The impact of dietary fatty acids in common sole larval (Solea solea , L.) nutrition

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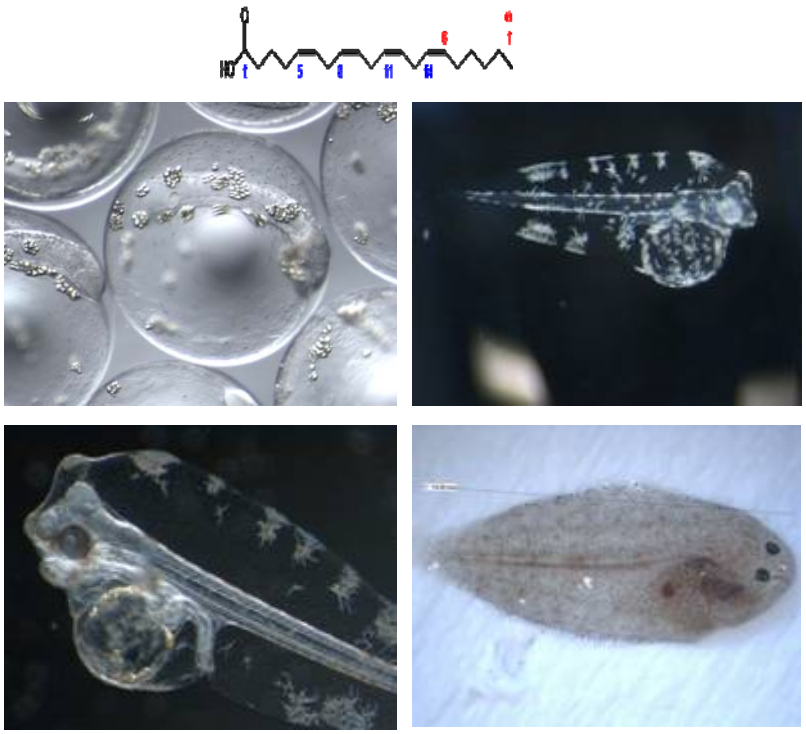

PhD thesis by Ivar Lund

December 2007

Roskilde University
Department of Environmental, Social and Spatial Change

Danish Institute for Fisheries Research
Danish Technical University

The impact of dietary fatty acids in common sole larval (*Solea solea* L.) nutrition

by

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Preface

The present thesis deals with the importance of fatty acids (FA) in larval nutrition of common sole (*Solea solea* L.); a species with a high commercial potential in aquaculture. Dietary fatty acids are found very important for the optimal development of most marine fish larvae and this has only been sparsely examined in common sole larvae. A main objective was to characterise the importance and influence of dietary polyunsaturated fatty acids (PUFAs) in relation to quality parameters of sole eggs, larval composition and larval requirements with a specific focus on pigmentation.

The thesis is submitted to Roskilde University, Department of Environmental, Social and Spatial Change, 4000 Roskilde, Denmark in partial fulfilment of the requirements for obtaining the Doctor of Philosophy (PhD) degree. The dissertation consists of 6 manuscripts.

I would like to thank Danish Institute for Fisheries Research for funding this work.

I wish to thank my supervisors Dr. Benni Winding Hansen, Roskilde University and Dr. Svend Jørgen Steenfeldt, Danish Institute for Fisheries Research (DIFRES) for general supervision, support and critical revision of manuscripts and in addition thanks to senior researcher Dr. Erik Hőglund, DIFRES.

A special thank to the laboratory staff at Roskilde University, Department of Environmental, Social and Spatial Change, especially chemist Peter Christensen for assistance with fatty acid analyses and GC-MS equipment. Further thanks to Head of Aquaculture section, Per Bovbjerg Pedersen, Danish Institute for Fisheries Research for general good advice.

Finally I wish to thank my wife, Birgitte for her patience, when my work was first priority… at the expense of the family.

Hirtshals, December 2007

Ivar Lund

Dansk resume

I bestræbelserne på at afdække mulighederne for opdræt af nye arter i akvakultur er der på Danmarks Fiskeriundersøgelser (DFU i Hirtshals) gennemført et flerårigt projekt med almindelig tunge (*Solea solea).* For mange marine fiskearter er larvefasen ofte den kritiske periode. Kvaliteten af marine fiskelarver er i stor udstrækning relateret til indholdet af og sammensætningen af fedtsyrer i foderet. Nærværende afhandling omhandler en undersøgelse af indvirkningen af fedtsyrer på kvaliteten af tunge æg samt på tunge larvers overlevelse, vækst, stress respons og pigmentering. Der blev specifikt fokuseret på betydningen af de langkædede flerumættede essentielle n-3 og n-6 fedtsyrer de såkaldte PUFAs.

Det blev påvist at tunge larver har et lavt krav til essentielle fedtsyrer, således var vækst og overlevelse af larver holdt under optimale fysiske betingelser ikke påvirket af forholdet mellem essentielle fedtsyrer eller af fodertyper med lavere indhold af PUFAs. Tunge larvers fedtsyresammensætning afspejlede fødens profil. Forsøgene viste dog, at de essentielle fedtsyrer kan være vigtige for øget stress tolerance under suboptimale forhold som hypoxi.
Den essentielle fedtsyre, arachidonsyre, C20:4n-6 forårsagede dorsal fejlpigmentering (hypomelanosis) som følge af biokemiske reaktioner der involverede hormonstoffer som prostaglandiner. Graden af fejlpigmentering øgedes med stigende koncentration af arachidonsyre og tungelarvers sensitivitet var væsentligt højere forud for metamorfosen. Andre essentielle fedtsyrer som eicosapentaensyre, C20:5n-3 og docosahexaensyre, C22:6n-3 var tilsyneladende ikke involveret i pigmenteringen. Stærkt fejlpigmenterede juvenile tunger havde en lavere vækst end ikke fejlpigmenterede, hvilket kan være forårsaget af processer under metamorfosen, idet disse fisk var kendetegnet ved ufuldendt abnormal øjevandring efter endt metamorfose. Fejlpigmentering på undersiden (overpigmentering, hypermelanosis) var specifikt relateret til enkelte larvehold og påvirket af fysiske forhold.

Summary

The thesis focuses on the importance of dietary fatty acids (FA) in relation to the quality of sole eggs, - larval pigmentation development and physiological requirements of sole larvae (*Solea solea* L.) held under experimental rearing conditions. Sole is a promising candidate for aquaculture, but fatty acid requirements have been sparsely examined. Dietary fatty acids are very important for most marine fish larval performance, especially the long chained polyunsaturated fatty acids,

PUFAs. A major problem in flatfish farming is skin malpigmentation, which may be influenced by unbalances in tissue n-3: n-6 PUFA composition. A main objective was therefore to elaborate knowledge on the effect of essential dietary n-3 and n-6 PUFAs on the pigmentation process of sole larvae.

The FA profile of cultured eggs was affected by the pelleted diet of the F1 cultured broodstock and wild or culture origin may be determined based on the egg FA profile. The differences in specific fatty acids profiles, however did not influence subsequent larval growth performance.
Common sole larvae may be start fed on *Artemia*. Unenriched *Artemia* strains of various FA qualities proved to be fully as good as essential fatty acid, EFA enriched *Artemia* with respect to larval growth, survival and pigmentation. Rotifers and the harpacticoid copepod *Tisbe holothuriae* should not be used in common sole larvi culture as they did not promote a good growth of the larvae or any gains to the culture. This may be related to their smaller size (i.e energy content per prey) more than any fatty acid deficiencies. In relation to the growth performance of common sole larvae highly expensive commercial dietary FA emulsions seemed only marginally better than a mixture of marine fish oil, E vitamin and soy lecithin. Both growth rate and survival of sole were not influenced by the dietary essential fatty acid (EFA) content. Hence, common sole larvae seem to have a relatively low requirement for dietary eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA in comparison to several other flatfish species. However, an increase in stress resistance was observed in sole larvae exposed to hypoxia when fed EFA enriched live prey contrary to unenriched live prey. This is believed to be related to a more optimal dietary content of essential fatty acids.
The FA profile in well growing common sole larvae highly reflected the dietary feed and the profile changed within a relatively few days in favour of a new diet.
Increasing dietary and tissue levels of arachidonic acid, ARA caused a displacement of EPA and DHA content, when these were not supplemented in the diet. This was probably related to a higher cell affinity to ARA. Sole larvae seemed to retain tissue ARA and especially starving sole retained the FA profile quite conservatively.
The degree of malpigmentation, - hypomelanosis in sole larvae fed ARA was dose dependent and highly related to the dietary ARA concentration. However, larval sensitivity to dietary ARA in relation to hypomelanosis was considered much higher during pre metamorphosis than during metamorphosis. Further, the sensitivity of sole larvae to dietary ARA induced hypomelanosis was

batch related. Live prey enriched by dietary ARA oil of 10 percent or more induced hypomelanosis with no interactive effects by a concomitantly dietary inclusion of EPA and / or DHA. Blind side pigmentation, - hypermelanosis was induced by environmental factors. Larvae may be predisposed to hypermelanosis, which may explain differences in batch related ARA induced hypomelanosis sensitivity.

The cause of malpigmentation induced by ARA may involve eicosanoids of the type prostaglandins, PGE_2, as a positive correlation was observed between an increase in larval tissue PGE_2 concentration, ARA tissue content and malpigmentation.

Initiation of metamorphosis defined as initiation of eye migration was related to the body length of the larvae (i.e. when larvae were 7-8 mm) and not to the diet or EFA treatment. The advance of metamorphosis (i.e. eye score) was similarly related to larval length. For the majority of albinic juveniles eye migration was impaired, and resulted in a permanent incomplete aberrant non symmetrical eye position. This abnormality was not solely related to the effect of dietary ARA or some of its metabolites, as it was also observed for albinic fish not treated with dietary ARA. It is regarded as a trait related to albinism and dietary caused albinism may therefore involve morphological changes in sole larvae.

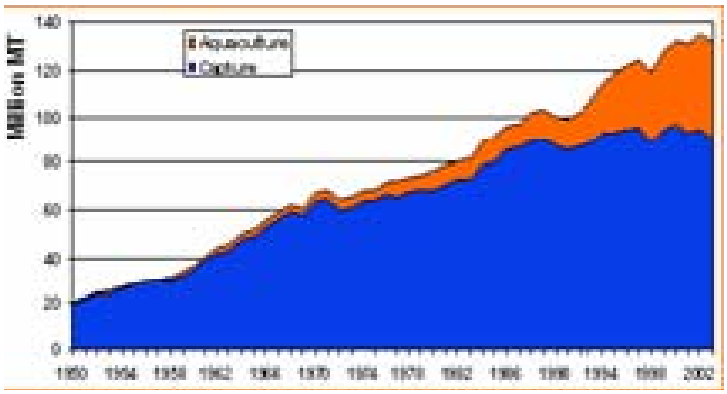
Highly malpigmented or albinic juvenile fish had a slower growth rate than normal pigmented juveniles, which may be related to visual abilities in detecting prey.

1.0 General introduction

The last two decades have witnessed a remarkable global increase in the culture of marine finfish and globally aquaculture is probably the fastest growing food producing sector accounting for 33 percent in 2004 increasing to 50 percent of the worlds food fish in 2006 (FAO, 2006).

Figure 1.

Illustration of development of aquaculture production and fisheries (FAO, 2003)



The future growth of aquaculture is expected to develop not only quantitatively, but also through the introduction of several new species many of which until now are harvested in the sea. Population growth, overfishing and heavy exploitation of many fish stocks are the main reasons for the increased focus towards aquaculture.

The fact, that the production of new marine species may be hampered by a shortage of larvae and nutritional knowledge, a continuous research into nutritional requirements has increased the aquaculture production of larvae of some marine species. This is exemplified by the rapid development of the commercial farming of the European Sea bass (*Dicentrarchus labrax*) and Sea bream (*Sparus aurata*), which until the late 80ies was still in its infancy and now are amongst the most important farmed marine warm water finfish species in Europe (Aires, 2000). Much of this improvement in production has been the result of breeding, improved production methods, live prey enrichment techniques and knowledge on larval nutrition.

Within the recent years research on larval nutrition on other marine species like cod (*Gadus morhua*) has resulted in a breakthrough. In Norway 10,000 tons cod were produced in 2006 and future estimates of a production of more than 300,000 tons year $^{-1}$.

The aquaculture production of flatfish is quantitatively limited, but turbot (*Schopthalmus maximus*) is commercially produced in Spain and France and Atlantic halibut (*Hippoglossus hippoglossus*) produced in Norway.

Common sole (*Solea solea*) is a prime candidate for intensive aquaculture. Gastronomically sole is highly appreciated and is a relatively isolated market product with no real substitutes (Rodgers et al., 2005). Common sole is widely distributed and the biogeographical distribution of sole ranges

from the northwest African coast and Mediterranean in the south to the Irish Sea, southern North Sea, Skagerrak and Kattegat in the north.

Figure 2. *Illustration of geographical distribution of common sole*

Common sole is a right eyed greyish brown flatfish with a distinctive shaped head, a rounded snout, and a small downturned mouth. It grows to 30-40 cm, and may reach 60 cm (Fig.3). Common sole usually live on sandy or muddy seabeds and are actively preying during the night time on crustaceans, bivalves and other invertebrates besides fish. During day time they remain buried in the sand.

Figure 3.

Illustration of cultured sole sampled from rearing tank at DIFRES and wild sole on sand bottom

As for sole stocks in the North Sea, ICES, 2006 classified the stock as being at risk of reduced reproduction capacity (30,000 t.) and below recommendations of 35,000 t.

The Danish Institute for Fisheries Research (DIFRES) initiated in 2002 a research project funded by the Danish Directorate for Food Fisheries and Agri Business in order to clarify the biological potential for a commercial farming of common sole. The main findings were observations, that sole demands relatively low stocking densities for optimal growth. The production per unit is low and optimisation of production is important to sustain economic feasibility of this species in land based recirculation systems as previously reported (Schram et al., 2006).

The present work on Common sole larvae was an extension of this research project, financially supported by a PhD fellowship from Danish Institute for Fisheries Research.
Broodstock and larval nutrition are central research areas within marine aquaculture; as because of limited knowledge within these segments; and due to the increasing need for superior quality fish fry for both the aquaculture industry and restocking. Unpredictable and variable egg quality is a major limiting factor for successful mass production of juveniles and dietary essential fatty acid, EFA content has been found to greatly affect spawning quality in fish (Izquierdo, 1988, Fernández-Palacios, et al., 1995).
In intensive larval culture the mass produced live feed species are not identical to prey species encountered in the wild, consequently fatty acid imbalances or deficiencies may address problems to the growth and quality of the larvae produced (Watanabe, 1983). Marine fish species differ in nutritional requirements and the underlying biochemical mechanisms are relevant for research in new species.
Dietary requirements of essential fatty acids have been regarded as one of the principal factors determining a successful larval development and much research has been concentrated on this topic. This has resulted in a better understanding of basal needs in marine larval rearing, however extended knowledge is limited to relatively few species. Despite extensive studies on common sole in the 1960s and 1970s a commercial viable cultivation technique has not been realised in contrast to the farming of the relative closely related Senegalese sole (*Solea senegalsensis*). During the larval stages of common sole quantitative requirements for lipids remains unknown (Howell, 1997) but as reported for Senegalese sole (Conceicao et al., 2007) the requirements for essential fatty acids may be less stringent than for many other marine species (Howell and Tzoumas, 1991). Nutritionally, EFAs (EPA, DHA and ARA) may be involved in the pigmentation process (Bell et al., 2003). Malpigmentation in flatfishes decreases marketability and lower the production, but fundamental aspects of development of malpigmentation in flatfish and defects that occur in hatcheries are not well understood (Bolker and Hill, 2000). A strong focus has recently been on the C20:4n-6, arachidonic acid (ARA) as this fatty acid apart from being an important essential fatty acid (Sargent et al.,1999a) is thought involved in induction of malpigmentation (hypomelanosis) in several flatfish species. (Estévez, 1997, Copeman et al., 2002; Villalta et al., 2005).

1.1 Objectives

One of the fundamental themes in hatchery production of marine fish larvae is the relation between quality of feed used and quality of larvae produced. Essential fatty acids (EFAs) play a central role in this relation and consequently receive the main focus in this thesis.The thesis summarises a series of papers on effects of EFAs in hatchery production of larvae of common sole. It includes work on broodstock and egg quality. This is important since batch variability may influence on larval quality and subsequent experimental output.

Quality has a pragmatic interpretation, but in the present thesis, quality was related to the effect of dietary fatty acids on egg and larval survival, larval growth rate, larval pigmentation and larval physiological responses to stressful conditions;

Specifically, egg fatty acid profiles may be related to egg and larval viability. Therefore an objective was to study the FA profiles of eggs of wild or cultured broodstock origin and to examine possible relations between dietary FAs (especially EFAs) - and egg fatty acid profiles / viability.

Another objective was to gain knowledge about the FA - and EFA requirements of pre feeding and early feeding sole larvae, combined with information on FA composition of larval prey and prey / larval interactions with respect to larval performance.

Sub optimal rearing conditions may stress fish larvae. By exposure of sole larvae to hypoxic stress, an objective was to examine if dietary EFA differences cause sublethal physiological effects on individual larval performance.

The role of EFAs and flatfish larval pigmentation success is *per se* unclear. A central objective was to examine the roles, - the dose dependent effects - and the interactive effects of dietary arachidonic acid, ARA, eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA in relation to pigmentation of common sole larvae.

Results showed that, arachicidonic acid induced malpigmentation in common sole, therefore an objective was an examination of the effect of dietary ARA at different stages of larval ontogeny in addition to examining possible (biochemical) factors involved in the pigmentation process.

Accordingly, these objectives resulted in 6 papers, referred to by their roman number **I-VI** in addition to an appendix (**IIA**). These are briefly presented below (1.2) and listed in (1.3).

1.2 Presentation of papers

A study (**I**) was performed to compare possible differences in the fatty acid composition and quality of eggs and larvae from batches of wild caught broodstock and cultured sole broodstock (F1 progeny), for which the cultured broodstock was fed an extruded pelleted diet for several years. Different live prey species were evaluated as first feed for common sole larvae (**II**). The aim was to investigate the impact of natural live food prey, copepods found in the diet of wild sole larvae on growth performance and larval FA composition in comparison to traditionally used live prey as *Artemia* and rotifers. Furthermore, we examined sole larval feed intake and prey preference, when offered a possibility of a preferential choice of these live prey (**IIA**).
A study was carried out to clarify the influence of *Artemia* enriched by different quantities and combinations of dietary ARA, EPA and DHA on sole larval pigmentation and growth performance (**III**). In addition a study was designed in order to investigate how the routine metabolic rate (RMR) and in-vivo tolerance of hypoxia of individual sole larvae were influenced by feeding either unenriched *Artemia* or *Artemia* that provided some of these different EFA quantities and combinations (**IV**).
Based on our findings (**III**) a dose response study was performed with low concentrations of dietary ARA and EPA, in order to search for a threshold level for the effect of ARA on pigmentation (**V**). It was moreover examined if a high dietary EPA in concert with low dietary levels may effect pigmentation or the eicosanoid production believed to be involved in pigmentation (**V**). A dose response study of ARA and EPA was carried out with the aim to determine a possible larval pigmentation window and to find evidence for whether ARA induced levels of eicosanoids, the 2-series of prostaglandins, (PGE_2) were involved in the pigmentation process (**VI**).

1.3 List of papers

I) A comparison of fatty acid composition and quality aspects of eggs and larvae from cultured and wild broodstock of common sole (*Solea solea* L.).
Submitted to Aquaculture Nutrition, August, 2007. Accepted, December 2007.

II) The consequence of the species of live prey and their FA composition on early fitness of common sole larvae (*Solea solea* L.). Submitted to Aquaculture International, September 2007.

IIA) The effect of prey species, abundance and light intensity on first feeding of common sole larvae (*Solea solea* L). Appendix. Manuscript.

III) Effect of dietary arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid on survival, growth and pigmentation in larvae of common sole (*Solea solea* L.). Aquaculture 273, 532-544.

IV) Metabolic rate and tolerance of hypoxia in common sole (*Solea solea*) larvae and juveniles raised on *Artemia* with different essential fatty acid compositions. Submitted to Marine Biology, September 2007. Accepted with revisions November, 2007.

V) Pigmentation sensitivity of common sole (*Solea solea* L.) larvae to low dietary inclusions of arachidonic acid. Manuscript.

VI) The influence of dietary concentrations of arachidonic acid and eicosapentaenoic acid at various stages of larval ontogeny on eye migration, pigmentation and prostaglandin content of common sole larvae (*Solea solea* L.). Submitted to Aquaculture, October 2007. Accepted subject to minor revisions, November 2007

The following chapters of the introduction serve the purpose of viewing these 6 papers in a broader perspective, to discuss the main findings and findings not presented in the papers, in addition to provide the reader with some additional background information**.**

2.0 Lipid nutrition in marine fish

Lipids and their constituent FAs are along with proteins, the major organic constituents of fish (Tocher, 2003). Fish like all other vertebrates require the essential three long chained polyunsaturated fatty acids (PUFAs); eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3) and arachidonic acid (ARA, C20:4n-6) for normal growth and development (Sargent et al., 1999b). The role of dietary EPA and DHA has been widely investigated, while ARA, as a minor constituent of marine fish oil, has not been examined nearly as intensively, despite that it deserves as much consideration as EPA and DHA.

Traditionally, marine lipids and fish oils have been standard sources for ensuring essential fatty acids to marine fish larvae production. ARA, EPA, DHA triacylglyceride oils are now produced commercially, thanks to new technology, which offers great possibilities of manipulating the PUFA content of live prey and thereby get insight into larval requirements and physiological effects of fatty acids (**II, III, IV, V, VI**).

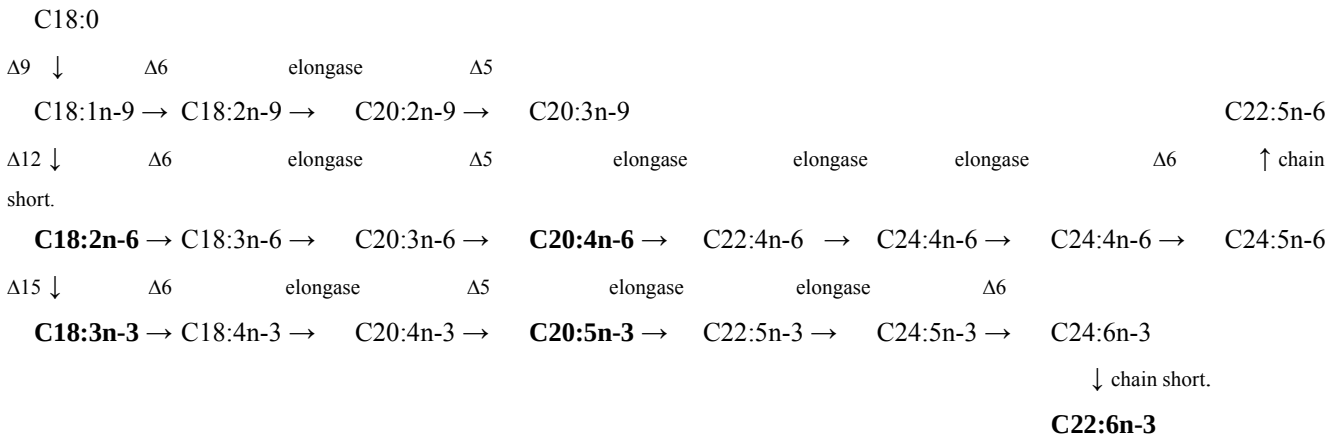
2.1 Synthesis, desaturation and elongation of fatty acids in fish

Fish are capable of desaturating saturated fatty acids, e.g. C16:0 and C18:0 to yield monounsaturated fatty acids as C16:1n-7, palmitoleic acid and C18:1n-9, oleic acid (Fig. 4). However, less is known about chain elongation to longer homologues, like C20:1n-9, C22:1n-9 and C24:1n-9 or further pathways of elongation (Tocher, 2003). Many marine species of fish, however, are well supplied with fatty acids in their natural diets, so *de novo* synthesis and chain elongation is likely to be repressed (Tocher, 2003)

De novo synthesis of polyunsaturated fatty acids in fish is hampered by lack of Δ15 desaturase (i.e. enzymes which removes 2 hydrogen bonds from an organic compound creating a double bond in the carbon chain) and therefore they can not form the two important short chained PUFAs, linoleic acid, C18:2n-6 (n-6 fatty acid) and linolenic acid, C18:3n-3 (n-3 fatty acid) from the monounsaturated fatty acid C18:1n-9. C18:2n-6, however, can be elongated (i.e. elongation: alternates with desaturases by adding an ethyl group) to the long chained PUFA, C20:4n-6, and C18:3n-3 to the long chained PUFAs, C20:5n-3 and C22:6n-3. The degree to which fish can carry out these conversions is dependent on the activity of two other enzymes, Δ5 desaturase and Δ6 elongase (Fig. 4).

Figure 4.

Pathways of biosynthesis of C20:4n-6, C20:5n-3 and C22:6n-3 from C18n-3, n-6, n-9 precursors. Δ5, 6, 9, 12; fatty acyl desaturases. Elongase; fatty acyl elongases. (Modified from Tocher, 2003)



The desaturation and elongation reactions occur in the liver and the same enzymes act on n-3 and n-6 fatty acids, but the activity for the n-3 series is higher. In turbot it was found that this species was unable to carry out the steps in the elongation / desaturation pathway described (Cowey et al., 1976) and in most marine fish species the reactions are believed not to be able to sustain larval essential fatty acid, EFA requirements (Sargent et al., 1995.; Tocher et al., 2003).

In fish, therefore, C20:4n-6, C20:5n-3 and C22:6n-3 are regarded as EFA as due to deficiencies of desaturases and / or elongases**.** While especially DHA is a major constituent of neural and visual membranes, EPA is present in cellular membranes and both EPA and ARA are precursors of different important eicosanoids with interacting effects (Sargent et al., 1993; Bell et al., 1995; Sargent et al., 1999 b).

2.2 Eicosanoids

In fish, DHA, EPA, and ARA are involved in maintaining cell membrane structure and function, where DHA and EPA are the major PUFAs of cell membranes.

One central role of C20 PUFAs (i.e. ARA, C20:4n-6 and EPA, C20:5n-3) are their role in production of hormone-like compounds i.e. eicosanoids. The eicosanoids are a range of highly active C20 compounds formed in small or even trace amounts by virtually every tissue in the body. They are produced in response to stressful situations, both at a cellular and whole body level. Eicosanoids have a wide range of physiological actions, for example in the immune response, inflammatory response and neural function (Tocher, 2003). The two main enzymes involved in the enzyme catalyzed oxidation of PUFAs are cyclooxygenase that produce cyclic oxygenated

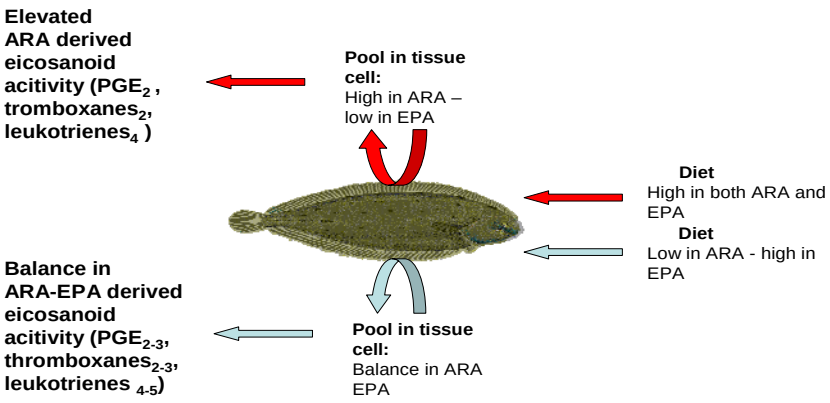
derivatives including prostaglandins, prostacyclins and thromboxanes - and lipoxygenases that produce linear oxygenated derivates including leukotrienes and lipoxins. EPA gives rise to prostaglandins of the 3-series and ARA to the prostaglandin 2-series and both ARA and EPA compete in eicosanoid production (Fig. 5), but at equal concentrations ARA is a stronger competitor. Therefore in fish as in mammals eicosanoid production is influenced by the cellular ratio of C20:4n-6 and C20:5n-3 and an imbalanced ratio of C20:4n-6: C20:5n-3 is damaging (Tocher, 2003).

High levels of dietary EPA in combination with low levels of ARA were tested (**V**), as this may reduce the efficacy of the 2-series prostaglandins (Bell et al., 1993; Bell et al., 1994).

Elevated PGE_2 levels may cause a biochemical- induced stress (Sargent et al., 1999a) and be involved in inducing malpigmentation (Bransden et al., 2005; Villalta et al., 2005). Consequently, EPA may have an important physiological function in modulating eicosanoid action.

Figure 5.

Possible links between dietary PUFA, - tissue PUFA and eicosanoid production. ARA and EPA compete for the same enzymes (see text) to produce either 2-series of prostaglandins and 4-series leukotrienes or 3-series prostanoids and 5-series leukotrienes, respectively. The ratio of C20:4n-6: C20:5n-3 determines the activity of the eicosanoids, at equal ratios ARA is a stronger competitor.



3.0 Broodstock and egg quality

Reproduction of many marine species still rely on wild caught broodstock for egg production (Gjedrem, 2000). In common sole experience with cultured breeders has been weakly described, despite that a better understanding of brood fish development is vital to produce eggs and sperm of the highest quality. The commercial production of Senegalese sole larvae, which takes place in Southern Europe has been seriously limited by the poor success of obtaining eggs from captive

breeders (Anguis and Cañavate, 2005) and reproduction of F1 broodstocks has failed (Cabrita et al., 2006).

In many marine species reared in aquaculture, the survival of egg to fry is less than 20 percent and can be highly variable, and it may be difficult to assess, as both biotic and abiotic factors may affect the quality (Baynes et al., 1993; Gallagher et al., 1998). Unpredictable and variable egg quality is a major limiting factor for the successful mass production of marine fish species (Kjørsvik, 1990). In fish farms and hatcheries the factors that affect fish egg quality, are several and dependent on complex interactions between genetics, physiology, nutrition and environment. Fertilization rate, egg and larval size, egg survival, larval mortality, and growth are features that have been used to assess egg quality in marine teleosts (Vallin, 1999). Kjørsvik (1990) suggested, that the morphological character of egg development is a good indicator of viability in marine teleost eggs and aberrations may be an indicator of the state of the environment (Westernhagen et al., 1988). Others have used lipid and fatty acid content as indicators of egg quality (Tocher et al., 1985, Gallagher et al., 1998) and it is therefore hypothesized, that much of the variation in egg quality is related to the biochemical FA composition (**I**).

3.1 Wild – and cultured sole breeders; egg viability

Wild caught sole broodstock and cultured sole broodstock readily spawns in captivity (Howell, 1997, **I**), but aquaculture related rearing conditions may be completely different from natural conditions. Factors affecting egg quality including FA composition may be related to the following:

Cultured sole, F_1 progeny, were fed extruded pelleted diet from late larval stages until matureness contrary to the wild breeders (**I**), which were caught in the North Sea just prior to the start of the spawning season and fed on blue mussels and sand worms.

Cultured sole reached maturity within the 2. year (authors observations), due to a fast growth rate (**V**). This is suggested to be much earlier than in wild sole. Egg viability may improve with the age of the spawners or in repetitive spawners (Imsland et al., 2003; Kamler, 2005).

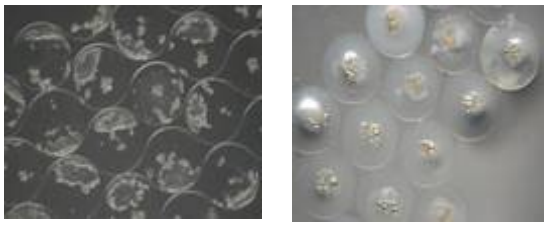
Egg quality, determined as percent of hatching eggs, increased in cultured 3. year old breeders as compared with eggs obtained from the same 2. year old breeders (**I**). However, egg quality was still very low and in general much lower in cultured eggs as compared to wild eggs (**I**) which however, showed significant variations between spawnings.

In cod (*Gadus morhua*), Vallin (1999) found a relationship between malformations at early blastula stage and hatching success, with some exceptions. However, egg malformation is a subjective judgement, which may vary between researchers thereby obstructing comparisons (Vallin, 1999). We did not evaluate malformations, but low egg viability was in general related to high mortality within 24 h after incubation (**I**), before end of the blastula stage.

Figure 6.

Illustration of development of viable sole eggs a) 12 hrs, b) 29 h and c) 54 h after fertilization. Lipid globules scattered around the yolk surface, characteristic for sole eggs. Egg size 1.3 mm. d) Non viable eggs within 24 h post fertilization, e) dead eggs within 48 h post fertilization.

a-c



d-e

Despite the variable egg quality of wild sole eggs (**I**), it is considered unlikely that wild broodstock in general suffer from a maternal or paternal reproduction disturbance to eggs, and this stresses the importance of evaluating wild egg quality in relation to environmental conditions.

Obvious factors affecting the quality of wild sole eggs (**I**) may be related to the broodstock handling and the short adaptation to tanks close to breeding, - stress, - lack of supply of sandy bottom substrate in the tanks, - male: female relations and broodstock density. For cultured sole, egg viability may also be related to reproductive disturbances. However, none of these factors were investigated.

3.2 Reproduction, - essential fatty acids. Egg and early larval quality

Fatty acids are the major source for metabolic energy in fish for growth, but are also the major source of metabolic energy for reproduction (Sargent et al., 1989). The failure of the recovery of cod populations in Newfoundland waters are believed to be caused by the absence of the oil rich capelin (*Malotus villotus*), which is a major contributor to the diet of cod. The capelin disappeared

and the consequent change in diet had a significant negative influence on the condition of the cod population causing a poorer reproductive potential (Rose, 2001).
Egg composition of fatty acids can be different from the body composition of the body oils of the broodstock, because of a selective catabolism or selective transfer of other fatty acids to the eggs. This may be the reason for a higher C22:6n-3, DHA: C20:5n-3 ratio in fish roe than in body triacylglycerols because C20:5n-3 is selectively catabolised relative to C22:6n-3 in oxidative processes producing energy for gonadogenesis (Tocher, 2003). Egg viability has been found to increase with an elevation of n-3 HUFA levels in eggs (Fernández-Palacios et al., 1995).
Fatty acid composition of either cultured or wild sole eggs was very consistent during the spawning season (**I**). Egg origin could be deduced based on the FA profile (**I**). Hence, fatty acid profile of eggs of cultured sole was affected by the broodstock diet for certain fatty acids, which indicated that egg composition and spawning quality may be improved by modifying the nutritional quality of the broodstock diets. Bromage (1995) reported, that the gonadal development and fecundity are greatly affected by the broodstock nutrition in several fish species and the spawning quality may be much improved by increasing the nutritional quality of broodstock diets (Watanabe and Kiron, 1995). In continuous spawners, with a short vitellogenic period, such as sparids spawning quality may even be affected by the dietary lipids during the verge of the spawn (Watanabe et al., 1985). However, the fatty acid composition or ratios of essential fatty acids as DHA: EPA did not seem to explain differences in survival and hatching rate between viable eggs and non viable eggs or between eggs of cultured and wild broodstocks (**I**). Accordingly, other nutritional components than the fatty acids, i.e dietary fish meal protein and amino acid quality, may be a part of an explanation for the low survival of the cultured sole eggs. In cultured sea bream, egg quality was markedly improved by substitution of fish meal with squid meal suggested to be related to the superior value of the squid protein (Fernández-Palacios et al, 1997).

Both cultured and wild sole eggs had a relative high content of DHA, C22:6n-3 (**I**) higher than reported for Senegalese sole (Conceicão et al., 2007) and much higher than in the diet of the cultured broodstock sole. Thus probably selectively accumulated and deposited in eggs of both cultured and wild sole broodstock as described (**I**). This indicates an importance of this fatty acid in early embryogenesis or in pre-feeding larvae and there may be a species specific accumulation or conservation of DHA to maintain it within a specific range, which is not observed for other PUFAs (Wiegand, 1996). A DHA: EPA of 1.6 was observed in feed for cultured broodstock, while it was

10.8 in eggs (**I**). As described, DHA accumulates at a faster rate in fish eggs than other EFAs. In Gilthead seabream (*Sparus aurata*) eggs, DHA was found to accumulate over 70 percent faster than EPA or linolenic acid, C18:3n-3 in response to a dietary increase (Harel et al., 1994).
DHA is a key component in membrane phospholipids of the cerebral cortex, heart and photoreceptor membranes (Harel and Place, 1998), which may explain the presumed higher need during early life stages. This is supported by the relative constant DHA content in pre feeding-larvae at dah 0 until start feeding at dah 3, (**II, III, V, VI**). This indicates that DHA is not utilized and most likely is retained in the membrane phospholipids in contrast to the utilization of other fatty acids exemplified by the monounsaturated C18:1n-9 content which decreased as well as the decrease in the total lipid content (**II, III**). Similarly, in Senegalese sole, monounsaturated fatty acids and saturated fatty acids have been reported as the main FAs metabolized for energy (Conceicão et al., 2007).
Early larva may have a specific requirement for ARA as reported for Senegalese sole (Mourente and Vázquez, 1996). The low arachidonic acid content in cultured eggs much lower than in wild eggs was probably related to a lower dietary content. ARA may be important for egg or larval survival as observed in other species (Bell et al., 1996, 1997; Evans et al., 1996; Conceicão et al., 2007). Broodstock limited in dietary ARA may not be able to produce phospholipids of the phosphatidylinositol class involving ARA as a constituting part. Moreover, ARA is involved in the production of prostaglandins, which are considered important during early development.
Observations during 2006 and 2007 indicated that the larvae from batches of cultured sole in general had a higher degree of malpigmentation defined as hypomelanosis than sole larvae obtained by wild broodstock, but it requires further investigations as whether this was partly related to a general lower ARA content in cultured eggs.

4.0 Live prey species, *Tisbe,* rotifers and *Artemia* as feed for sole larvae

The larval production of most commercial marine species is dependent on live feed, for which production is a labour intensive and costly affair and may put constraints to the rapid development of this segment of aquaculture (Bengtson, 2003). The development of suitable formulated feeds is an even greater future challenge, which will require both innovative and technical breakthroughs for a successful complete replacement of live feed.
At the Danish Institute for Marine Research, DIFRES an area of focus has been on the mass production of the harpacticoid copepod *Tisbe holothuriae* as an alternative to traditional prey as

rotifers and *Artemia* as a feed for the very early larval stages. This copepod species could be a valuable supplement for a range of marine larval species as due to the wide range of nauplii and copepodite sizes and nutritionally superiority with regard to composition; *Tisbe holothuriae,* is known to synthesize EFAs (C20:5n-3, C22:6n-3) (Støttrup, 2003) and therefore need no enrichment.

The natural prey of sole larvae is various copepod species (Drake and Arias, 1993). In general copepods have been considered too difficult to cultivate intensively. Therefore nearly all intensive production of marine larvae rely on rotifers or *Artemia*. However, this may be a bottleneck for the juvenile production of some marine species.

Intensive production of copepods is though very labour demanding. Studies have been carried out on both calanoid copepods from the genera *Centropages, Labidocera, Acartia* and *Eurytemora* (Marcus, 2005) and harpactocoid copepods in which species as *Tisbe* spp, *Tigriopus* spp., *Schizopera elatensis*, *Euterpina acutifrons*, *A. atopus*, and *Nitokra lacustris* have shown a potential to be cultured at densities to be scaled to the needs of aquaculture (Fleeger, 2005). Copepods will, however, not be a major live feed organism in hatcheries for finfish larvae unless mass-production technology is established (Lee, 2005).

At DIFRES, it is possible to produce substantial quantities to support the requirement of first feeding larvae and with success *Tisbe* has been introduced as a start organism for very small marine larvae of various warm water species (i.e. groupers and snappers, personal experience) prior to the feeding of rotifers. The success seemed to be a combination of optimal prey size, behaviour and nutritional superiority. *Tisbe* has 6 nauplii stages and 5 copepodite stages besides the adult stages with a size range of 0.1 to 0.4 mm lengths. Copepods move in jerks, which is further believed to trigger larval attacks. These fators are important, as one major difficulty in larvae rearing is the period around first feeding, in which larvae quickly have to require the skills of exogenous feeding before an energetic depletion of yolk sac and lipid globules results in a point of no return. - The harpacticoid nature of copepodites and adults, may however, be a drawback for the availability to pelagic fish larvae.

When feeding has been established, the change to less demanding prey species will often be less troublesome, and mortality lower. In terms of size a start feeding sole larvae is relatively large (i.e ≈ 3 mm) and is capable to prey on newly hatched AF grade *Artemia* nauplii.

4.1 Larval prey capture

The ability of first feeding larvae to capture a particular prey may be influenced by the perception of the prey, catchability (size, shape and predator avoidance) and palatability (Chesney, 2005). *Tisbe*, rotifers and *Artemia* are different in size, shape, colour, contrast, and behaviour (**II**). The sole larvae were fed a similar density of 5 ml^{-1} twice daily, this density is frequently used when feeding rotifers in marine larval studies (Reitan et al., 1994; Fernández-Diaz et al., 2001; Morais et al., 2004).

The similar density (**II**) was in favour of *Artemia*, but if larvae instead were fed a similar dry weight of prey, the density of *Artemia* would have been much lower, causing possible higher larval energetic cost in search for feed. Especially as sole larvae is a relatively poor swimmer and slow moving predator (personal observations).

