22:5 n-6	0,66	n.d	n.d	n.d	1,38	n.d.	n.d.	n.d.
22:4n-3	n.d	n.d	n.d	n.d	n.d.	n.d.	1,03	n.d.
22:5 n-3	1,65	n.d	0,61	n.d	n.d.	0,20	1,05	0,51
22:6 n-3	12,21	5,69	4,66	3,97	9,42	0,34	4,81	3,04
Saturados	39,02	47,39	46,24	47,89	41,49	22,32	39,10	42,82
Monoinsaturados	27,68	25,55	29,91	26,79	26,83	50,25	41,37	28,66
n-3	22,01	8,58	11,87	6,62	13,58	3,82	12,60	8,23
n-6	10,46	13,09	9,23	16,29	15,57	21,30	3,90	15,30
n-9	16,14	16,64	22,43	18,50	16,76	29,47	21,12	20,38
n-3HUFA	20,68	7,88	6,99	5,25	12,20	1,65	9,79	5,44
EPA/DHA	0,49	0,25	0,10	0,32	0,30	1,00	0,25	0,29

Tabla III. Ácidos grasos presentes en lípidos totales de cerebro y ojos de larvas de dorada de 20 días alimentadas con las diferentes dietas

Comportamiento

1.- Dorada

a) Estímulo Sonoro

La figura 1 muestra el efecto de la alimentación sobre la velocidad de crucero (mm/s) adquirida por las diferentes larvas. En el día 19 se obtuvieron diferencias significativas entre las dietas, siendo las larvas alimentadas con rotíferos ricos en FO las que obtuvieron una mayor velocidad de crucero.

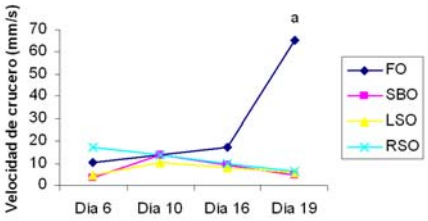

Figura 1. Desarrollo temporal de la velocidad de crucero (mm/s) en condiciones de aislamiento luminoso y previa al estímulo sonoro

La figura 2, muestra el efecto de la alimentación sobre la velocidad de huída (mm/s) conseguida por las diferentes larvas. Como se puede observar, no se encontraron diferencias significativas entre dietas a lo largo del estudio.

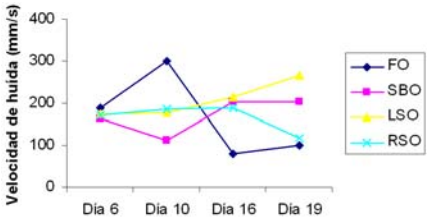

Figura 2. Desarrollo temporal de la velocidad de huída (mm/s)

b) Estímulo Visual

En la figura 3 se puede observar el desarrollo temporal de la velocidad de crucero en mm/s obtenida por las diferentes larvas. Se observaron diferencias significativas entre las dietas a partir del día 16 siendo las larvas alimentadas con rotíferos ricos en FO las que presentaron mayores resultados.

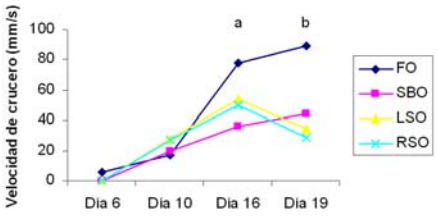

Figura 3. Desarrollo temporal de la velocidad de crucero (mm/s) en condiciones de aislamiento luminoso y previa al estímulo visual

Con respecto a la velocidad de huída (figura 4), en el día 16 las larvas mantenidas con rotíferos enriquecidos con FO y SBO mostraron diferencias significativas con el resto de las dietas, siendo las alimentadas con rotíferos enriquecidos con FO las que obtuvieron un mayor resultado. Por otro lado, en el día 19 indicaron diferencias significativas y resultados mayores, las larvas alimentadas con rotíferos enriquecidos con FO y LSO.

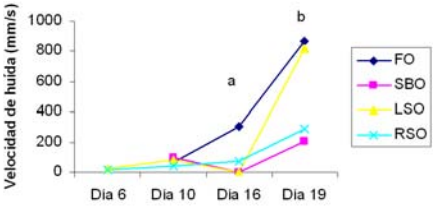

Figura 4. Desarrollo temporal de la velocidad de huída (mm/s)

2. Bocinegro

a) Estímulo Sonoro

La velocidad de crucero (figura 5) y la velocidad de huída (figura 6) obtenida por las larvas, no mostraron diferencias significativas durante los días de experimentación.