Start feeding sole larvae, followed until 9 days post hatch, had a much better growth when fed *Artemia* than *Tisbe,* while overall growth was negative when fed rotifers (**II**). It was examined if first feeding sole larvae actively selected or developed a preference of any of these prey (**IIa**). Preference has been reported in several fish species (Miller et al., 1997), accordingly, Walleye larvae (*Stizostedion vitreum*) fed different prey species preferred for a certain intermediate size (Mayer et al., 1997).

Neither *Artemia* nor rotifers were unambiguously preferred when fed to sole larvae in a preference test at dah 4-6, but *Tisbe* were ingested in a lower number of larvae. None of the prey species were however completely discriminated. However, when tested 10 days after hatching (prey density 1:1 and 1:10) sole larvae seemed to show an active preference for *Artemia* at an even 10 times higher rotifer concentration (**IIa**).

The results indicated that first feeding sole larvae actively were preying on all 3 prey species, but later during development either preferred larger prey, or did not recognize the small energetically unsuitable prey (rotifers). This is characteristic for many species (Miller et al., 1997) such as turbut (van der Meeren, 1991). Sole larvae may have a lower capture success of *Tisbe* as due to characteristics associated with their harpactocoid nature and primary benthic lifestyle, and therefore possible distribution in the hatchery tanks. Growth differences were probably a combination of size, availability and energy intake, and not related to fatty acid composition. Out of the three offered prey, *Artemia* was therefore considered a very good start feed for larvae of common sole and *Tisbe* and rotifers were not suitable (**II**) in contradiction to observations for many other larvae of marine fish species (Lubzens and Zmora, 2003).

4.2 EFA in live prey and dietary EFA requirements in start feeding sole larvae

Harpacticoid copepods have superior nutritional FA qualities especially for essential fatty acids, EFAs than both unenriched *Artemia* and rotifers (Cutts, 2002). In (**II**) *Tisbe* showed a relatively high content of C20:5 n-3 and C22:6n-3, while C22:6n-3 was absent in the *Artemia* strains tested and in rotifers (**II, III, V**). In *Artemia* the levels of EPA seemed to be inversely related to the level of linolenic acid, C18:3n-3 (**V**) as previously reported (Dhont and van Stappen, 2003, **V**).

The higher content and better EFA profile in *Tisbe* is suggested to be related to the presence of enzymes (i.e. Δ- 5 desaturase and Δ- 6, elongase) in these copepods necessary for conversion of shorter chain n-3 polyunsaturated fatty acids to longer chained essential fatty acids such as C22:6n-3 and C20:5n-3 (Nanton and Castell, 1998).

It is suggested that the fatty acid composition of eggs (or perhaps egg yolk) may reflect the requirements of start feeding marine larvae (Morais et al., 2004) and therefore dietary EFAs and especially DHA are considered very important for larval performance during the initial life stage (Takeuchi, 1997).

Sole larval growth was not affected by comparative tests of IH *Artemia* strains with low TFA, EPA and DHA contents, in comparison to larvae fed AF *Artemia* with a higher natural content or *Artemia* enriched with DHA or EPA (**III, V, VI**). Despite the very high DHA content in sole eggs, *Artemia* proved to be an excellent live feed (**II**), despite that these are known selectively to catabolise DHA (Macqueen et al., 2003). Hence, *Artemia* contained at most 13 percent DHA even when enriched by emulsions containing up to 30 percent DHA (% TFA). We did not register an obvious advantage of excess dietary DHA in relation to growth, survival or pigmentation in exogenously feeding larvae until the end of the larval period (**III, V, VI**). The relative low DHA requirement of feeding common sole larvae and Senegalese sole larvae as previously reported (Morais et al., 2004; Villalta et al., 2005b), is similar to other marine flatfishes but contrary to pelagic carnivorous marine fishes such as cod (*Gadus morhua*) (Park et al, 2006). It may be caused by the relative predominance of EPA rather than DHA in the wild benthos prey (Villalta et al., 2005b). As mentioned (3.2), the high deposition of DHA in eggs may therefore be to ensure vital neuronal development, or to sustain physiologically important pathways in the egg or very early larval stage.

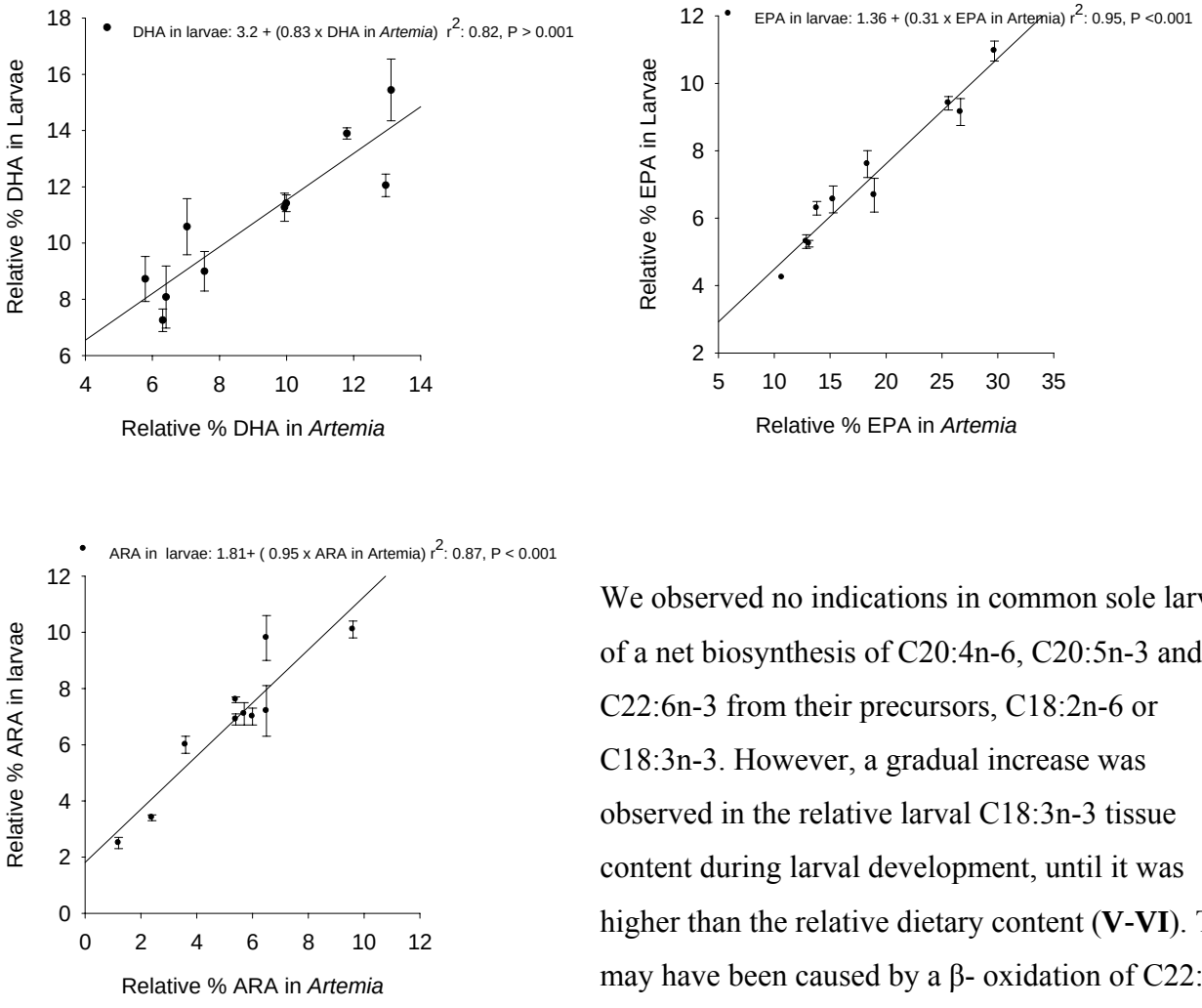
4.3 Transfer of FA between trophic levels and tissue FA interactions

Tissue FA composition in feeding sole larvae was related to the dietary FA profile **(III, V, VI)** as reported for other species (Conceicão et al., 2007). In most studies (**III, V, VI**), larval specific growth rates extended 15 -20 percent day $^{-1}$, accordingly, the tissue composition changed within quite few days in favour of the dietary composition, which is similar for other marine fish larvae with fast growth rates such as Gilthead seabream (Mourente et al.,1993).

Apart from a possible β- oxidation of DHA in larvae (**V** and **VI**) (described below) and that *Artemia* selectively catabolised the dietary DHA in the emulsions **(III**), the EFA content in sole larvae was highly correlated to the dietary content (Villalta et al., 2005, **II, III, V, VI**) as exemplified in Fig. 7.

Figure 7.

Illustration of correlation between dietary EFAs content in Artemia and 19 day old larvae. Modified from paper III.



We observed no indications in common sole larvae of a net biosynthesis of C20:4n-6, C20:5n-3 and C22:6n-3 from their precursors, C18:2n-6 or C18:3n-3. However, a gradual increase was observed in the relative larval C18:3n-3 tissue content during larval development, until it was higher than the relative dietary content (**V-VI**). This may have been caused by a β- oxidation of C22:6n-

3 or C20:5n-3. This process does not require the enzymes described in figure 4, but follows a catabolism involving other enzymes in the cellular organelles or mitochondria. The β- oxidation of PUFAs in fish is however not well studied and may vary between different PUFA molecules. At a cellular level the chain shortening is believed to be initiated in the peroxisomes.

As mentioned DHA was the most prevalent fatty acid in sole eggs and early larvae. The native DHA content in feeding sole larvae decreased rapidly over time when DHA was not dietary supplemented (**II, V-VI**). Hence, as suggested, DHA probably has en essential function during early embryogenesis, while in older larvae it may partly act as a source of energy similarly to EPA as suggested for other marine species (Watanabe, 1993). EPA, however, is expected to be more readily β- oxidized, as DHA catabolism may require both mitochondrial and peroxisomal oxidation (Tocher, 2003).

The fish oils used (**III, V, VI**) were particularly abundant in the saturated FA, C16:0 and the monounsaturated C18:1n-9, which are often abundant in so called northern fish oils. These together with other C18 fatty acids are heavily catabolised in fish for energy (Tocher, 2003), suggesting that these fatty acids are the preferred fuels in swimming muscles (McKenzie et al., 1998). In support of this (**II, III**), we observed that pre- feeding sole larvae seemed selectively to use C18:1n-9.

We observed a gradual decrease in C18:0 content of feeding larvae over time and a concomitantly relative increase in C18:1n-9 (**III**), accordingly. This was probably related to dietary prey content and may not have involved desaturation of C18:0.

During starvation common sole larvae were not particularly selective in their use of fatty acids as fuel as only the content of a few fatty acids seemed to decline slightly (C16:1n-9, C20:5n-3) in comparison to a highly significant decrease in total tissue lipid content (**II**). DHA and ARA seemed retained (**II**). The different lipid classes were not analysed, which may have revealed a selective catabolization in favour of specific fatty acids within the triacylglycerols, phospholipids or esters. In starving larvae of Senegalese sole (Mourente and Vazquez, 1996) mainly saturated fatty acids and monounsaturated fatty acids were catabolised, whereas EFAs, as DHA and ARA were similarly retained. The retaining of important fatty acids in starving larvae may be a mechanism related to increasing the likelihood of survival.

5.0 The influence of essential fatty acids in larval fish respiration

Cultivated fish larvae are frequently exposed to stressors induced by fluctuations in environmental factors e.g. level of dissolved oxygen, salinity, and water temperature (Ishibashi et al., 1992;

Ishibashi, 1994). The term stress may be caused by physical or chemical factors and may contribute to disease or death in flatfish. Prolonged stress may reduce the effectiveness of the immune system. Hence, biochemical stress in flatfish may be inevitable, during the radical morphological changes of metamorphosis. A stress response includes the rapid release of stress hormones, catecholamines and cortisol, into the circulatory systems (Iwama et al., 2004).

High survival and sustained growth usually indicates that a diet satisfies the minimum nutritional requirements of the species being fed. Survival and growth alone may not be indicative of a fishs physiological condition (Dhert et al., 1992), but may still affect its response to stressful conditions. Several studies have investigated the effects of dietary EFAs upon stress resistance in marine fish larvae (Kanazawa, 1997; Weirich and Reigh, 2001; Koven et al., 2001). In gilthead sea bream, dietary deficiencies of essential fatty acids of marine fish raised the basal cortisol levels (an indicator of stress) and altered the pattern of cortisol release after stress (Ganga et al., 2006). For common sole it has been reported that deprivation of EPA and DHA increased mortality rates upon exposure to stressors; such as hypoxia, low temperature, and low salinity (Loque et al., 2000). Most studies have used mortality as an end point of measurements of stress tolerance, due to difficulties in probing the *in-vivo* physiology of minute marine fish larvae. In **IV** sub lethal *in-vivo* regulation of metabolism in hypoxia by individual marine fish larvae was demonstrated in individual sole larvae fed with live *Artemia,* that provided different EFA quantities and combinations (from **III**). Accordingly, sole larvae fed unenriched *Artemia* had a higher routine metabolic rate (RMR) than the other dietary groups in normoxia. They exhibited visible signs of respiratory distress at higher water oxygen partial pressures (PO_2) than the other groups during progressive hypoxia, and had a significantly higher mean critical oxygen partial pressure (P_{crit}) for the regulation of RMR (**IV)**. The results indicated (**IV**) that enriching live feeds with EFA can have significant effects upon the respiratory physiology of fish larvae and juveniles, and improve their *in-vivo* tolerance of hypoxia as previously suggested (Loque, 2000). The underlying mechanisms for the differences are not known, but as EFAs and particular DHA are important in the neural network a more optimal tissue concentration or FA balance may have lowered larval sensitivity to oxygen related stress responses. Studies have indicated, that DHA in phospholipids were more efficient than EPA to cope with stress tolerance in Japanese flounder exposed to increased temperatures and decreased DO_2 levels (Tago et al., 1999) similar to results on Red seabream (*Pagrus major*) and Marbled sole (*Pleuronectes yokohamae*) (Kanazawa, 1997). Larval fishes may therefore respond negatively to slight unbalances

or deficiencies of EFAs during stress related conditions, which may not be registered in laboratory scale experiments under optimal rearing conditions (**III, V, VI**).
As mentioned, stress induced by means of fluctuating environmental factors, overcrowding or sub-optimal husbandry practice, is not unusual in marine (flat) fish larval production (Sargent et al.,1999a). In settling sole larvae, fin bites in tails and fins were very common (**II**). Indeed, stress may be inevitable given the high stocking densities required for economically viable larval production, which emphasizes the importance of the present findings.

6.0 Pigmentation

Malpigmentation is one of the most frequent abnormalities in cultured flatfish. Malpigmented juveniles are usually seen as undesirable for future cultivation (Imsland et al., 2006). The pigmentation of the fish is directly related to its market price (Yamanome and Amano, 2005), and is used as a quality criterion Malpigmentation in flatfishes is very unpredictable, and vary from 0 to 100 % (Imsland et al., 2006). Pigmentation development has been described in several flatfishes as flounders (*Paralichthys olivaceus*, *P. dentatus*, *P. lethostigma*), turbot (*Schopthalmus maximus*), plaice (*Pleuronectes platessa*) and halibut (*Hippoglossus hippoglossus*), but the fundamental aspects of developmental defects are poorly understood (Bolker et al., 2005).
Pigmentation is determined by the number and distribution of three types of pigment cells; dark melanophores, yellow xanthophores and reflective iridophores; and originates from the neural crest cells, that migrate over the embryo during early ontogeny (Bagnara et al., 1979). Around the period of metamorphosis, pigment cells differentiate further and produces additional melanophores and other pigment cells necessary for the pigmentation pattern (Matsumoto and Seikai, 1992). The development of normal pigmentation, depends on the processes that initiates metamorphosis, and thereby the asymmetry of flatfish, which involves the differentiation of pigment cells along with local signals from their histological environment (Zuasti, 2002).
Usually 2 types of malpigmentation may occur in hatchery populations. Excessive pigmentation of the skin, usually as a result of increased epidermal or dermal melanin pigmentation, "hypermelanosis" / ambicolouration or a deficiency of pigment cells of the ocular side, hypomelanosis / albinism (Bolker and Hill, 2000). It is suggested that hypermelanosis and hypomelanosis may reflect distinct phenomena in the normal pigmentation system (Bolker and Hill, 2000). However, both nutritional and environmental factors be may be important for development of ambicolouration / hypermelanosis (Haga et al., 2004).

The degree of malpigmentation may vary from various patterns of ambicolouration (i.e. complete pigmentation on the blind side) to albinism (i.e. complete lack of pigmentation on both sides). We classified degree of malpigmentation into a few categories (**III, V**, **VI)**. Fig.8.

Figure. 8

*Illustration of three different levels of hypomelanosis in sole juveniles, a) tail malpigmentation, b) body malpigmentation, c) albinos - complete malpigmentation (**III**). Notice eye position. d) Illustration of hypermelanosis; blind side pigmentation on the lower body part of a sole juvenile (**III**).*

a,b,c)

d)

Hypermelanosis mainly appeared in one study (**V**). In this study no juveniles were observed with excess pigment of the blind side and any degree of hypomelanosis on the ocular side (**V**). In addition ARA did not induce malpigmentation in sole sensitive to hypermelanosis (**V**). Both factors may indicate, that ocular side albinism and blind side pigmentation may be mutually coupled. However, for Japanese flounder (*Paralichthys olivaceus*), diet seems to be the most important factor in determining ocular side pigmentation, while blind side pigmentation is suggested controlled by environmental factors such as light or substratum (Seikai, 1991, Iwata and Kikuchi, 1998).

6.1 The inducement of malpigmentation in sole larvae by dietary EFAs

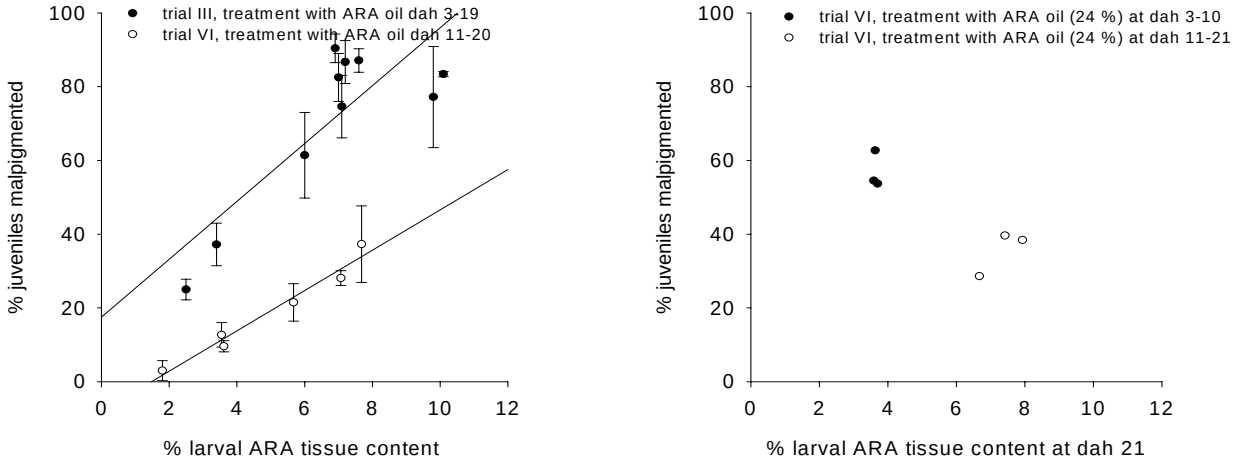
Nutrients and therefore diet composition is believed to be a critical factor in flatfish pigmentation development, and EPA, DHA and ARA may be involved (Bolker and Hill, 2000; Hamre et al., 2002, Villalta et al., 2005a).

Artemia and rotifers enriched by essential fatty acids as DHA has been reported to improve pigmentation success in flatfish (Dickey-Collas, 1993; Reitan et al., 1994; Estevez and Kanazawa, 1995). More recent studies have indicated that pigmentation may be related more to ARA. Estevez et al. (1999) fed turbot larvae with rotifers and *Artemia* enriched with increasing levels of ARA, and found that the frequency of normal pigmentation decreased. This is similar to results on a number of species including; Senegalese sole (Villalta et al., 2005a), common sole (**III, VI**), Yellow tail flounder (*Limanda ferruginea*) (Copeman et al., 2002), turbot and Atlantic halibut (McEvoy et al., 1998). Of interest is, that dietary EPA and DHA did not influence pigmentation on common sole (**III, V, VI**), and consequently their role in pigmentation is therefore questioned. A recent study on Senegal sole indicated that C18 PUFAs especially C18:4n-3, perhaps in combination with C18:3n-6 and C20:4n-3 or their metabolites may have an anti- ARA activity effect, hence a positive effect on pigmentation rate (Villalta et al., 2007). The perhaps more powerful effect of C18:4n-3 on suppressing ARA induced malpigmentation than EPA, however, needs further investigation.

6.2 Larval sensitivity to ARA and pigmentation window

The degree of sole juvenile malpigmentation was related to larval tissue concentrations of ARA as illustrated in Fig. 3a (**III**). However, the sensitivity to ARA was not only related to the dietary concentrations or duration of ARA treatment as indicated in Fig. 9a, the sensitivity was also related to the stage of larval metamorphosis and was higher during premetamorphosis (**VI**) Fig. 9b.

Figure 9, a-b. *a) Illustration of juvenile sole malpigmentation as a function of larval ARA tissue content treated with ARA at different stages of larval ontogeny (**III, VI**). b) Illustrates that larvae exposed to dietary ARA during premetamorphosis had a relative higher incidence of malpigmentation despite a lower ARA tissue content at the end of metamorphosis (dah 21) in comparison to larvae fed ARA during metamorphosis (**VI**).*



A specific sensitive pre-metamorphic period for dietary supplementation, to reduce later incidence of albinism has similarly been identified in Japanese flounder (Seikai et al., 1987); and Atlantic halibut (Næss and Lie, 1998). The mechanisms are unknown, but the process may disrupt the differentiation of pigment cells.

Sole larval pigmentation related sensitivity to ARA was batch related. In one study malpigmentation in sole larvae was induced (**VI**) but not in another study (**V**), despite a similar dietary treatment of 8 percent ARA oil and despite similarities in ARA larval tissue concentrations. As mentioned, in the study, in which the larvae were not affected (**V**), a high percentage of juveniles were ambicoloured / hyperpigmented; a characteristics vaguely observed in our other studies (**III,VI**). A large replicate variation in hypermelanosis demonstrated, that this abnormality was not treatment related or related to ARA or other FA concentrations. Hence, environmental conditions had an influence. The rearing conditions were apparently identical (**V** and **VI**), which means that the triggering of hypermelanosis was considered related to local delicate environmental and physiological balances in the rearing tanks, on a scale of the individual tank. Hypermelanosis was almost non existing in juveniles (**II, III** and **VI**) and susceptibility to hypermelanosis (**V**) may have lowered larval sensitivity to ARA or its eicosanoid derivatives for inducement of hypomelanosis (**V**). Consequenly, we observed no significant relation between larval tissue ARA and prostaglandin content (**V**).

Differences in live prey, cultured vs. wild zooplankton, fatty acid or amino acid composition have been suggested as important nutritional factors in reducing malpigmentation (Bolker and Hill, 2000). Flatfish larvae fed natural reared zooplankton, generally exhibits higher rates of normal pigmentation than larvae fed *Artemia* nauplii (Seikai et al., 1987b, Japanese flounder, Næss, et al.,

1995, Atlantic halibut, McEvoy et al., 1998, Atlantic halibut). However, different live prey did not affect sole pigmentation (**II**), despite that the feed was provided during the sensitive period of larval ontogeny (**VI**). This indicates either differences in batch related sensitivity, and / or that sole larvae pigmentation may be under hormonal control (**VI**).

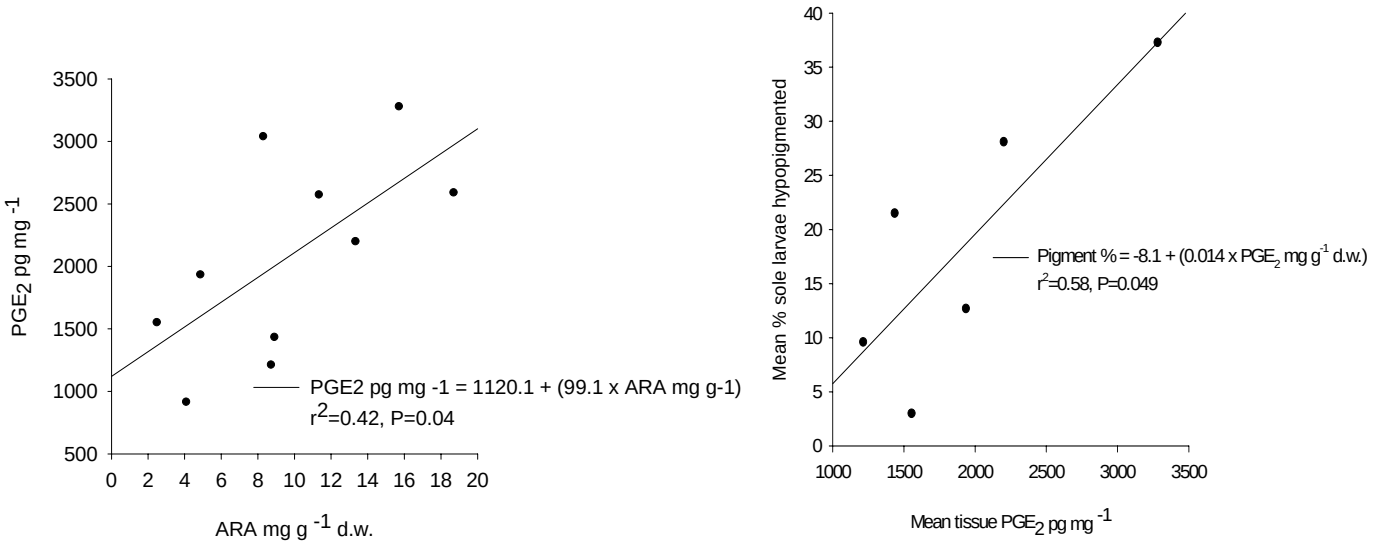
6.3 Hormonal influence on pigmentation

Relatively few studies have reported the eicosanoid production or eicosanoid function in fish (Tocher, 2003). In larval fish only recently a few studies have demonstrated a relationship between pigmentation and eicosanoids in cod and Senegalese sole (Bransden et al., 2005, Villalta et al., 2007). We examined the prostaglandin levels of the 2-series derived by ARA in relation to the dietary level and larval tissue content of ARA and EPA (**V** and **VI**). The results indicated, that prostaglandins, PGE_2 may be related to tissue ARA, and involved in malpigmentation (Fig 10 a,b), while dietary or larval tissue levels of DHA and EPA had no influence on the tissue levels of PGE_2.

Figure 10, a-b.

a) Larval PGE_2 tissue concentration as a function of tissue ARA concentration, b) Juvenile malpigmentation as a function of PGE_2 larval tissue concentration at dah 21.

a) *b)*

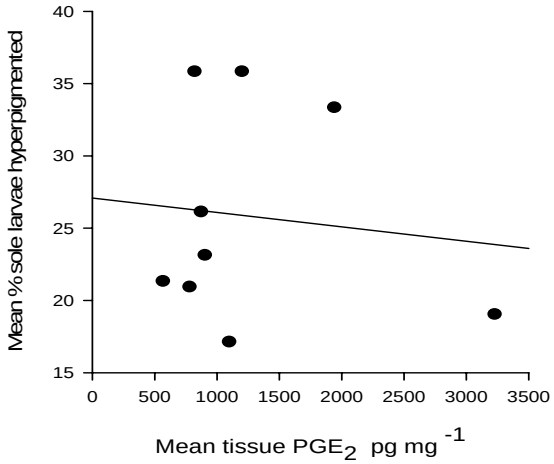


The results supported the eicosanoid hypothesis (**VI**), that ARA lead to an increased PGE_2 activity, which was involved in the inducement of hypomelanosis. However, a large variation between replicates diminished the statistical differences, which may be related to the nature of prostaglandins as signal compounds.

The inducement may be related to biochemical stress as previously hypothesized (Sargent et al., 1999b). The results further documented Fig. 11, that PGE_2 tissue concentrations had no influence on the degree of hypermelanosis (**V**) supporting that this form of malpigmentation is induced by environmental factors.

Figure 11.

*Juvenile hypermelanosis as a function of PGE_2 larval tissue concentration at dah 19. (**V**)*



6.4 Mechanisms of PGE_2 and pigmentation

Several studies have suggested that the fatty acid arachidonic acid (ARA, 20:4*n* − 6) is involved in the release of cortisol in fish, although the actual mechanisms have not been investigated (Harel et al., 2001; Koven et al., 2003).

It is speculated if cortisol levels may be linked to pigmentation. It has been suggested that PGE_2 derived from ARA is involved in the regulation of cortisol production through the hypothalmus-pituitary-interrenal axis. Alternatively ARA may directly mediate the stress response (Lutzky et al., 2002; Ganga et al., 2006). Consequently, cortisol may induce stress leading to malpigmentation, however, high levels of cortisol is likely to reduce larval growth performance, as shown for tilapia larvae (*Oreochromis mossambicus*) (Mathiyalagan et al., 1996). This was not observed here (**III, V, VI**). Another hypothesis suggest, that eicosanoids regulate the mechanisms involved in the release of melanophore stimulating hormone (Koven et al., 2003; Bransden et al., 2005). Possible effects of prostaglandins on tyrosinase activity may be responsible for the differences in pigmentation, as tyrosinase is one of the initial enzymes that catalyses the production of melanin from l-tyrosine (Slominski et al., 2004). It has been demonstrated that mouse melanoma cells incubated with PGE_1 or PGE_2 increased tyrosinase activity (Abdel-Malek, 1987). This is likely to cause a higher degree of pigmentation. However, the

contrary was observed here (**VI**). Hence, the biochemical mechanisms linking eicosanoids and pigmentation in fish has not been substantiated, and needs further attention.

7.0 Eye migration

Metamorphosis in flatfish is characterized by an anatomical transformation during development, and involves 90 ° rotation of the body and the migration of one eye to the ocular side (Fernandez – Diaz et al., 2001), as well as ossification and skeletal development (Sæle et al., 2003). This is further associated with a benthic life and consequently changes in diet (Fernandez -Diaz et al., 2001). Size is believed to be the determinant factor in the onset of metamorphosis (Fernandez-Diaz et al., 2001, **V,VI**). Studies have suggested that nutritional factors; such as EFA may influence both onset of eye migration and the advance of eye migration in Senegalese sole (Villalta et al., 2005ab). This is in contradiction to findings in common sole, where size was the determining factor (**V,VI**) and contrary to studies on Atlantic halibut (Shields et al., 1999). Studies on Atlantic halibut and Japanese flounder have reported a higher success of complete metamorphosis and dorsal pigmentation for larvae fed copepods than enriched *Artemia*. (Koven et al., 2003). Malpigmentation frequencies were not reported, but high degrees of malpigmentation may be related to incomplete eye migration in common sole (**III**, **V, VI**) not observed in partially pigmented individuals (**III, V, VI**), Fig.12.

Figure 12.

*Illustration of eye position in sole juveniles fed Artemia (**III**). The two albino specimen to the left show incomplete eye migration or an abnormal final position of both eyes in comparison to the highly malpigmented individual (right) with a complete normal eye position.*

Normal eye migration may involve a series of linked events and in Atlantic halibut juveniles with an arrested eye migration, the dermal bone (part of the cranium) does not develop (Sæle et al., 2006). Therefore correct metamorphosis (i.e. eye migration, skeletogenisis) may be determined during a critical nutritional window (Sæle et al., 2003), being identical to the pigmentation window (7.2). Also these aspects should be further investigated.

8.0 Conclusions

- Sole eggs of wild and cultured broodstock could be discriminated by their FA profile, related to the diet of the broodstock, but egg quality was not related to FA profiles.
- Common sole larvae had a relatively low requirement for essential PUFAs and could be start fed on unenriched *Artemia.*
- Malpigmentation (hypomelanosis) in common sole larvae was positively correlated to increased concentrations of dietary ARA and the sensitivity to ARA highest during pre-metamorphosis.
- The inducement of malpigmentation involved ARA derived eicosanoids such as PGE_2 prostaglandins.
- EPA and DHA were not involved in pigmentation in common sole.
- Eye migration was not related to dietary FA composition but to larval size.
- Albinism was related to an incomplete abnormal eye migration as well as lower larval growth.

9.0 References

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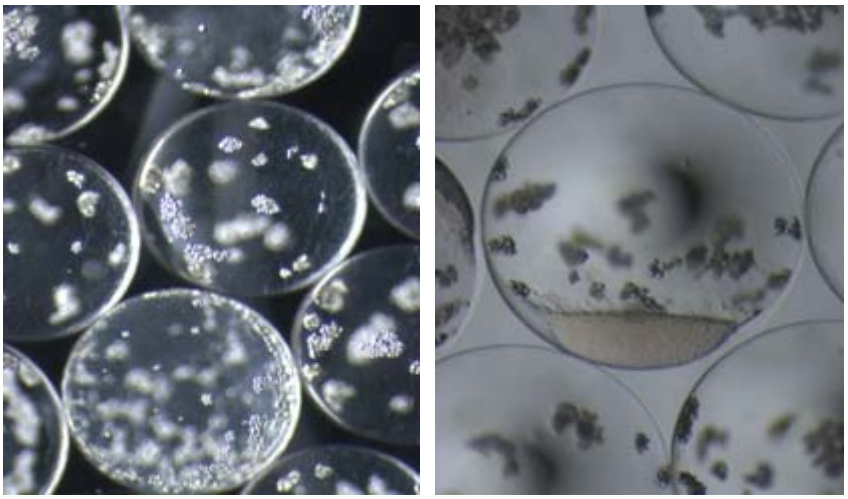

A comparison of fatty acid composition and quality aspects of eggs and larvae from cultured and wild broodstock of common sole (*Solea solea* L.).

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A comparison of fatty acid composition and quality aspects of eggs and larvae from cultured and wild broodstock of common sole (*Solea solea* L.).

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Running title:
Quality and fatty acid composition of sole eggs
Keywords: Common sole, egg quality, larvae, fatty acid composition, cultured broodstock.

Abstract

Eggs from a F_1 cultured broodstock of sole were compared to eggs from wild caught breeders throughout one spawning season to evaluate if egg quality may be affected by culture related conditions. Fourteen batches of eggs from cultured broodstock and 17 batches from wild caught sole were compared with respect to fatty acid (FA) composition, egg size, fertilization rate and hatching rate. Based on a multivariate analysis of the FA profiles, it was possible to discriminate between culture and wild inheritance. Eggs from cultured broodstock had high levels of C20:1(n-9), C18:2(n-6) and C18:3(n-3), whereas eggs from wild fish had high levels of C16:1(n-7), C20:4(n-6) and C20:5(n-3). Differences in FA profiles were most likely related to dietary differences. Fertilization- and hatching rates were generally low and lowest in eggs from cultured broodstock, but not related to FA composition. Larval growth of one batch from each group was compared. Larval growth was not correlated to broodstock origin, FA-composition or egg or larval size. However, larval survival was significantly lower for larvae from cultured broodstock.

Introduction

Common Sole (*Solea solea*), Linnaeus 1758, is a potential candidate for aquaculture, due to its high market price and demand. The commercial production of common sole is still limited. This may be explained by its high temperature requirement (Fonds, 1979, Howell, 1997, Imsland et al., 2003); temperatures which in Northern Europe are feasible only by use of recirculation technology.
Sole production in Europe is still based on wild caught broodstock and, in culture of senegalese sole (*Solea senegalensis*), large scale production has been limited by poor success in obtaining eggs

from captive breeders (Anguis and Cañavate, 2005). Reproduction of F1 broodstock of senegalese sole has still not been achieved (Cabrita et al., 2006).

As with other new marine species a main issue for further development of culture is the production of good quality eggs (Sargent et al., 2002), as egg quality influences fertilization rate, hatching rate and subsequent larval survival (Rainuzzo et al., 1997, Lavens et al., 1999, Giménez et al, 2006).

A quantification of larval nutritional fatty acid requirements is notorious difficult. It is often simulated by the lipid content of eggs or better the yolk reserves of the species in culture (Mourente and Vázquez, 1996; Sargent et al 1999). Lipid content and fatty acid composition - especially of essential fatty acids (EFA) may therefore be important parameters in evaluating egg quality as they are important for marine fish larval performance (Salze et al., 2005). Sole larval performance may be affected by the quantity and biochemical composition of fatty acid reserves in the yolk sac or the biochemical composition during endogenous feeding, and this may again depend on the broodstock nutritional status throughout oogenesis (Sargent et al., 1999, Morais et al., 2004).

Lipid content and fatty acid composition of broodstock diets have been identified as major dietary factors that determine successful reproduction and survival of offspring (Izquierdo,et al.2001). Some marine fish species with a short vitellogenic period such as sparids (for instance Gilthead seabream, *Sparus aurata*) readily incorporate dietary unsaturated fatty acids into eggs, even during the course of the spawning season (Fernández-Palacios et al., 1995). Polyunsaturated fatty acids (PUFAs) with 20 or more carbon atoms affect directly, or through their metabolites, fish maturation and steroidogenesis. In some marine fish species, PUFA levels in broodstock diets increase the fecundity, the fertilization and the egg quality (Pavlov et al, 2004, Salze et al., 2005).

In sole no gap between the unyolked reserve oocytes and maturing oocytes are found. It is likely that the oocyte development is continuous, and it is therefore difficult until right before the spawning season to know which oocytes will remain for future years and which will develop in the actual spawning season (Imsland et al., 2003), which indicates that feeding close to maturation may affect oocyte composition. Devauchelle et al. (1987), however, described that half of the ovarian growth in common sole occurs during starvation in the winter period, therefore, the composition of the developing oocytes may be much affected by the nutritional conditions of the broodstock prior to the breeding season.

Accordingly, the history of breeders of culture origin, in relation to rearing conditions, feed compositions and growth rates may affect the biochemical composition of the oocytes, and a better

understanding of egg quality in relation to wild or culture brood-fish origin must precede a strategy for improving the quality of eggs produced in culture.

The primary objective of this study was therefore to compare eggs from culture and wild broodstocks during a spawning season with a specific focus on their fatty acid profiles in relation to egg and larval quality. This to elucidate if offspring quality was affected by culture related conditions.

Materials and Methods

Broodstock holding

The broodstock facility was an unisolated building with natural overhead light creating a natural photoperiod.

The facility included 3 rectangular tanks of 9 m^3 each (i.e. 3 by 3 metres with a one meter water depth). The tank water was aerated and exchanged continuously with unheated, sand filtered water from the North Sea (salinity 33 -34 g L^{-1}). The exchange rate was 3.2 times day^{-1}.

Wild broodstock was caught in gill nets in the North Sea in late April, 2006. Sole were caught during night time and the nets were inspected with intervals of 2-3 hours to minimize stress and possible damage of trapped fish. Within one hour after capture sole were released into a broodstock tank. Fifty females with abdominal swelling and 10-15 presumed males (i.e. smaller with no external signs of matureness) were selected from 200 fish, individually weighed and transferred to one broodstock tank.

Broodstock from culture were kept in another broodstock tank. These 32 fish originated from a batch of F_1 produced offspring and were 3 years old. Males were identified (7 individuals) as fish without swollen abdomens. The broodstock had been in the facility for more than one year prior to the study and spawned as 2 years old, without any egg survival.

Broodstock size, feeding and survival

Selected wild caught breeders in the range 0.6-1.5 kg individual $^{-1}$, mean weight 0.764 ± 0.219 kg individual $^{-1}$ were offered fresh blue mussels and sand worms, *Nereis* sp, once a day, in excess. Uneaten feed was removed daily. The feed fed wild caught spawners was not subjected to FA analysis, as the feed intake during the spawning season was negliable.

Cultured sole broodstock were in the size range 0.4-0.6 kg individual $^{-1}$ with a mean weight of 0.489 ± 0.067 kg $individual^{-1}$. Since juvenility they were kept on an extruded INVE feed (INVE

Aquaculture, Dendermonde, Belgium). The latter year prior to the study, the fish were fed INVE IDL Solea Go extruded feed (at 1 percent of biomass); with a declared content of 55 % protein and 16 % fat. The analysed fatty acid composition of this feed is shown in Table 2.

During the spawning season no mortality was observed for the cultured breeders, while for wild breeders 2.5 percent died. These few fish died within one week after capture and were not contributing to the following egg material.

Egg spawning, incubation and hatching survival

Spawning of wild fish started on 3 May approximately one week after the fish were caught, and lasted until 17. June. Cultured broodstock fish spawned from May 10 till June 17. Spawnings were observed in the late night / early morning, and eggs were collected every morning by 900 μm mesh egg collectors, connected to the surface outlet from the tanks.

Spawnings with more than 10.000 eggs were transferred to the incubation system.

The number of eggs per spawning was estimated by 6 representative subsamplings of 10 ml from a 10 l bucket holding the eggs in suspension with a gentle aeration. Representative subsamples of eggs were taken for analysis. Eggs from wild fish were separated into two groups by buoyancy in 33 g L^{-1} salinity seawater in a 5 l measuring cylinder. Good quality eggs floated immediately (according to Anguis and Cañavate, 2005). In eggs from cultured sole in general a much larger fraction of these eggs drifted in the water column. Consequently, eggs were not separated.

Eggs were immediately transferred to the incubators. The incubators were 160 litres cylindric conical tanks with a flow through water system based on water from the North Sea. Gentle aeration was used to keep eggs uniformly distributed in the water column and dimmed light was provided by fluorescent light tubes in the room.

On a daily basis aeration was temporarily stopped and sinking pale eggs were flushed out. For each group of wild and cultured eggs all hatched within 2-4 hours after initiation of hatching. Hatching rate was estimated by a gentle skimming off the newly hatched larvae from the surface. The number of larvae was estimated by subsamplings, similarly as described for the eggs.

Larval rearing and characterization

Eggs collected mid-periodically in the spawning season were kept for further larval characterization. Hence, 10800 eggs from cultured sole and 9600 eggs from wild broodstock were incubated on June 1 at 11.2 º C (the ambient sea temperature).

An equal density of approximately 1750 larvae from each batch was transferred to 160 l tanks. Each tank was a part of a recirculation unit. The temperature was slowly increased to 16.1 º C until start feeding was inititiated and the temperature was subsequently gradually increased to 19.1 º C. The water exchange rate was kept at approximately 30 l h^{-1} $tank^{-1}$.

Four days after hatching the larvae were fed newly hatched *Artemia,* AF strain from INVE *Artemia* systems (INVE, Belgium). Each tank was administered *Artemia*, in the morning and in the afternoon at a density of 2 ml^{-1}. *Artemia* were kept refrigerated until the second feeding to avoid moulting into instar stage II nauplii and thereby to become inappropriate large in relation to the size of the larvae. Water flow was temporary increased daily to clear out uneaten leftovers from the tanks. Representative subsamples of larvae were examined at 3, 6, 10, 14 and 21 days after hatching for standard length and dryweight (d.w.).

Egg, larval sampling and analyses

Duplicate samples from each batch of unsorted eggs (100 x 2) or larvae (20 x 2) were rinsed by distilled water. Egg fertilization was identified microscopically as those that were either transparent or had initiated cell division. Duplicate representative samples of each batch of eggs (i.e 50-100 eggs $sample^{-1}$) and larvae (i.e. 30-50 $sample^{-1}$) were collected for measurement of size (egg diameter, larval standard length), d.w. / ash content and fatty acid (FA) composition. Egg diameter and larval standard length were measured to the nearest 0.01 mm by a Leica MZ6 dissecting microscope connected to a Digital high resolution DFC 320 Leica camera and a Leica IM50 software programme. Samples for d.w. were dried in an oven at 80 ° C for 24 h and then weighed on a Mettler Toledo d=0.1 µg. Ash content was determined by combustion at 450° C for 16 h.

In total 17 batches of wild eggs and 14 batches of cultured eggs were examined in addition to four batches each of newly hatched wild and cultured larvae.

Fatty acid analyses

Duplicate samples of egg (50-100 $sample^{-1}$) and larvae (30-50 $sample^{-1}$) for each batch were flushed with nitrogen and stored in 5 ml sterile cryo vials at – 80 º C.

Prior to extraction, the samples were torn with an Ultraturrax, probe diameter 4.5 mm (Biospec Products Inc, USA. The fatty acid composition of the egg and larval samples was determined by total extraction of the lipids in a chloroform/ methanol (2:1.v/v) for 24 h (Folch et al.,1957). The samples were transferred to clean glass test tubes of 3 ml and 1 ml chloroform /methanol was

poured until the samples were covered followed by addition of 40 µl internal standard of methyl tricosanoate (C23:0) in chloroform (1 mg ml^{-1}). Following transesterification of the lipids in toluene, methanol, acetyl chloride (40:50:10), the fatty acid methylesters were analyzed by gas chromatography - mass spectrometry (GC-MS) on an Agilent 6890 series gascromatograf. The FAs were identified with a standard fame mix (Supelco 18919, Sigma Aldrich) and quantified by means of the target response factor of the FA to the C23:0 internal standard. FA concentrations in samples were calculated (ChemStation®) from the quantified peaks of a diluted series of the standard fame mix, and expressed as ng $sample^{-1}$, and the relative percentage of the total amount was calculated. The method has been thoroughly described elsewhere (Lund et al., In press).

Statistics

Discriminant analysis by Partial Least Squares regression (PLSD) was used for descriptive modelling and comparison of the fatty acid content of the eggs from the two broodstocks. The PLSD method was decribed by Wold et al. (1983). Indicator variable (Y-matrix) was the binary designation of wild or cultured origin, and response variables (X-matrix) were the fatty acid profiles. Multivariate statistics was done by The Unscrambler (version 9.1, 2004, Camo, Oslo, Norway). Statistical tests for sole larvae growth and individual FA composition were carried out by one-way ANOVA and a pairwise multiple comparison of means using Tukey`s test. A Pearson Product moment correlation analysis was used to identify correlation between temperature and egg size.

Results

Spawnings and hatching rate

Twenty eight spawnings (>10000 eggs $spawning^{-1}$) were obtained from the wild broodstock and 14 spawnings from the cultured breeders (Table 1). During May twenty one batches of wild eggs and 7 batches of cultured eggs were incubated. All eggs died within 48 h after incubation, except for one batch of wild eggs in which 5 percent larvae hatched. A mean hatching rate of 32 percent was observed for the 7 batches of wild eggs obtained during June and some hatching success was registered in all of them despite large variations. For eggs from cultured fish, some hatching success was observed in 5 out of 7 batches. In batches with no survival all eggs died within 48 h after incubation. The water temperature during May increased from 8.4 to 11.0 ° C, and during June from 11.0 -14.3 ° C.

Eggs of wild fish were significantly larger than eggs from cultured broodstock, both with respect to diameter ($P < 0.001$) and dryweight ($P = 0.003$). Ash content was not significantly different between groups (Table 1).

Table 1.

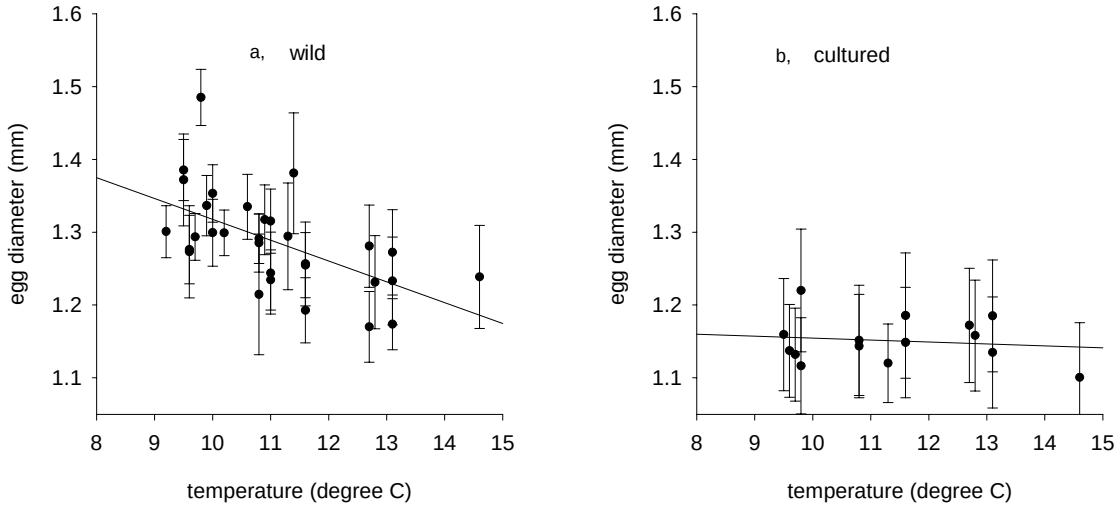
Total number of spawnings and number of eggs spawned of common sole primo May to medio June of wild and cultured broodstock. Size of eggs shown as egg diameter (mm egg^{-1}) and egg d.w (mg egg^{-1}). Illustrated mean fertilization estimate (%) and hatching success (%) given as means ± s.d.

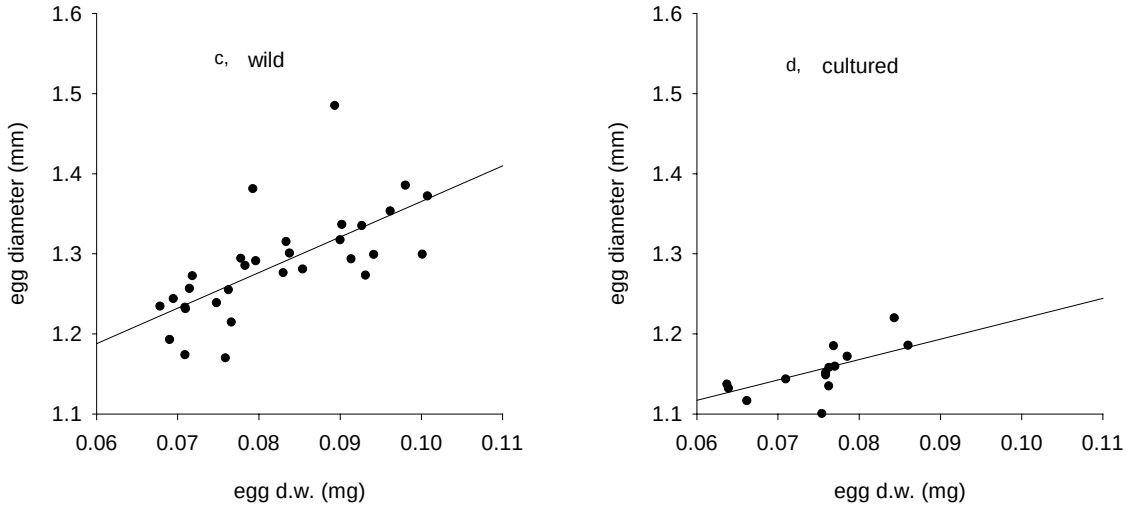
Eggs	Spawnings	Total spawned	Eggs day^{-1}	Egg diameter	Egg d.w.	Egg ash	Overall fertilization estimate	Hatching rate (all batches)	Hatching rate (batches with viable eggs)
Wild	28	1008220	36007 ± 31328	1.30 ± 0.05	0.08 ± 0.01	0.009 ± 0.003	> 50	8.9 ± 20.3 (n= 28)	31.7 ± 28.1 (n=9)
Cultured	14	423200	32170 ± 24420	1.15 ± 0.05	0.07 ± 0.01	0.007 ± 0.004	< 2	1.4 ± 4.1 (n=14)	4.8 ± 7.5 (n=5)

Diameter and weight of eggs from wild caught fish decreased with time and was significantly negatively correlated to temperature ($P < 0.001$) (Fig.1a). However, this relation was not observed for cultured sole eggs ($P = 0.3$-0.8) (Fig 1b). Diameter of both wild and cultured eggs were significantly positively correlated to an increase in d.w. ($P<0.001$, $r^2=0.46$ and $P = 0.004$, $r^2=0.48$ respectively) Fig 1c-d.

Figure 1 a-d.

Egg diameter (mm) versus both temperature (a-b) and dry weight (c-d), during the spawning season, primo May to medio June for batches of eggs of wild and cultured common sole.





Fatty acid composition of eggs

The relative TFA content (mg g^{-1} d.w.) was significantly higher for eggs from cultured broodstock. ($P > 0.001$) (Table 2). In both groups, the relative fatty acid composition (% TFA) was dominated by PUFAs of the (n-3) series, accounting for 36.3 percent of TFA in eggs from wild fish and 34.3 percent in eggs from cultured broodstock ($P = 0.029$). Docosahexaenoic acid (DHA), C22:6(n-3) was the most abundant fatty acid and comprised 31.4 percent and 30.4 percent in the two groups respectively. EPA content, was significantly lower ($P < 0.001$) in eggs from cultured broodstock than in eggs from wild fish (2.8 percent as compared to 4.6 percent). Eggs from cultured sole, however, contained a slightly higher relative proportion of C20:3(n-3) and C18:3(n-3).
PUFAs of the (n-6) series were significantly higher in eggs from cultured fish (9.4 percent) than the 4.3 percent found in eggs from wild fish ($P < 0.001$). A 10 fold higher proportion of linoleic acid, C18:2(n-6) was observed in eggs from cultured broodstock (7.8 percent) as compared to the content in wild eggs (0.7 percent). The content of ARA, C20:4(n-6) was significantly lower ($P < 0.001$) in eggs from cultured broodstock (0.9 percent) than in eggs from wild fish (3.1 percent). The contents of saturated fatty acids were overall similar between the groups, while the monounsaturated fatty acids overall were significantly higher in eggs from wild fish.

The PLS discriminant analysis of the fatty acid profiles gave a satisfactory model for classifying samples to either wild or cultured origin (Fig. 2). The first principle component (PC) explained 95% of the Y-variance (distinguishing wild from cultured samples, Fig 2a), while the 2. PC explained 2% of the Y-variance (and mainly subdividing samples within the cultured and wild groups). The

Table 2.

Mean total fatty acid content (TFA) (mg g^{-1} d.w), and fatty acid composition (in % TFA) of eggs and larvae from wild and cultured common sole during a spawning season, primo May to medio June. A different superscript in a horizontal row between comparable groups defines a significant difference (P<0.05). P values indicated. No superscript or P value indicates no significant difference.

	Eggs Wild	Eggs Cultured	P value eggs	INVE feed	Larvae Wild	Larvae Cultured
Batches (n)	n = 17	n = 14		n = 1	n = 4	n = 4
TFA (mg g^{-1} d.w.)	92.7± 14.0^{a}	124.9 ± 17.7^{b}	P < 0.001	499.0	95.0± 28.0	132 ± 50.4
FA (% TFA)						
C14:0	3.3±0.4^{b}	2.9±0.3^{a}	P = 0.004	6.2	3.2±0.8^{b}	2.5±0.6^{a}
C15:0	0.9±0.1^{b}	0.6±0.3^{a}	P < 0.001	0.7	1.2±0.3^{b}	0.8±0.3^{a}
C16:0	21.5±1.0	21.0±1.1		17.9	20.5±1.2	22.0±0.6
C17:0	0.8±0.2	0.7±0.6		0.6	1.2±0.3	1.0±0.7
C18:0	6.7±1.8	6.4±1.2		4.1	7.9±0.5	5.8±1.0
C20:0	0.1±0.0	0.2±0.2		0.3	0.3±0.2	0.4±0.2
C21:0	0.0±0.0	0.0±0.1		0.0	0.0±0.0	0.0±0.0
C22:0	0.0±0.0	0.1±0.2		0.2	0.1±0.0	0.1±0.0
C24:0	0.0±0.0	0.0±0.0		0.1	0.1±0.0	0.0±0.0
total saturated	33.3±2.6	31.9±2.1		30.1	34.6±0.9	33.9±2.1
C14:1 (n-5)	0.0±0.0	0.0±0.0		0.1	0.0±0.0	0.0±0.0
C15:1 (n-5)	0.0±0.0	0.0±0.0		0.0	0.1±0.0	0.0±0.0
C16:1 (n-7)	9.8±0.7^{b}	5.5±0.3^{a}	P < 0.001	5.4	8.8±3.1^{b}	4.6±0.7^{a}
C17:1 (n-7)	0.8±0.2^{b}	0.2±0.1^{a}	P < 0.001	0.4	0.7±0.5^{b}	0.1±0.1^{a}
C18:1 (n-9) t	0.0±0.0	0.0±0.0		0.0	0.0±0.0	0.0±0.0
C18:1 (n-9) cis	14.5±0.9^{a}	15.9±1.2^{b}	P < 0.001	14.5	13.4±0.4^{a}	13.8±1.4^{b}
C20:1 (n-9)	0.7±0.2^{a}	2.4±0.3^{b}	P < 0.001	7.7	0.5±0.0^{a}	2.4±0.1^{b}
C22:1 (n-9)	0.1±0.0^{a}	0.2±0.1^{b}	P < 0.001	0.0	0.2±0.0^{a}	0.2±0.1^{b}
C24:1 (n-9)	0.2±0.1^{a}	0.3±0.1^{b}	P = 0.008	0.0	0.3±0.1	0.3±0.1
total monounsaturated	26.1±1.2^{b}	24.5±1.4^{a}	P = 0.002	28.1	22.9±2.9^{b}	21.4±2.0^{a}
C18:2 (n-6) t	0.0±0.0	0.0±0.1		0.0	0.0±0.0	0.0±0.1
C18:2 (n-6) c	0.7±0.1^{a}	7.8±0.5^{b}	P < 0.001	11.2	0.8±0.1^{a}	8.6±0.9^{b}
C18:3 (n-6)	0.1±0.0	0.1±0.0		0.1	0.0±0.0	0.1±0.0
C20:2 (n-6)	0.4±0.1	0.4±0.1		0.5	0.4±0.1	0.4±0.0
C20:3 (n-6)	0.1±0.0	0.1±0.0		0.2	0.1±0.0	0.3±0.3
C20:4 (n-6), ARA	3.1±0.8^{b}	0.9±0.2^{a}	P < 0.001	0.9	3.3±0.9^{b}	0.8±0.3^{a}
C22:2 (n-6)	0.0±0.0	0.1±0.1		0.0	0.0±0.0	0.0±0.0
total (n-6) PUFA	4.3±0.8^{a}	9.4±0.6^{b}	P < 0.001	12.9	4.6±0.9^{a}	10.1±0.6^{b}
C18:3 (n-3) c	0.2±0.1^{a}	0.8±0.1^{b}	P < 0.001	2.8	0.2±0.1^{a}	1.1±0.5^{b}
C20:3 (n-3) c	0.1±0.0^{a}	0.2±0.1^{b}	P < 0.001	0.2	0.1±0.1^{a}	0.1±0.0^{b}
C20:5 (n-3), EPA	4.6±0.8^{b}	2.8±0.4^{a}	P < 0.001	10.1	4.6±1.1^{b}	2.7±0.6^{a}
C22:6 (n-3), DHA	31.4±2.3	30.4±3.1		15.8	33.0±1.2	30.7±3.2
total (n-3) PUFA	36.3±2.1^{b}	34.3±3.3^{a}	P = 0.029	28.9	37.9±2.1^{b}	34.6±3.9^{a}
DHA/EPA	7.1±1.5^{a}	10.8±1.4^{b}	P < 0.001	1.6	7.2±1.2^{a}	11.3±1.3^{b}
ARA/DHA	0.1±0.0	0.0±0.0	P < 0.001	0.1	0.1±0.0	0.0±0.0
ARA/EPA	0.7±0.1^{b}	0.3±0.1^{a}	P < 0.001	0.1	0.7±0.2^{b}	0.3±0.1^{a}
(n-3)/(n-6)	8.7±1.8^{b}	3.7±0.5^{a}	P < 0.001	2.2	7.8±1.3^{b}	3.0±0.4^{a}

regression coefficients for the significant variables obtained by leave-one-samples-out cross-validation are shown in table 3. Three cultured samples were regarded as outliers due to

extraordinary high C17:0 and C15:0 content (compared to the rest of the cultured group) and left out of the analysis.

Table 3

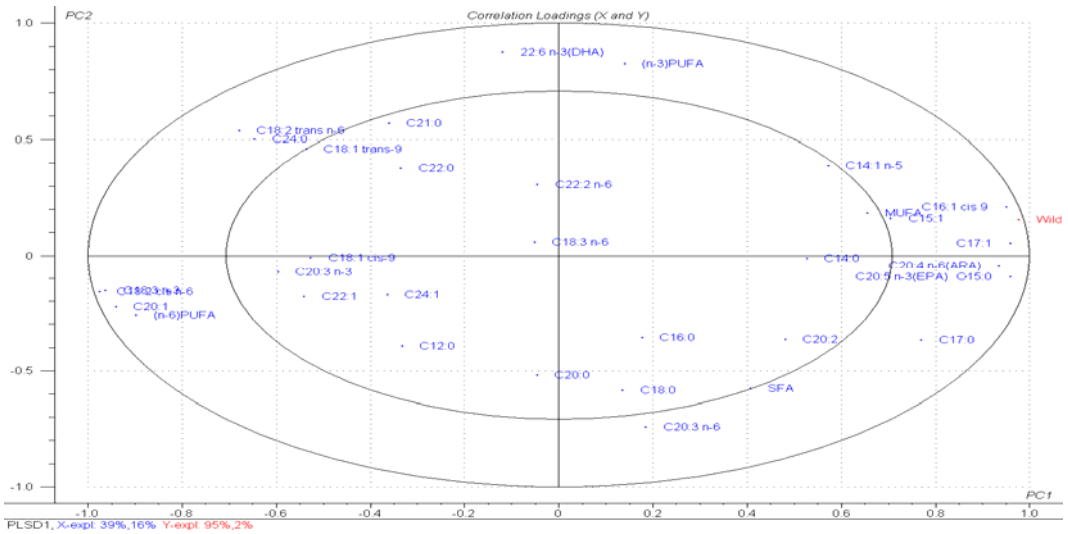
Regression coefficients (positive,negative) for the most important significant variables in the DPLSR model discriminating wild from cultured FA profiles. ([a]1 PC model)

Fatty acid Variable	Regression coefficient[a]
C15:0	0.071
C16:1n-7	0.074
C17:1	0.071
C20:1n-9	- 0.074
Total MUFA	0.054
C18:2 trans	-0.046
C18:2 cis	-0.076
C20:4 n-6, ARA	0.067
Total n-6 PUFA	-0.073
C18:3 cis n-3	-0.075
C20:5 n-3, EPA	0.060

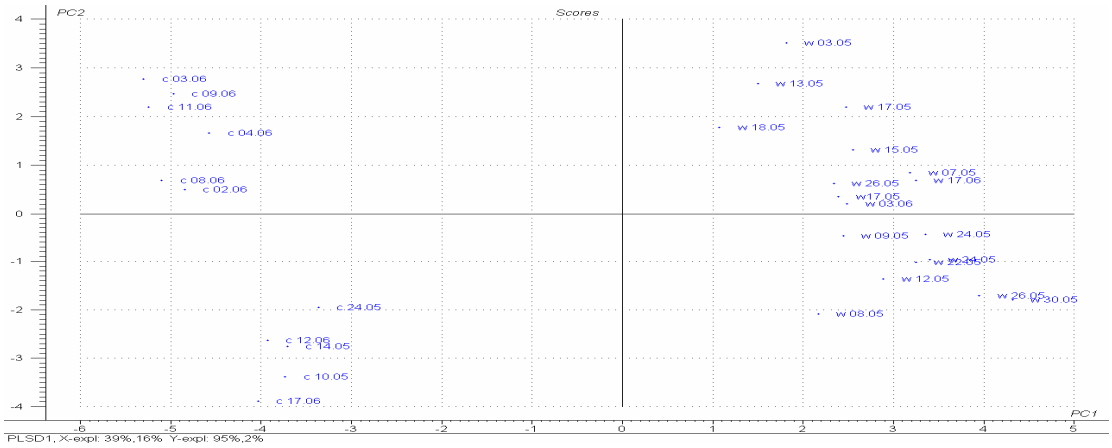
Thee discriminate function very clearly shows the differences between the wild and cultured groups as also formerly described and listed in table 2. Thus, most negatively on the 1.st PC (X-axis) the cultured group are correlated to high linoleic acid and overall total (n-6), high C20:1 and C18:3(n-3) (Fig.2b). Opposite is the wild group characterized by a high monounsaturated acid content, in particular C16:1 cis 9 and C17:1, and a high ARA and EPA content. The model also shows that total (n-3), and thus in particular DHA, were not good discriminatory factors for separating wild from cultured. EPA (n-3) was, however, characteristically higher levelled in wild samples, as C18:3 (n-3) was in the cultured. The Y-axis (PC 2) divides the two groups into subgroups explained by their differences in particularly C20:3 (n-6), DHA and total (n-3) content. This subgrouping or variability within the cultured or wild groups did not seem to be directly correlated with time, survival or other experimental recordings.

Figure 2.a-b.

Scores (a) and Correlation loadings (b) plot of discriminant-PLS analysis of fatty acid profiles of eggs from wild and cultured broodstock. Ellipsoids indicate 50 and 100 % explained variance for X- and Y-variables. Clear distinction of wild from cultured samples is achieved with 1 PC (X-axis) correlating high content of C18:2(n-6), total (n-6) PUFA, C18:3(n-3), C20:1 with cultured origin, and high C16:1, C17:1, C15:0, ARA and EPA with wild.



a)



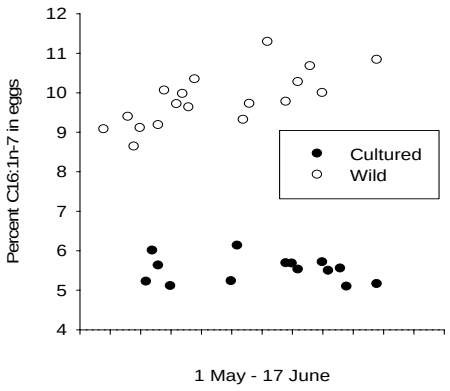
b)

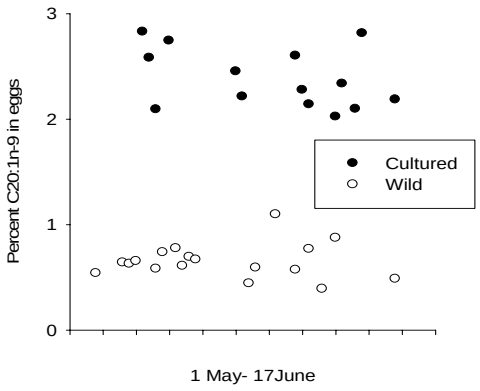
The FA profiles of wild eggs and cultured eggs were very consistent during the spawning season as illustrated for the specific fatty acids C16:1n-7, C20:1n-9, C18:2n-6, C20:4n-6, C18:3n-3 and C20:5n-3, characteristic for each group (Fig. 3a-f). ($P < 0.001$ for all illustrated groups).

Figure 3 a-f.

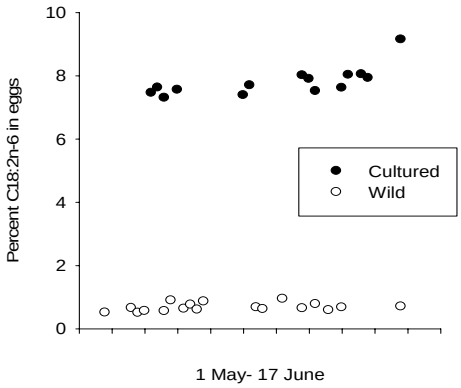
Fatty acids profiles in obtained batches of eggs of wild or culture origin during the spawning season The illustrated fatty acids were characteristic for the differences in profiles

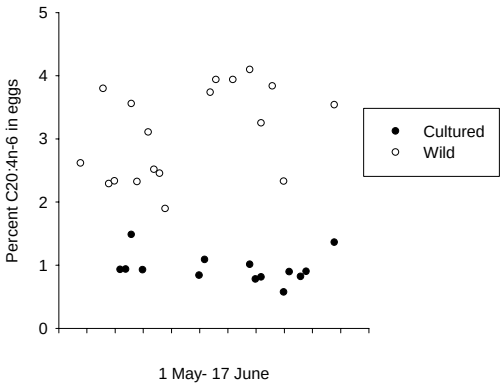
a) *b)*



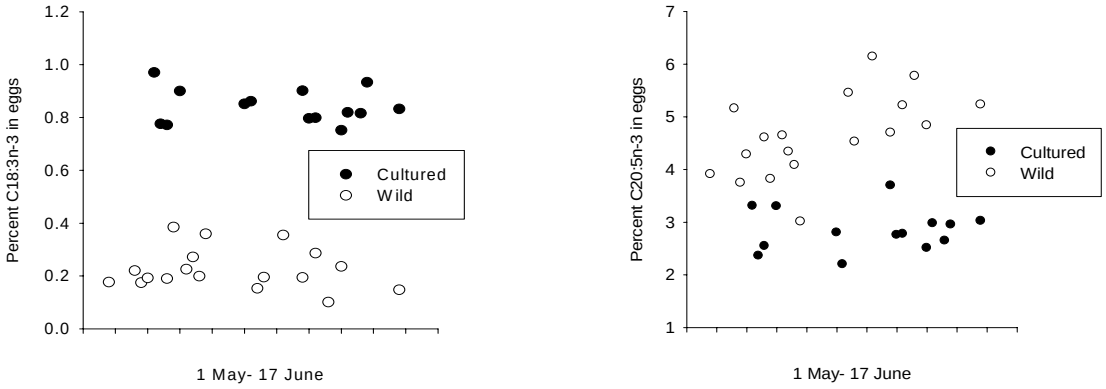


c) *d)*





e) *f)*



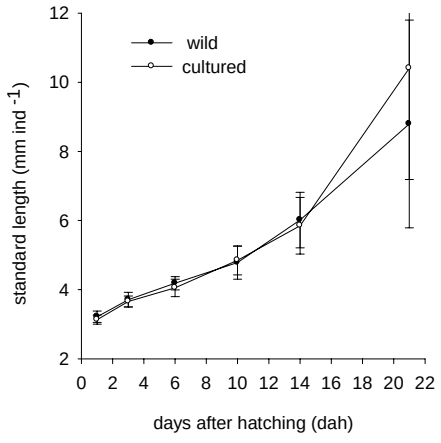
Hatching rate and growth of larvae

Hatching rate was 72 percent in larvae from wild fish and 17 percent in larvae from cultured broodstock (i.e. June 1 incubated egg batches). Dry weight of larvae from cultured broodstock (0.035 ± 0.003 mg individual^{-1}) and wild fish (0.037 ± 0.002 mg individual^{-1}) did not differ. The standard lengths were 2.34 ± 0.20 mm and 2.91 ± 0.26 mm, respectively. At the end of the larval period the mean survival of larvae from cultured broodstock was significantly lower than for larvae from wild sole. ie. 14 percent as opposed to 50 percent.

There were no significant differences ($P > 0.1$) in growth (length or weight) throughout the larval rearing period (Figure 4).

Figure 4.

Standard length (mm ind^{-1}) of wild and cultured larvae until 21 days after hatching.



Discussion

Fish egg quality such as fertilization success, hatching rate and larval growth may be related to the size of the eggs (Trippel, 1998, Lavens et al., 1999, Palumbi, 2004). For many fishes female age or size may determine the size of eggs (Bromage et al.,1990, Kamler, 2005) and therefore may be indicators of reproductive success in fishes, determining the amount of metabolic reserves in the eggs (Berkely et al., 2004). The smaller size and presumably younger age of the

cultured spawners in the present study, (i.e 3 years old) therefore most likely explain the lower mean size of eggs and the lower fertilization success and hatching rate compared to eggs of wild origin. However, individual relations between spawner and egg size were here prevented by the fact that natural spawning and fertilization took place in tanks holding all broodstock individuals.
Both egg size and larval survival increased in the 3 year old cultured sole as compared with a complete lack of hatching success in eggs from the same 2 year old breeders (own observations). As suggested, this indicates a crucial effect of maternal age/size.
The low fertilization rate in this study may also have been related to a low sperm production or inferior sperm quality (i.e total number of spermatozoa), as observed for cultured senegalese sole (Cabrita et al., 2006), but was not studied at present.
The low but variable survival rates in eggs from wild breeders were presumably related to other factors than female age or size. Low viability in sole eggs has been observed when broodstock was transferred from the wild to small tanks (Devauchelle et al., 1987). In the present study the wild breeders initiated to spawn less than one week after capture, but an extended acclimatization period to tanks may have improved egg viability (Baynes et al.,1993). Other stress factors may have been the lack of sandy substrate or the relatively high density of individuals in the tank (Baynes et al. 1993).
Early indicators of egg viability may be egg transparency, as in turbot (McEvoy, 1984) or symmetrical early cleavage as in sea bream (Sakai et al.,1985). Egg morphology parameters, however, may be difficult to assess in case of natural fertilization in tanks due to lack of the earlier stages of embryonic development (Giménez et al., 2006) and was therefore not examined. In order to test if egg quality may be adversely affected by culture related conditions, instead, egg quality was tried recorded by their buoyancy. Eggs of sole will float when fertilized (Anguis and Cañavate, 2005), and sink if development ceases (Unuma et al.,2005). Egg buoyancy seems therefore to be a useful indicator in assessment of egg quality. In support of this, eggs from cultured broodstock were characterized by a very low percentage of fertilized floating eggs and a low hatching rate
In batches of eggs with no survival microscopical analyses revealed an initial cell cleavage, but eggs died before the neurula stage. In marine fish eggs, especially two periods of elevated mortality have been reported; just after fertilization and during hatching (Kamler, 2005), this pattern was however, not supported by our observations here. In fact most eggs died before completion of gastrulation. The reasons for this failure are not known, but stress may cause overripening of eggs,

ovulated but not oviposited eggs can result in a lack of oxygen in the abdominal cavity. This has been reported to cause an increased mortality during the morula stage (Kamler , 2005).

The positive correlation between increasing water temperature (8.4 to 14.6 º C) and decreasing diameter of released eggs from wild fish has previously been reported for both common sole and senegalese sole (Houghton et al., 1985; Devauchelle et al.,1987; Baynes and Howell, 1996; Dinis et al.,1999). Higher metabolism at higher temperatures may reduce available reserves for vitellogenesis which affects egg size as previously suggested for common sole(Devauchelle et al.,1987). Another explanation may be, that larger and older individuals are more likely to spawn at lower temperatures than smaller individuals (Rijnsdorp and Vingerhoed, 1994; Ramsey and Witthames, 1996).Visual observations in this study, however, could not confirm this, as large individuals with swollen abdomens also were observed at high temperatures. The fact, that the size of cultured eggs was not related to the temperature increase, supports the hypothesis that the size of eggs mainly relates to the age or size of the female spawner.
The higher viability in June than in May indicates that temperature may have had an influence on the egg maturation process in this study, but should be further investigated. The optimal temperature for incubation of sole eggs has been reported to be 13-15 º C, which is higher than the optimal temperature reported for spawning, i.e. 10.5 º C (Devauchelle et al., 1987). However, common sole reproduction in the North Sea usually initiates at 7-9 º C (Fonds, 1979) and others have reported temperatures from 8 to 12.5 º C to be optimal (Anguis and Cañavate, 2005). This suggests a certain stock/strain specific spawning behaviour.

The relative fatty acid content was higher in eggs from cultured broodstock, than in eggs from wild breeders, which indicates a higher total lipid content in cultured sole eggs as most fatty acids were quantified. Wild sole prey mainly on polychaetes, molluscs and crustaceans (Cabral, 2000) with a lower fat content (Saborowski and Buchholz, 1996) compared to the extruded broodstock feed used (i.e 16 % lipids) and wild sole have a general lower tissue fat content than aquaculture reared (personal observations). A 16 % total lipid content has been reported for eggs of senegalese sole (Mourente and Vázquez, 1996 in comparison to the present 9 % and 12 % TFA (% d.w.) in eggs of wild and culture origin. It is therefore suggested, that the lipid levels deposited in sole eggs may be correlated with the level in the broodstock diet as reported for gilthead seabream (Harel et al.,1992).

Using total lipid content as an indicator of egg quality is however controversial as literature contains contradictory results (Rainuzzo, 1993).

Based on the FA profiles of wild and cultured common sole eggs, PLS discriminant analysis showed that the relation of a few specific FA clearly distinguished the two groups, and thus egg origin could be determined solely by the FA composition. Fatty acid profiling therefore seem to be a highly accurate method for differentiating eggs of wild and cultured sole as previously reported for differentiating cultured and wild striped bass (*Morone saxatilis*), (Seaborn et al., 2000) and as indicated for eggs of wild and cultured fish of red snapper (*Lutjanus campechanus*), (Papanikos, 2005). A relatively high content of C20:1, C18:2(n-6), C18:3(n-3) characterized the cultured eggs / larvae, whereas high C16:1, ARA and EPA signified wild eggs / larvae. Overall, the FA profile of the cultured eggs correlated to the dietary composition fed the cultured broodstock (Kjoersvik et al.,1989), with only DHA and EPA showing some inconsistencies. The FA profile of both cultured and wild eggs was very consistently different during the spawning season (Fig.3a-f). In addition, the TFA content or FA profiles were similar in batches of wild eggs with high or low viability as reported for common dentex (*Dentex dentex*) (Giménez et al., 2006). This indicates, that FA composition of developing oocytes may remain relatively unaffected by factors such as a low feed intake or post capture stress and therefore FA composition may be determined prior to the spawning season. This further implies, that FA composition may be a poor determinant of egg viability within culture or wild origin, but may be useful when comparing the quality of cultured eggs with wild eggs.

The FA composition of both wild and cultured common sole eggs indicated a predominance of n-3 PUFAs, followed by saturated fatty acids, monounsaturated fatty acids, and finally n-6 PUFAs (Table 2). This order of appearance was also analysed in one day old senegalensis larvae (Villalta et al., 2005). On the contrary, results by Morais et al. (2004) indicated that total PUFA content was the lowest FA fraction in eggs from wild caught senegalese sole.

The FA profile of the eggs and larvae from cultured broodstocks showed a high resemblance to the FA profile in the broodstock feed, except that the relative EPA content was much lower - and the DHA content much higher than in the feed. The inconsistency in the relative much higher DHA content in cultured eggs than in the pelleted feed, combined with the findings of no significant difference between DHA content in cultured and wild eggs, suggests that DHA plays an essential role in egg / larvae development, and is accumulated or retained during maturation of broodstock. A high proportion of DHA is consistent with general findings in marine fish larvae (Reitan et al.,

1994; Mourente and Vázquez, 1996; Næss and Lie, 1998; Morais et al., 2004; Villalta et al., 2005), and a high content has been related to specific requirements for DHA during the early larval development of neural tissues as brain and retina, as these tissues are highly enriched in DHA (Silversand et al., 1996).

The dominant role of linoleic acid C18:2(n-6) in the cultured eggs/larvae was due to the high content of this FA in the feed. High presence of linoleic acid in egg lipids is typical for fish given a formulated diet containing vegetable meals (Silversand et al., 1996, Gallagher et al., 1998). The high content of C20:1n-9 in broodstock feed and cultured eggs indicates the presence of lipids originating from calanoid copepod feeding fish such as herring (*Clupea harengus*) and capelin (*Mallotus villosus*) in the feed formulation, as these fish store this particular fatty acid (Sargent et al., 1995; Silversand et al., 1996).

ARA is an important constituent of phospholipids and an important precursor of prostaglandins, and may therefore be involved in determining general egg quality (Gallagher et al., 1998). Bell et al. (1996; 1997) related a lower viability of sperm and eggs, and a decreased hatching rate in sea bass (*Dicentrarchus labrax*) eggs, to a decreased ARA: EPA ratio in pelleted broodstock diets. Moreover, increased egg quality in cod (*Gadus morhua*) has been related to increased levels of ARA (Salze et al., 2005) and an improved egg quality in repeat spawning Atlantic halibut (*Hippoglossus hippoglossus*) has been related to increased levels of DHA and ARA, as compared to first time spawners (Evans et al., 1996). In the present study, eggs and larvae from wild fish were comparably rich in ARA, as has been shown for wild Striped bass (*Morone saxatilis*) (Harrell and Woods, 1995). The relative ARA content in the cultured eggs was relatively lower and similar to the content in the broodstock feed. Apparently the cultured broodstock did not accumulate ARA in the eggs by retaining, or elongation of C18:2(n-6) and the desaturase activity in marine fish may be too low to produce appreciable amounts of ARA from C18:2(n-6) (Harrell and Woods, 1995). The higher ARA proportion in wild sole eggs is therefore suggested related to a natural high dietary content more than accumulation or elongation. Based on the present results, no association was observed between improved egg viability and ARA content within batches of cultured or wild eggs. However, both egg quality and ARA content was much lower in cultured eggs which indicates a possible relation, perhaps masked by other factors. Hence, a consideration of ARA supplementation to the broodstock diet may be required as similarly suggested for farmed cod (Salze et al., 2005).

The fatty acid requirement for larvae may reflect the fatty acid composition of the yolk profile of the eggs, (Morais et al., 2004). As ARA may be an important fatty acid in determining egg quality

in sole, the function of dietary ARA should be investigated in relation to the content in egg lipids and phospholipids of the yolk sac.

Egg quality is usually estimated not only from the study of the egg, but also from the evaluation of larval performance. Newly hatched wild larvae were significantly larger than cultured larvae, corresponding to previous reports on sole (Baynes and Howell, 1996). This was related to differences in egg size as egg and larval size is correlated (Lavens et al.,1999).Growth was very similar for the two groups though, as the small initial difference in length continued until larvae were 15 days old. However, the mortality of larvae from cultured broodstock was much higher than of larvae from wild broodstock. The lower survival of cultured larvae may be related to the smaller size of eggs (Rijnsdorp and Vingerhoed, 1994; Kamler 2005) and consequently the lower amount of yolk reserves, which may decrease the time period for larvae to develop feeding skills before irreversible starvation (Baynes et al., 1996).

TFA and the relative FA profile from newly hatched larvae of both groups appeared similar to their egg profiles as has been reported for Senegalese sole (Parra et al., 1999), except for a slight decrease in C16:1n-7 and C18:1n-9 for both larval groups. It is likely that these two FAs are used as energy source during early development from fertilized eggs to yolk sac larvae, as recorded for Senegalese sole and common sole (Vázquez et al., 1994, Lund et al, unpublished data).

Conclusions and recommendations

Wild or culture origin could be clearly determined by PLS analysis of FA composition of sole eggs, but egg quality based on hatching rate was not related to fatty acid profiles within batches of eggs from wild or cultured broodstock. It was demonstrated, that it is possible to obtain viable eggs and good quality larvae from cultured sole broodstock. The growth rate of larvae was not related to egg or larval size, FA composition or broodstock origin. Conclusively, aquaculture related conditions seemed not to affect cultured offspring quality.