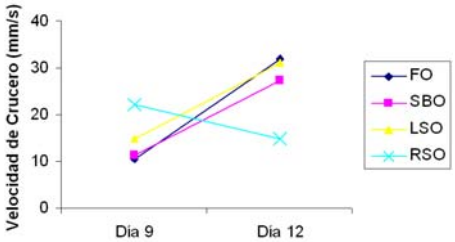

Figura 5. Desarrollo temporal de la velocidad de crucero (mm/s) en condiciones de aislamiento luminoso y previa al estímulo sonoro

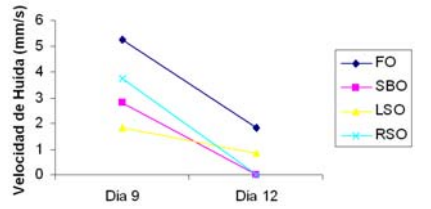

Figura 6. Desarrollo temporal de la velocidad de huída (mm/s)

b) Estímulo Visual

La evolución temporal de la velocidad de crucero (mm/s) obtenida por las diferentes larvas en estas condiciones no mostró diferencias significativas (figura 7).

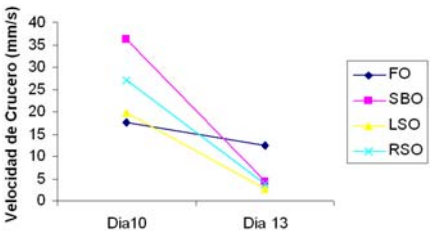

Figura 7. Desarrollo temporal de la velocidad de crucero (mm/s) en condiciones de aislamiento luminoso y previa al estímulo visual

Sin embargo, en el día 13, las larvas mantenidas con rotíferos enriquecidos con FO mostraron diferencias significativas con el resto de las dietas, presentando un mayor resultado (figura 8).

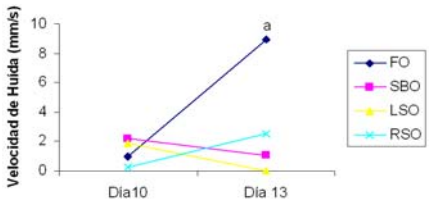

Figura 8. Desarrollo temporal de la velocidad de huída (mm/s)

DISCUSIÓN

Las larvas de dorada de 10 días de edad manifiestan, respecto a las larvas de 72 horas, una pérdida de lípidos totales, salvo en las larvas alimentadas con rotíferos enriquecidos con aceite de pescado. De esta forma, por ejemplo, el grupo de los n-3 HUFA es uno de los que sufre variaciones más acusadas. Estos cambios son provocados principalmente por el grupo de los ácidos grasos monoenoicos que aumentan en los rotíferos a medida que disminuye el aporte de n-3 HUFA. Estas variaciones indican deficiencias de los ácidos grasos correspondientes al grupo n-3 HUFA en la dieta (Izquierdo *et al*., 1989). Sin embargo, en las larvas alimentadas con rotíferos enriquecidos con aceite de pescado se conserva el nivel de DHA, lo que indica unos buenos niveles en la dieta para el correcto desarrollo de la larva. En larvas de 20 días de edad alimentadas con rotíferos ricos en aceites vegetales, se observa también una disminución en el contenido de n-3 HUFA por el aumento del grupo de ácidos grasos monoenoicos. Del mismo modo, se detecta un aumento de los n-3 HUFA, en especial en DHA, en las larvas alimentadas con rotíferos ricos en aceite de pescado, llegando incluso a presentar niveles muy similares a los obtenidos en las larvas iniciales. Siendo el DHA tan importante para el desarrollo del sistema neural y los ojos, este ascenso estaría relacionado íntimamente con una mejora de la capacidad predativa de las larvas, ya que las larvas de dorada, como la mayoría de los peces, son predadores visuales (Blaxter, 1986).

A lo largo del desarrollo larvario, varios autores indican que los requerimientos de ácidos grasos esenciales en dorada tienen que estar en torno al 1.5 % n-3 HUFA en materia seca, cuando las larvas son alimentadas con cualquier tipo de presa (Rodríguez *et al*., 1998) o microdietas (Salhi *et al*., 1999), independientemente del contenido de lípidos en la dieta (Salhi *et al*., 1994). Los resultados del presente estudio indican que el contenido de n-3 HUFA en los rotíferos enriquecidos con aceites vegetales fueron menores de 0.19 para el aceite de soja, lino y colza, encontrándose por debajo del nivel mínimo necesario para cubrir los requerimientos de ácidos

grasos de esta especie. Muchos de los requerimientos son apreciados en la literatura cuando el contenido de EPA es 2 o 3 veces superior al de DHA (Rodríguez *et a*l., 1994, 1997), debido a la gran incorporación de EPA en los lípidos polares y el reemplazo de DHA de ciertos lípidos polares (Izquierdo *et al*., 2000).