Improvement of general egg quality along with the causes which affect this, however, must be further investigated based on analyses of biochemical composition and morphological characteristics of eggs.

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II

The consequence of the species of live prey and their FA composition on early fitness of common sole larvae (*Solea solea* L.).

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The consequence of the species of live prey and their FA composition on early fitness of common sole larvae (*Solea solea* L.)

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Running head: The influence of live prey on sole larvae

Key words: Sole larvae, *Tisbe*, *Artemia*, rotifers, growth, fatty acids, pigmentation

Abstract

Early larval growth, fatty acid (FA) composition and pigmentation success was examined in common sole larvae *Solea solea* (Linnaeus 1758) feeding on 3 species of prey from day 3 to day 9 post hatch; the harpacticoid copepod *Tisbe holothuriae,* the rotifer *Brachionus plicatilis,* and the crustacean *Artemia* sp**.** Starving sole larvae were used as a control. Sole larval survival was not affected by the treatments. Larval growth was significantly higher for larvae fed *Artemia* than *Tisbe* and negative for starving sole larvae and larvae fed rotifers. Prey fatty acid composition was different for the 3 species. Sole larval FA composition gradually approached the composition of *Artemia,* while larvae fed rotifers resembled the fatty acid composition of starving larvae. C22:6n-3, docosahexaenoic acid, DHA was the most dominant FA and was conserved in larvae feeding on rotifers and *Tisbe* and in starving larvae. C20:4n-6, arachidonic acid, ARA was retained in all larval groups. Subsequent juvenile survival was highly affected by the initial feed and growth was inversely related to the final juvenile density in the rearing tanks. Pigmentation was not affected by initial dietary treatment as more than 95 percent of all individuals were normally pigmented.

Introduction

Common sole (*Solea solea*) is a potential candidate for aquaculture, due to its high market price and demand. The commercial production of common sole is still limited. This may be explained by its high temperature requirement (Fonds, 1979, Howell, 1997, Imsland et al., 2003); temperatures which in Northern Europe are feasible only by the use of recirculation technology. The culture of

larval sole is considered relatively easy (Howell, 1997*),* but larval and juvenile response to dietary fatty acids are not fully understood.

Failure to provide adequate quantities of dietary fatty acids is a prime cause for unsuccessful rearing of marine fish larvae (Watanabe and Kiron, 1994). Lipids are very important, as both providing energy and as structural components (Vázquez et al., 1994). The level of essential fatty acids (EFAs) especially eicosapentanoic acid (EPA, C20:5n-3) and docosahexanoic acid (DHA, C22:6 n-3) is considered important for growth (Watanabe and Kiron, 1994, Sargent et al., 1995) and for the formation of cell membranes important for normal development of the vision and the neural system (Mourente and Tocher, 1992, Sargent et al., 1993).

The traditional types of live feed for first feeding Senegalese sole larvae are rotifers and *Artemia,* whereas *Artemia* is used for common sole larvae (Howell, 1997, Caňavate and Fernanděz, 1999). A comparison of the two prey types on larval growth has hitherto not been carried out.

Enrichment of *Brachionus* and *Artemia* may create unbalances in n-3, - n-6 HUFAs leading to low rearing performance (Støttrup and Attramadal, 1992, Støttrup, 1993). On the contrary, copepods have been recognized as having a more suitable nutritional profile (Heath and Moore, 1997, Støttrup, 2000, Drillet et al., 2006). Calanoid and harpacticoid copepods are the natural food items for sole larvae and newly settled juveniles (Amara et al., 2001, Villalta et al., 2005). Copepods are difficult to mass cultivate, but *Tisbe holothuriae* are produced in a recirculation production system at DIFRES. *Tisbe holothuriae* as opposed to *Brachionus* and *Artemia* are able to synthesize significant amounts of EPA and DHA, when fed PUFA poor diets (Støttrup, 2003) and is able to elongate and desaturate fatty acids of the linolenic acid n-3 family 18:3n-3 to 20:5n-3 and 22:6n-3 (Støttrup, 1993, Nanton and Castell, 1998a). The effect by the live feed types *Tisbe* and *Artemia* were compared on sole by Heath (Heath and Moore, 1997).The results were far from conclusive, but *Tisbe* seemed to be an acceptable food source. However, the results indicated a better growth and less juvenile malpigmentation by a mixed diet of *Artemia* and *Tisbe* when compared with a *Tisbe* diet. In other studies, *Tisbe* were concluded superior to rotifers in rearing of haddock (*Melanogrammus aeglefinus*) and plaice (*Pleuronectes platessa*) (Nanton and Castell, 1998b).

The n-3 HUFA requirement is lower for sole than for many other marine fish species, and *Artemia* strains rich in eicosapentaenoic acid 20:5(n-3) may be nutritionally sufficient for *Solea solea* as earlier indicated (Howell and Tzoumas, 1991).

The main objective of the present study was therefore to evaluate early growth, 3-9 days after hatching (dah) of common sole larvae reared on 3 different live prey species i.e. *Tisbe holothuriae*,

Brachionus plicatilis or either enriched or unenriched AF strain *Artemia* sp. FA analyses of eggs and endogenous feeding larvae were conducted to study the utilization of FA`s at these development stages. The influence of the prey FA profiles on sole larval profiles and physiological performances was evaluated. Finally, it was examined, if the initial fed prey species would have any carry – over influence on the later survival, growth and pigmentation success of juveniles at day 39.

Materials and methods

Live prey

Tisbe holothuriae were reared in 12 cylindrical 150 l tanks. The copepods were cultured on an excess diet of *Rhodomonas salina* algae. Copepods passing a 120 µm nylon mesh sieve were used as feed in the experiment.

Artemia of the AF type was used (*Artemia* Systems, INVE, Belgium). *Artemia* cysts were incubated at a concentration of 2 g l^{-1} at 28 -30 ° C. As instar I nauplii of AF *Artemia* thrive on their energy reserves until moulting approximately 8 hrs after hatching (Lavens and Sorgeloos 1996, Dhont and Van Stappen 2003,), nauplii were harvested 8 -10 hrs post hatch at a mean body length of 0.54 ± 0.1 mm ind^{-1}. The *Artemia* were separated into two groups. One group was enriched for a minimum of 8 hrs with a stirred emulsion of Easy DHA Selco, at a concentration of 0.6 g l^{-1}. The other group was left unenriched for a similar period. The body length of *Artemia* at 20 hrs posthatch was similar for both groups (Table 1).

Table 1

Prey species used as feed, size and dryweight, d.w. given as a mean ± standard deviation. Energy content J mg^{-1} calculated based on analysed TFA, d.w. and ash content. Protein and carbohydrate contents estimated from literature values.

Prey species used	Stages	Body length (mm)	D.w.(mg ind^{-1})	Est. Energy content (J mg^{-1})
Tisbe (nauplii, copepodites)	all	0.20 ± 0.09	1.4 ± 5.5 x 10^{-3}	21.0
Rotifers	÷ eggs	0.12 ± 0.02	3.7 ± 2.2 x 10^{-4}	18.9
AF Artemia nauplii unenriched	II-III (20 hrs post hatch)	0.60 ± 0.08	2.3 ± 1.1 x 10^{-3}	21.1
AF Artemia nauplii enriched	II-III (20 hrs post hatch)	0.61 ± 0.07	2.8 ± 0.8 x 10^{-3}	21.4

Artemia as well as *Tisbe* were harvested once a day in the morning and fed to the sole larvae. One half was refrigerated at 5 ° C with a slight aeration until the second feeding.

Rotifers *(Brachionus plicatilis*) were reared (25° C) on marine oils (0.02 g Super Selco, INVE, Belgium) and yeast, 1.0 g paste $million^{-1}$ rotifers supplemented by the phytoplankton T- *Isochrysis* sp. in a concentration of 0.1-0.2 x 10^{6} cells $million^{-1}$ rotifers. Rotifers were enriched for 10-12 hrs with a concentration of 0.4 g Easy DHA Selco and one l T- *Isochrysis* sp. algae per million rotifers.

Enriched rotifers were harvested and used twice daily. Rotifers passing a 90 µm nylon mesh were used to avoid rotifers with eggs. The microalgae *Isochrysis* (T-Iso) was used as green water at dah 3-5. Algae were added 2 times a day at a density of 130.000 cells ml $^{-1}$.

Prey was sampled at dah 3, dah 4, dah, 6 and dah 8 prior to feeding. Prey size, fatty acid composition and dry weight, d.w. were analysed. The prey for analyses were filtered on a 60 µm filter net and gently washed with 0.2 µm seawater (>15°C) to remove excess emulsion of enriched *Artemia* and rotifers. A representative sub sample of prey (>100 sample^{-1}) was counted, filtrated and frozen until analysis.

Unenriched and enriched *Artemia* had an almost similar d.w. (table 1). The d.w. of rotifers and *Tisbe* constituted about 16 and 59 percent of the d.w. of unenriched *Artemia,* respectively (table 1). The body length was different between prey species and smallest for rotifers. The *Tisbe* population contained all different stages and sizes from nauplii with a mean length of 0.13 ± 0.03 mm ind $^{-1}$ and copepodites / copepods of 0.30 ± 0.04 mm ind^{-1} (including spines; 0.40 ± 0.05 mm).

Broodstock handling and spawning conditions

Broodstock sole were caught from the North Sea, Skagerak in gill nets at the end of April. 350 fish in the size range 0.3 to 1.2 kg pcs. $^{-1}$ were transferred to a broodstock holding facility. The system consisted of three 9 m^3 flow through tanks with dimmed natural overhead light. The fish were offered fresh mussels (*Mytilus edulis*) and sand worms (*Aremicula marina*) in excess daily. The spawning started at the beginning of May.

Eggs were collected by 900 µm mesh egg collectors installed in a reservoir connected to the surface outlet from the tanks. Approximately 68000 eggs were transferred to a 160 l upflow cylindrical incubator, where they were kept in suspension by a gentle aeration. The eggs were incubated at the following conditions: 24 hrs dim light; temperature between 11.1 - to 11.4 º C, a water exchange of 100 percent hr $^{-1}$, pH between 7.96 and 7.98, no detectable levels (< 0.1 mg l^{-1}) of nitrate, nitrite or ammonia, and the oxygen level was close to 100 percent.

A total of 6600 dead eggs (sinking) were removed during incubation. Hatching began after 48 hrs. The temperature was gradually elevated to 16.2 º C.

Larval rearing

The larvae were moved to the rearing facility at day 2 after hatching (dah 2) and kept in the system until dah 10. The rearing facility consisted of 30 transparent cylindrical - conical tanks containing 46 l each, connected to a flow through water system of 1 µm filtrated seawater. Each tank was

equipped with a separate inlet tap with a flowmeter, a 500 µm outlet filter, and filtrated aeration (0.45 µm) through airstones. An equal number of approximately 2150 larvae were transferred to each of 15 tanks (triplicate setup) giving a density of approximately 47 larvae l^{-1}. Rearing temperature was adjusted to approximately 18 ° C. The water flow was kept at 8-10 l $hr.^{-1}$. However, water exchange was increased to 80 l $hr.^{-1}$ one to two hrs prior to feeding to remove old prey items remaining in the rearing tanks.

Rearing water chemistry conditions were measured daily. Temperature 18.1 ± 0.3 º C, pH 8.0 ± 0.2, nitrite, nitrate and ammonia below kit detection levels, and oxygen content between 7.3 and 8.5 mg l^{-1}.

The photo period was 24 hrs. light. Live prey was administered at 8 a.m. and 8 p.m at a density of 5 ml^{-1} for all live prey. This despite differences in prey size, but to avoid possible density related effects. A live feed concentration of 5 ml^{-1} is often used for marine larvae (Temple et al., 2004) and considered in excess no matter prey type.

The larval sole were moved to a recirculation system at dah 11 and kept in separate tanks (triplicate setup for each treatment) at an initial similar density. The starving group of larvae was not transferred as due to a high mortality. All larvae received 24 hrs Easy DHA Selco enriched EG *Artemia* 3 times a day. At dah 39 all fish in all tanks were evaluated to estimate survival, growth and pigmentation rate. Larvae were classified as fully pigmented, or malpigmented.

Larval samplings

Larvae from each rearing tank were sampled at dah 3, 5, 7 and 9. All samples of larvae were collected prior to feeding in the morning. Fifty to 60 larvae were randomly sampled from each tank. Eggs (100 pcs. $sample^{-1}$) and larvae (10-15 pcs. $sample^{-1}$) were collected in duplicate pooled samples. They were counted and filtrated on Whatman Ø 25 mm 0.7 µm GF/F glass fibre filters. Five ml of 0.5 M v/v ammonium formate in distilled water was added to remove seawater from the dry matter samples. Filters for determination of d.w. were precombusted at 450 °C for 24 hrs and weighed on a Mettler Toledo MT5 d =0.1 µg. The filter samples were dried in an oven at 80 °C for 24 hrs until constant d.w. and then weighed. Samples of larvae for lipid analyses were filtrated and frozen at – 80° C until analysis.

Diameter of sole eggs and length of larvae was registered by use of a digital high resolution DFC 320 Leica camera connected to a dissecting microscope (MZ6) and measured by a Leica IM50 Image manager.

Lipid analysis

The fatty acid composition was determined by extraction of the lipids by a chloroform / methanol mixture, Folch (Folch et al., 1957) and sonicated in an ultrasound cleaner, model Branson, 2510. Then followed trans esterification of the lipids by acetyl chloride in methanol. The fatty acid methyl esters were analyzed by gas chromatography - mass spectrometry (GC-MS). Peaks were quantified by means of the target response factor of the fatty acids to a C23:0 internal standard. Peaks on a given chromatogram were identified by comparison with the retention time of a commercial mix of a known FAME standard, SUPELCO 18919 (C4:0-C24:0), from SIGMA. Fatty acid concentrations were calculated (Chem. Station programme) based on the quantified peaks of the standard series and the samples as well of dry weight of prey and larvae and expressed as ng sample^{-1}.

Statistics and growth calculations

All statistical tests for sole larvae growth and FA composition were carried out using a one- way ANOVA and a pairwise multiple comparison of means using Tukey`s test ($P < 0.05$). Variance is given as standard deviation (SD) of the mean of three replicates. Growth rate (SGR) was calculated as $SGR= (\ln W_f - \ln W_i \times 100)/ t$, Where $\ln W_{f\,i}$ = the natural logarithm of the final and initial the weight, t = time (days) between ln W_f and ln W_i.

Results

Larval survival

Sole larval survival was not statistically different ($P>0.501$) between treatments and ranged from 40.9 to 55.3 percent (table 2). The survival estimate included the about 200 larvae tank^{-1} sampled for analyses during the trial.

Table 2.

Larval survival and juvenile survival (%). Body length (mm ind^{-1}) and Specific growth rate (SGR) at dah 39. Data presented as a mean for each treatment group ± standard deviation. (n=3) A value followed by a different superscript in a column denotes a significant difference (P<0.05). No superscript indicates no significance.

Treatment	Malpigmentation	Survival (dah 0-9)	Survival (dah 11-39)	Survival (dah 0-39)	Length	SGR (dah 10-39)
Tisbe	4.2 ± 0.5	40.9 ± 8.7	57.6 ± 8.8^{b}	19.1	22.5 ± 3.1^{b}	20.2 ± 1.7^{b}
Rotifers	3.3 ± 2.1	47.3 ± 2.8	15.0 ± 1.87^{c}	7.1	24.9 ± 2.6^{b}	23.1 ± 1.0^{c}
AF *Art.* unenriched	2.9 ± 0.2	55.3 ± 25.1	92.1 ± 9.6^{a}	50.9	18.9 ± 2.1^{a}	13.4 ± 2.5^{a}
AF *Art.* enriched	3.8 ± 0.4	42.5 ± 8.4	100.0 ± 0.0^{a}	42.5	18.1 ± 1.6^{a}	12.8 ± 2.0^{a}
No feed	-	46.7 ± 5.4	-	-	-	-

Larval growth and carry over effect on juvenility

The mean egg diameter was 1.32 ± 0.03 mm. Larval length at hatching was 2.70 ± 0.23 mm ind $^{-1}$ and dry weight (d.w.) was 8.2 x 10^{-2} ± 4.2 x 10^{-4} mg ind. $^{-1}$. At dah 3 the body length had increased to 4.00 ± 0.16 mm whereas the d.w. was almost similar (7.1 x 10 $^{-2}$ ± 1.1 x 10^{-2} mg ind. $^{-1}$.)

At dah 5, larval body length was significantly larger when fed unenriched *Artemia* than larvae not fed (i.e. starving larvae) (P =0.05) (table 3). At dah 7 and dah 9 both the d.w. and body length of larvae fed unenriched and enriched *Artemia* were significantly larger (P < 0.001) than larvae fed with *Tisbe*, rotifers or starving larvae.

At day 7 the length of larvae fed *Tisbe* and rotifers was larger than for starving sole larvae (P <0.001). At dah 9, both larval d.w. and body length were significantly larger for larvae fed *Tisbe* than larvae fed rotifers or starving larvae (P <0.001). Larvae fed rotifers were not different from starving larvae.

Consequently, at the end of the trial starving larvae and larvae fed rotifers had a negative increase in d.w. and in daily specific growth rate (SGR) (table 3). Larvae fed *Tisbe* grew slightly, whereas larvae fed *Artemia* had highly significantly improved growth rates, (P> 0.001).

Table 3.

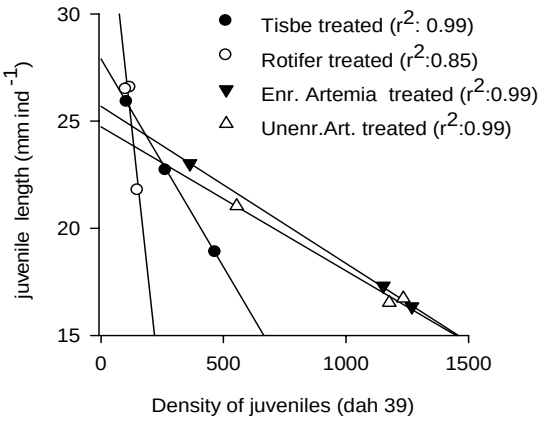
Larval d.w. (mg ind $^{-1}$) and body length (mm ind^{-1} at dah 5 - 9 given as a mean ± standard deviation. Specific daily growth rate (SGR) calculated based on the increase in larval d.w. A different superscript following a value denotes a significant difference (P < 0.05) between treatments at a particular day. No superscript indicates no significance

	dah 5		dah 7		dah 9		dah 3-9
Treatment	d.w.	length	d.w.	length	d.w.	length	SGR
Tisbe	0.070 ±0.003^{ab}	4.19±0.20	0.066± 0.005^{a}	4.49±0.30^{b}	0.079±0.005^{b}	4.63±0.36^{b}	1.7± 0.9^{b}
Rotifers	0.075±0.009^{ab}	4.30±0.21	0.057±0.002^{a}	4.48±0.24^{b}	0.050±0.001^{a}	4.32±0.29^{a}	-5.7±0.3^{a}
Artemia unenriched	0.084±0.009^{b}	4.15±0.22	0.126±0.011^{b}	5.10±0.27^{c}	0.242±0.010^{c}	5.83±0.39^{c}	22.7±0.8^{c}
Artemia enriched	0.078±0.003^{ab}	4.28±0.21	0.122±0.015^{b}	5.12±0.32^{c}	0.238±0.033^{c}	5.83±0.37^{c}	22.3±2.7^{c}
No feed	0.066±0.001^{a}	4.23±0.24	0.049±0.004^{a}	4.31±0.35^{a}	0.047±0.004^{a}	4.25±0.31^{a}	-6.5±1.2^{a}

Juveniles pre-fed with *Artemia* until dah 9 had a significantly higher survival than when fed *Tisbe* or rotifers (P < 0.001) lowest for the rotifer treated larvae (table 2). Survival affected growth rate (table 3), but growth was clearly density dependent (Fig. 1). Juvenile malpigmentation was low for all treatments (table 3) and there were no statistical differences in pigmentation between any of the treatments (P > 0.52)

Figure 1.

Correlation between larval length (mm ind. $^{-1}$) of sole juveniles and the density (number) of juveniles at the end of the trial (dah 39) illustrated for each replicate for the 4 live feeds.



Live feed FA

Total fatty acid content (TFA, mg g $^{-1}$ d.w.) in the prey organisms was significantly different ($P < 0.001$) (table 4) and highest in the *Artemia* prey. C16:0 and C18:0 were the most abundant of the saturated fatty acids (SFAs). The relative content of C18:0 varied significantly ($P< 0.001$) between the prey groups and was highest in rotifers and *Tisbe*. The monounsaturated fatty acids (MUFAs) C16:1 and C18:1 were highest in *Artemia* and significantly lowest in *Tisbe* ($P <0.001$).

Table 4.

Total fatty acid content (TFA, mg g^{-1}d.w.) analysed in the 4 live feeds and fatty acid composition (% TFA) given as a mean for each treatment ± standard deviation. n=4.

Totals include minor fatty acids not shown. A value followed by a different superscript denotes a significant difference (P<0.05). No superscript indicates no significance.

	Treatment			
	Tisbe	Rotifer	*Artemia* Unenriched	*Artemia* enriched
TFA	19.1±12.2^{a}	81.4±34.8^{b}	117.3±33.6^{bc}	126.4±23.4^{c}
FA (%TFA)				
C14:0	0.8±1.0	0.9±0.7	0.4±0.2	0.5±0.3
C16:0	13.0±6.2^{ab}	18.4±7.5^{b}	10.0±0.5^{ab}	9.5±1.7^{a}
C18:0	26.4±14.2^{bc}	39.9±13.5^{c}	6.5±3.7^{a}	4.5±1.8ab
Total SFA*	44.9±19.6^{b}	63.9±17.7^{c}	18.4±3.8^{ab}	15.9±1.6^{a}
C16:1 (n-7)	1.1±1.1^{a}	2.2±1.4^{a}	9.9±2.7^{b}	9.4±3.0^{b}
C18:1 (n-9) cis	4.3±2.1^{a}	10.9±4.6^{b}	14.6±1.0^{bc}	15.1±0.5^{c}
C22:1 (n-9)	0.8±0.6	2.0±2.5	0.2±0.2	0.6±1.6
Total MUFA*	7.4±2.4^{a}	17.4±7.2^{b}	26.6±3.3^{b}	27.0±3.7^{b}
C18:2 (n-6) c	4.8±2.6	4.3±2.3	3.8±0.4	4.0±0.5
C20:4 (n-6), ARA	1.0±0.7^{a}	0.3±0.3^{a}	2.7±0.2^{b}	2.7±0.3^{b}
total (n-6) PUFA*	6.6±4.1	4.8±2.6	7.1±0.4	7.6±1.1
C18:3 (n-3) c	4.4±2.5^{ab}	1.9±1.3^{a}	5.2±0.8^{b}	4.4±1.0^{b}
C20:5 (n-3), EPA	17.9±8.0^{b}	5.5±3.9^{a}	42.5±1.8^{c}	42.1±3.2^{c}
C22:6 (n-3), DHA	18.5±7.7^{b}	6.6±3.4^{a}	0.1±0.1^{a}	2.8±2.0^{a}
total (n-3) PUFA	41.1±16.5^{b}	14.0±8.4^{a}	47.9±1.7^{b}	49.6±3.4^{b}
DHA:EPA	1.11±0.37^{b}	1.48±0.82^{b}	0.00±0.00^{a}	0.07±0.04^{a}
ARA:DHA	0.05±0.04^{a}	0.03±0.03^{a}	25.12±10.98^{b}	0.97±0.63^{a}
ARA:EPA	0.05±0.02	0.04±0.03	0.06±0.00	0.06±0.01
(n-3):(n-6)	7.27±3.64^{b}	2.94±0.63^{a}	6.79±0.42^{b}	6.64±0.85^{b}

The main difference of the n-6 polyunsaturated fatty acids (PUFAs) was related to C20:4n-6, (arachidonic acid, ARA), which was significantly highest in *Artemia* ($P < 0.001$).

EPA, eicosapentaenoic acid, C20:5n-3 content was highest in *Artemia* constituting almost 43 percent of TFA while only 18 percent in *Tisbe* and 5.5 percent in rotifers ($P < 0.001$). Docosahexaenoic acid, DHA, C22:6n-3 was much higher in *Tisbe* (19 percent), than in rotifers (6.5 percent) or

Artemia (0.1 and 2.8 percent, respectively) ($P < 0.001$).

Consequently the DHA: EPA and ARA: DHA differed significantly between the live prey ($P < 0.001$), (table 4). The absence of DHA in unenriched *Artemia* lead to a much higher ARA: DHA. There appeared to be no significant differences in the ARA: EPA for any of the prey groups.

Larval FA

TFA in sole larval eggs and larvae (table 5) decreased significantly until dah 5. At dah 9 the TFA content was lowest for rotifer fed larvae and starving larvae ($P = 0.04$), reflecting the poor growth. In eggs, newly hatched larvae and pre feeding larvae the FA composition was quite similar and constant for most fatty acids with the exception of a marked decrease of the MUFA, C18:1 and a minor decrease of EPA (table 5).

Larvae fed *Artemia* in general approached the FA composition of the prey, while especially larvae fed rotifers resembled the profile of the starving larvae (table 5). This is exemplified by 16:1n-7 and C18:1n-9 MUFAs. These were significantly lower ($P < 0.001$) in starving larvae or larvae fed *Tisbe* or rotifers as compared with larvae fed *Artemia* for which the relative contents increased significantly ($P<0.001$). The progressive development in percent similarity of C16:1 in prey and larvae is illustrated in Fig. 2a, which shows an approximation for sole larvae treated with *Artemia*. As compared with the EPA content in pre feeding larvae, the relative EPA content decreased ($P < 0.001$) in larvae fed rotifers or when starved. The percent similarity between prey and sole larval EPA profiles (Fig. 2b) indicated a rapid approximation for larvae fed *Artemia* as tissue EPA increased very significantly ($P > 0.001$). In larvae fed *Tisbe* the tissue content was 2.5-3 percent despite a high EPA proportion in *Tisbe* itself.

C22:6n-3, DHA was the absolute most dominant tissue FA and constituted about 46-53 percent of TFA in eggs, newly hatched larvae, start-feeding larvae and larvae at dah 5. The relative proportion of this FA remained very high in starving larvae and larvae fed *Tisbe* and rotifers, but decreased significantly with age for larvae fed *Artemia* ($P < 0.001$). Hence, the degree of similarity (%) between C22:6 (n-3) in diets and common sole larvae was low, but increased slightly with age for larvae treated with enriched *Artemia*

DHA: EPA and the ARA: EPA were significantly different ($P < 0.001$) between treatments. The ratios gradually increased for rotifer treated larvae and starving larvae. In comparison a significant decrease ($P < 0.001$) was observed in larvae fed *Artemia* especially for DHA: EPA.

Table 5.

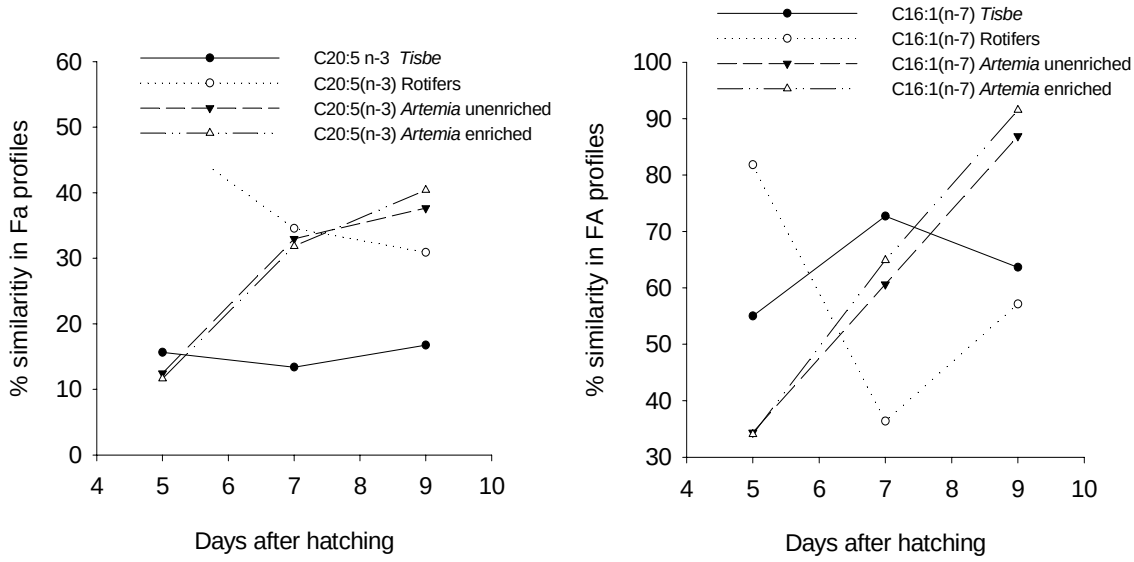
Total fatty acid content (TFA mg g^{-1}d.w.) in eggs and larvae and FA composition (% of TFA) given as a mean for each live feed treatment ± standard deviation. n=3, TFA; total fatty acids, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, ARA; arachidonic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid. Totals include minor fatty acids not shown. A value followed by a different superscript in a horizontal row denotes a significant difference (P<0.05).

				Treatment														
				Tisbe			Rotifers			*Artemia* unenriched			*Artemia* enriched			No feed		
	Eggs	dah 0	Pre feeding	dah 5	dah 7	dah 9	dah 5	dah 7	dah 9	dah 5	dah 7	dah 9	dah 5	dah 7	dah 9	dah 5	dah 7	Dah 9
TFA content	168.0	135.3	105.1±18.0^{d}	61.4± 2.9^{c}	57.5±1.5^{bc}	39.5±22.1^{ab}	60.2±12.5^{bc}	44.4±8.0^{ab}	39.2±1.3^{a}	72.9±5.5^{c}	66.9±6.3^{bc}	53.3±8.5^{b}	72.6±8.9^{c}	76.8±2.3^{c}	65.3±9.5^{bc}	67.7±8.0^{c}	50.5±8.8^{b}	34.7±3.6^{a}
FA (%TFA)																		
C14:0	2.2	2.5	2.5±0.3^{c}	0.5±0.4^{a}	0.8±0.2^{ab}	1.3±0.6^{ab}	1.1±0.4^{ab}	0.4±0.2^{a}	0.9±0.2^{ab}	1.0±0.1^{ab}	0.6±0.3^{ab}	0.8±0.2^{ab}	1.1±0.7^{ab}	0.8±0.2^{ab}	0.6±0.1^{ab}	1.5±0.6^{b}	0.3±0.2^{ab}	0.2±0.3^{ab}
C16:0	14.4	12.4	15.2±0.6^{ab}	17.8±2.2^{c}	16.8±0.3^{b}	17.1±0.3^{bc}	18.3±0.6^{c}	16.9±0.3^{bc}	17.5±1.0^{c}	16.9±1.1^{bc}	13.9±0.6^{a}	14.3±1.2^{a}	16.9±0.5^{bc}	13.5±0.6^{a}	12.6±1.5^{a}	18.9±1.4^{c}	17.2±0.1^{bc}	16.8±0.5^{b}
C18:0	3.0	3.1	4.6±0.3^{a}	8.3±0.9^{b}	9.8±0.3^{bc}	10.4±0.8^{c}	7.5±0.5^{b}	10.8±1.6^{c}	10.8±0.2^{c}	6.9±0.3^{b}	7.4±0.5^{b}	7.4±0.2^{b}	6.7±0.5^{b}	6.9±0.2^{b}	6.6±0.6^{b}	7.2±0.6^{b}	11.2±0.5^{c}	11.3±0.7^{c}
Total SFA	20.8	19.3	23.7±0.9^{ab}	28.4±2.8^{cd}	29.4±0.6^{cd}	31.3±0.7^{d}	28.6±0.5^{cd}	30.0±1.8^{cd}	31.4±1.1^{d}	26.3±0.9^{b}	23.8±0.6^{ab}	25.0±1.3^{ab}	25.2±0.7^{b}	23.0±1.0^{ab}	21.9±2.2^{a}	29.2±3.0^{cd}	30.9±0.6^{d}	30.5±0.9^{d}
C16:1 (n-7)	6.0	6.4	5.7±0.2^{c}	2.0±0.6^{a}	0.8±0.1^{a}	0.7±0.2^{a}	1.8±0.1^{a}	0.8±0.2^{a}	0.8±0.1^{a}	3.4±0.3^{b}	6.0±0.6^{c}	8.6±1.3^{d}	3.2±1.0^{b}	6.1±0.6^{c}	8.6±0.8^{d}	1.9±0.1^{a}	0.8±0.1^{a}	0.7±0.1^{a}
C18:1 (n-9) cis	14.9	10.0	8.8±0.2^{c}	6.9±0.9^{ba}	6.2±0.4^{a}	6.1±0.2^{a}	6.5±0.2^{b}	6.4±0.1^{ba}	7.2±0.2^{ba}	7.9±0.1^{c}	11.6±0.5^{d}	14.2±0.1^{de}	7.6±0.6^{b}	11.8±1.0^{d}	15.1±0.8^{e}	6.5±0.2^{ba}	6.4±0.1^{ba}	6.9±0.1^{ba}
Total MUFA	22.8	18.4	16.1±0.2^{c}	10.3±0.8^{ab}	8.5±0.4^{a}	8.7±0.9^{a}	10.5±0.3^{ab}	8.9±0.4^{a}	10.0±1.0^{a}	12.9±0.4^{b}	19.3±0.9^{d}	25.1±2.1^{e}	12.4±1.7^{b}	19.5±1.7^{d}	25.7±1.7^{e}	9.0±0.4^{a}	8.7±0.3^{ab}	9.4±0.2^{a}
C18:2 (n-6) c	0.6	0.9	0.8±0.0^{a}	0.9±0.2^{ab}	1.7±0.7^{bc}	3.3±0.4^{cd}	0.5±0.4^{ab}	0.6±0.5^{ab}	0.9±0.8^{ab}	1.3±0.3^{ab}	2.6±0.1^{cd}	3.7±0.1^{e}	1.0±0.2^{ab}	2.9±0.3^{cd}	4.0±0.2^{e}	0.4±0.4^{ab}	0.4±0.4^{ab}	0.5±0.4^{ab}
C20:4 (n-6), ARA	3.4	3.1	3.3±0.1^{a}	4.6±0.4^{bc}	5.0±0.4^{cd}	4.5±0.1^{bc}	4.4±0.5^{bc}	5.5±0.0^{d}	5.4±0.2^{d}	4.0±0.1^{b}	5.4±0.2^{d}	5.1±0.4^{cd}	4.2±0.2^{b}	5.4±0.2^{d}	4.8±0.7^{bc}	4.3±0.3^{bc}	5.6±0.2^{d}	5.6±0.2^{d}
Total (n-6) PUFA	4.4	4.5	4.5±0.1^{a}	6.0±0.7^{b}	7.5±0.4^{c}	9.0±0.5^{d}	5.5±0.8^{b}	6.6±0.5^{bc}	7.1±1.0^{bc}	5.7±0.4^{b}	9.0±0.3^{d}	10.7±0.6^{e}	5.6±0.1^{ab}	9.1±0.2^{d}	10.8±0.2^{e}	4.9±0.3^{ab}	6.7±0.4^{bc}	7.3±0.5^{bc}
C18:3 (n-3) c	0.1	0.3	0.3±0.0^{ab}	0.2±0.2^{a}	0.6±0.3^{c}	1.1±0.2^{c}	0.2±0.0^{a}	0.1±0.2^{ab}	0.2±0.2^{b}	0.8±0.1^{bc}	2.4±0.3^{d}	3.6±0.1^{e}	0.5±0.3^{ab}	2.5±0.5^{d}	3.8±0.1^{e}	0.1±0.1^{ab}	0.1±0.2^{ab}	0.0±0.0^{a}
C20:5 (n-3), EPA	6.0	5.6	4.3±0.1^{c}	2.8±0.3^{bc}	2.4±0.3^{a}	3.0±0.3^{bc}	2.7±0.0^{bc}	1.9±0.1^{a}	1.7±0.0^{a}	5.3±0.5^{d}	14.0±1.0^{e}	16.2±1.2^{f}	4.9±1.1^{cd}	13.4±2.0^{e}	17.0±1.1^{f}	2.9±0.3^{c}	1.8±0.1^{a}	1.6±0.1^{a}
C22:6 (n-3), DHA	46.0	51.7	50.2±1.0^{c}	52.1±3.8^{c}	51.4±0.4^{c}	46.3±2.2^{c}	52.3±1.0^{c}	52.3±1.4^{c}	49.2±1.2^{c}	48.8±0.9^{c}	31.2±1.7^{b}	19.1±3.7^{a}	50.2±3.7^{c}	32.1±3.3^{b}	20.6±2.8^{a}	53.8±2.4^{c}	51.6±0.8^{c}	50.9±1.1^{c}
Total (n-3) PUFA	52.1	57.9	55.6±0.9^{c}	55.2±3.6^{c}	54.6±0.5^{c}	50.9±1.8^{bc}	55.4±1.0^{c}	54.4±1.6^{c}	51.4±1.0^{bc}	55.1±0.9^{c}	47.9±0.6^{ab}	39.2±4.9^{a}	55.8±2.3^{c}	48.3±0.9^{ab}	41.7±3.8^{a}	56.8±2.5^{c}	53.7±0.8^{bc}	52.8±1.2^{bc}
DHA:EPA	7.7	9.2	11.7±0.2^{b}	18.7±3.0^{c}	21.3±2.6^{c}	15.8±2.3^{c}	19.1±0.4^{c}	27.8±1.3^{d}	28.9±1.2^{d}	9.3±0.9^{b}	2.2±0.3^{a}	1.2±0.2^{a}	10.6±3.2^{b}	2.5±0.7^{a}	1.2±0.1^{a}	18.7±2.4^{c}	28.7±1.1^{d}	31.8±1.5^{d}
ARA:DHA	0.1	0.1	0.1±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{ab}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{ab}	0.2±0.0^{c}	0.3±0.0^{e}	0.1±0.0^{ab}	0.2±0.0^{c}	0.2±0.0^{d}	0.1±0.0^{ab}	0.1±0.0^{b}	0.1±0.0^{b}
ARA:EPA	0.6	0.6	0.8±0.0^{b}	1.6±0.1^{c}	2.1±0.4^{d}	1.5±0.2^{c}	1.6±0.2^{c}	2.9±0.2^{e}	3.2±0.1^{ef}	0.8±0.1^{ab}	0.4±0.0^{a}	0.3±0.0^{a}	0.9±0.2^{b}	0.4±0.1^{a}	0.3±0.0^{a}	1.5±0.1^{c}	3.1±0.1^{ef}	3.5±0.2^{f}
(n-3):(n-6)	11.8	13.0	12.3±0.2^{f}	9.2±1.4^{cd}	7.3±0.3^{c}	5.7±0.5^{ab}	10.1±1.6^{e}	8.2±0.7^{cd}	7.3±1.3^{c}	9.7±0.4^{de}	5.3±0.1^{b}	3.7±0.6^{ab}	10.0±0.5^{de}	5.3±0.1^{ab}	3.9±0.3^{ab}	11.5±0.2^{ef}	8.0±0.6^{cd}	7.2±0.6^{c}

ARA: DHA remained at almost zero despite the various treatments. In all larval groups the tissue n3: n6 decreased significantly ($P < 0.001$) with the progress of the trial as compared to the initial proportion in eggs and pre-feeding sole larvae.

Figure 2a-b.

Percent similarity of C16:1 (n-7 and C20:5 (n-3) in live prey and sole larvae at dah 5, 7, 9



Discussion

The larval survival (dah 3-10) was not influenced by the various live feed treatments, but was lower than (80-100 %) reported in a study of effects of *Tisbe* and *Artemia* on common sole larvae (Heath and More, 1997).

Common sole is known to have a high resistance to starvation (Boulhic and Gabaudan, 1992). Still, it was quite remarkable that starving sole larvae at dah 9 had a similar survival as the other codes. However, the larvae could not survive, when rotifers and *Artemia* were supplied at dah 11. This was possibly caused by an irreversible starvation damage as described by Baynes and Howell, (1996), where sole larvae are too weak to seek for prey. Rotifer treated larvae exhibited the lowest survival in the period from dah 11 until dah 39. This was probably related to the negative growth during the initial larval period and consequently poor nutritional status.

First feeding sole larvae seem to posses the necessary enzymes required to digest the ingested prey (Boulhic and Gabaudan, 1992, Morais et al., 2004 b). The fact, that larvae feeding on *Artemia* had a superior growth than when fed *Tisbe* was different than findings by Heath and More, (1997), who reported no difference in length growth between sole larvae fed *Artemia* or *Tisbe.* There may be

several reasons for the observed discrepancy especially as pelagic wild Senegalese sole larvae have been found to prey mainly on copepods prior to metamorphosis (Drake and Arias, 1993). Larvae of common sole are relatively large i.e. capable of handling *Artemia* nauplii from first feeding (Boulhic and Gabaudan, 1992, Caňavate and Fernández-Diaz, 1999). The prey density of *Tisbe* and *Artemia* was identical and in surplus, (i.e. 5 ml $^{-1}$), however, sole larvae were favoured by the larger *Artemia* prey size despite that *Tisbe* were offered as a mixture of nauplii stages, copepodites and adults *Tisbe* is a harpactocoid and adult stages mainly occupy surfaces and sides / bottoms of a tank; and only the very small nauplii stages are pelagic (Støttrup and Norsker, 1997). It is likely that nauplii were too small to be detected (0.128 mm ind -^{1}). Sole larvae were observed also to aggregate at surfaces and tank walls in agreement with an observation by Fonds (Fonds, 1979).We therefore advocate, that the main reasons for the low larval growth may be related to *Tisbe* prey size and the fact that early sole larvae are very slow moving predators (authors observations). This may have reduced the predator / prey encounters as *Tisbe* often move very fast with short active bursts, despite that adult and juvenile copepods are relatively poor swimmers (Fleeger, 2005).

A variety of species of marine larval fish are start reared on rotifers (Lubzens et al., 2001). In the present trial, the d.w. and length of rotifers (i.e sorted on a 90 µm to remove eggs) were only about 20 percent the size of *Artemia.* Despite a similar energy content in all prey species (J mg^{-1}), rotifers may have provided insufficient energy, as a combination of small size and low feed intake. Similar poor growth results on rotifers have previously been observed for common sole (Dendrinos and Thorpe, 1987). In support of our findings, they concluded, that the observed low growth rate may be either nutritional or mechanical related. The initial similarity in larval sizes (length, d.w.) at dah 5 indicated a general low initial feed intake. Therefore, we can not conclude if early larvae assimilated sufficient energy from rotifers. The subsequent negative growth (dah 7-9), however, suggests that rotifers soon became inferior. This may indicate, that larvae either did not prey on rotifers or within a few days selected larger prey. In the close sister relative, Senegalese sole (*Solea senegalensis*), however, this was not observed (Marin-Magan et al., 1995, Magalhães and Dinis, 1996). First feeding Senegal sole (d.w. 39 µg ind^{-1}) has been found to select rotifers over *Artemia* (Canavate, Fernăndez- Diaz,1999), which however may be attributable to the smaller size of Senegal sole i.e. smaller oral diameter at first feeding in comparison to common sole (i.e d.w. 71 µg ind^{-1}, present study).

In spite *Solea solea and Solea senegalensis* accept *Artemia* as first prey culturists prefer to give rotifers at dah 3 (Dinis et al., 1999). Based on the present results, it may be possible, that common

sole offered a combination of rotifers and *Artemia* will select *Artemia,* as larvae of some fishes are able to discriminate among prey items (e.g. Pryor and Epifanio, 1993). This advocates for a study on common sole larvae on prey intake and selectivity.

Sole larval ability to detect rotifers (or *Tisbe*) may have been further reduced by the use of transparent tanks as speculated by Dendrinos (Dendrinos and Thorpe, 1987). The orange colour of *Artemia* may have been more visible for the fouraging larvae (Canavate et al., 2006) especially as light intensity was relatively low (100 lux at surface).

Juvenile survival was depressed in larvae initially fed rotifers and *Tisbe*, and juvenile growth was clearly negatively related to an increased density as reviewed by Howell (1997) and as reported for larger sole (Schram, 2004). In the present study, juveniles showed a higher degree of fin abrusions with an increased density. This may be associated with stress. Settled common sole seem therefore to be quite territorial and require a certain bottom area of the rearing tank or sand for burying in order to develop optimally.

The physiological performance was similar for larvae preying on unenriched or enriched AF *Artemia,* which may have been the consequence of a relatively short enrichment time, too short to create large PUFA differences in the two *Artemia* groups. However, in Senegalese sole (Morais et al., 2004a) a better growth was reported using unenriched *Artemia* as compared with enriched *Artemia*, which suggests a similar low requirement of PUFAs in common sole larvae.

The relative high PUFA levels of C20:5 n-3 and 22:6 n-3 in *Tisbe* is consistent with other FA data reported for this species (Støttrup, 2003) and support findings, that harpacticoid copepods are believed to have a superior nutritional composition (Fleeger, 2005). The present findings, that *Tisbe* (i.e. mainly nauplii and copepodites) had a low total fatty acid content (TFA) is supported by other studies; Støttrup (Støttrup, 2003), states that lipid levels in harpacticoids are generally low, and Evjemo (Evjemo et al, 2003) has reported lower levels in harpacticoid nauplii and copepodites than in adults.

Rotifers did not reflect the FA profile of the algae diet T- *Isochrysis* or the enrichment product, EASY DHA SELCO, suggesting a low uptake of nutrients, which may be related to the size and therefore age. TFA in rotifers was approximately 8 % d.w., in comparison to previous findings of 9-28 % d.w. (Støttrup, 2003), hence, the lower content may additionally be a result of rearing conditions at 25 ° C as rotifers accumulate more lipids at 10 º C than at 25 º C (Lubzens and Zmora, 2003).

The obvious differences in composition of especially the esential n-3 PUFAs in *Tisbe*, rotifers and AF *Artemia* suggest, other things being equal, that a combination of live feed organisms for fish larvae most likely would enhance larval performance. A relative constant lipid level was observed in sole larvae with a high growth rate (i.e fed *Artemia*).

In general larval lipids will be depleted during reabsorption of the larval yolk around dah 5, and a gradually re-increase during exogenous feed intake (Boulhic and Gabaudan, 1992). Opposite to this, in undernourished or starving sole larvae lipid levels decreased progressively, which suggests utilization for energy related purposes.

A relatively high similarity in the profile of some main FAs, by a comparison of starving larvae with rotifer fed larvae (r^2 = 0.38-0.99) and *Tisbe* fed sole larvae (r^2 = 0-0.66), supports the growth data, that at least rotifer fed larvae actually were not ingesting rotifers in sufficient quantities to sustain energetic requirements and hence were functionally starving**.** In comparison sole larvae fed *Artemia* gradually approached the relative dietary prey FA composition as reported in studies on Senegalese sole larvae and common sole larvae (Morais et al., 2004a, Lund et al., 2007).

The present observations of 46-52 % DHA content in sole eggs and pre feeding sole is higher than at most 35 % observed in eggs the following year by a comparison of different batches (Lund et al., in press). In the close sister relative, Senegal sole ≤ 31 % DHA has been reported, (Morais et al., 2004a, Villalta et al., 2005,). The reason may be related to broodstock diet or general nutritious health. The relative DHA content remained unchanged in unfed and undernourished sole larvae fed rotifers or *Tisbe*, while DHA decreased rapidly for larvae with a high growth rate, probably as a consequence of a low dietary content and incorporation of other dietary fatty acids. In support of the present findings studies have shown that starving Senegalese sole larvae retain DHA (Mourente and Vázquez, 1996, Villalta et al., 2005) as well as ARA (Vázquez et al., 1994, Morais et al., 2004a, Villalta et al., 2005), allowing the preservation of valuable essential components of biological membranes (Izquierdo et al., 2000, review).

Contrary to this tissue C20:5 n-3 in starving /undernourished larvae were gradually depleted probably to sustain energy related purposes as reported for first feeding Senegal sole larvae (Vázquez et al., 1994, Mourente and Vázquez, 1996) illustrating the different function of PUFAs. Similarly the monounsaturated FAs C16:1 and C18:1n-9 obviously were used as energy supply both in pre feeding and feeding larvae as previously reviewed for common sole (Imsland et al., 2003), and reported for Senegalese sole (Morais et al., 2006). This was especially observed until dah 5, where levels seemed depleted to a certain physiological threshold.

The very low percent malpigmented individuals (i.e. < 5 percent) found at dah 39 for all feed treatments, suggests that pigmentation either is not determined during pre metamorphosis or that the present dietary differences or even starvation had any effect on the pigmentation process of the present larval batch. In a previous trial (Lund et al, 2007) pigmentation was determined within 20 days after hatching. Based on the present study a possible pigmentation "window" is therefore suggested to be during the period dah 10 until - 20.

Conclusions

Rotifers and *Tisbe* should not be used as first feed for common sole larvae. Based on the present study unenriched *Artemia* nauplii may be used as first feed, as common sole larvae seem to have a low requirement for PUFAs. Growing larvae seem to reflect the FA composition of the diet, while in undernourished and starving larvae specific FAs as C16:1n-7 and C20:5n-3 are utilized and others especially C22:6n-3 is conserved.

Early dietary treatment influenced on later survival, but did not affect pigmentation.

Recommendations

The obtained differences in larval growth were most likely related to differences in prey size. Hence, encounter, prey capture and ingestion of various live prey organisms indeed need to be further investigated. The importance of early dietary levels of n-3 and n-6 PUFAs in common sole in relation to a possible larval pigmentation window should also be pursued.

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IIA

The effect of prey species, abundance and light intensity on first feeding of common sole larvae (*Solea solea*).

Appendix

Appendix

The effect of prey species, abundance and light intensity on first feeding of common sole larvae (*Solea solea* L).

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Keywords: Sole larvae, *Artemia*, *Tisbe* , rotifers, preferences, selectivity, light

Abstract

Previous studies have indicated a poor growth of common sole larvae when offered rotifers or the copepod, *Tisbe holothuriae* from first feeding until 9 days post hatch. The present study was carried out to demonstrate if sole larvae discriminate between prey species.

Three prey species were used as feed. The species were *Artemia*, rotifers and *Tisbe holothuriae.* Sole larvae did not eat until after complete utilization of yolk sac and oil globules. First feeding sole larvae seemed not to discriminate between prey species of rotifers and *Artemia*, but may have a lower intake of *Tisbe*. Ten days old sole larvae actively preferred *Artemia* when fed in combination with rotifers. *Artemia* cysts were readily ingested at dah 4 and were ingested in a much larger proportion than their abundance in the tanks. Sole larvae ability to eat in darkness was low and gradually improved by an increased illuminance.

Introduction

In a study carried out by Lund et al (unpublished data, a) on start feeding common sole larvae, 3 species of live prey were compared as to their applicability as start feed and their effect on growth performance and biochemical composition of sole larvae. The species tested were *Tisbe holothuriae, Brachionus plicatilis and Artemia* sp. The results revealed a much better growth rate when sole larvae were fed *Artemia* as compared to larvae fed rotifers or *Tisbe*.

Feeding ability of first feeding marine fish larvae is dependent on many factors such as the size of the larvae especially width of mouth and esophagus, larval motility, prey size, prey colour, contrast and shape - as well as environmental conditions (i.e. light, turbidity, turbulence, temperature) (Pryor and Epifanio, 1993, Chesney, 2005).

In the present setup it was examined whether the previous reported growth differences, (Lund et al, unpublished data, a) may be influenced by differences in feed intake when fed *Artemia*, *Tisbe* or rotifers and related to larval prey preference.

Common sole are capable of ingesting *Artemia* from the day of mouth opening, normally 3 days post hatch. An objective was therefore to observe if sole larvae may discriminate between prey species at first feeding. This was tested by allowing different batches of first feeding larvae to select on pairs of prey species for a few hours. Subsequently larval stomach content was evaluated. Further the effect of light intensity was tested on the feeding ability of common sole larvae 8 to 11 days after hatching.

Materials and Methods

Prey

Three prey species were used as prey; rotifers (*Brachionus plicatilis*) retained on a 90 µm sieve (large rotifers), rotifers passing a 90 µm sieve and retained on a 48 µm sieve (small rotifers), *Artemia* nauplii (AF strain) sampled 8 hrs post hatch and *Tisbe* (*Tisbe holothuriae*) passing a 120 µm sieve and retained on a 38 µm sieve. The intake of sole larvae was tested by allowing sole larvae to feed on prey pairs. The 6 prey pairs tested are shown in table 1.

Table1.

Prey pairs tested.

Prey pair
Large rotifers / small rotifers
Large rotifers / *Tisbe*
Large rotifers / AF *Artemia*
Small rotifers / *Tisbe*
Small rotifers / AF *Artemia*
Tisbe / AF *Artemia*

The total length of all food organisms were measured by a digital high resolution DFC 320 Leica camera connected to a dissecting microscope (MZ6). The mean length and width of the prey is given in the Table 2.

Larvae, sampling and experimental setup

The larvae used for the first test were obtained from one batch of hatching larvae and larvae were start fed from 4 days after hatching (dah). Larvae tested at dah 4, 5 and 6 were not fed prior to the test to avoid larvae with stomach contents and to avoid that pre fed larvae would favour or discriminate certain prey species. However, larvae tested at dah 10 were fed with rotifers prior to the tests (dah 6 to 9).

At dah 4 the larvae had developed an open functioning mouth 20-24 prior to the test. At dah 5 and 6 the larvae were 24 and 48 hrs older than the dah 4 larvae.

At dah 4 a high percentage of the sole larvae had almost depleted yolk sac reserves, while at dah 4 about 1/3 it was still present. Contrary to many other marine larvae sole have numerous small oil

globules clustered around in the yolk of the developing larvae (Marinaro, 1991), consequently these were not measured.

At each test day the standard length of 30 individuals replicate $^{-1}$ was measured before preservation in 70 % alcohol. Prior to the trial the larvae were kept in a 46 l conical transparent tank connected to a flow through water system of 1 µm filtrated seawater. 4 hrs before start of the trial larvae were moved to 2 x 6 beakers of 2 l each (duplicate trial). The density of larvae in each beaker was 25 pcs l $^{-1}$ (i.e. 50 pcs beaker $^{-1}$). The temperature and light intensity was kept constant, 17.5 ± 0.6 º C and a light intensity of 700 lux was provided at the water surface. An airstone provided upwelling.

The density of prey was 5 ml $^{-1}$ for each prey species, resulting in a total density of 10 ml^{-1}.

Larvae were allowed to eat undisturbed for 2 hrs and afterwards fixed in cold 5 % formaldehyde in seawater and transferred to 70 % alcohol for gut content determination. Gut content was dissected and the number of prey was counted for each species. Dead larvae, at the end of the trial, were excluded from evaluation.

Results

Prey size

The mean size of the tested prey is indicated in Table 2.

The size variation, within the *Tisbe* population, was largest as due to various development stages, i.e. nauplii stages 1-6 with a mean length of 0.13 ± 0.03 mm individual $^{-1}$. Copepodites / adults had a body length of 0.30 ± 0.04 mm individual^{-1} (including spines 0.4 ± 0.05 mm individual $^{-1}$).

In the population of large rotifers a high percentage contained eggs, the length of which is included in Table 2. The largest prey was *Artemia* and the width was wider than the body length due to their locomotory second antennae.

Table 2.

Mean standard length (mm) and width (mm) of prey species.

*n.m.: not measured but less than length

Species	Length (mm)	Width (mm)
Small rotifers	0.14 ± 0.03	0.10 ± 0.02
Large rotifers	0.22 ± 0.03	0.12 ± 0.01
Tisbe	0.20 ± 0.09	n.m.*
AF *Artemia*	0.43 ± 0.03	0.55 ± 0.07

At dah 4,5,6 the standard length of the sole larvae was 3.60 ± 0.15 mm ind^{-1}, 3.97 ± 0.12 mm ind^{-1} and 4.12 ± 0.13 mm ind^{-1}, respectively. At dah 10 larvae were 5.89 ± 0.63 mm ind $^{-1}$.

The effect of prey species on preference

Results from the preference experiments are shown in Table 3a, b, c. The results are presented as the overall percentage of larvae with stomach contents at dah 4, 5 and 6 and the percentage of larvae which preferred one of the prey pair species. The percentage of larvae feeding on both prey species is not shown.

Table 3 a-c.

Proportion of larvae with ingested prey and percentage of larvae feeding on each species of a prey pair at dah 4, 5 or 6, as well as proportion of larvae feeding on AF empty cysts. Density of larvae n = 50 for each replicate. The results are given as a mean ± standard deviation.

a)

Days after hatching 4					
Prey pair	Larvae feeding %	Larvae feeding on either prey (%)	Larvae with AF cysts (%)	Prey larvae^{-1}	Cysts larvae $^{-1}$
Large rotifers / small rotifers	1.9 ± 2.6	1.9 ± 2.6 (not distinguishable)		< 0.1	
Large rotifers / *Tisbe*	3.3 ± 0.8	3.3 ± 0.8 / 0.0 ± 0.0*		<0.1	
Large rotifers / AF *Artemia*	4.6 ± 3.2	4.6 ± 3.2 / 0.0 ± 0.0*	- / 7.2 ± 10.2	<0.1	<0.1
Small rotifers / *Tisbe*	6.7 ± 9.4	6.7 ± 9.4 / 0.0 ± 0.0*	-	<0.1	
Small rotifers / AF *Artemia*	0.0 ± 0.0	1.2 ± 1.0 / 0.0 ± 0.0*	- / 4.5 ± 2.3	0	
Tisbe / AF *Artemia*	0.0 ± 0.0	0.0 ± 0.0 / 0.0 ± 0.0*	- / 2.6 ± 3.6	0	

b)

Days after hatching 5					
Prey pair	Larvae feeding %	Larvae feeding on either prey (%)	Larvae with AF cysts (%)	Prey larvae^{-1}	Cysts larvae $^{-1}$
Large rotifers / small rotifers	39.7 ± 12.2	(not evaluated)		0.5	
Large rotifers / *Tisbe*	34.0 ± 7.7	22.8 ± 2.0 / 6.9 ± 3.1*		0.3 / 0.1	
Large rotifers / AF *Artemia*	56.3 ± 14.4	10.3 ± 14.6 / 20.0 ± 1.0*	8.6 ± 12.2	0.3 / 0.8	0.3
Small rotifers / *Tisbe*	27.6 ± 31.6	19.7 ± 24.1 / 8.0 ± 7.6*		0.2 / 0.1	
Small rotifers / AF *Artemia*	67.9 ± 3.0	15.7 ± 15.1 / 12.8 ± 3.2*	25.4 ± 13.6	0.3 / 0.4	0.5
Tisbe / AF *Artemia*	69.1 ± 18.1	9.6 ± 5.4 / 26.3 ± 4.6*	22.6 ± 6.6	0.2 / 0.3	0.5

C)

Days after hatching 6					
Prey pair	Larvae feeding %	Larvae feeding on either prey (%)	Larvae with AF cysts (%)	Prey larvae^{-1}	Cysts larvae $^{-1}$
Large rotifers / small rotifers	60.4 ± 15.6	(not evaluated)		0.6	
Large rotifers / *Tisbe*	30.9 ± 18.3	29.1± 15.8 / 8.6 ± 7.1*		0.3 / 0	
Large rotifers / AF *Artemia*	61.2 ± 5.9	12.5 ± 1.2 / 26.5 ± 9.1*	19.8 ± 4.9	0.2 / 0.8	0.5 ± 0.2
Small rotifers / *Tisbe*	33.9 ± 25.5	28.8 ± 22.8 / 5.1 ± 2.7*		0.3 / < 0.1	
Small rotifers / AF *Artemia*	55.1 ± 13.0	9.2 ± 0.5 / 18.8 ± 13.9*	26.1 ± 3.3	0.1 / 0.3	0.6 ± 0.2
Tisbe / AF *Artemia*	65.8 ± 1.1	19.9 ± 4.7 / 22.5 ± 0.6*	18.4 ± 0.8	0.2 / 0.4	0.7 ± 0.1

* Not shown percentage of larvae feeding on both prey

At dah 4 the percentage of larvae which had ingested prey was very low despite the prey pairs offered. Rotifers (large and small) were the only prey ingested at dah 4, but results revealed a large

variation within replicates of the same prey pair offered and between different prey pairs. However, none of the examined larvae had ingested *Artemia* or *Tisbe*.

Despite a low density of (empty) *Artemia* cysts in the tanks, 2.6 to 7.2 percent of the larvae had ingested these in pair groups with *Artemia*. *Artemia* cysts typically floated on the water surface. Similarly, rotifers tended to accumulate at the surface as they are photo positive. Consequently, prey intake per larvae was very low during the 2 hrs trial. Only a very few larvae had ingested more than 1 prey or 1 cyst.

The percentage of larvae which had initiated feeding at dah 5 was much higher as compared to dah 4 no matter the type of prey pair offered. A mean of 28 to 69 percent of the larvae had ingested some prey, but results revealed a large variation between replicates. For larvae which fed on both the small and large rotifers it was impossible to distinguish between the two sizes when sampled from the gut.

Tisbe seemed to be the least selected prey, but large differences between the prey pairs were observed. The overall highest percentage of feeding larvae was observed for prey pairs in which one of the species was *Artemia.* The data showed, however, that in these prey pairs 9 to 25 percent of all larvae which were feeding had ingested *Artemia* cysts, despite the relative low density of these. The number of cysts per larvae was also similar or higher than the intake of the various prey species. None of the prey species were unambiguously preferred, but larvae seemed to prefer *Artemia* in prey pairs with large rotifers or *Tisbe*.

At dah 6 the yolk and oil globules were completely absorbed. The overall ingestion pattern and percent of feeding larvae were similar to dah 5. Apart from the larvae which fed on large and small rotifers, the highest overall percent of feeding larvae was observed in prey pairs with *Artemia*. However, this was mainly caused by a high percentage of larvae feeding on *Artemia* cysts.

All prey species were ingested, and no preferences were observed, but an overall lower percent of larvae seemed to ingest *Tisbe*, when offered another alternative. The variation between replicates was high at dah 6, as similar to results for dah 4 and 5. The larvae showed a high preference for cysts despite the low density of these. The number of prey larvae^{-1} was similar to dah 5, and relatively much higher for *Artemia* cysts.

10 days after hatching larvae were fed a prey pair of rotifers and *Artemia,* table 4.

Table 4.

Larvae feeding on each species of a rotifer / Artemia prey pair 10 days after hatching .

Days after hatching 10						
	Larvae feeding	Larvae on either prey	Larvae on both	Larvae on *Artemia* and cysts	Prey larvae^{-1}	Cysts larvae $^{-1}$
Prey pair	(%)	(%)	(%)	%		
Rotifers / *Artemia*	85.7 ± 11.7	0 ± 0 / 57.1 ± 9.6	14.2 ± 4.7	14.4 ± 6.2	0.2 / 3.5	0.14

About eighty five percent of the larvae ingested prey. Fifty seven percent of the larvae fed on *Artemia,* while 0 percent of the larvae had ingested rotifers only. The number of *Artemia* per larvae increased as compared to larval intake 4 to 6 days after hatching while the number per larvae was very low for rotifers and cysts. The results indicate that larger larvae actively preferred *Artemia* over rotifers, when fed at similar densities.

Additional tests

The larvae used for the tests below were obtained from different batches of larvae spawned during the breeding season 2006. The results are means of several tests carried out in a triplicate set up. The set up and density of larvae were similar to previous descriptions.

4 to 6 days old larvae were not fed prior to the trials to avoid larvae with stomach content and to avoid discrimination of prey. Prior to the trials 8-11 dah old larvae were fed rotifers at a concentration of 5 ml $^{-1}$ and tests were carried out with 8 h post hatched *Artemia.*

Prey selection at different densities

In one of the trials, 6 days old sole larvae (standard length 4.18 ± 0.14 mm ind $^{-1}$) were fed prey pairs of rotifers and *Artemia.* The total density of prey was 10 ml $^{-1}$. The ratios of rotifers and *Artemia* were, 1:1, 10:1 or 20:1, table 5.

Table 5.

Percentage of larvae feeding on each species of rotifer / Artemia prey pairs at increasing rotifer concentrations 6 days after hatching. Results given as a mean ± sd.

Days after hatching 6					
Prey pair ratio	Larvae feeding (%)	Larvae feeding on either prey (%)	Larvae with cysts (%)	Prey larvae^{-1}	Cysts larvae $^{-1}$
Rotifers / *Artemia* 1/1	78.8 ± 3.0	13.1 ± 9.2 / 32.8 ± 7.3	31.1 ± 11.3	0.3 / 0.7	1.2
Rotifers / *Artemia* 10/1	78.9 ± 6.5	30.9 ± 7.3 / 13.7 ± 6.4	19.8 ± 2.1	0.8 / 0.5	0.8
Rotifers / *Artemia* 20/1	68.7 ± 14.3	25.5 ± 12.7 / 14.3 ± 6.6	13.7 ± 7.4	0.8 / 0.3	0.5

The percentage of larvae which were feeding was almost similar at each density. When rotifers and *Artemia* were fed at a similar density of 5 prey ml^{-1} the percentage of larvae which had ingested *Artemia* was much higher than when offered rotifers, but decreased when the ratio was 1:10 and did

not seem to decrease further. Similarly the percentage of larvae which were feeding on *Artemia* cysts decreased when the *Artemia*: rotifer: ratio decreased. The number of prey larvae^{-1} was related to the prey density. A relatively high number of cysts were ingested larvae $^{-1}$ despite the low density of these.

Larvae from the same batch were used in a non preference trial at dah 7, Hence, the larvae were offered either *Artemia* or *Tisbe* at a density of 4 individuals ml^{-1}, Table 6.

Table 6.

Percentage of larvae preying on Artemia or Tisbe at dah 7

Days after hatching 7		
	Larvae preying (%)	Prey larvae^{-1}
Artemia	54.0 ± 4.1	0.7
Tisbe	34.0 ± 2.1	0.5

A higher percentage of larvae did ingest *Artemia* as compared to *Tisbe*, and the number of *Artemia* ingested larvae^{-1} was slightly higher than larvae fed on *Tisbe*.

Larvae from another batch were fed prey pairs of rotifers and *Artemia* either with or without algae (*Isochrysis* sp. 30000 cells ml^{-1}) at 6 days after hatching, table 7. Larval density was 5 ml^{-1} for each species.

Table 7.

Percentage of larvae feeding on each species of Artemia / rotifer prey pairs at dah 6

Days after hatching 6						
Prey pair	Larvae feeding (%)	Larvae feeding on either prey (%)	Larvae feeding on both (%)	Larvae with cysts %	Prey larvae^{-1}	Cysts larvae $^{-1}$
Rotifers / *Artemia* + T-iso	55.5 ± 10.6	3.2 ± 4.6 / 38.0 ± 5.5	7.0 ± 1.0	6.0 ± 1.0	0.1 / 0.6	0.3
Rotifers / *Artemia*	42.0 ± 7.6	7.6 ± 6.7 / 26.2 ± 9.5	6.6 ± 6.4	3.5 ± 1.3	0.05 / 0.5	0.2

The percentage of feeding larvae increased slightly by supplementation of algae and more larvae ingested *Artemia* than rotifers in this study. The number of ingested prey larvae^{-1} was similar with or without supplementation of algae.

Feed intake of sole larvae exposed to different light intensities

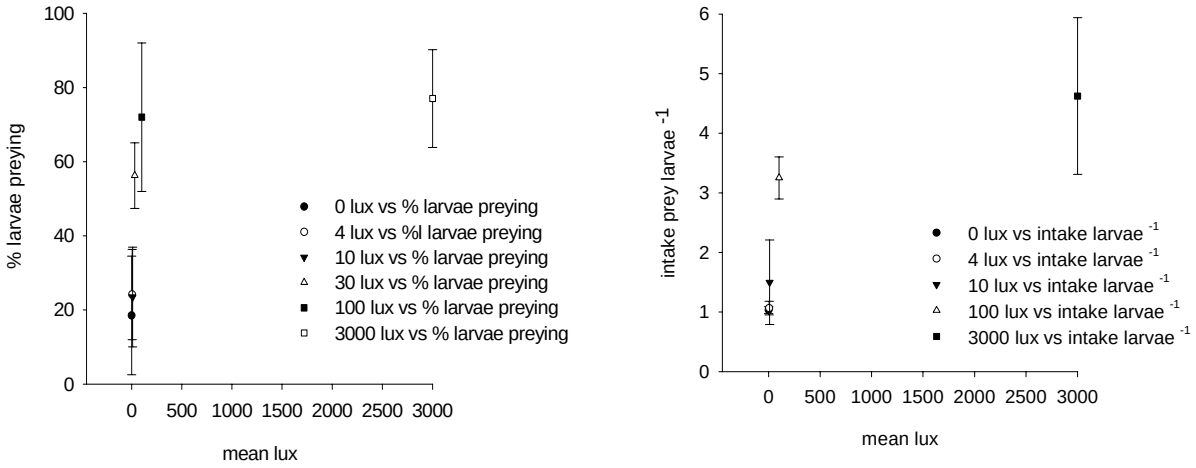
Previous reported growth trials on sole were performed with an surface illuminance (lux = 1 lumen /m^2) of 100 lux measured as by a lux meter (Lund et al.,unpublished data a, Lund et al, in press).

In the present trial, the ability of sole larvae to detect prey was tested at different light intensities and was examined on 8 to11 days old sole larvae. Prior to the trial larvae were fed rotifers and the test was performed with the use of AF *Artemia*. An experimental duplicate set up was established with a mean lux intensity of 0, 4, 10, 30, 100, 3000.

The results from the test are shown as the mean percentage of feeding sole larvae at 8 to11 days after hatching for each light intensity (Fig. 1a) as well as the mean number of prey ingested per larvae (Fig. 1b).

Figure 1. a- b

a) The mean percentage of sole larvae preying at surface light intensities of 0, 4, 10, 30, 100 or 3000 lux. b) The mean number of prey ingested per larvae at each light intensity.



The percentage of larvae with had ingested prey was lower at light densities of 0 and 4 lux as compared with 30 lux. The highest percentage of feeding larvae was observed in concert with lux intensities of 100 and 3000 respectively. However, a relatively large variation was observed between replicates.

The number of prey consumed per larvae increased with an increase in light intensity, but no differences were observed in larvae at dah 8 or dah 11 despite the increase in age and size.

A similar test was carried out for another batch of 4 days old larvae, which not had been fed prior to the trial. The result revealed that only about 9 % of the larvae were feeding at 0 lux, 12 % at 4 lux and 46 % at 100 lux.

Discussion

First feeding sole larvae are slow moving predators, poor swimmers and are observed to float /swim near the surface. This is especially characteristic when tank aeration is switched off, as all larvae will drift to the surface (personal observations).

The reason, that larvae only ingested rotifers and cysts at dah 4, may be related to the relative ease at which larvae catch rotifers or cysts in comparison to *Artemia* and *Tisbe*. Empty cysts tend to float at the surface, have a suitable size and a good contrast, which all may explain the relative much higher feed intake in first feeding sole larvae, despite the low density. Rotifers have a narrow radius of action, which may improve larval success of prey capture.

In the present trials the initiation of first feeding may be related to the time of the final depletion of the oil globules. Observations have indicated that 3 days old sole larvae with a functioning mouth and remaining oil globule reserves do not eat (personal observations). In support of this, a relatively low percentage of larvae were feeding at dah 4, while at dah 4 and 6 the percentage of feeding larvae was much higher, when the yolk and oil globules were depleted.

A clear larval preference was observed in favour of *Artemia* cysts but not for any of the three live prey species, however, a lower percentage of larvae ingested *Tisbe*. Cysts have a suitable size for sole larvae, but unhatched cysts or empty shells cannot be digested by fish larvae and may obstruct the gut, when ingested (Dhont and Van Stappen). In terms of suggesting a preferable prey, a floating microencapsulated formulated feed a “magic bullet” resembling the physical properties of an *Artemia* cyst may be readily ingested by sole larvae.

Apparently, difference in prey size was not related to the number of prey ingested per larvae. At most 70 percent of the larvae were feeding in the trials, which may be related to the short duration of the trials and the relatively short acclimatization to the experimental conditions.

In conclusion, all 3 species were initially ingested by the larvae (at dah 4-6), with no clear discrimination of prey species. In a previous study, in which sole larvae were fed either *Artemia*, rotifers or *Tisbe* until 9 days after hatching, larvae fed rotifers revealed a negative growth. Larvae on *Tisbe* had a slight positive growth and larvae on *Artemia* had a very high growth (Lund et al., unpublished data a), and it was questioned if sole larvae were actually preying on rotifers.

The present results indicate, that start feeding sole larvae **do eat** rotifers and *Tisbe*, but that *Artemia* were actively preferred at dah 10, when fed in combination with rotifers.

The suppression of growth observed in the referred study by Lund et al is therefore suggested mainly related to the overall energy intake pr larvae. The caloric content in J mg^{-1} d.w. was estimated to be quite similar for all three prey species, but the prey size much different (Lund et al., unpublished data a).

At prey densities of 1:1 a much higher proportion of larvae ingested *Artemia* when fed a combination of rotifers and *Artemia.* The results indicated an active preference for *Artemia* when fed a concentration of rotifers 10 or 20 times higher that of *Artemia*.
The combination of algae and prey did not improve the proportion of feeding larvae, despite that algae may modify the visibility of prey and increase the rate of feed intake as observed for turbot and halibut (Øie et al., 1997, Reitan et al., 1997), but the short duration of the trial may have had an influence. This batch of larvae clearly preferred *Artemia* for rotifers in contradiction to the previous observation, which may indicate batch related differences.

Feed intake and illuminance
The ability of larvae to eat in complete darkness was low and intake was gradually improved by an increased illuminance. In Senegal sole the ability to feed in darkness seemed to improve with the age of the larvae (Canavate et al., 2006). The present results revealed that sole larvae, close to the start of metamorphosis, are capable to feed in complete darkness, however, with a limited success, as the majority of larvae contained at most 1 prey per larvae. Therefore sole larvae at these stages seem to rely on their visual ability to detect and catch prey more than on chemical cues.
3000 lux may provide an even better visual contrast for sole larvae to detect and catch prey in transparent tanks, than the 100 lux used in previous studies (Lund et al.,unpublished data a, Lund et al, 2007) but may affect larval pigmentation, as light intensity may be involved in the pigmentation process.(Lund et al., unpublished data, b)

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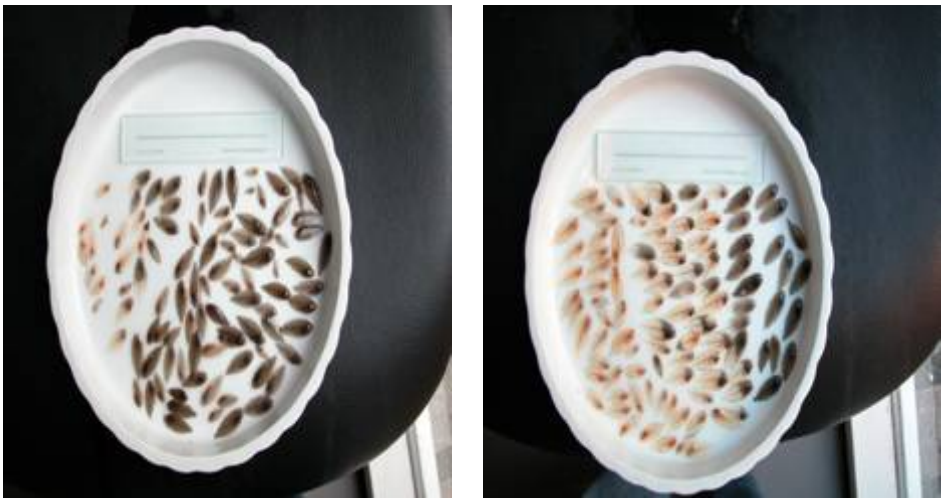

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Effect of dietary arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid on survival, growth and pigmentation in larvae of common sole (*Solea solea* L.)

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Abstract

Evidence confirms that polyunsaturated fatty acids (PUFAs), arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid, DHA are involved in growth as well in pigmentation of marine fish larvae.

In the present study we examined the performance of common sole larvae reared on *Artemia* enriched with 10 formulated emulsions, differing in inclusions of ARA, EPA, and DHA. The specific growth rate of the sole larvae until late metamorphosis, 21 days after hatching (dah) was 20 to 27% d^{-1}. Even though the relative tissue essential fatty acid (EFA) concentrations significantly reflected dietary composition, neither standard growth nor larval survival were significantly related to the absolute concentrations of ARA, EPA and DHA or their ratios. This suggests low requirements for essential polyunsaturated fatty acids (PUFAs) in common sole. Malpigmentation was significantly related to increased dietary ARA content. However, pigmentation was not affected by inclusion levels of EPA or DHA when ARA was high. This, and no relation between DHA: EPA or ARA: EPA ratios and pigmentation and only a weak relation to ARA: DHA ratio, advocate for that it is the absolute concentration of ARA in larval tissues, that is responsible for malpigmentation rather than the relative concentration to other PUFAs.

Within malpigmentation, the trait "albinism" was characterised by an abnormal incomplete eye migration, but this trait is suggested not to be related to dietary ARA. Furthermore, albinism resulted in a lower growth rate, which suggests that visual aberrations affected prey capture.

Keywords: Sole larvae; Growth; Arachidonic acid; Eicosapentaenoic acid; Docosahexaenoic acid; Pigmentation; Eye migration

1. Introduction

Common sole are an esteemed, high priced fish for consumption with a relatively fast production cycle of 1–1 1/2 years at 20 °C (Howell, 1997; Imsland et al., 2003). Furthermore, sole larvae are regarded fairly easy to rear (Howell, 1997), making it a prime candidate for the expanding intensive recirculation fish farms in northern Europe.

Reduced marketability due to malpigmentation is a well known problem in culture of common sole and in

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other flatfish species (McEvoy et al., 1998; Venizelos and Benetti, 2000). Development of pigmentation is influenced by the larval diet until the end of metamorphosis (Seikai, 1985; Reitan et al., 1994). An increasing body of evidence suggests an important role of n-3 polyunsaturated fatty acids (PUFAs) on survival, growth and pigmentation of fish larvae (Izquierdo et al., 2000; Morais et al., 2004; Bransden et al., 2005). Dietary deficiency or imbalances in n-3 PUFAs may induce mortality and delay growth in fish larvae (Izquierdo, 1996) as these are important for the formation of cell membranes important for normal development of vision and neural systems. The n-3 PUFA requirement is however, lower for sole than for many other marine fish species, and diets rich in eicosapentaenoic acid 20:5n-3 may be nutritionally sufficient (Howell and Tzoumas, 1991).

Until recently most of the studies on the dietary effect of manipulation of PUFAs on growth and pigmentation have been focused on n-3 PUFAs, probably due to their predominance in marine fish (Bell et al., 2003; Villalta et al., 2005). However, recent studies have indicated the importance of n-6 PUFAs on pigmentation, in particular arachidonic acid, ARA (Bell and Sargent, 2003) which is an essential fatty acid (EFA) for marine fish.

Studies of dietary manipulations of ARA, and the n-3 PUFAs, EPA and docosahexaenoic acid (DHA) suggest that they affect pigmentation in flat fish, however the actual role of each of these essential fatty acids is still under debate, and contradicting results have been reported. DHA is known to be vital for vision and membrane fluidity, while ARA and EPA are involved in eicosanoid synthesis, hormones which are believed involved in malpigmentation (Koven, 2003). Elevated levels of ARA or high ARA: EPA ratio in diets has been reported to result in significantly reduced pigmentation rates in turbot (McEvoy et al., 1998; Estevez et al., 1999) and Senegalese sole (Villalta et al., 2005). This is in contrast to previous studies in fish larvae, indicating that the DHA: EPA ratio imposes the main effect on pigmentation success (Rainuzzo et al., 1997).

The objective of the present study was to examine different dietary combinations and EFA quantities of ARA, EPA and DHA on sole larval survival, growth and pigmentation. Graduated inclusions of ARA oil up to 30% in emulsions were examined. Inclusions of 30% ARA oil has been shown previously to cause malpigmentation in Senegalese sole, but with no negative effect on growth and survival (Villalta et al., 2005). EPA and DHA oil were supplemented from 0 to 27%, in order to obtain diets with different concentrations of ARA, EPA and DHA and different ratios.

2. Materials and methods

2.1. Broodstock handling and hatchery conditions

Approximately 350 wild (mature) broodstock common sole (*Solea solea*) were caught from the North Sea, Skagerak with gill nets at the end of April 2005. The fish were transferred to two 9 m^3 circular tanks and fed fresh blue mussels once a day. The broodstock spawned naturally in the tanks.

On the 28 May approximately 55,000 fertilized eggs were transferred to 2 cylindrical incubators with 160 l of 1 μm filtered seawater and gentle aeration. The eggs were kept at semi dark (dimmed light) conditions during incubation and the temperature was increased gradually from 12.8 to 13.7 °C. Water exchange was above 100% h^{-1}, oxygen saturation was close to 100%, pH was 7.93–7.95. The eggs hatched on 1 June. Temperature was slowly elevated from 13.7 to 16.5 °C. The larvae were moved to the rearing facility at dah 3.

The rearing facility consisted of 30 cylindrical–conical tanks each holding 46 l and connected to a flow-through water system with 1 μm filtered water. Each tank had a separate inlet through a flowmeter, a 500 μm outlet filter, and (0.45 μm) filtrated aeration through an airstone.

Larvae were transferred to the 30 rearing tanks by multiple subsampling from a 60 l bucket holding all larvae, triplicate tanks were used. An expected 1068 larvae were added to each rearing tank, equalling a density of 27 larvae l^{-1}.

Oxygen saturation, temperature and pH were monitored daily by an Oxyguard/pH meter from Oxyguard, Birkerød, Denmark and NH_4^+, NO_3^-, NO_2^- by a Merckquant test kit from Merck, Darmstadt, Germany.

The temperature from dah 4–22 was 17.9±0.5 °C and from dah 22–36 it was kept at 20.5±0.6 °C. pH values was during the trial approximately 7.8. Levels of nitrite, nitrate and ammonia were below kit detection. Oxygen content for all tanks throughout the trial was kept above 7.1 mg l^{-1} (7.6±0.3).

The photo period was 24 h light.