La composición de ácidos grasos registrada en las muestras de cerebro y ojo, revela que existe una retención del grupo n-3 HUFA y en especial del DHA, incluso en las larvas que han sido alimentadas con dietas deficientes en estos ácidos grasos de gran interés. Estos resultados demuestran la importancia que tiene este ácido graso para el desarrollo del cerebro y ojo. En ambas muestras, se observan variaciones en los niveles de n-3 HUFA según la cantidad de ácidos grasos monoenoicos presentes. Trabajos como los de Mourente y Tocher, (1993a) y Mourente y Tocher (1993b) revelan que el cerebro de los peces contiene largas cadenas de DHA. Bell *et al*., 1995 trabajando con larvas de arenque (*Cuplea harengus*) criadas con dietas pobres en DHA, detectaron que las funciones de los bastones no se desarrollaban con normalidad y como consecuencia, aconteció un deterioramiento en el desarrollo visual. Como resultado, estos peces sufrieron una disminución en la eficiencia de captura de presas en condiciones de intensidades de luz baja. Por lo que dietas pobres en DHA impiden el correcto desarrollo de la retina. Como las larvas de los peces son predadores visuales, el comportamiento trófico de las larvas está íntimamente relacionado con el desarrollo de la habilidad visual (Izquierdo, en prensa). En espáridos, como en la dorada y besugo (Roo *et a*l., 1999) los cambios más importantes en la estructura ocular del ojo ocurren a lo largo del estado lecitotrófico así como la preparación para capturar presas. Los fotorreceptores de los bastones necesarios para una correcta visión en intensidades de luz baja aparecen en dorada sobre el día 18 de vida. Esto concuerda con el marcado incremento en la velocidad de huída ante el estímulo luminoso y en la velocidad de crucero después del día 16 de vida de larvas de dorada en el presente estudio. Dentro de los n-3 HUFA, el DHA juega un papel crítico en las funciones del tejido neural y retinal. Es más, aumentos en el contenido de DHA y EPA, incrementan el diámetro del ojo en dorada (Izquierdo *et al*., 2000; Roo *et al*., en prensa) y este hecho, junto con la alta densidad de conos fotorreceptores en estas larvas, implica un alto número de conos y una mejora potencial de la visión (Roo *et al*., en prensa). Esto explicaría la mayor velocidad de huída frente al estímulo luminoso de las larvas de dorada y bocinegro alimentadas con rotíferos ricos en FO, aunque con valores absolutos inferiores para larvas de bocinegro. Esta consecuencia también se ha encontrado en otras especies de peces. Masuda *et al*., 1999 trabajando con seriola (*Seriola quinqueradiara*) alimentada bajo condiciones nutricionales deficientes en DHA, hallaron una incapacidad en el sentido de la visión.

La composición de ácidos grasos registrada en las muestras de cerebro y ojo, revela que existe una retención del grupo n-3 HUFA y en especial del DHA

Comparando los resultados de la velocidad de crucero obtenidos por las larvas de dorada alimentadas con aceites vegetales entre el estímulo sonoro y el estímulo visual, se observa que la velocidad de crucero aparece antes en el estímulo sonoro. Sin embargo, los resultados conseguidos en la velocidad de huída revelan que la reacción ante el estímulo aparece en el mismo día, apreciando en este día resultados superiores en el estímulo sonoro, excepto para las larvas alimentadas con rotíferos ricos en aceite de soja.

La velocidad de crucero obtenida en el estímulo sonoro aparece muy temprano en las larvas de dorada alimentadas con rotíferos enriquecidos con aceite de pescado. En estas larvas se observa en el día 16 un cambio en la velocidad de crucero. El

momento en el que se produce este cambio de la velocidad puede ser debido a un mayor desarrollo del cerebro y de la línea lateral. No obstante, la velocidad de crucero obtenida en el estímulo visual en el día 16 es diferente a la lograda en el estímulo sonoro, ya que las larvas ven lo que hay alrededor. El incremento que se produce en la velocidad es debido al desarrollo de los conos y bastones (Roo, 1999). Sin embargo, en las larvas alimentadas con rotíferos ricos en aceites vegetales, se produce un cambio de la velocidad de crucero conseguida en el estímulo visual a partir del día 16. Esto es debido a que existe un retraso en la formación de los conos y bastones o del diámetro del ojo (Roo, 1999). La aparición de la velocidad de crucero obtenida en el estímulo sonoro por las lavas alimentadas con rotíferos ricos en aceite de pescado presenta similitudes con otra especie, la caballa (*Scomber japonicus)* (Masuda *et al*., 2002), pero con valores absolutos inferiores. Lo mismo ocurre con la velocidad de huída. El momento en el cual se empieza a detectar respuesta ante el estímulo es muy similar pero los resultados son totalmente opuestos, ya que en caballa van incrementándose a lo largo de todo el experimento.

Comparado los resultados de la velocidad de crucero obtenida por las larvas de dorada y bocinegro en condiciones del estímulo sonoro, se puede observar que ambas parten con velocidades muy similares al inicio del experimento, exceptuando las larvas alimentadas con rotíferos enriquecidos con aceite de colza, que presenta valores absolutos superiores en larvas de bocinegro. Sin embargo, en el día 12 de estudio los resultados son diferentes ya que las larvas de bocinegro presentan una mayor velocidad. Este hecho podría estar relacionado con un temprano desarrollo del cerebro y de la línea lateral, comparado con el de dorada. En el estímulo visual, los resultados obtenidos por las larvas de bocinegro alimentados con rotíferos ricos en aceite de pescado y lino en el día 10 son muy parecidos a los logrados por la dorada. Sin embargo, las velocidades de crucero del día 13 conseguidas por las larvas de bocinegro son muy inferiores a las conseguidas por la dorada. Esto puede estar relacionado con un retraso en la formación de conos y bastones o del diámetro del ojo (Roo, 1999).

La velocidad de huída lograda por las larvas de dorada alimentadas con rotíferos ricos en aceite de pescado a partir del día 16 en el estímulo visual, indica un desarrollo del cerebro y ojo. Hay que destacar la velocidad de huída obtenida por estas larvas alimentadas con rotíferos enriquecidos con aceite de lino en el día 19 en el estímulo visual. Estos resultados indican que existe una mayor reacción en condiciones estresantes (Montero *et al*., 2003). Los resultados de velocidad de huída de este estudio señalan que cuando se estudian las larvas bajo las condiciones de preparación del estímulo sonoro, y el vaso de experimentación está cubierto con una funda negra que impide la interferencia con estímulos de tipo visual, la reacción de huída de las larvas es menor que frente a un estímulo visual. Así, en las larvas de dorada y bocinegro alimentadas con rotíferos enriquecidos con aceite de pescado, las velocidades obtenidas en el estímulo visual son superiores a las logradas en el estímulo sonoro. Estos resultados confirman la importancia de la visión durante este periodo del desarrollo no sólo para la prelación sino también para la huída. Este mismo hecho se observa en el experimento de larvas de bocinegro. Se podría suponer que la tendencia sería la misma y corroborar la importancia de la visión.

Sin embargo la reducción en el contenido dietético de ácidos grasos esenciales, por el enriquecimiento con aceites vegetales, retra-

sa la aparición del estímulo visual, lo cual sugiere un retraso en el desarrollo funcional del cerebro y la visión y estaría de acuerdo con el menor contenido de DHA en los ojos y cerebros de estas larvas. Además, las larvas que son alimentadas con rotíferos enriquecidos con aceite de pescado, con niveles de n-3 HUFAs adecuados para cubrir sus requerimientos para el desarrollo del cerebro, presentan una mayor velocidad de crucero que las larvas donde los requerimientos y en especial el DHA, no han sido cubiertos. Estos resultados concuerdan con los cambios anatómicos de los componentes del cerebro de seriola encontrados por Ogawa (1967), y en los que el desarrollo notable del cerebelo coincide con la mejora de los cambios de natación, demostrándose una íntima relación entre las modificaciones del comportamiento y los cambios morfológicos de algunos componentes del cerebro. Así, el presente estudio demuestra como al no suministrarse las cantidades idóneas de DHA para que se desarrolle el cerebro con toda normalidad, la natación de esta especie, el movimiento natural de la larva, y su velocidad de crucero se verán afectadas. Esto va a tener consecuencias tróficas, puesto que variaciones en el comportamiento como es la velocidad de natación, van a repercutir en la estrategia de captura de presas. Con lo que larvas "más lentas" van a presentar menores capturas y menores tasas de crecimiento, sin perjuicio de que las muchas otras funciones que los ácidos grasos esenciales realizan para el correcto crecimiento de la larva se vean también afectadas.