2.2. Preparation of emulsions

Nine prepared emulsions and one commercial emulsion Easy DHA Selco (INVE, Belgium) were used in the experiment (Table 1). Easy DHA Selco was used as a reference diet. The emulsions were prepared by mixing freshly prepared marine fish oil (sprat/blue whiting) from Fiskernes Fiskeindusti (FF) Skagen, Denmark with various amount of concentrated Incromega eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) oils from Croda Chemicals Europe, Snaith, UK, and natural arachidonic acid (ARA) (VEVODAR) oil, produced by means of fermentation of a fungus (*Mortierella alpina)* from DSM Food Specialities BV, Holland. The emulgator was soy lecithin (BioMar, Brande, Denmark) added (7%) as a growth promoter (Macqueen et al., 2003) and an E-vitamin mixture (BioMar, Brande, Denmark) was added (4%) as an antioxidant. All oils were freshly prepared and stored at −30 °C until usage.

Table 1
Formulation of diets,% inclusion of ARA, EPA and DHA

	Reference	*Control*	ARA effects			Interactive effect				
	DHA Selco	Fish oil	ARA supplementation							
			10%	20%	30%	20%	20% DHA supplementation	20%	20%	20%
						7.5%	17.5% EPA supplementation	27.5%	0%	20%
						7.5%	17.5%	27.5%	20%	0%
Total FA (TFA)	359.2	438.1	391.7	403.3	442.2	476.3	446.5	477.5	436.5	418.8
FA composition (%TFA)										
C16:0	17.5	18.7	19.1	18.5	19.3	16.5	10.6	5.7	14.0	12.8
C18:0	3.4	1.5	2.3	3.0	3.6	2.4	2.4	2.1	2.5	2.8
total SFA*	26.2	26.9	28.0	27.9	29.1	24.1	16.3	10.0	21.3	19.9
C16:1 (n-7)	4.4	5.1	4.4	3.8	3.3	2.9	1.8	0.9	2.8	2.5
C18:1 (n-9) cis	13.9	20.3	19.4	18.0	16.7	14.3	10.3	6.3	13.8	13.0
total MUFA*	21.1	29.3	27.2	24.8	22.4	19.9	14.2	9.0	18.6	18.4
C18:2 (n-6) c	5.4	5.0	4.9	5.8	6.3	4.9	5.3	3.5	4.9	4.8
C18:3 (n-6)	0.1	0.1	0.4	0.8	1.1	0.7	0.7	0.7	0.8	0.7
C20:3 (n-6)	0.1	0.0	0.5	0.9	1.2	0.7	0.8	0.8	0.9	0.8
C20:4 (n-6), ARA*	1.8	0.8	6.2	12.3	17.7	11.4	11.2	11.3	12.2	11.3
total (n-6) PUFA*	7.7	6.4	12.5	20.3	26.7	18.1	18.4	16.6	19.1	18.1
C18:3 (n-3) c	1.1	2.8	2.4	2.0	1.6	1.5	1.2	0.7	1.6	1.4
C20:5 (n-3), EPA*	10.0	10.2	8.8	7.2	5.8	12.5	19.2	26.0	23.0	7.2
C22:6 (n-3), DHA*	33.8	24.2	20.9	17.5	14.2	23.9	30.5	37.5	16.3	34.8
total (n-3) PUFA	45.1	37.4	32.3	27.0	21.7	37.9	51.0	64.4	41.0	43.6
DHA:EPA	3.4	2.4	2.4	2.4	2.5	1.9	1.6	1.4	0.7	4.8
ARA:DHA	0.1	0.0	0.3	0.7	1.2	0.5	0.4	0.3	0.7	0.3
ARA:EPA	0.2	0.1	0.7	1.7	3.1	0.9	0.6	0.4	0.5	1.6
(n-3):(n-6)	5.9	5.8	2.6	1.3	0.8	2.1	2.8	3.9	2.1	2.4

Total fatty acid content (TFA) (mg g^{-1} d.w) and, fatty acid composition (% TFA) of the 10 diets. (n=1).
*TFA; total fatty acids, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, ARA; arachidonic acid, EPA; eicosapentaenoic acid, DHA;docosahexaenoic acid. Totals include minor fatty acids not shown. ($P<0.05$).

During preparation of the emulsions the oils were heated to 25 ° C, the ingredients were mixed with a stir bar on a magnetic stirrer for 5 min before the emulsions were flushed with nitrogen. Each emulsion was then sucked up into four 20 ml sterile plastic syringes; the air was removed and the emulsion frozen at −80 °C. The commercial Selco emulsion was treated similarly.

At usage one syringe was thawed at a time and kept in a refrigerator at + 4 °C. The daily amount of emulsion used for enrichment was weighed and mixed with 100 ml distilled water by homogenisation with a Büchi mixer B-400 for 40 s at 9000 rpm.

To examine effects of ARA supplementation on pigmentation, three emulsions were formulated, containing 10, 20 and 30% Vevodar, ARA oil. Interactive effects between ARA, EPA and DHA on survival, growth and pigmentation were investigated by formulating three emulsions with 20% ARA oil and 7.5, 17.5 or 27.5% of EPA and DHA oil, and two emulsions containing 20% ARA oil and either 20% EPA oil *or* 20% DHA oil to isolate the influence of EPA and DHA. One emulsion was formulated with only fish oil, as a control.

The analysed FA content of the 10 different emulsions is given in Table 1.

2.3. Production of live feed

Rotifers were enriched for 8–12 h at 25 °C with each of the 10 emulsions by a concentration of 0.4 g emulsion per million rotifers. The second stage AF nauplii *Artemia,* harvested 10 h after hatching and newly hatched EG grade *Artemia* were washed with 0.2 μm filtered seawater followed by freshwater. They were counted and divided into 10 buckets of 5 l filtered seawater each at a similar density of 1.2×10^5 l^{-1} and enriched with 0.6 g emulsion l^{-1} at 21–22 °C for 22–24 h.

Artemia was harvested once a day in the morning *Artemia* for the second daily feeding was kept refrigerated with aeration in separate beakers.

2.4. Larval rearing

Larval feeding was initiated in the afternoon at dah 3. Live feed was administered 2 times a day at 0010–1100 and 2100–

2200 h, at a concentration of 5 rotifers ml^{-1} or 2 *Artemia* ml^{-1}. Prior to each feeding, water exchange in each tank was increased for 1–2 h to avoid possible leftovers of prey from the previous feeding. Larvae were fed rotifers at dah 3–4 followed by AF grade *Artemia* on dah 5–7 and EG grade *Artemia* hereafter (dah 8–21). Post settling (dah 22–36), all fish were fed EG *Artemia* enriched with Easy DHA Selco.

2.5. Estimation of size, pigmentation rate and eye migration

Standard length of larvae was measured, by a digital high resolution DFC 320 Leica camera connected to a dissecting microscope (Leica MZ 6).

The evaluation of pigmentation was carried out by evaluating all fish individually. The juvenile fish were categorized into 4 subgroups; 1) normally pigmented, 2) malpigmented tails, 3) malpigmented part of body, or 4) "albinos" (>95% malpigmentation). Hyperpigmented (ambicolourated) fish were registered but categorized as normal. The overall malpigmentation was evaluated as (sum of malpigmented fish/total surviving number) × 100.

Eye migration was evaluated for all fish at dah 37. An abnormal incomplete migration of the eyes was defined as when the migrating eye was on or close to the dorsal midline, or between final position and dorsal midline on the ocular side. According to a traditional index (Fernàndez-Diaz et al., 2001) the positions found in this trial could be defined as stage 2 or 3, respectively. Stage 4 defines a complete and normal eye translocation.

2.6. Dry weight (dw) and ash content

Larvae (10–15 larvae $sample^{-1}$) and prey (50–100 prey $sample^{-1}$) were collected, counted and filtrated on Whatman Ø 25 mm 0.7 μm GF/F glass fibre filters. 5 ml of 0.5 M v/v ammonium formate was filtrated through the filters to remove seawater from the dry matter samples. The filter samples were dried in an oven at 80 °C for 24 h then weighed on a Mettler Toledo MT5 d=0.1 μg, and combusted at 450° for 16 h and reweighed.

2.7. Sampling of live feed and larvae

Samples of *Artemia* were collected at dah 5, 12 and 18. After collection they were washed with distilled water, counted and filtrated on sample filters and kept in 5 ml sterile cryo vials, covered with nitrogen and frozen at −80 °C until extraction. Enriched rotifers and AF *Artemia* were only used as feed for 2 and 3 days respectively, therefore their composition is not presented.

Biochemical analyses were carried out for larvae from each rearing tank at dah 6, 13 and 19, but only data at dah 19 are presented for clearness. Samples of sole larvae were collected in duplicate prior to feeding in the morning. Fifty to 60 larvae were randomly sampled from each tank. Each subsample (10–15 larvae) was gently washed on a 60 μm filter net with 0.2 μm seawater (>15 °C).The samples were stored in nitrogen at −80 °C as similarly to *Artemia.*

For estimation of survival the 200 larvae sampled during the trial were included as live individuals.

2.8. Extraction and analysis of fatty acids

Total lipids from the emulsions, the enriched live feed and larvae fed the emulsions were extracted in chloroform/methanol (2:1.v/v) for 24 h (Folch et al., 1957). The −80 °C frozen filters were transferred to 3 ml clean glass test tubes and 1 ml chloroform/methanol mixture was poured until the filter was covered followed by addition of either 60 μl internal standard of methyl tricosanoate (C23:0) in chloroform (0.046 mg ml^{-1}) (for prey samples) or 40 μl (1 mg ml^{-1}) (for fish larvae/eggs etc.). The test tubes were flushed with nitrogen and closed with an aluminium lid. The samples were sonicated for 10–15 min (42 KHz, 100 W) in a 2510 Branson ultrasound cleaner and kept for 24 h at −25 °C in order to extract lipids.

The liquid/solvent in the samples were transferred to 2 ml autosampler vials with butyl/teflon lids followed by evaporation of the solvent at 60 °C by applying a flow of nitrogen for ca. 20 min by a 9 port Reacti-Vap Evaporator in a Pierce Reacti-Therm heating module. The vials then received a reagent solution (1 ml) composed of a mixture of toluene, methanol and acetyl chloride (40: 50: 10) before they were heated for 2 h at 95 °C. Then aqueous $NaHCO_3$ (5% by weight, 0.5 ml) was added to the vial and after vigorous shaking the upper layer containing the fatty acid methyl esters was removed. The water phase was extracted twice with heptane (0.5 ml) and the combined organic layers were collected and evaporated at 60 °C by a gentle stream of nitrogen. Finally, the fatty acid methyl esters were re-suspended in chloroform (0.5 ml) and an aliquot of the sample (5 μl) was analysed by GC-MS. The GC-MS instrument was composed of an Agilent 6890 series gas chromatograph equipped with a PTV inlet and an Agilent 5973 mass selective detector. The column was a 60 m Agilent DB23 with an inner diameter of 250 μm and a film thickness of 0.3 μm. Peaks on a given chromatogram were identified by comparison with the retention time of a commercial available mixture of a known FAME standard, SUPELCO (C4:0–C24:0), from SIGMA, No 18919.

Peaks were quantified by means of the target response factor of the fatty acids to the C23:0 internal standard. Fatty acid concentrations were calculated (Chem. Station program) based on the quantified peaks of the standard series and the samples and expressed as ng $sample^{-1}$.

A total of 34 fatty acids were analysed, but only the main saturated fatty acids, monounsaturated fatty acids, n-6 and n-3 PUFAs are presented in the tables for clearness.

2.9. Survival and growth of larvae

Survival was estimated by counting all individuals at dah 21, including 200 larvae $replicate^{-1}$ sampled for analyses.

Growth rate was calculated as Specific Growth Rate (SGR) based on d.w. of sampled larvae according to the formula; $SGR = (\ln W_f - \ln W_i \times 100)/ t$, Where $\ln W_{f\ i}$ = the natural logarithm of the final and initial the weight, t = time (days) between $\ln W_f$ and $\ln W_i$.

2.10. Statistics

All statistical tests ($P<0.05$) for sole larvae growth, and FA composition were carried out using a one-way ANOVA and a pairwise multiple comparison of means using Tukey`s post hoc test.

Linear regression analysis was used to identify the relationship between larval FA contents, eye migration and malpigmentation. The variance of data are given as standard deviation (s.d.), ($n=3$), unless otherwise stated.

3. Results

3.1. Fatty acid composition of emulsions and live prey

All emulsions had a similar mg g^{-1} dw content of total fatty acids (TFA) (Table 1). The substitution with high inclusions of ARA, EPA and DHA oils lead to considerably reduced relative concentrations of e.g. C16:0 and C18:1. The n-6 and n-3 PUFAs were also highly affected by the various inclusion levels of the EFA oils, Table 1.

EG *Artemia,* used as feed from day 8–21 were affected by the composition of the emulsions (Table 2).

3.2. Larval fatty acid composition

3.2.1. ARA

Pre-feeding larvae at dah 3 had a relative ARA content of 4–5% (data not presented). Differences in larval ARA content became gradually prevalent with time. Larval ARA tissue contents were significantly different at dah 19 ($P<0.001$) and significantly ($P<0.001$) related to the dietary content (Table 3). Larval treated with identical ARA implementations had similar tissue contents.

3.2.2. EPA

At dah 19 the EPA content in larval groups differed significantly ($P<0.001$), and was significantly correlated ($P<0.001$) to the dietary content (Table 3). At dah 19 larvae fed *Artemia* with 27.5% inclusion of both EPA and DHA oil had a significant higher EPA content ($P<0.004$) than for any of the other larvae groups followed by larvae treated with 17.5 and 20% EPA oil. The significantly lowest EPA content (4.2%) at dah 19 ($P<0.001$) was observed for larvae treated with the highest ARA content (30%).

3.2.3. DHA

Pre-feeding larvae at dah 3 had a relative predominance of DHA (>51%) (data not shown). Larval content decreased extensively throughout the trial for all larval groups regardless of treatment, probably both as a consequence of larvae utilization and the very much lower content in the nauplii analysed. Hence, the relative DHA proportion gradually decreased from >30% at dah 6 to <15% at dah 19, (Table 3). At dah 19 larval DHA contents were significantly different ($P<0.001$) and significantly correlated to the dietary content despite the overall gradual decrease.

3.3. Larval survival

Larval survival was evaluated at dah 21 (Table 4). Larval survival was not significantly affected by treatments ($P>0.6$) and not correlated to tissue ARA, EPA or DHA at dah 19 ($P>0.08$, $r^2<0.33$).

3.4. Larval growth dah 3–21

Larval at hatching were 3.38±0.13 mm and 3 days after hatching 3.93±0.11 mm with a dw of $5.4\times10^{-2}\pm1.9\times10^{-3}$ mg ind^{-1}. At dah 19, DHA Selco treated larvae (Table 4) were significantly larger than all other groups of larvae except larvae fed 20,−17.5–17.5% ARA, EPA, DHA ($P<0.001$). Furthermore at dah 19 the length of larvae fed fish oil enriched *Artemia* (control) was smaller than 6 of the larval groups fed on *Artemia* enriched by the ARA, EPA, DHA combinations ($P<0.017$).

Larval dry weights did not depend on enrichment type ($P>0.15$). However, at dah 19 there was a tendency towards higher values for larvae fed with *Artemia* enriched by the commercial Easy DHA Selco (Table 4).

Growth in dw calculated as specific daily growth rate at dah 3–21 was 19.6–26.6% d^{-1}, but not significantly different among treatment groups ($P=0.14$) or significant correlated to dietary ARA, EPA or DHA contents ($P>0.33$, $r^2<0.12$).

3.5. Juvenile pigmentation

Juvenile pigmentation depended on dietary treatment ($P<0.001$) and overall malpigmentation increased by increases in dietary levels of ARA (Table 4). Larvae fed prey enriched on the reference feed, DHA Selco, or the control feed, fish oil, had a significantly better pigmentation ($P<0.001$) than any of the other treatment groups. There was no significant difference between the reference DHA Selco and the control fish oil enriched groups of larvae ($P>0.57$).

Larval overall malpigmentation was highly significantly correlated to increasing levels of arachidonic acid in tissues at dah 19, ($P\leq0.003$). (Fig. 1)

Pigmentation was neither correlated to dietary contents of EPA, DHA ($r^2=0.22$), ($r^2=0.07$) nor to EPA ($P>0.6$, $r^2=0.04$) or DHA contents in sole larval tissues at dah 19 ($P=0.26$, $r^2=0.15$).

Pigmentation was not related to dietary or tissue ARA: EPA ratio ($P>0.06$, $r^2<0.38$), DHA: EPA ratio ($P>0.2$) or ARA: DHA ratio, but correlated to larval ARA: DHA ratio at dah 19 ($P=0.04$, $r^2=0.42$). Pigmentation was not correlated to prey n-3: n-6 ratio, but to larval n-3: n-6 ratio at dah 19 ($P=0.02$, $r^{2=}0.53$).

Table 2
Total fatty acid content (mg g^{-1}d.w.) and fatty acid composition (% FA) of EG *Artemia* given as a mean±standard deviation ($n=3$)

	Reference	Control	ARA effects			Interactive effects				
	DHA Selco	Fish oil	ARA supplementation							
			10%	20%	30%	20%	20% DHA supplementation	20%	20%	20%
						7.5%	17.5% EPA supplementation	27.5%	0%	20%
						7.5%	17.5%	27.5%	20%	0%
Total FA (TFA)	226.0±4.0	189.6±22.9	167.1±34.0	149.5±12.2	201.0±5.6	161.9±39.5	201.3±52.7	188.1±17.2	288.5±36.1	236.8±57.6
FA composition (%TFA)										
C16:0	10.1±0.1^{abc}	11.9±0.9^{bc}	11.1±1.9^{abc}	10.2±2.1^{abc}	12.8±0.5^{c}	9.4±0.2^{abc}	7.9±0.4^{ab}	6.6±0.1^{a}	9.5±1.3^{abc}	11.4±1.9^{b}
C18:0	4.8±0.0	4.5±0.3	5.8±1.3	4.9±0.0	6.5±0.4	5.6±0.3	4.6±0.4	4.3±0.8	5.3±0.1	7.0±3.1
total SFAs*	16.7±1.9^{abc}	18.3±1.6^{abc}	18.8±1.0^{abc}	16.9±2.6^{abc}	21.9±1.1^{c}	16.6±0.1^{abc}	13.9±0.7^{ab}	12.1±1.0^{a}	16.5±1.8^{abc}	20.6±5.2^{bc}
C16:1 (n-7)	2.6±0.3^{bc}	2.6±0.2^{bc}	2.8±0.1^{c}	2.5±0.1^{bc}	2.4±0.1^{bc}	1.8±0.1^{ab}	1.7±0.1^{a}	1.3±0.0^{a}	1.9±0.3^{ab}	2.1±0.1^{b}
C18:1 (n-9) cis	18.1±0.9^{cd}	21.1±0.1^{d}	21.0±2.0^{cd}	20.6±1.1^{cd}	18.7±1.3^{bcd}	17.2±0.9^{bcd}	14.6±0.9^{a}	12.5±0.1^{a}	15.5±1.4^{ab}	16.5±1.5^{ab}
total MUFAs*	22.5±1.5^{cd}	26.5±0.2^{d}	26.2±1.7^{d}	25.3±0.9^{cd}	23.6±1.4^{cd}	21.0±1.0^{bc}	18.1±0.8^{ab}	15.3±0.1^{a}	19.3±1.0^{b}	20.8±1.7^{bc}
C18:2 (n-6) c	6.4±0.6	6.8±0.1	7.6±0.4	7.7±1.4	7.6±0.1	6.9±0.0	6.1±0.4	6.4±0.4	6.2±0.5	6.2±0.1
C18:3 (n-6)	0.4±0.0^{ab}	0.3±0.0^{a}	0.5±0.1^{bc}	0.6±0.0^{c}	0.9±0.1^{d}	0.6±0.0^{c}	0.6±0.0^{c}	0.6±0.0^{c}	0.6±0.1^{c}	0.6±0.0^{c}
C20:3 (n-6)	0.1±0.0	0.6±0.7	0.3±0.0	0.5±0.1	0.8±0.1	0.5±0.0	0.5±0.0	0.5±0.1	0.6±0.0	0.5±0.0
C20:4 (n-6), ARA*	2.4±0.8^{ab}	1.2±0.0^{a}	3.6±0.0^{b}	6.5±0.7^{c}	9.6±0.8^{d}	5.7±0.2^{c}	5.4±0.7^{c}	5.4±0.1^{c}	6.5±0.2^{c}	6.0±0.2^{c}
total (n-6) PUFA*	9.8±0.1^{a}	9.8±0.8^{a}	12.8±0.4^{b}	16.2±0.7^{d}	19.7±0.8^{e}	14.5±0.2^{bc}	13.4±0.2^{bc}	13.5±0.2^{bc}	14.6±0.1^{c}	14.1±0.3^{bc}
C18:3 (n-3) c	19.0±0.2^{ab}	19.5±1.8^{ab}	20.7±0.6^{ab}	22.1±0.9^{b}	17.2±1.6^{ab}	20.8±0.5^{ab}	18.4±1.2^{ab}	17.0±1.0^{ab}	16.0±0.9^{a}	18.1±3.6^{ab}
C20:5 (n-3), EPA*	18.3±0.1^{c}	15.3±0.3^{b}	13.8±0.5^{b}	13.1±1.6^{b}	10.7±0.2^{a}	18.9±1.2^{c}	25.6±0.3^{d}	29.7±0.4^{e}	26.7±0.2^{d}	12.9±0.9^{b}
C22:6 (n-3), DHA*	13.1±0.3^{d}	9.9±0.4^{c}	7.0±0.2^{a}	5.8±0.2^{a}	6.3±0.9^{a}	7.5±0.3^{a}	10.0±1.0^{b}	11.8±0.3^{cd}	6.4±0.4^{a}	13.0±0.8^{d}
total (n-3) PUFA	51.1±0.3^{c}	45.4±2.5^{bc}	42.2±0.3^{b}	41.7±2.4^{b}	34.8±0.5^{a}	47.9±1.0^{d}	54.6±0.3^{de}	59.1±1.0^{e}	49.6±0.6^{cd}	44.5±3.8^{bc}
DHA:EPA	0.7±0.0^{c}	0.7±0.0^{bc}	0.5±0.0^{bc}	0.4±0.1^{b}	0.6±0.1^{bc}	0.4±0.0^{b}	0.4±0.0^{b}	0.4±0.0^{b}	0.2±0.0^{a}	1.0±0.1^{d}
ARA:DHA	0.2±0.1^{a}	0.1±0.0^{a}	0.5±0.0^{b}	1.1±0.2^{d}	1.5±0.1^{e}	0.8±0.0^{c}	0.5±0.0^{b}	0.5±0.0^{b}	1.0±0.0^{d}	0.5±0.0^{b}
ARA:EPA	0.1±0.0^{b}	0.1±0.0^{a}	0.3±0.0^{c}	0.5±0.0^{d}	0.9±0.1^{e}	0.3±0.0^{c}	0.2±0.0^{bc}	0.2±0.0^{abc}	0.2±0.0^{c}	0.5±0.0^{d}
(n-3):(n-6)	5.2±0.0^{e}	4.7±0.6^{e}	3.3±0.1^{c}	2.6±0.3^{ab}	1.8±0.1^{a}	3.3±0.0^{c}	4.1±0.1^{cd}	4.4±0.0^{d}	3.4±0.0^{bc}	3.2±0.3^{c}

*TFA; total fatty acids, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids; PUFA; polyunsaturated fatty acids, ARA; arachidonic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid. Totals include minor fatty acids not shown. A different superscript in a horizontal row denotes significance ($P<0.05$).

Table 3
Total fatty acid content (mg g^{-1} d.w.) and fatty acid composition (% of TFA) of larvae at 19 days after hatching given as a mean±standard deviation ($n=3$)

	Reference	Control	ARA effects			Interactive effects				
	DHA Selco	Fish oil	ARA supplementation							
			10%	20%	30%	20%	20% DHA supplementation	20%	20%	20%
						7.5%	17.5% EPA supplementation	27.5%	0%	20%
						7.5%	17.5%	27.5%	20%	0%
Total FA (TFA)	82.6±16.6	64.6±9.2	95.7±2.8	73.4±8.7	82.6±10.4	68.2±11.3	65.5±18.7	115.8±26.9	88.2±2.6	62.9±17.8
FA composition (% TFA)										
C16:0	11.4±0.3	12.3±0.8	11.6±0.5	12.0±0.7	12.2±0.3	11.9±0.7	11.3±0.4	10.7±0.2	11.6±0.7	11.3±0.4
C18:0	5.2±0.1^{a}	6.3±0.1^{d}	5.2±0.2^{a}	5.8±0.3^{cd}	6.0±0.1^{cd}	5.6±0.1^{bc}	5.3±0.1^{ab}	5.0±0.1^{a}	5.3±0.2^{ab}	5.9±0.2^{cd}
total SFA*	18.8±0.3^{ab}	21.0±0.8^{c}	19.0±0.5^{ab}	20.2±0.5^{c}	20.6±0.3^{c}	19.8±0.5^{b}	18.8±0.5^{ab}	17.6±0.3^{a}	19.1±0.7^{ab}	19.5±0.3^{b}
C16:1 (n-7)	2.3±0.0^{b}	2.1±0.4^{ab}	2.3±0.1^{b}	2.0±0.1^{ab}	1.9±0.0^{ab}	1.9±0.1^{ab}	2.1±0.1^{ab}	1.5±0.0^{a}	2.1±0.4^{ab}	1.8±0.2^{ab}
C18:1 (n-9) cis	18.9±0.2^{c}	19.9±0.3^{d}	21.3±0.4^{e}	19.9±0.4^{cd}	19.5±0.1^{d}	18.6±0.3^{c}	17.2±0.1^{b}	16.0±0.2^{a}	19.0±0.6^{c}	18.3±0.2^{c}
total MUFA*	1.1±0.0^{bcd}	1.2±0.0^{d}	1.1±0.0^{cd}	1.0±0.1^{b}	1.0±0.0^{b}	1.0±0.0^{b}	0.9±0.0^{b}	0.9±0.0^{a}	0.9±0.0^{ab}	1.1±0.0^{bc}
C18:2 (n-6) c	23.1±0.1^{c}	24.2±0.5^{c}	25.7±0.5^{d}	23.7±0.5^{c}	23.3±0.1^{c}	22.3±0.4^{bc}	21.0±0.2^{b}	19.1±0.3^{a}	22.8±1.0^{c}	22.1±0.3^{bc}
C18:3 (n-6)	7.4±0.0^{a}	8.6±0.1^{e}	8.3±0.1^{d}	7.8±0.1^{c}	8.7±0.1^{e}	7.7±0.1^{b}	7.2±0.1^{a}	7.2±0.1^{a}	8.1±0.0^{c}	8.0±0.1^{c}
C20:3 (n-6)	0.4±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{b}	0.6±0.0^{c}	0.8±0.0^{d}	0.6±0.0^{c}	0.6±0.0^{c}	0.6±0.0^{c}	0.6±0.0^{c}	0.6±0.0^{c}
C20:4 (n-6), ARA*	3.4±0.1^{a}	2.5±0.2^{a}	6.0±0.3^{b}	9.8±0.6^{e}	10.1±0.3^{e}	7.1±0.4^{bc}	6.9±0.2^{bc}	7.6±0.1^{cd}	7.2±0.9^{cd}	7.0±0.3^{bcd}
total (n-6) PUFA*	11.8±0.1^{a}	12.3±0.2^{a}	15.8±0.3^{b}	19.3±0.5^{d}	20.8±0.4^{e}	16.4±0.4^{c}	15.7±0.2^{bc}	16.5±0.1^{c}	17.0±0.8^{c}	16.6±0.3^{bc}
C18:3 (n-3) c	21.3±0.1^{abc}	22.6±0.3^{cd}	20.9±0.7^{ab}	21.1±0.5^{c}	22.0±0.6^{ab}	23.9±0.5^{d}	22.0±0.2^{abc}	20.5±0.0^{a}	22.2±0.7^{bc}	22.4±0.2^{c}
C20:5 (n-3), EPA*	7.6±0.4^{e}	6.6±0.4^{d}	6.3±0.2^{d}	5.2±0.1^{bc}	4.2±0.0^{a}	6.7±0.5^{d}	9.4±0.2^{f}	11.0±0.3^{g}	9.1±0.4^{f}	5.3±0.2^{c}
C22:6 (n-3), DHA*	15.4±1.1^{1d}	11.3±0.5^{c}	10.6±1.0^{bc}	8.7±0.8^{ab}	7.3±0.4^{a}	9.0±0.7^{ab}	11.4±0.3^{c}	13.9±0.2^{cd}	8.1±1.1^{a}	12.1±0.4^{c}
total (n-3) PUFA*	46.2±0.6^{de}	42.4±0.9^{c}	39.4±0.4^{b}	36.8±0.5^{a}	35.4±0.3^{a}	41.5±0.6^{c}	44.5±0.4^{d}	46.9±0.5^{e}	41.1±0.8^{c}	41.7±0.5^{c}
DHA:EPA	2.0±0.2^{e}	1.7±0.0^{d}	1.7±0.2^{cd}	1.7±0.1^{cd}	1.7±0.1^{d}	1.3±0.0^{bc}	1.2±0.0^{b}	1.3±0.0^{b}	0.9±0.1^{a}	2.3±0.1^{e}
ARA:DHA	0.2±0.0^{a}	0.2±0.0^{a}	0.6±0.0^{b}	1.1±0.0^{e}	1.4±0.0f	0.8±0.0^{c}	0.6±0.0^{b}	0.5±0.0^{b}	0.9±0.0^{d}	0.6±0.0^{b}
ARA:EPA	0.4±0.0^{a}	0.4±0.0^{a}	1.0±0.1^{c}	1.9±0.1^{e}	2.4±0.1^{f}	1.1±0.0^{c}	0.7±0.0^{b}	0.7±0.0^{b}	0.8±0.1^{b}	1.3±0.0^{d}
(n-3):(n-6)	3.9±0.1f	3.5±0.0^{e}	2.5±0.0^{c}	1.9±0.0^{b}	1.7±0.0^{a}	2.5±0.0^{c}	2.8±0.0^{d}	2.8±0.0^{d}	2.4±0.1^{c}	2.5±0.0^{c}

*TFA; total fatty acids, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, ARA; arachidonic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid. Totals include minor fatty acids not shown. A different superscript in horizontal rows defines a significant difference ($P<0.05$).

Table 4
Larval survival (%), larval dry weight (mg.ind^{-1}), larval body length (mm), growth calculated as specific growth rate (SGR)

	Reference	Contrrol	ARA effects			Interactive effects			
	DHA Selco	Fish oil	ARA supplementation						
			10	20	30	20	20 DHA supplementation	20	20
						7.5	17.5 EPA supplementation	27.5	0	20
						7.5	17.5	27.5	20	0
Survival (dah 3–21))	34.1±9.8	29.0±11.2	33.7±2.9	37.2±20.6	42.9±5.4	28.1±2.2	29.9±10.1	28.8±3.7	28.0±7.6	27.5±13.1
d.w. (dah 19)	1.902±0.52	0.842±0.38	1.210±0.20	1.350±0.13	1.286±0.55	1.404±0.1	1.371±0.12	1.303±0.23	1.127±0.1	1.236±0.55
Length (dah 19)	9.30±0.78^{c}	7.55±0.70^{a}	8.46±0.17^{b}	8.56±0.19^{b}	8.17±0.70^{ba}	8.35±0.22^{ba}	8.98±0.60^{bc}	8.54±0.26^{b}	8.54±0.67^{b}	8.30±0.85^{ba}
SGR (d.w.) (dah 3–19)	26.6±2.4	19.6±3.5	22.9±1.4	23.9±0.8	23.1±3.3	24.2±0.5	24.0±0.7	23.6±1.5	22.4±0.6	22.5±4.4
Length dah 37	2.19±0.51^{c}	2.09±0.65^{bc}	2.02±0.43^{b}	1.80±0.42^{a}	1.82±0.44^{a}	2.15±0.45^{c}	2.09±0.48^{bc}	2.18±0.52^{c}	2.17±0.60^{c}	2.01±0.65^{b}
Length (normal pigmented) dah 37	2.26±0.53^{b}	2.14±0.51^{a}	2.20±0.39^{b}	1.95±0.54^{a}	1.98±0.51^{a}	2.37±0.44^{b}	2.11±0.57^{ab}	2.30±0.59^{b}	2.39±0.43^{b}	2.11±0.59^{ab}
Length (albino groups) dah 37	1.83±0.39^{ab}	1.77±0.49^{ab}	1.65±0.42^{a}	1.63±0.37^{a}	1.66±0.37^{a}	1.88±0.37^{b}	1.96±0.40^{b}	1.98±0.39^{b}	1.98±0.49^{b}	1.84±0.49^{b}
Mal pigmented dah 37	37.2±5.8^{a}	25.0±2.8^{a}	61.4±11.6^{b}	77.2±3.7^{bc}	83.4±0.7^{c}	74.6±8.5^{bc}	90.4±3.9^{c}	87.1±3.2^{c}	86.7±5.8^{c}	82.5±6.5^{c}

Length of juveniles and length of normalpigmented juveniles as well as albinos (cm) and percent malpigmented for all treatments at days of sampling given as a mean±standard deviations. n=3.
SGR=Specific growth rate. A different superscript in a horizontal row denotes a significant difference $P<0.05$.

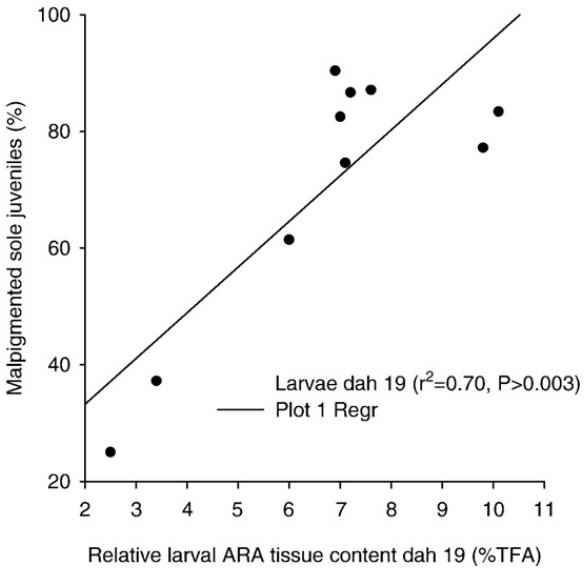

Fig. 1. Regression correlation between the relative larval percentage ARA in sole larvae at dah 19 and malpigmentation.

3.6. Malpigmented subgroups and larval eye migration

The frequency of overall malpigmented juveniles, classified as "tail malpigmented" or "body malpigmented" was not significantly different among treatments ($P>0.61$) or related to EFA enrichment. The frequency of juveniles classified as "albinos" was lowest in larvae treated with the reference DHA Selco followed by the fish oil control diet. This was significantly lower than in larvae treated with 30% ARA oil ($P<0.048$). The frequency of larvae showing hyper pigmentation was very low for most treatments; less than 1% of the total number of fish.

An abnormal incomplete eye translocation was far most frequent in albino juveniles (Fig. 2) as 46 to 76% of the albino juveniles had this aberration. This trait was less frequent in body malpigmented juveniles (2–11%) and even lower in the tail malpigmented juveniles (0–5%), or normal pigmented juveniles (0–2%). Incomplete eye translocation in albinos was not related to larval ARA, EPA or DHA contents (Fig. 2a–c).

4. Discussion

In the present study larval survival was not affected by any of the dietary treatments. The average larval survival of 28–43% was lower than 70%, obtained in other larval studies on *Solea solea* (Howell, 1997), or 57–71% survival, as reported for the close relative, Senegalese sole (Cañavate and Fernández-Diaz, 1999). The reason for this discrepancy is not clear, it may have been batch related, as egg quality may influence larval quality (Lund et al., unpublished data). The similarity in survival of larvae fed formulated emulsions and the

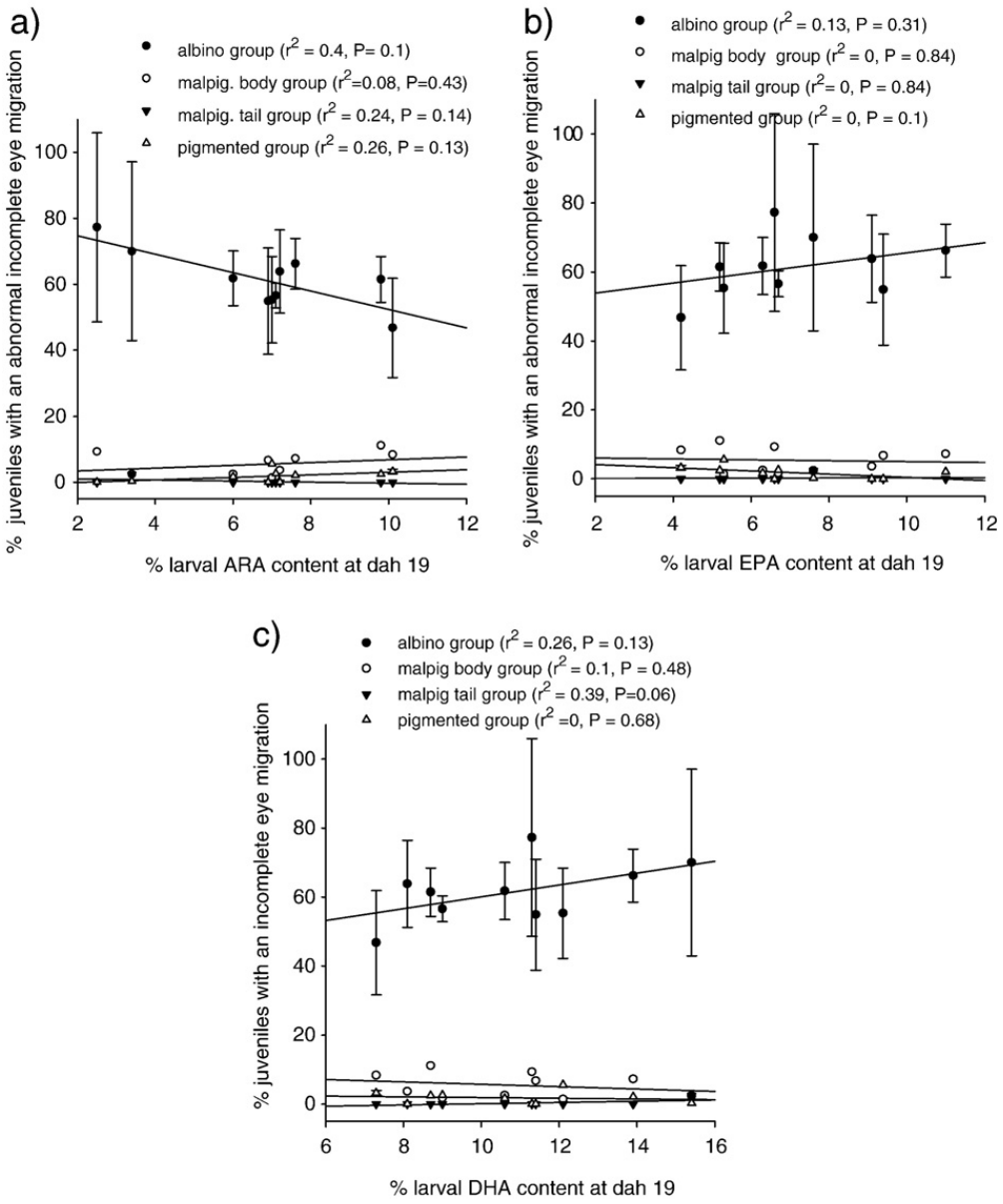

Fig. 2. a–c Percent juveniles with and incomplete abnormal eye migration versus tissue concentration of ARA,EPA or DHA at dah 19 in the 4 malpigmented subgroups.

reference diet, DHA Selco, however indicates that survival was not influenced by dietary effects.

Larval length at dah 19 was highest for larvae fed the reference, DHA Selco feed, which may have been caused by the presence of various growth promoters such as fat soluble vitamins and proteins in this diet, similar to results obtained by other commercial emulsions (Izquierdo, 1996; Villalta et al., 2005). Similar to the present results, Villalta et al. (2005) did not find any improvements in terms of growth and survival, when Senegalese sole larvae were treated with DHA. Larvae fed prey enriched with only fish oil (control) were the smallest. These were significantly smaller than 6 of the ARA, DHA, EPA combinations and were considerably lower in ARA content, than larvae fed the other formulated diets. However, larval length was not correlated to final larval ARA content similar to results on Senegalese sole (Villalta et al., 2005), in which larval growth was not related to dietary ARA. Studies on the effect of ARA on survival and growth are scarce. In a study on striped trumpeter (*Latris lineata*), results also indicated, that ARA had no effect neither in survival nor in growth (Bransden et al., 2004b) but Ishizaki et al. (1998) found a negative effect of ARA on survival and growth of *Seriola quinqueradiata*. In gilthead sea bream larvae, growth and survival were improved by ARA (Bessonart et al., 1999), but the ARA levels used were much lower (0.1–1.8% dw) than in the present study.

Zheng et al. (1996) reported that cod larvae fed enriched *Artemia* with different ARA levels (3.7–7.6% dw) showed poorer survival and slower growth than when fed with less than 0.5% of dw.

Overall high larval specific growth rates of 20–27% d^{-1} from dah 3 to dah 21 is similar to growth rates reported for Senegalese sole (26% d^{-1}) (Yùfera et al., 1999). The overall similarity in growth and survival, despite highly formulated differences of n-6 /n-3 PUFAs indicate a relatively low requirement /effect of PUFAs on growth as previously reported (Howell, 1997).

In the present study, the length of the albino juveniles were significantly smaller ($P<0.001$) than juveniles with a normal pigmentation (Table 4), for 9 of the 10 treatment groups and not related to overall survival. A possible cause of the lower growth rate could be the high occurrence of incomplete impaired eye migration of albino juveniles, and a resulting aberrant visual ability as found by Bolker and Hill (2000) influencing the hunting performance. It is therefore suggested that the visual ability when feeding on live feed is more important than chemoreception, despite that chemoreception is believed to be an integrated part of sole larval / juvenile feeding strategy (Imsland et al., 2003). A similar growth of highly malpigmented (<25% pigmentation on the ocular side) and normal pigmented juvenile turbot as well as halibut has been reported (Imsland et al., 2006), but the authors did not examine the growth of albino juveniles in relation to a possible impaired eye migration.

The proportional fatty acid content in *Artemia* e.g. ARA, EPA, DHA very well reflected the composition of the emulsions ($r^2=0.89$–0.96) as shown in other studies (Villalta et al., 2005).

The fatty acid composition in larvae at dah 19 reflected very well the dietary fatty acid composition, which is consistent with other studies on larval fish (Castell et al., 1994; Bransden et al., 2004a).

The relative ARA content in sole larvae exposed to the fish oil control with the lowest dietary level of ARA (1.2%) decreased progressively from the 4% in start feeding fish to 2.5% in dah 19 larvae. In contradiction, in Senegalese sole, a close relative, a strong retention of tissue ARA has been reported in case of a low dietary supply (Vàzquez et al., 1994; Villalta et al., 2005). However, Villalta et al. (2005) used a diet with almost 0% ARA, so retention of ARA may only occur at very low dietary ARA levels to sustain important biochemical processes involving ARA.

At dah 19 EPA and DHA tissue contents decreased significantly in larvae fed 10, 20 and 30% ARA, i.e ARA effects without EPA or DHA supplements. Part of the decrease was related to the significant increase in the fish larval tissue content of ARA, and an actively displacement of EPA and DHA as reported for other marine fish (Estevez et al., 1999, Willey et al., 2003). This may be due to a competitive cellular interaction between EPA, ARA and DHA (Tocher and Sargent, 1986; Bell et al., 1995; Villalta et al., 2005) and a higher affinity of ARA to the cell enzymatic binding site competing for the fatty acids (Bell et al., 1995; Whelan, 1996). However when EPA and /or DHA were supplemented in diets, (i.e. interactive effect), their content in tissue seemed not to be displaced by inclusion of 20% ARA.

The use of linear regressions in the present study, to calculate the displacement or the accumulation rates of each fatty acid in sole larval tissue in relation to either ARA, EPA or DHA tissue levels as described by Harel et al. (2000), showed no competition between tissue fatty acids with increasing ARA, EPA or DHA levels, as similar to results on Senegalese sole (Villalta et al., 2005). In general, the relative larval tissue content of most analysed fatty acids, (i.e not only PUFAs) reflected the relative dietary composition.

Fatty acid requirements of marine fish larvae, especially PUFAs, may reflect fatty acid profiles in the eggs or egg phospholipids (Izquierdo, 1996; Silversand et al., 1996; Bell et al., 2003). In the present study the relative EPA egg content was 5–6% (data not presented). The uptake of 30% EPA in *Artemia*, increased the relative EPA levels in the sole larval tissue to 11% at most and indicated that EPA was only retained to a certain extent. Larval tissue composition did not indicate major chain shortening of EPA to other n-3 metabolites, so excess dietary EPA may have been utilised for other requirements, such as energy. The discrepancy in EPA content between *Artemia* and larvae may be related to general differences in uptake, digestibility/metabolism and the storage of nutrients and fatty acids between the species, and the fact, that sole larvae had empty guts whereas *Artemia* had filled guts at time of analysis.

DHA content in eggs and pre feeding sole larvae (i.e. 50% TFA) was very high in comparison to the content in studies of other flatfishes. Eggs of Senegalese sole, turbot and halibut have been reported to contain 24–35% DHA (of TFA) (Reitan et al., 1994; Næss and Lie, 1998; Villalta et al., 2005). The difference may be related to the composition of the broodstock diet (Izquierdo, 1996). A high DHA content in eggs has been related to the specific requirements for DHA during the early larval development of neural tissues as brain and retina, as these tissues are highly enriched in

DHA (Silversand et al., 1996). Obviously an effect of DHA might have been expected. However, dietary DHA did not improve sole larval performance, which is consistent with results reported by Tzoumas (1988) and also for Senegalese sole reported by Morais et al. (2004).

The degree of malpigmentation was positively correlated to an increase in dietary ARA. Despite the fact that we observed highly significant positive correlations between an increase in malpigmentation and in dietary ARA: DHA ratio ($r^2=0.90$, $P<0.03$) or in ARA: EPA ratio ($r^2=0.78$, $P<0.05$), which is similar as to results on turbut (McEvoy et al., 1998),—normal pigmentation was not improved by interactive effects of EPA or DHA, i.e. a high dietary EPA: ARA ratio (>5.5) or an increase in dietary DHA: ARA ratio (0.6 → 2.2) by high inclusions of DHA.

It seems therefore likely that pigmentation success cannot simply be explained by the ratios of ARA: DHA or ARA: EPA. A dietary DHA: EPA of >2:1 and EPA: ARA>5:1 has been suggested to improve pigmentation in turbot and halibut (Reitan et al., 1994; Bell et al., 2003), but could not be confirmed in the present study. None of the inclusion levels of EPA and DHA had any influence on the pigmentation rate, when *Artemia* with an ARA content of 5–6% were used. It was the actual concentration of ARA (or concentration of possible ARA metabolites) that was responsible for the malpigmentation.

It is believed that ARA and EPA compete for binding to the enzymes cycloxygenase and lipoxygenase (Hamre et al., 2005). The ratio of EPA and DHA binding to these enzymes will ultimately determine the ratio of n-2 and n-3 eicosanoids in the tissue of the fish larvae. These two series of eicosanoids have different effects, which are of physiological significance. Enhanced levels of the bioactive 2-series eicosanoids (prostaglandins) derived from arachidonic acid are involved in various pathological conditions like thrombosis, tumor growth, and immune inflammatory disorders (Logue et al., 2000). As an excess production of eicosanoids is involved in fish experiencing biochemically induced stress (Sargent et al., 1999), it is hypothesised that malpigmentation could be caused by this (Sargent et al., 1999). It is speculated that increasing ratios (>1) of EPA: ARA and or DHA: ARA could minimize these effects by a reduction / inhibition of the synthesis of ARA derived prostaglandins (Whelan, 1996). Consequently, the ratio of ARA: EPA might influence the rate- and the type of eicosanoids produced. The data in the present experiment, however, did not support that an increase in the concentration or in the EPA: ARA ratio improved the pigmentation. This may be explained by the high inclusion levels of ARA used, as it has been demonstrated that when ARA and EPA are present together fish cells convert more of the ARA to eicosanoids than what EPA does.

The albino group juveniles in all treatments had high incidence of incomplete eye migration at dah 37. The albino groups exhibited lower growth rate than the other pigmentation subgroups, and it could be questioned whether or not the albino group in the present study had completed metamorphosis. In a study on Senegalese sole (Fernàndez-Diaz et al., 2001) only 80% of the sole larvae had finished metamorphosis at dah 37. On the contrary the eye migration was successfully completed in 38 days old Senegalese sole exposed to high levels of arachidonic acid in the juvenile diet (Villalta et al., 2005). However, strongly malpigmented specimens from the present study, at an age of 3 months and a total length of 5–10 cm, also showed an abnormal eye position, which strengthens evidence that the impaired eye migration in albinos was not simply related to a delayed metamorphosis.

For all treatments, a similar non significant incomplete eye migration in the albino subgroup was observed including the fish oil control having a relatively low ARA level (Fig. 2a–c). This suggests that impaired eye migration is not solely related to high ARA levels and that the aberration seems to be related to albinism itself and not to arachidonic acid. Contrary to this, it has been reported, that increasing dietary ARA levels slowed the eye migration at dah 15 and dah 20 in Senegalese sole larvae (Villalta et al., 2005).

Abnormal eye translocation may instead be an irreversible injury caused by nutritional factors during or just before the metamorphosis. It suggests that common sole are restricted by an early “pigmentation window” where the final characteristics of both the pigmentation and the morphology of the fish are determined (Solbakken et al., 1999).

Further trials might elucidate, if common sole larvae have a “window of pigmentation sensitivity” in relation to dietary levels and inclusions of ARA and EPA, and whether a relationship between pigmentation and eicosanoids could be established as has been reported for cod, *Gadus morhua* (Bransden et al., 2005).

5. Conclusions

In the present study growth rate and survival in common sole larvae was not related to ARA or dietary EFA combinations. Malpigmentation was determined during the larval stage and was significantly related to

dietary levels of ARA, but not related to dietary DHA: EPA or ARA: EPA ratio or to dietary concentrations of EPA, DHA. Albino juveniles had a lower growth than normal pigmented, and in contrast had an abnormal incomplete eye migration presumably not related to ARA.

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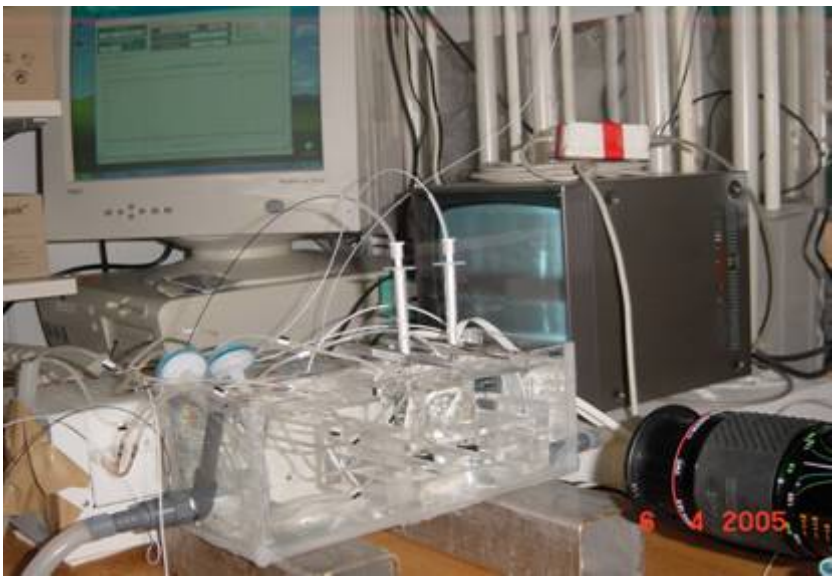

Metabolic rate and tolerance of hypoxia in common sole (*Solea solea*) larvae and juveniles raised on *Artemia* with different essential fatty acid compositions.

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Metabolic rate and tolerance of hypoxia in common sole (*Solea solea*) larvae and juveniles raised on *Artemia* with different essential fatty acid compositions.

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Abstract

Common sole *(Solea solea*, Linneaus 1758) were raised from first feeding upon *Artemia* with different contents and compositions of the essential fatty acids (EFA) arachidonic acid (ARA, 20:4n-6); eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3), and their metabolic rate and tolerance of hypoxia measured prior to and following metamorphosis and settlement. Four types of *Artemia* were compared: 1) un-enriched; 2) enriched with a commercial mixture of EFAs (Easy DHA SELCO Emulsion); 3) enriched with a marine fish oil containing a high ratio of ARA to DHA (VEVODAR oil), and 4) enriched with a marine fish oil containing a low ratio of ARA to DHA (Incromega DHA oil). Prior to settlement, larvae fed upon un-enriched *Artemia* had a significantly higher routine metabolic rate (RMR) than the other dietary groups in normoxia; exhibited visible signs of respiratory distress at higher water oxygen partial pressures (PO_2) than the other groups during progressive hypoxia, and had a significantly higher mean critical oxygen partial pressure (P_{crit}) for the regulation of RMR. Larval metamorphosis and settlement was associated with an overall decline in RMR and increase in P_{crit}, but post-settlement juveniles raised upon un-enriched *Artemia* still exhibited a higher P_{crit} than the other groups, although this was not associated with a significant difference in RMR. The sole fed un-enriched *Artemia* had significantly lower contents of EFA in their tissues than the other dietary groups both before and after settlement. The results indicate that enriching live feeds with EFA can have significant effects upon the respiratory physiology of sole larvae and juveniles, and improve their in-vivo tolerance of hypoxia. We found no evidence, however, for any effect of the ratio of ARA to DHA.

Introduction

Marine fish larvae have an absolute requirement for three physiologically essential fatty acids (EFAs), namely the long-chain highly unsaturated arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (DHA, C22:6n-3) (Sargent et al., 1999a; Bransden et al., 2005). These EFAs maintain the normal structure and function of cell membranes and are precursors of the autocrine eicosanoids (Sargent et al. 1999a,b). Dietary deficiency or imbalances in EFA during larval stages can have various negative effects including abnormal behaviour, malpigmentation, depressed immunity, tissue pathologies and, consequently, reduced growth and survival (Salhi et al., 1999; Sargent et al., 1999b; Bell et al., 2003, Morais et al. 2004; Bransden et al., 2005).

A variety of studies have investigated the effects of dietary EFAs upon stress resistance in marine fish larvae (Tago et al. 1999; Logue et al. 2000; Koven et al. 2001; Weirich and Reigh, 2001). For example, a previous study on larvae and juveniles of the common sole (*Solea solea*) reported that deprivation of EPA and DHA increased mortality rates upon exposure to stresses such as hypoxia, low temperature, and low salinity (Logue et al., 2000). These studies have all used mortality as the end point for the measurement of stress tolerance (Weirich and Reigh 2001; McKenzie 2005), because of the difficulties inherent to probing the in-vivo physiology of minute marine fish larvae (Weiser 1995; Bang et al. 2004). There is, however, much evidence to suggest that dietary EFAs can have significant sub-lethal effects upon the physiology of fishes (Randall et al. 1992; McKenzie 2001; 2005; Chatelier et al. 2006). In particular, diets rich in EPA and DHA appear to improve tolerance of respiratory stresses such as hypoxia and hypercapnia (Randall et al. 1992; McKenzie et al. 1995; 1997; 2000). Further increases in EPA:ARA or DHA:ARA may reduce the production of ARA derived prostaglandins and thereby minimize a possible biochemical stress response (Sargent et al. 1999a,b).

The purpose of the current study was, therefore, to investigate how the routine metabolic rate (RMR) and in-vivo tolerance of hypoxia of individual sole larvae was influenced by feeding with live brine shrimp (*Artemia* sp) nauplii that provided different EFA quantities and combinations. The experiment comprised two sequential trials, in an initial trial larvae were fed upon *Artemia* that were either not enriched at all (unenriched) or enriched with a standard commercial EFA mixture (Easy DHA SELCO oil, INVE, The Netherlands). In the second trial, larvae were fed upon *Artemia* that had been enriched with one of two marine oils that provided different EFA combinations, to compare a high ratio of ARA to DHA against a low ratio of these EFA. Routine metabolic rate

(RMR) was measured as instantaneous oxygen consumption (M_{O2}), and tolerance of hypoxia was assessed as by examining the ability of a larva to maintain RMR during progressive reductions in oxygen availability. Animals were also filmed to monitor behavioural responses to hypoxia, for comparison with the respirometry data.

Materials and methods

Experimental animals and rearing conditions

Common sole larvae were obtained from a wild-caught broodstock that was held in large tanks (volume 9 m^3) provided with a flow of seawater at prevailing environmental temperatures at the North Sea Centre, Hirtshals, Denmark. The broodstock spawned spontaneously and floating eggs were collected. In the two sequential larval rearing trials larval densities were between 27 and 47 larvae l^{-1}. Three replicates were used for each diet, and larvae were randomly selected from these tanks. Larvae were reared at a photoperiod of 24 h light within a temperature controlled room (17.9-18.1 ° C) within 30 flow-through conical tanks (volume 46 l). The tanks were provided with 1 µm filtered seawater (exchange rate 8-10 l h^-1) and filtered (0.45µm) aeration with airstones. Oxygen content was maintained between 7.1 and 8.5 mg l^{-1}. Water pH was measured daily and was between 7.8 - 8.0, water content of nitrite, nitrate and ammonia were below detection levels.
Feed for the first trial consisted of AF grade *Artemia* harvested at 8 to10 h post-hatch. The *Artemia* were separated into two groups, one left unenriched and the other enriched for a minimum of 8 h with a stirred emulsion of Easy DHA SELCO at a concentration of 0.6 g l^{-1}. Feed for the second trial consisted of EG grade *Artemia* harvested at 24 h post-hatch and enriched with two different types of oils providing different dietary contents of ARA, EPA and DHA, each for 24 h at a concentration of 0.6 g l^{-1} (Table 1).

	Emulsion oil	
	High ARA/DHA	Low ARA/DHA
Inclusion %		
Sprat,blue whiting fish oil[b]	59.0	54.0
Vevodar (ARA) oil [c]	30.0	20.0
Incromega EPA fish oil [d]	0.0	7.5
Incromega DHA fish oil [d]	0.0	7.5
Soy lecithin[e]	7.0	7.0
Vitamin E mix[e]	4.0	4.0

Table 1. Formulation of the 2 experimental emulsions used in ARA/DHA Artemia enrichment trial.
[a] INVE, Netherlands,[b] FF, Skagen, Denmark, [c] DSM Food Specialities, Netherlands, [d] Croda Chemicals, UK, [e] BioMar, Denmark

The larvae were fed twice a day, in the morning and afternoon, with a concentration of prey of 3-5 ml^{-1} for each feeding. Feed for the second daily feeding was kept refrigerated with aeration. For both trials, individual sole were sampled between 13-16 days after hatching (dah), therefore just prior to metamorphosis (characterised as "pre-settlement"), and then between 21-23 dah and hence after metamorphosis and eye translocation (characterised as "post-settlement"). Pre-settlement larvae were free-swimming in the water column, and animals were sampled that did not show any pronounced evidence of an asymmetrical position of the left and right eyes. For the post-settlement larvae, eye translocation was completed and the larvae lay upon the bottom or sides of the tank for the vast majority of the time.

Respirometry setup

For measurements of oxygen uptake, individual larvae were transferred to borosilicate glass chambers containing seawater. Two sizes of chambers were used, with a volume of either 710 µl for pre-settlement larvae, or 1710 µl for post-settlement larvae. Great care was taken to avoid air-exposing the animals during transfer to the chambers, this was achieved by capturing them from their holding tanks into a 50 ml beaker, gently pipetting single larvae from the beaker into a glass tube, and then releasing them into the chamber. Each chamber had a watertight borosilicate glass stopper, that was fitted with two stainless steel ports (inner diameter 800 µm) that were used as the inlet and outlet for a flow of 0.2 µm-filtered autoclaved aerated seawater supplied by a peristaltic pump (ISMATEC IPC-12) via tygon tubing (inner diameter 800 µm). The chambers were thermostatted at 19.0 ± 0.1ºC by immersion in a plexiglass reservoir (80 x 80 x 200 mm) provided with a continuous recirculating flow of distilled water from a heater/cooler system (Heto 8-30). The autoclaved seawater provided to the respirometry chambers was also thermostatted at the same temperature by immersion in a large outer tank also receiving a recirculating flow of water from the heater / cooler system (Figure 1).

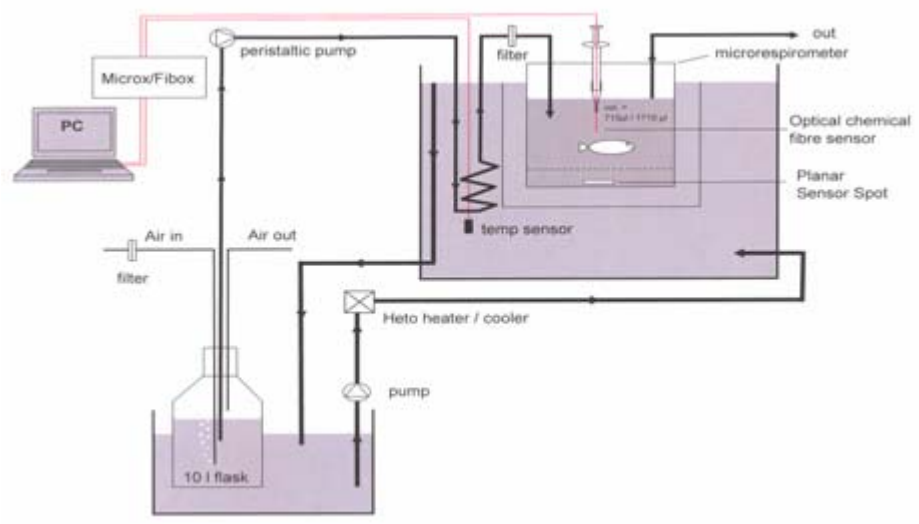


Figure 1.
Illustration of experimental set up.

Instantaneous oxygen consumption (MO_2, mmol $g_{dw\ ind.}^{-1}$ h^{-1}, where

the subscript dw ind. refers to dry weight individual $^{-1}$) was measured by intermittent stop-flow respirometry (Steffensen 1989) as described in McKenzie et al. (1995; 2007a). Water oxygen partial pressures (PO_2) were measured with temperature-compensated fiber optic sensors coated with an oxygen quenchable fluorophore immobilised in PVC (PreSens, Precision Sensing GmbH, Regensburg, Germany). The advantage of these sensors is that they have no intrinsic oxygen consumption, and hence no correction was required when measuring oxygen consumption of the larvae in small water volumes. Two types of PreSens probe were used, either an optical chemical fibre sensor attached to a Microx TX3 meter (McKenzie et al. 2004) or a planar sensor spot (Behrens et al. 2007) attached to a Fibox 3 meter. When the fibre sensor was used, this was advanced into the chamber via a third stainless steel port in the stopper, otherwise this third port was sealed with silicon. The signals from these probes were displayed on a PC with PreSens Oxyview software, and then transferred into an automated data acquisition system (LoliDAQ, Loligo Systems ApS) and associated software (LoliResp, Loligo Systems ApS), which calculated MO_2 at defined cycles of recirculation versus flushing (McKenzie et al. 1995; 2007). Microsensors were calibrated according to the manufacturers' instructions. Two respirometry chambers were run in parallel, and the whole setup was shielded below an opaque black cloth, to avoid disturbing the larvae. The system was, however, illuminated with an IR spotlight beneath the cloth, which allowed the larvae to be filmed through the glass walls of their chambers, with a Minitron CCD camera (MTV-1802CB), fitted with a Computar TV Macro zoom lens, (MLH-10x) and the image displayed on a monitor. The background oxygen consumption of the water was measured at the end of each day and never amounted to more than 5% of larval MO_2. No corrections were, therefore, applied.

Experimental protocol

All sole larvae used in the respirometry studies were collected in the morning, prior to the daily provision of *Artemia*, to reduce potential errors associated with large stomach contents. All larvae were allowed 3 h recovery from transfer to the chambers, with cyclical measurements of normoxic routine metabolic rate (RMR) made once each 20 min. The peristaltic pump was then turned off, so that the chamber was no longer flushed and the larva progressively consumed the oxygen in the water. Thus, the larva themselves created progressive hypoxia, and MO_2 was measured every 10 min throughout this period. The behaviour of the larva was monitored throughout and, as soon as the larvae lost equilibrium, the peristaltic pump was turned on again to provide a flow of aerated seawater to the chamber. All measurements were made between 09:00 and 18:00 to reduce any

potential effects of circadian rhythms in MO_2, and, for each trial, a comparison was made of two different diets (i.e. larvae fed either enriched versus unenriched *Artemia*, or larvae fed *Artemia* enriched with either high or low ARA/DHA ratio oils). As previously described by McKenzie et al. (2000; 2007), the water PO_2 beyond which the larvae showed a consistent decline in their MO_2, of at least 10 % below their normoxic RMR, was taken as an indicator of their critical PO_2 for regulation of normoxic RMR (P_{crit}) , and hence their tolerance of hypoxia.

Measurements and analysis of larvae

Following each trial, larvae were measured for standard length, dry weight (dw) and ash content. Standard length was measured to the nearest 0.01 mm by a Leica MZ6 dissecting microscope connected to a digital high resolution DFC320 Leica camera and treated by a Leica software programme. Samples for dw were dried in an oven at 80 °C for 24 h then weighed, and combusted at 450 °C for 16 h and reweighed (Mettler Toledo balance, MT5 d =0.1 µg).

Fatty acid analysis

For each trial, representative samples of *Artemia* were taken (> 40 individuals). Larvae (> 15 individuals) were sampled in duplicate from each tank and analysed for their FA composition during the pre and post-settlement periods of the respirometry trial. Fatty acids were extracted in a chloroform/ methanol mixture (Folch et al., 1957) and then trans-esterified by acetyl chloride in methanol. The FA methyl esters were analyzed by gas chromatography - mass spectrometry (GC-MS). Peaks were quantified by means of the target response factor of the fatty acids to the C23:0 internal standard. The FA concentrations were calculated (Chem. Station programme) based on the quantified peaks of a standard series with a known composition (SUPELCO, 18919, SIGMA) and the samples and expressed as ng $sample^{-1}$.

Statistics

For those physiological and tissue FA variables that were measured both pre- and post-settlement, a two-way analysis of variance (ANOVA) was performed where settlement and dietary group were the two factors. For those behavioural variables in hypoxia that were only compared between dietary groups at a single stage (see below), a one-way ANOVA was performed. A probability of less than 5 % ($P < 0.05$) was taken as the fiducial level of significance for the ANOVAs and, in those cases where the ANOVA revealed a significant difference amongst groups, the groups that differed were identified post-hoc by Holm-Sidak tests.

Results

Larval metabolic rate and responses to hypoxia

A total of 8 pre-settlement larvae and 8 post-settlement juveniles were tested for the first trial, and 7 larvae and 6 juveniles for the second trial, each group with the mean dry weights shown in Table 2.

Table 2.

Mean (± SEM) normoxic routine metabolic rate, critical PO_2 and thresholds for behavioural responses to hypoxia in sole fed various types of Artemia as live prey. For each variable, a different superscript denotes a significant difference ($P < 0.05$) between means.

	Larval diet			
	Unenriched	Selco	High ARA/DHA	Low ARA/DHA
Pre-settlement				
Mass (mg dw)	0.99 ± 0.30	1.06 ± 0.23	1.00 ± 0.52	0.78 ± 0.24
RMR ($nmolO_2\ mg_{dw}^{-1}\ h^{-1}$)	115 ± 9^{a}	87 ± 8^{b}	82 ± 14^{b}	104 ± 17^{a,b}
P_{crit} (kPa)	13.6 ± 1.5^{a}	8.5 ± 2.8^{b}	8.4 ± 2.1^{b}	11.4± 2.5^{a,b}
Agitated (kPa)	15.3 ± 2.3^{a}	9.9 ± 1.8^{b}	9.3 ± 1.9^{b}	12.7 ± 1.7$^{a??}$
Gulping (kPa)	8.1 ± 1.9^{a}	5.1 ± 1.4^{b}	5.3 ± 1.5^{b}	7.1 ± 1.4^{a,b}
Loss of equiibrium (kPa)	7.0 ± 1.7^{a}	3.8 ± 0.9^{b}	3.9 ± 0.9^{b}	5.0 ± 0.7^{b}
Post-settlement				
Mass (mg dw)	7.36 ± 1.96^{a}	7.25 ± 1.36^{a}	3.44 ± 0.83^{b}	3.74 ± 0.96^{b}
RMR ($nmolO_2\ mg_{dw}^{-1}\ h^{-1}$)	60 ± 4	64 ± 3	69 ± 13	55 ± 11
P_{crit} (kPa)	9.8 ± 1.6^{a}	8.9 ± 2.2^{a,b}	7.0 ± 2.2^{b}	5.7 ± 1.4^{c}
Wriggled (kPa)	11.0 ± 1.2^{a}	8.9 ± 1.9^{a}	12.8 ± 1.8^{b}	12.5 ± 3.4^{b}
Agitated (kPa)	7.3 ± 1.1^{a}	5.4 ± 1.9^{b}	5.9 ± 1.5^{b}	5.6 ± 1.4^{b}

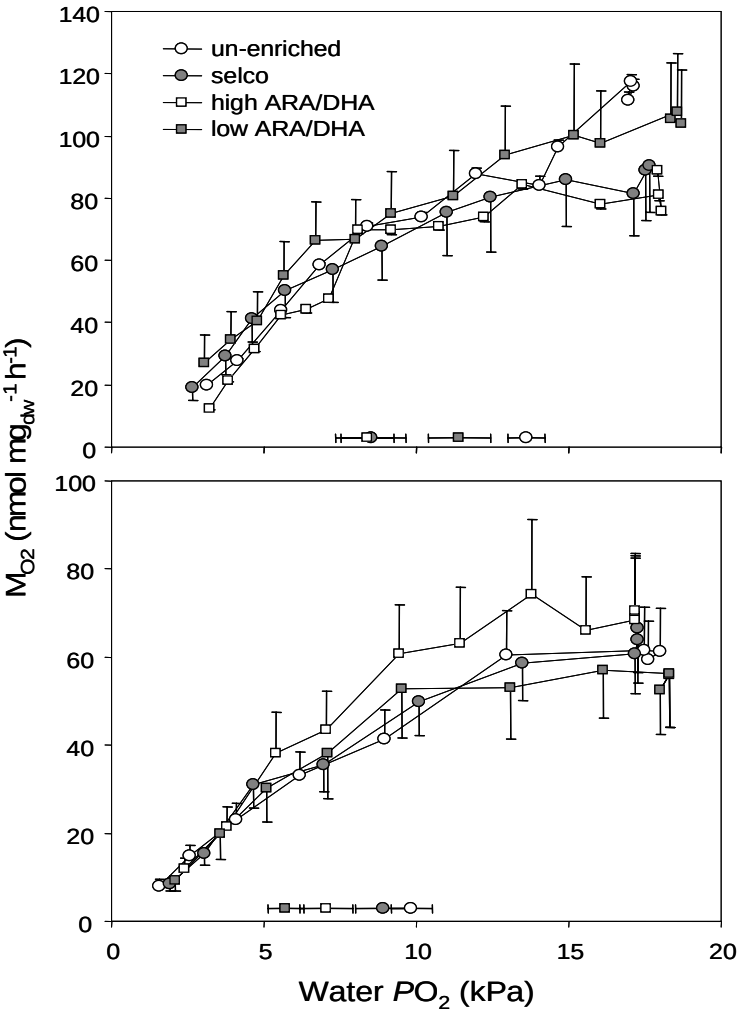
There were no significant differences in mean larval dry weight pre-settlement, but the post-settlement juveniles from the first trial all had a greater dry weight than those from the second trial.

Table 2 also shows the RMR and elements of the mean responses to hypoxia in the four groups, while Figure 2 shows the pattern of respiratory metabolism in progressive hypoxia. Pre -settlement larvae fed upon un-enriched *Artemia* had a significantly higher RMR than those fed *Artemia* enriched either with Selco or the high ARA/DHA oil (Table 2). This difference in RMR was associated with clear differences in responses to hypoxia. As is visible on Figure 2A, the larvae fed unenriched *Artemia* showed a constant progressive decline in M_{O2} from their elevated normoxic RMR, whereas the other groups tended to regulate M_{O2} relatively unchanged from normoxic RMR until a degree of hypoxia beyond which they exhibited a progressive decline in metabolic rate. These different responses were reflected in different estimated P_{crit}'s for each group of pre-settlement larvae (Figure 2A, Table 2). Those fed un-enriched *Artemia* had the highest mean $P_{crit,}$ significantly higher than larvae fed *Artemia* enriched either with Selco or with the oil containing a high ARA/DHA ratio.

All of the pre-settlement larvae showed pronounced behavioural responses to the progressive hypoxic stress. The initial response was to become very agitated with rapid movements in random directions. As they consumed the oxygen in their chamber, the next response was exaggerated

Figure 2.

Mean (± SEM) rates of instantaneous oxygen uptake (M_{O2}) in progressive hypoxia by pre-settlement larvae (A) and post-settlement juveniles (B) of sole that were raised upon Artemia that were either un-enriched or enriched with one of three oils (see text for details). P_{crit} values near the abscissa show the mean (± SEM) PO_2 beyond which the larvae from each group exhibited a progressive decrease in M_{O2} of at least 10% below their normoxic values. n = 8 for unenriched and Selco groups, 7 for ARA: DHA groups pre settlement and 6 post settlement.



gulping ventilation, presumably indicating respiratory distress. This behaviour was then followed by loss of equilibrium accompanied by a visible drop in ventilatory effort and general cessation of movements. As shown in Table 2, the mean PO_2 thresholds for these behaviours differed significantly between the dietary groups. The larvae fed un-enriched *Artemia* became agitated (often in normoxia) and exhibited "gulping" and loss of equilibrium at significantly higher PO_2 compared to other diet groups (Table 2). The two-way ANOVA revealed that overall RMR for all the groups considered together, was significantly lower post-settlement compared to pre-settlement, being 6.22 ± 0.34 mmol O_2 g_{dw}^{-1} h^{-1} versus 9.70 ± 0.32 mmol O_2 g_{dw}^{-1} h^{-1} (n = 28 and 30), respectively. Within the individual groups, RMR was significantly lower in all except for the low ARA/DHA group (Table 2). There were no longer any significant differences in RMR among

the groups, although the difference in dry weight confounds the comparison somewhat (Table 2). As is visible from Table 2 and Figure 2B, metamorphosis and settlement were associated with an increase in hypoxia tolerance. All groups exhibited regulation of their M_{O2} around RMR until a degree of hypoxia beyond which they exhibited a progressive decline in metabolic rate (Figure 2B). The two-way ANOVA revealed that overall P_{crit}, for all the groups considered together, was significantly lower post-settlement compared to pre-settlement, being 7.87 ± 0.40 kPa versus 10.47 ± 0.39 kPa (n = 28 and 30), respectively. Within each group, however, the post-settlement reduction in P_{crit} was significant only for the sole fed un-enriched *Artemia* or the *Artemia* enriched with the low ARA/DHA mixture (Table 2), There were still significant differences amongst the groups for their mean P_{crit} following settlement, whereby the juveniles fed un-enriched *Artemia* had the highest mean P_{crit} and this was significantly higher than those fed *Artemia* enriched in either of the ARA/DHA mixtures. Furthermore, the sole fed the low ARA/DHA mixture also had a lower P_{crit} than the other two enriched groups, and were the most hypoxia-tolerant group following settlement.

For the post-settlement juveniles, behaviour was also slightly different. In normoxia, the fishes lay on the bottom of their chamber. As they consumed the oxygen in the chamber and created the progressive hypoxic stress, their initial response was to gently wriggle their body, raising portions off the glass surface of the chamber. This was followed, as PO_2 dropped, by intermittent periods of intense agitation. The PO_2 at which the juveniles lost equilibrium was difficult to assess because they tended naturally to come to rest on their left (blind) side. Thus, these animals were recovered to normoxia as soon as they seemed to have lost reactivity, i.e. when a long delay occurred after the last period of agitation. The sole from the second trial, fed the ARA/DHA mixtures, wriggled at higher PO_2's than the other two groups (Table 2). Finally, the two-way ANOVA revealed that overall PO_2 at which agitation was observed, for all the groups considered together, was significantly lower post-settlement compared to pre-settlement, being 6.05 ± 0.35 kPa versus 11.80 ± 0.34 kPa (n = 28 and 30), respectively, and all groups exhibited a significant decline in the PO_2 at which they became agitated post-settlement (Table 2). The post-settlement group with the highest P_{crit}, those fed the un-enriched *Artemia*, also had the highest PO_2 at which they became distressed and agitated in hypoxia (Table 2).

Fatty acid composition

Table 3 shows the total FA (TFA) content and its associated content of selected FAs in the *Artemia* from the four different preparations. As expected, the unenriched *Artemia* had the lowest total FA

content, although there was great variability and the difference was only significant against the *Artemia* fed the high ARA/DHA mixture (Table 3). The total content of saturated, monounsaturated and n-6 fatty acids was not significantly different between un-enriched and enriched *Artemia*, but was significantly higher in *Artemia* enriched by the high ARA/DHA ratio (Table 3). The un-enriched *Artemia* also had, as expected, a significantly lower total content of ARA and DHA, although EPA was similar to the other groups, and was actually lowest in the *Artemia* fed the high ARA/DHA ratio. The ARA content in *Artemia* was highest in those enriched with the high ARA/DHA oil. On the other hand, DHA contents were similar in the two groups fed the ARA/DHA ratios, and significantly higher than in the other two groups (Table 3). The ARA/DHA ratio was actually highest in un-enriched *Artemia* by comparison with all of the other groups and lowest, as expected, in the group fed the low ARA/DHA ratio. Finally, the n-3/n-6 ratio also differed between the groups, being lower in those fed the two ARA/DHA combinations, and lowest of all in the group fed the high ARA/DHA ratio (Table 3).

Table 3.

Mean (± SEM) total fatty acids and fatty acid composition of the Artemia from the four preparations used to feed the sole larvae. For each variable, a different superscript denotes a significant difference ($P < 0.05$) between means.

	Unenriched	Selco	high ARA/DHA	low ARA/DHA
TFA (mg g_{dw}^{-1})	117.3 ± 33.6^{a}	126.4 ± 23.4^{a}	201 ± 5.6^{b}	161.9 ± 39.5^{a}
FA (mg g_{dw}^{-1})				
Total saturated	20.3 ± 5.3^{a}	20.6 ± 4.8^{a}	44.1 ± 3.5^{b}	26.9 ± 6.4^{a}
Total monounsaturated	32.0 ± 12.1^{a}	32.9 ± 8.3^{a}	47.4 ± 1.6^{b}	33.8 ± 6.7^{a}
Total n-6 PUFA	9.2 ± 3.2^{a}	10.0 ± 2.0^{a}	39.7 ± 2.8^{c}	23.4 ± 6.0^{b}
Total n-3 PUFA	55.1 ± 17.3^{a}	63.0 ± 9.8^{a}	69.9 ± 0.9^{a}	77.8 ± 20.6^{a}
C20:4n-6,ARA	2.6 ± 1.2^{a}	3.6 ± 0.6^{a}	19.4 ± 2.2^{c}	9.3 ± 2.6^{b}
C20:5n-3,EPA	$37.3 \pm 24.8^{a,b}$	53.6 ± 8.8^{b}	21.5 ± 1.0^{a}	$30.9 \pm 9.4^{a,b}$
C22:6n-3,DHA	0.1 ± 0.1^{a}	3.5 ± 2.7^{b}	12.7 ± 2.2^{c}	12.3 ± 3.5^{c}
DHA/EPA	0.0 ± 0.0^{a}	0.1 ± 0.1^{a}	0.6 ± 0.1^{b}	0.4 ± 0.1^{b}
ARA/DHA	27.4 ± 10.5^{c}	$1.0 \pm 0.9^{a,b}$	1.5 ± 0.1^{b}	0.8 ± 0.0^{a}
ARA/EPA	0.1 ± 0.1^{a}	0.1 ± 0.0^{a}	0.9 ± 0.1^{c}	0.3 ± 0.0^{b}
n-3/n-6	6.2 ± 1.0^{c}	6.4 ± 0.7^{c}	1.8 ± 0.1^{a}	3.3 ± 0.0^{b}

In the pre-settlement sole larvae, TFA content was least in the group fed unenriched *Artemia*, although this was only significant for the larvae fed the high and low ARA/DHA mixture, due to much variability amongst the larval samples (Table 4).

Settlement was associated with a significant increase in TFA for the juveniles fed unenriched and Selco-enriched *Artemia*, but this was not true of the juveniles raised on *Artemia* enriched with the ARA/DHA mixtures (Table 4).

The ARA content in larvae and juveniles was directly influenced by the content in the *Artemia*, with the highest ARA content in the larvae fed on *Artemia* enriched with the high ARA/DHA mixture (Table 4).

Table 4.

Mean (± SEM) total fatty acids and fatty acid composition in pre-settlement and post-settlement larvae that were raised on the four different Artemia preparations. For each variable, a different superscript denotes a significant difference (P < 0.05) within pre settlement larvae or post settlement larvae. For each variable, a different superscript denotes a significant difference (P < 0.05) between means.