En cuanto a la velocidad de huída es interesante resaltar que el mayor movimiento de la velocidad de huída se produce en las cuatro primeras secuencias tras la actuación del estímulo. Este mismo hecho se ha encontrado en otras especies de peces (Masuda *et al*., 2002). Por otro lado, en larvas de bocinegro este mayor movimiento se produce entre las siete y diez primeras secuencias tras la ejecución del estímulo. Los resultados obtenidos en velocidad de huída para el estímulo visual en larvas de dorada y bocinegro fueron mucho mayores que los obtenidos en el estímulo sonoro. De nuevo estos resultados indican la mayor importancia del estímulo visual y la agudeza del mismo durante este periodo del desarrollo larvario. Hay que resaltar que en su medio natural el estímulo visual se transmite en el agua mucho más rápido que el sonoro, y que posiblemente el estímulo visual sea más vital para la larva a la hora de situarse, encontrar alimento, huir de predadores, etc. Así, mientras con el estímulo sonoro no se encontraron diferencias significativas en la respuesta de las larvas de alimentadas con diferentes dietas ante el estímulo visual, las larvas de dorada presentaron una mayor velocidad de huída cuando habían sido alimentadas con rotíferos enriquecidos con aceite de pescado y lino que las alimentadas con aceite de soja y colza. Comparando el DHA retenido por cada una de las larvas, se observa que las larvas alimentadas con rotíferos ricos en aceite de pescado y lino, presentaron las mayores concentraciones de este ácido graso en ojo. Por lo que se puede relacionar, la velocidad de huída ante un estímulo visual con la cantidad de DHA acumulada en ojo. Es interesante denotar por otra parte, que la inclusión de aceite de lino en los piensos para dorada origina una reacción más rápida y mayor, expresada en niveles de cortisol plasmático, frente a un estrés agudo (Montero *et al*., 2003). Si este hecho sucediese igualmente en los estadíos larvarios de esta especie contribuiría a explicar la mayor velocidad de huída de las larvas alimentadas con lino como una mayor y más rápida respuesta ante el estrés que supone el estímulo visual.

Así, la mayor velocidad de huída y de crucero en las larvas de dorada

La mayor velocidad de huída y de crucero en las larvas de dorada alimentadas con aceite de pescado, está relacionada con los niveles de DHA en el ojo y cerebro de las larvas

alimentadas con aceite de pescado, está relacionada con los niveles de DHA en el ojo y cerebro de las larvas, dadas las importantes funciones que este ácido graso cumple en dichos tejidos. Así por ejemplo, parte de las posibles funciones del DHA en el cerebro implican la mielinación de los neurocistes y la construcción de la sinapsis; ambas funciones consideradas esenciales para la formación de la red neural. Además, ambas funciones son presentadas por ser sensibles a una deficiencia nutritiva u hormonal (Krigman y Hoga, 1976). Una comparación histológica del jurel (*Longirostris delicatissimus*) mantenido con dietas enriquecidas con DHA, EPA y ácido oleico demuestran que los juveniles alimentados con dietas ricas en DHA presentaron un mejor desarrollo superficial de la zona blanca y gris sobre el tectum óptico que con los otros dos grupos (Masuda, 1995). Considerando que la zona superficial blanca y gris está principalmente compuesta de axones neurales y dendrites (Northcutt, 1983), el DHA puede estar involucrado en la composición de la red neural, algunos de los cuales están implicados en muchos comportamientos complejos, como el de formación de escuela (Masuda *et al*., 1999). Las regiones del cerebro tectum óptico y cerebelo influyen en la habilidad visual y en el funcionamiento de natación en seriola (Ogawa, 1967)

En conclusión, los resultados del presente estudio demuestran que la dieta va a repercutir en la recepción y reacción de estímulos externos tales como luz y sonido, y en la capacidad de natación de las larvas y por ello en la habilidad para la predación y la huída y finalmente en el crecimiento. Deficiencias en DHA van a dar lugar a un inadecuado desarrollo del sistema nervioso central y ojo, con lo que va a inducir a un incorrecto desarrollo del comportamiento, y más concretamente el comportamiento de alimentación. Esto sugiere que los requerimientos de DHA en la dieta pueden estar relacionados con los cambios fisiológicos y de comportamiento durante el desarrollo larvario de estas especies. Son necesarios futuros experimentos para establecer el efecto del DHA de la dieta con el crecimiento volumétrico de distintas regiones del cerebro de las larvas de dorada y bocinegro, y enlazarlo con el desarrollo del comportamiento en la vida de estas especies.

Estos resultados revelan la importancia de la alimentación en el comportamiento larvario. Este estudio sobre el comportamiento larvario del bocinegro podrá servir a futuros experimentos para mejorar la calidad de la dieta así como optimizar tanto las condiciones de manejo como las técnicas de producción de larvas y alevines de esta especie.

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