	Pre-settlement larvae				Post-settlement juveniles			
	Unenriched	Selco	High ARA/DHA	low ARA/DHA	Unenriched	Selco	high ARA/DHA	Low ARA/DHA
TFA (mg g_{dw}^{-1})	23.3 ± 10.5^{a}	40.3 ± 26.1^{ab}	66.9 ± 25.7^{b}	69.6 ± 15.6^{b}	78.3 ± 8.7^{a}	94.3 ± 16.1^{a}	82.6 ± 10.4^{a}	68.2 ± 11.3^{a}
FA (mg g_{dw}^{-1})								
Total saturated	5.7 ± 2.2^{a}	6.9 ± 5.2^{a}	14.3 ± 5.0^{a}	12.3 ± 3.0^{a}	20.7 ± 2.3^{b}	23.6 ± 2.9^{b}	17.0 ± 1.9^{ab}	13.5 ± 2.6^{a}
Total monounsaturated	5.7 ± 2.3^{a}	8.1 ± 6.4^{a}	13.5 ± 5.5^{a,b}	11.7 ± 3.0^{a,b}	19.3 ± 1.4^{ab}	22.9 ± 3.2^{b}	19.3 ± 2.4^{ab}	15.3 ±2.8^{a}
Total n-6 PUFA	2.5 ± 1.0^{a}	3.6 ± 2.9^{a}	12.4 ± 4.1^{b}	9.4 ± 2.4^{b}	9.2 ± 1.1^{a}	11.0 ± 2.2^{a}	17.2 ± 2.3^{b}	11.2 ±1.6^{a}
Total n-3 PUFA	9.5 ± 5.0^{a}	14.3 ± 11.6^{a}	26.9 ± 10.4^{b}	27.2 ± 7.1^{b}	28.8 ± 3.8^{a}	37.0 ± 7.7^{a}	29.2 ± 3.8^{a}	28.3 ± 4.3^{a}
C20:4n-6, ARA	1.2 ± 0.6^{a}	1.7 ± 1.4^{a}	6.6 ± 1.5^{c}	4.7 ± 1.2^{b}	3.6 ± 0.4^{a}	3.8 ± 0.3^{a}	8.4 ± 1.1^{b}	4.8 ± 0.5^{a}
C20:5n-3, EPA	3.9 ± 1.9^{a}	5.7 ± 4.6^{a}	4.0 ± 1.5^{a}	5.2 ± 1.4^{a}	26.2 ± 2.7^{b}	25.4 ± 3.1^{b}	3.5 ± 0.4^{a}	4.5 ± 0.5^{a}
C22:6n-3, DHA	4.7 ± 2.7^{a}	7.2 ± 6.0^{ab}	10.3 ± 3.5^{b}	10.4 ± 2.9^{b}	3.1 ± 1.7^{a}	5.7 ± 0.9^{b}	6.0 ± 0.8^{b}	6.1± 0.6^{b}
ARA/DHA	0.3 ± 0.0^{b}	0.2 ± 0.0^{a}	0.6 ± 0.1^{d}	0.5 ± 0.0^{c}	1.2 ± 0.2^{c}	0.7 ± 0.1^{a}	1.4 ± 0.0^{d}	0.8 ± 0.0^{b}
ARA/EPA	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	1.7 ± 0.1^{c}	0.9 ± 0.1^{b}	0.1 ± 0.0^{a}	0.2 ± 0.0^{b}	2.4 ± 0.1^{d}	1.1 ± 0.0^{c}
DHA/EPA	1.2 ± 0.2^{a}	1.3 ± 0.1^{a}	2.7 ± 0.6^{b}	2.0 ± 0.2^{b}	0.1 ± 0.0^{a}	0.2 ± 0.1^{b}	1.7 ± 0.1^{c}	1.3 ± 0.0^{c}
n-3/n-6	3.7 ± 0.6^{a}	3.9 ± 0.6^{a}	2.2 ± 0.0^{a}	2.9 ± 0.0^{a}	3.2 ± 0.6^{ab}	3.4 ± 0.3^{b}	1.7 ± 0.0^{a}	2.5 ± 0.0^{ab}

Consequently, of the relative ARA enrichment, pre-settlement larvae on low ARA/DHA nonetheless also had a higher ARA content than the larvae fed either unenriched or Selco-enriched *Artemia*, although this effect on ARA was no longer visible post-settlement (Table 4). Settlement itself was associated with an increase in tissue ARA in all of the dietary groups except the sole fed the low ARA/DHA ratio.

For DHA, both larvae and juveniles treated with the high and low ARA/DHA *Artemia* had a similar content, and this was significantly higher than the other two dietary groups. In contrast to the pattern observed for ARA, DHA content fell post-settlement, in all groups. In post-settlement juveniles, the group fed Selco-enriched *Artemia* had higher DHA content than those fed unenriched *Artemia*, which had the lowest overall DHA content (Table 4).

The EPA content was not significantly affected by the *Artemia* treatments in the pre-settlement larvae. Post-settlement, however, both the Selco-enriched and unenriched groups exhibited an enormous increase in EPA content that was not observed in the groups fed the ARA/DHA mixtures (Table 4).

As expected, the highest ARA/DHA and ARA/EPA ratios were observed in sole fed *Artemia* with the high ARA/DHA mixture, both pre- and post-settlement. The lowest ARA/DHA ratios were observed in the Selco-enriched sole, both pre- and post-settlement (Table 4). The two-way ANOVA revealed that settlement itself was associated with an overall increased ARA/DHA ratio (Table 4). The DHA/EPA ratio was significantly higher in sole fed the ARA/DHA mixtures compared to the other two dietary groups, both pre- and post-settlement. Settlement was associated with an overall decline in DHA/EPA ratio. The n-3/n-6 ratio was higher in Selco-enriched post settlement larvae as compared to larvae fed the high ARA/DHA diet (Table 4).

Discussion

The current study is the first report of in-vivo regulation of metabolism in hypoxia by individual marine fish larvae. The results indicated that pre-settlement sole larvae already possess the ability to regulate metabolism in hypoxia, although only to a limited extent, and this ability improves significantly following metamorphosis and settlement. The results also indicated that enriching live feeds (*Artemia)* with ARA, DHA and EPA leads to a significant accumulation of these EFA in the tissues of the sole, with significant effects upon the respiratory metabolism and tolerance of hypoxia in the earliest life stages.

Marine fish larvae are the smallest vertebrates (Weiser, 1995) and so there are relatively few published reports on the metabolic rate of individuals, due to the difficulties of measuring the low absolute rates of M_{O2} on such minute animals (e.g. Finn et al. 1995a; 2002; Hunt von Herbing and Boutilier 1996; Bang et al., 2004). One source of error in such measurements can be the relatively large intrinsic rate of oxygen consumption by polarographic oxygen electrodes, and this is why the majority of respirometry studies have been performed upon groups of larvae (e.g. Finn et al. 1995b; Parra and Yufera 2001; Ishibashi et al. 2005; Cunha et al. 2007). The range of normoxic RMR's measured on the individual common sole in the current study are in fact very similar to those reported by Parra and Yufera (2001) for groups of the Senegalese sole (*Solea senegalensis* Kaup 1858) prior to and following metamorphosis and settlement at the same water temperature. The intrinsic O_2 consumption by polarographic electrodes may explain why there are no published reports, to our knowledge, of how individual marine fish larvae regulate M_{O2} during hypoxia, as electrode oxygen consumption would become increasingly difficult to control for as environmental oxygen levels fall. The fluorophore optode sensors used in the current study have no intrinsic

oxygen consumption, and provided the first demonstration that pre-settlement flatfish larvae have some ability to regulate their metabolic rate during progressive hypoxia.

The pronounced agitation exhibited by all pre-settlement larvae when PO_2 fell below an average of about 12 kPa in hypoxia may have represented a hypoxia avoidance behaviour, which has been reported for marine fish larvae including the common sole (Macquart-Moulin 1997; Weltzien et al. 1999) and it is interesting that this agitation always occurred above the P_{crit}. The pronounced gulping hyperventilation exhibited by the same larvae below their P_{crit}, when PO_2 fell below an average of about 6.5 kPa, was presumably a reflex response that reflected massive stimulation of O_2-sensitive chemoreceptors, as observed in adult fish (Burleson et al. 1992). It is not clear, however, how effective this might be at increasing water flow through such the minute bucco-opercular channel and branchial basket of the pre-settlement larva, where viscosity would be such an important issue (Couturier et al. 2007).

The current data are the first demonstration that flatfish metamorphosis and settlement is associated with an increase in the ability to regulate metabolism in hypoxia. The wriggling behaviours in the post-settlement juveniles may have improved cutaneous oxygen uptake by "ventilating" the blind side. The skin is an essential site for gas-exchange in fish larvae and early life stages (Rombough 1998) and cutaneous oxygen uptake continues to make a significant contribution to RMR in adult flatfish (Nonnotte and Kirsch 1978). Larger sole, with a wet body mass of approximately 12g, have a much more pronounced ability to regulate metabolism in hypoxia, with a P_{crit} of approximately 2 kPa (Couturier et al. 2007).

The larvae fed un-enriched *Artemia* were notable for their increased levels of activity (and often agitation) in normoxia by comparison with the other groups. The energetic costs of spontaneous activity can represent an important proportion of RMR in marine fish larvae, (Hunt von Herbing and Boutilier, 1996; Hunt von Herbing et al., 2001), and so the increased activity and tendency to agitation in the larvae fed the un-enriched *Artemia* may well have been a contributing element to their increased normoxic RMR relative to the other dietary groups. In fishes, an elevated normoxic RMR is often associated with an almost linear decline in MO_2 in progressive hypoxia (McKenzie et al., 2007b; Steffensen, 2007) and this was indeed the response observed in the pre-settlement sole fed un-enriched *Artemia*. The larvae in this dietary group quite clearly had trouble sustaining their elevated RMR when oxygen availability was reduced because they showed signs of gulping respiratory distress and lost equilibrium at higher PO_2. However, although the elevated RMR might explain the higher P_{crit} and reduced tolerance of the pre-settlement larvae fed un-enriched *Artemia*,

there were no differences in RMR to explain the higher P_{crit} and threshold for hypoxic agitation in the post-settlement juveniles from the same dietary group.

The current finding that enrichment of *Artemia* with EFA improved in-vivo regulation of metabolism in hypoxia is consistent with a study by Logue et al. (2000) on common sole larvae, which found that those fed *Artemia* enriched in EPA and DHA exhibited less mortality during an acute hypoxic stress than did larvae fed an n-3 EFA-deficient diet. The current study indicates that there are also significant differences in the sub-lethal effects of hypoxia, with an earlier onset of agitation and respiratory stress that might reflect, at least in pre-settlement larvae, differences in levels of spontaneous activity in normoxia. Randall et al. (1992) reported that dietary and tissue enrichment with EPA and DHA made 1-year old Adriatic sturgeon (*Acipenser naccarii*) less agitated when the fish created progressive hypoxia by consuming the oxygen in a sealed respirometer chamber. As a consequence of the reduced degree of agitation, the animals fed these EFA had a lower P_{crit} than their conspecifics fed an un-enriched control diet (Randall et al. 1992). These previous results on sturgeon are, therefore, qualitatively similar to those of the current study. Although the current study demonstrates that enriching the tissues of sole with EFA during their earliest life stages can improve their tolerance of hypoxia, these data do not provide any clear evidence for differences in response that were linked to the ratio of the three EFAs in the *Artemia* or the animals' tissues. In particular, there was no clear evidence for any effect of the ratio of ARA to DHA, or indeed for an effect of any ratio of the EFAs to each other. Post-settlement, the most hypoxia-tolerant groups were those from the second trial, which accumulated the highest content of both ARA and DHA in their tissues. Unfortunately, at the same age, these groups also had a lower dry mass than those from the first trial and, although it might be expected that a smaller mass should be associated with lower hypoxia tolerance and not the opposite, the difference in mass confounds comparisons until future studies are performed. This difference in mass at age was measured post-hoc and impossible to detect by eye during the respirometry trials. Within the two groups fed the high versus low ARA/DHA ratios, the most hypoxia-tolerant post-settlement was that fed the most DHA, although this was not associated with significantly higher tissue DHA content than the other two groups fed enriched *Artemia*, or with the lowest overall tissue ARA/DHA ratio. Thus, speculation about the potential mechanisms by which the EFA might exert their beneficial effects, which necessarily relate to the actions of the individual FA (Sargent et al., 1999a,b; Bell et al., 2003; McKenzie, 2005), seem beyond the scope of the current study.

Nonetheless, the data do indicate that enrichment of *Artemia* with EFA should improve tolerance to the stresses of intensive aquaculture by the earliest life stages of sole. There were, however, no significant diet-related differences in larval survival or growth within either of the rearing trials (Ivar Lund, unpublished data). In these trials, however, cultivated larvae were reared under optimal conditions, with no stresses associated with variations in dissolved oxygen, salinity or temperature. Indeed, although high survival and growth indicates that a diet provides for all basic nutritional requirements, this might not be indicative of physiological conditions that influence responses to stress (Dhert et al., 1992). All previous studies have demonstrated that the effects of the EFA on fish respiratory physiology in aquaculture are subtle and rarely related to differences in growth or survival (McKenzie, 2001; 2005). Indeed, diet quality may be most significant for early life stages of sole in their natural environments. Flatfish nursery habitats, such as inter-tidal mudflats, may often be physiologically stressful environments (e.g Couturier et al., 2007) and, therefore, investigating the effects of EFA availability in natural foodwebs is an interesting area for future research.

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Pigmentation sensitivity of common sole (*Solea solea* L.) larvae to low dietary inclusions of arachidonic acid.

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Pigmentation sensitivity of common sole (*Solea solea* L.) larvae to low dietary inclusions of arachidonic acid.

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Keywords: Sole larvae, arachidonic acid, eicosapentaenoic acid, malpigmentation, hypomelanosis, hypermelanosis, prostaglandins.

Abstract

Recent studies have demonstrated induced malpigmentation in sole larvae fed *Artemia* enriched by emulsions with dietary inclusion of arachidonic acid, ARA (i.e. ≥ 8 percent inclusion of ARA oil in emulsion). In the present study a possible threshold level of dietary ARA (≤ 8 percent of emulsion) was examined in relation to malpigmentation in sole larvae.

Malpigmentation defined as hypomelanosis at 43 days after hatching was low and not significantly affected by dietary treatment, consequently no tissue threshold level could be deduced for ARA inducement of malpigmentation. An additional implementation of dietary EPA had no influence on pigmentation. Tissue PGE_2 prostaglandin concentrations was not significantly related to larval ARA contents. Permanent blind side pigmentation i.e. ambicolouration was high for all treatments, but not related to diet type. Ambicolouration made up from 3 to 55 percent of the individuals within replicates indicating induction by environmental factors. Ambicolouration may decrease ARA related sensititvity in inducing malpigmentation. The advance of metamorphosis defined by the eye migration was not related to age, ARA or EPA contents, but to larval standard length. Survival or growth was not different or related to dietary ARA or EPA.

Introduction

Previous studies have demonstrated that common sole larvae (*Solea solea*) fed *Artemia* enriched by arachidonic acid, ARA (≥ 8 percent of diet) induced malpigmentation. Furthermore this malpigmentation was related to the tissue concentration of ARA and to the 2- series prostaglandins during a sensitive period prior to metamorphosis (Lund et al., 2007; Lund et al., in press, a).

A relatively high ARA content in eggs and pre feeding sole larvae may be related to the function of ARA as a preferred substrate for producing eicosanoids and their role in regulating the neuronal activity during initial development (Estévez et al., 1999). ARA plays an important role in feeding larvae thus demonstrated by the fact that ARA is retained in larvae fed prey low or deficient in ARA (Bell and Sargent.,2003, Harel and Place, 2003), which is suggested related to ARA involvement in eicosanoid synthesis as previously reviewed (Koven, 2003). This indicates a delicate physiological balance in which even slightly elevated or unbalanced ARA concentrations may have severe effects such as the mechanisms involved in pigmentation. The aim of this study was to examine if malpigmentation in sole larvae was related to a threshold level of dietary ARA. Larvae were fed *Artemia* enriched with increasing dietary inclusions of ARA (0, 2, 4, 8 percent of diet). Furthermore, a possible interactive effect on pigmentation was tested by implementation of EPA in some of the diets. This was done because increased incorporation of EPA relative to ARA in cells may reduce the production of 2- series prostaglandins (Smith, 2005, Wada et al., 2007), which have been suggested to be involved in the mechanisms inducing malpigmentation (Lund et al., in press a).

Materials and Methods

A total of 6 emulsions were prepared for enrichment of *Artemia* fed for start feeding sole larvae. High quality marine sprat fish oil from Fiskernes Fiskerindustri (FF), Skagen, Denmark was gradually replaced by natural Arachidonic acid (ARA) (VEVODAR) oil, produced by means of fermentation of a fungus (*Mortierella alpina*) from DSM Food Specialities BV, Holland and /or concentrated Incromega Eicosapentaenoic acid (EPA) oil from Croda Chemicals Europe, Snaith, UK. The emulgator was Soy lecithin (BioMar, Brande Denmark) added as a growth promoter (7 %) (Macqueen et al, 2003) and an E- vitamin mix. (BioMar, Brande, Denmark) was added as an antioxidant (4 %). The ingredients were weighed and mixed and homogenised with a Büchi mixer B- 400 for 1 min. at 9000 rpm.

4 of these emulsions were prepared with increasing ARA oil inclusions (0, 2, 4, 8 percent) in concert with 20 percent EPA oil. In addition 2 emulsions were prepared with 2 and 8 percent ARA oil but without inclusion of EPA oil. The emulsions were flushed with nitrogen and stored in 20 ml sterile plastic syringes at – 80 ° C. Table 1 illustrates the supplementation of ARA and EPA formulation and the analysed FA content in emulsions and EG *Artemia*.

Table 1.

Analysed TFA content (mg g d.w.$^{-1}$) and FA content (percent of TFA) in the 6 different emulsions and in EG Artemia Incl. minor fatty acids omitted.*

	ARA supplementation											
	0	2	4	8	2	8	0	2	4	8	2	8
	EPA supplementation											
	20	20	20	20	0	0	20	20	20	20	0	0
	Emulsion						*Artemia*					
Total analysed FA	726.4	1094.7	1035.8	1032.9	976.9	944.8	236.0	229.0	164.0	299.0	192.0	178.0
FA (% TFA)												
C14:0	3.1	3.4	3.0	2.4	3.8	3.4	1.2	1.4	1.6	1.5	1.4	1.2
C16:0	13.2	14.1	12.3	11.5	15.9	14.8	12.5	12.2	13.6	12.7	12.8	13.3
C18:0	7.3	7.9	8.3	10.0	9.5	11.6	9.2	8.6	9.1	9.2	9.6	9.1
total SFA*	24.7	26.6	25.0	25.3	30.8	31.3	25.3	25.0	27.1	26.2	28.1	26.8
C16:1 (n-7)	3.8	4.0	3.7	3.1	4.6	4.2	2.6	2.3	2.0	2.0	3.3	3.0
C18:1 (n-9) cis	16.6	16.5	15.6	14.7	19.8	18.6	17.7	18.7	17.1	17.3	23.1	21.6
C20:1 (n-9)	1.7	1.6	1.6	1.5	2.1	2.0	1.1	1.0	1.0	0.9	1.3	1.2
C22:1 (n-9)	1.2	2.0	2.1	1.9	0.4	0.4	0.5	0.6	0.6	0.7	0.4	0.2
Total MUFA*	23.7	24.4	23.4	21.5	27.4	25.6	23.0	23.9	22.1	22.7	28.8	26.8
C18:2 (n-6) c	5.7	5.5	4.2	5.5	6.0	5.6	5.9	5.8	6.1	6.3	7.3	7.4
C18:3 (n-6)	0.1	0.2	0.3	0.4	0.2	0.4	0.4	0.5	0.5	0.5	0.4	0.6
C20:2 (n-6)	0.4	0.4	0.4	0.4	0.5	0.6	0.4	0.5	0.5	0.5	0.5	0.5
C20:3 (n-6)	0.1	0.2	0.3	0.5	0.1	0.4	0.2	0.2	0.3	0.4	0.1	0.3
C20:4 (n-6)	1.4	2.2	3.5	5.6	1.6	4.9	1.4	1.6	2.1	3.2	1.2	3.1
total (n-6) PUFA*	7.9	8.6	8.8	12.5	8.6	11.9	8.4	9.1	10.0	11.6	9.6	11.9
C18:3 (n-3) c	2.5	2.4	2.3	2.2	3.0	2.7	15.3	16.7	14.3	14.4	17.8	18.7
C20:3 (n-3) c	0.2	0.1	0.1	0.1	0.2	0.2	0.7	0.8	0.7	0.7	0.8	0.9
C20:5 (n-3)	21.1	19.9	21.4	20.7	8.0	7.5	22.3	20.4	21.1	20.4	10.0	10.1
C22:6 (n-3)	20.0	17.9	19.1	17.8	22.1	20.8	5.0	4.2	4.7	4.1	5.0	4.8
total (n-3) PUFA*	43.8	40.3	42.9	40.7	33.3	31.2	43.2	42.1	40.8	39.6	33.6	34.5
DHA/EPA	1.0	0.9	0.9	0.9	2.8	2.8	0.2	0.2	0.2	0.2	0.50	0.5
ARA/DHA	0.1	0.1	0.2	0.3	0.1	0.2	0.3	0.4	0.5	0.8	0.2	0.7
ARA/EPA	0.1	0.1	0.2	0.3	0.2	0.7	0.1	0.1	0.1	0.2	0.1	0.3
(n-3)/(n-6)	5.7	4.7	4.9	3.3	3.9	2.6	5.1	4.7	4.1	3.4	3.5	2.9

The rearing facility consisted of 30 cylindrical-conical tanks holding 46 l each connected to a flow through water system including a 1 µm filtration unit. Each tank had a separate inlet tap with a flowmeter, a 700 µm drainage filter and aeration. The photo period was 24 hrs light. The light intensity at the surface was approximately 100 lux.

The water flow was 8 l hr $^{-1}$. Oxygen saturation, temperature and pH were monitored daily by a handy Oxyguard meter (Oxyguard, Birkerød, Denmark) followed by measurements of NH_4^+, NO_2^-, NO_3^- by a Merckquant test kit from Merck, Darmstadt, Germany.

The temperature was kept at 18.6 ± 0.6 º C during the trial. Levels of NH_4^+, NO_2^-, NO_3^- were below detection. Oxygen content was kept above 6.5 mg l^{-1} in all tanks.

Larval rearing

The sole larvae were obtained from Institute for Marine Resources and Ecosystem Studies (IMARES) Ijmuiden, Holland. Eggs were packed in polystyrene boxes with ice and transported to the rearing facility at the North Sea Centre, Denmark. About 1227 larvae were added to each tank, equalling a density of 26 larvae l^{-1}. Larvae were start fed at 3 days after hatching (dah 3). The study was carried out with 3 tanks treatment $^{-1}$ at dah 3-21. An IH *Artemia* strain was used to feed larvae 3 -10 days after hatching (dah) and an enriched EG strain at dah 11-21. *Artemia* were enriched with 0.6 g emulsion l^{-1} for 18-24 hrs. Live feeds were harvested once a day in the morning and fed to the larvae at 10 a.m. and 8 p.m. Live feed for the second daily feeding was kept refrigerated. Prey concentration was 2 ml^{-1} at each feeding.

According to table 2, four groups of larvae were fed unenriched IH *Artemia* at dah 3-10 followed by EG *Artemia* enriched with either 0, 2, 4, 8 percent ARA and 20 percent EPA at dah 11-21. Similarly two groups were fed unenriched IH *Artemia* at dah 3-10 followed by *Artemia* enriched with either 2 or 8 percent ARA at dah 11-21. One group was fed unenriched IH *Artemia* at dah 3-10, then EG *Artemia* enriched with 20 percent EPA at dah 11-15 followed by EG *Artemia* enriched with 8 percent ARA at dah 16-21. One group was fed IH or EG *Artemia* enriched with 8 percent ARA at dah 3-21. One group was fed IH *Artemia* enriched with 8 percent ARA and 20 percent EPA at dah 3-10 followed by 20 percent EPA at dah 11-21.

At dah 22-42 all larvae were fed *Artemia* enriched with a commercial Selco emulsion from INVE, Holland. The evaluation of pigmentation was carried out at dah 43 in all individuals. The overall malpigmentation (hypomelanosis) was evaluated as the number of malpigmented fish / total number of fish x 100. Juveniles with a normal ocular side, but in which the blind side displayed either completely or partially ocular-side pigmentation, hypermelanosis (Bolker et al., 2005), were registered separately.

Samples

All samples of sole larvae were collected in duplicate prior to feeding in the morning. Prey and larval samples were washed gently on a 60 µm filter net with 0.2 µm seawater. Duplicate pooled samples of larvae (10 pcs $sample^{-1}$) and *Artemia* (50-100 pcs. $sample^{-1}$) were collected, counted and filtrated on preweighed and precombusted Whatman Ø 25 0.7 µm GF/F glass fibre filters and weighed on a Mettler Toledo MT5 d =0.1 µ

Larval length and fatty acid analyses were carried out on pre feeding larvae at dah 3, and larvae from each rearing tank at dah 11, 16, 19, 22. Prostaglandin PGE_2 analyses were carried out on

larvae sampled at dah 22. Standard length of larvae was measured by a digital high resolution DFC 320 Leica camera connected to a dissecting microscope (MZ6). Each subsample for fatty acids analysis was kept in sterile cryo-vials, covered with nitrogen and frozen at – 80 ° C until extraction. Larval samples for prostaglandin analyses were stored in 5 ml sterile cryo-vials in 4 ml of Hanks solution containing 0.6 ml of absolute alcohol and 0.2 ml of 2M Formic acid and immediately frozen in liquid nitrogen (-196 ° C).

Eye migration

The degree of metamorphosis was evaluated at dah 11, 16 and 21. The process was characterized by the position of the migrating left eye modified according to a traditional index (Fernández-Diaz et al, 2001). The 5 sub stages were: 0, symmetrical left and right eye position, 1, an asymmetrical position of the left eye and right eye; the right eye starts to migrate, 2, the migrating eye reached at maximum the midline of the dorsal surface, 3, the migrating eye could be seen from the right ocular side or migrated within the dorsal side, 4, eye translocation was completed and the orbital arch was visible. At dah 43 eye migration was evaluated as normal or abnormal for all juveniles. An abnormal position of the eyes was defined as when the migrating eye was in position 2 or 3.

Extraction and fatty acid analysis

The fatty acid composition was determined by extraction of the lipids by a chloroform/ methanol mixture, Folch (Folch et al.1957) homogenized by a tissue tearor, Biospec Products, Inc., USA. Then followed trans esterification of the lipids by acetyl chloride in methanol. The fatty acid methyl esters were analyzed by gas chromatography - mass spectrometry (GC-MS). The method is thoroughly described in a previous study (Lund et al, in press). More than 34 fatty acids were analysed per sample at each day of sampling, however only ARA, EPA and DHA contents are shown in larval tables.

Prostaglandin E_2 content was performed by Cayman Chemicals, USA according to a Cayman monoclonal Competitive Enzyme Immunoassay (EIA) kit; Cat. No 514010, (pp. 1-17) as previously described (Lund et al, unpublished data a).

Statistics

All statistical tests for sole larval growth and FA composition were carried out using a one - way ANOVA and a pairwise multiple comparison of means using Tukey`s post hoc test ($P< 0.05$). Percentage data were normalized by arcsine transformation prior to analysis. Malpigmentation was compared by a Kruskall Wallis test. Linear regression analysis was used to identify the relationship

between diet, larval FA contents and PGE_2 content. The variance of data is given as standard deviation, sd, and (n=3).

Survival and growth

Survival was estimated by counting all individuals at dah 21, including larvae sampled for analyses. Growth rate was calculated as Specific Growth Rate (SGR) based on d.w. according to the formula; $SGR = (\ln W_f - \ln W_i \times 100)/ t$, Where $\ln W_{f\,i}$ = the natural logarithm of the final and initial d.w., t = time (days) between $\ln W_f$ and $\ln W_i$.

Results

Growth and Survival

Specific growth rate calculated as the increase in larval d.w. or standard length at dah 3 to 22 was not significantly different between any of the treatments. ($P \geq 0.87$) and varied from 17.9 to 20.1 percent d.w. day $^{-1}$. The survival estimate included about 140 larvae tank $^{-1}$ sampled for analyses. Survival at dah 43 (i.e from 32-45 percent larval group $^{-1}$) was not significantly different between treatments ($P > 0.2$).

Table 2.

Larval survival (%), dry weight (d.w.ind^{-1}), standard length (mm ind^{-1}) and growth calculated as specific growth rate (SGR) until dah 21. Data given as a mean ± standard deviation for all larval groups. A different superscript in a horizontal row denotes a significant difference $P < 0.05$.

	Time interval for dietary manipulation (dah)								
	11 to 21							3 to 21	
	ARA supplementation								
	0	2	4	8	2	8	8 (dah 16-21)	8	8 (dah 3-10)
	EPA supplementation								
	20	20	20	20	0	0	20 (dah 11-15)	0	20 (dah 11-21)
Survival(dah 3-43)	32.6 ± 3.0	32.7 ± 2.2	41.1 ± 18.1	45.8 ± 15.2	40.1 ± 3.8	28.2 ± 14.5	27.9 ± 9.5	29.8 ± 6.9	29.5 ± 3.7
d.w. (dah 11)	0.19±0.02	0.183±0.06	0.191±0.02	0.204±0.02	0.134±0.03	0.148±0.02	0.159±0.04	0.179±0.02	0.164±0.04
d.w. (dah 15)	0.59±0.38	0.44±0.09	0.46±0.11	0.42±0.08	0.35±0.01	0.37±0.11	0.36±0.09	0.38±0.07	0.37±0.07
d.w. (dah 19)	1.06±0.53	0.96±0.27	0.82±0.19	1.07±0.33	0.78±0.09	0.70±0.22	0.83±0.15	0.80±0.21	0.78±0.08
d.w. (dah 22)	1.77±0.71	1.45±0.27	1.33±0.06	1.48±0.38	1.27±0.24	1.43±0.35	1.29±0.17	1.30±0.20	1.27±0.23
length (dah 11)	5.55±0.60	5.02±0.64	5.40±0.49	5.39±0.54	5.11±0.58	5.13±0.58	5.06±0.65	5.21±0.63	5.11±0.63
length (dah 15)	6.94±1.02	6.79±0.65	6.49±0.78	6.56±0.73	6.25±0.75	6.27±0.65	6.34±0.60	6.26±0.67	6.31±0.62
length (dah 19)	8.24±1.89	8.00±1.1	7.99±0.87	7.99±0.98	7.81±0.86	7.61±0.85	7.60±0.89	8.00±0.82	7.76±0.95
length (dah 22)	9.38±1.52	9.42±1.31	9.09±1.02	9.33±1.42	8.72±1.14	9.16±1.19	8.94±1.03	8.79±1.10	9.14±1.2^{b}
SGR (length)	5.37±0.62	5.12±0.17	4.92±0.13	5.05±0.62	4.68±0.62	4.96±0.40	4.82±0.42	4.72±0.23	4.96±0.27

Prey and larval FA relations

At the days of sampling the ARA, EPA larval tissue content was significantly related to the *Artemia* content (table 3), Fig. 1 (P<0.001). At dah 22 similar relative tissue ARA contents of 3.8 to 4 percent was observed for larvae fed *Artemia* enriched by 8 percent ARA (+ EPA) at dah 11-21 or larvae enriched by 8 percent ARA (-EPA) from dah 3, table 3. The content in both groups was significantly higher than for the remaining larval treatments (P < 0.001). This was followed by larvae fed *Artemia* enriched with 8 percent ARA (- EPA) from dah 11.

The ARA tissue contents were similar in larval groups fed *Artemia* enriched with 20 percent EPA, larvae fed *Artemia* enriched with 2 percent ARA at dah 11-21, and larvae fed *Artemia* enriched with 20 percent EPA at dah 11-21 changing from *Artemia* enriched with 8 percent ARA at dah 3-10. Tissue EPA content was similar in larval groups fed *Artemia* enriched by 20 percent EPA and significantly higher than in the larval groups fed *Artemia* without inclusion of EPA (P<0.001).

Figure 1.

The relation between the relative ARA (1a) and EPA contents (1b) (% TFA) in larval tissues sampled at dah 15, 19, 22 and EG Artemia.

a) b)

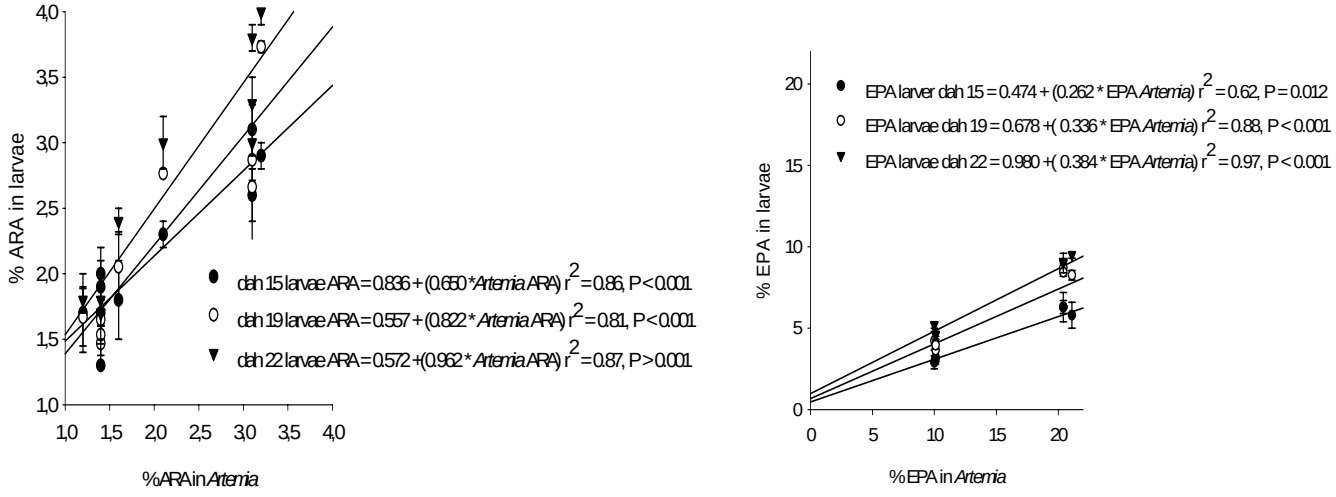


Table 3.

*Larval fatty acid content (TFA mg g^{-1} d.w.) and composition of TFA) given as means ± standard deviation in larvae at 3, 11, 15 and 22 days after hatching. n=3 unless otherwise stated. * Including fatty acids cut out for clearness A different superscript in a horizontal rows defines a significant difference ($P < 0.05$)*

		Time interval for dietary manipulation (dah)								
		11 to 21							3 to 21	
		ARA supplementation								
		0	2	4	8	2	8	8 (dah 16-21)	8	8 (dah 3-10)
		EPA supplementation								
		20	20	20	20	0	0	20 (dah 11-15)	0	20 (dah 11-21)
	dah 3	dah 11								
		n=2	n=2	n=2				n=2		
TFA	51.1±7.7	91.4±35.4	86.7±41.9	87.9±9.7	81.5±12.1	87.1±20.7	71.0±10.7	79.1±10.4	100.8±6.7	119.0±26.8
FA (% TFA)										
total SFA*	26.5±1.2	33.3±2.3	36.9±2.6	35.5±3.0	39.6±2.8	36.3±0.5	37.7±0.7	40.3±2.6	37.2±6.5	34.8±1.7
total MUFA*	28.8±1.8	26.1±1.0	23.4±2.0	24.1±1.7	23.1±0.8	22.8±1.0	23.9±1.2	22.5±1.8	22.7±3.2	23.4±0.4
C20:4 (n-6)	3.4±0.1	1.5±0.2^{a}	2.1±0.1^{ab}	2.3±0.3^{ab}	2.2±0.2^{ab}	2.7±0.4^{ab}	2.1±0.7^{ab}	2.3±0.5^{ab}	2.7±0.2^{ab}	2.8±0.2^{ab}
total (n-6) PUFA*	8.1±0.9	10.3±0.4^{ab}	10.0±0.2^{ab}	10.3±0.4^{ab}	10.3±0.2^{ab}	10.6±0.5^{b}	10.4±0.1^{ab}	10.0±0.5^{ab}	10.4±1.0^{b}	10.9±0.0^{b}
C20:5 (n-3)	3.5±0.5	1.4±0.3^{a}	1.7±0.1^{a}	2.0±0.5	1.6±0.2^{a}	2.0±0.3^{a}	1.8±0.2^{a}	1.5±0.2^{a}	2.2±0.2^{a}	2.3±0.2^{a}
C22:6 (n-3)	32.5±3.4	5.3±0.7^{a}	6.6±0.5^{ab}	7.5±0.3	6.9±0.3^{ab}	7.7±0.4^{ab}	7.9±0.8^{ab}	7.2±0.4^{ab}	9.1±0.1^{b}	9.9±0.6^{b}
total (n-3) PUFA*	36.5±3.9	29.9±1.9	29.7±2.3	30.1±2.0	27.0±1.8	30.3±1.1	28.0±1.0	27.1±0.4	29.6±2.7	30.9±1.9
DHA/EPA	9.4±0.3	3.9±0.9^{b}	3.8±0.1^{ab}	3.8±0.2	4.5±0.3^{b}	3.9±0.6^{b}	5.2±1.8^{b}	5.5±0.6^{b}	4.2±0.5^{b}	4.4±0.4^{b}
ARA/DHA	0.1±0.0	0.3±0.1	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.1	0.2±0.1	0.3±0.0	0.3±0.0	0.3±0.0
ARA/EPA	1.0±0.1	1.1±0.1^{ab}	1.2±0.1^{ab}	1.2±0.1	1.4±0.1^{b}	1.3±0.1^{b}	1.1±0.4^{b}	1.5±0.2^{b}	1.2±0.1^{b}	1.2±0.1^{b}
(n-3)/(n-6)	4.6±1.0	2.9±0.3	3.0±0.3	2.9±0.1	2.6±0.1	2.9±0.1	2.7±0.1	2.7±0.1	2.8±0.2	2.8±0.2

Table 3.-continued

	Time interval for dietary manipulation (dah)								
	11 to 21							3 to 21	
	ARA supplementation								
	0	2	4	8	2	8	8 (dah 16-21)	8	8 (dah 3-10)
	EPA supplementation								
	20	20	20	20	0	0	20 (dah 11-15)	0	20 (dah 11-21)
	dah 15								
TFA	113.3±7.6	128.0± 12.4	124.0 ±16.1	108.7±11.8	108.0±11.0	98.2±30.2	92.1±40.9	94.5±31.8	102.0±22.5
FA (% TFA)									
total SFA*	28.4±6.2	29.7±4.6	29.7±4.9	27.5±2.7	33.0±1.8	32.1±1.8	34.2±4.0	34.9±2.8	36.2±1.5
total MUFA*	26.2±0.9	24.3±0.9	24.2±1.1	23.9±1.1	24.3±1.0	24.2±1.0	22.4±0.5	23.3±0.3	23.7±1.2
C20:4 (n-6)	1.3±0.0^{a}	1.8±0.3^{ab}	2.3±0.1^{bc}	2.9±0.1^{d}	1.7±0.3^{a}	2.6±0.2^{cd}	1.9±0.3^{ab}	3.1±0.0^{d}	1.7±0.1^{ab}
total (n-6) PUFA*	10.0±0.2^{a}	10.1±0.5^{a}	10.9±0.2^{ab}	11.7±0.3^{bc}	11.0±0.4^{c}	11.9±0.1^{bc}	10.4±0.7^{a}	12.3±0.5^{c}	10.3±0.1^{a}
C20:5 (n-3)	5.2±1.7^{ab}	6.3±0.9^{bc}	5.8±0.8^{b}	6.3±0.4^{bc}	2.9±0.4^{a}	3.1±0.2^{a}	5.7±1.1^{b}	3.2±0.4^{a}	4.4±0.1^{ab}
C22:6 (n-3)	3.9±1.4	5.7±1.5	5.6±0.3	6.2±0.9	5.7±1.2	5.9±0.1	6.6±1.4	7.0±0.4	5.4±0.5
total (n-3) PUFA*	35.4±7.3	35.9±3.8	35.2±3.8	36.9±1.4	31.7±1.6	31.8±1.0	32.9±3.0	29.5±2.1	29.8±0.2
DHA/EPA	0.7±0.0^{a}	0.9±0.2^{a}	1.0±0.1^{a}	1.0±0.2^{a}	2.0±0.2^{b}	1.9±0.1^{b}	1.2±0.0^{a}	2.2±0.1^{b}	1.2±0.1^{a}
ARA/DHA	0.4±0.1^{ab}	0.3±0.1^{a}	0.4±0.0^{ab}	0.5±0.0^{b}	0.3±0.0^{a}	0.4±0.0^{ab}	0.3±0.0^{a}	0.4±0.0^{ab}	0.3±0.0^{a}
ARA/EPA	0.3±0.1^{a}	0.3±0.0^{a}	0.4±0.1^{abc}	0.5±0.0^{c}	0.6±0.1^{c}	0.8±0.0^{d}	0.3±0.0^{ab}	1.0±0.1^{d}	0.4±0.0^{abc}
(n-3)/(n-6)	3.6±0.8^{b}	3.6±0.3^{b}	3.2±0.3^{ab}	3.1±0.1^{ab}	2.9±0.1^{ab}	2.7±0.1^{ab}	3.1±0.1^{ab}	2.4±0.1^{a}	2.9±0.0^{ab}

Table 3.-continued

	Time interval for dietary manipulation (dah)								
	11 to 21							3 to 21	
	ARA supplementation								
	0	2	4	8	2	8	8 (dah 16-21)	8	8 (dah 3-10)
	EPA supplementation								
	20	20	20	20	0	0	20 (dah 11-15)	0	20 (dah 11-21)
	dah 22								
TFA	117.0±20.4	79.0±13.1	118.3±23.9	98.7±22.1	121.0±34.7	93.6±16.7	116.7±13.0	111.3±19.4	114.6±12.6
FA (% TFA)									
total SFA*	23.2±1.8	24.8±4.1	22.8±0.2	22.4±0.5	24.2±0.3	24.2±1.4	23.4±0.6	23.3±1.1	23.0±0.5
total MUFA*	25.4±0.6	25.7±0.3	24.9±0.7	25.2±0.1	26.0±0.2	26.0±0.4	26.0±0.3	26.3±0.4	24.8±0.1
C20:4 (n-6)	1.7±0.1^{a}	2.4±0.1^{a}	3.0±0.2^{b}	4.0±0.1^{c}	1.8±0.1^{a}	3.3±0.2^{b}	3.0±0.1^{b}	3.8±0.1^{c}	1.7±0.2^{a}
total (n-6) PUFA*	11.7±0.3^{ab}	11.4±0.4^{ab}	12.6±0.4^{a}	14.2±0.2^{d}	12.3±0.0^{bc}	14.5±0.9^{d}	13.4±0.3^{cd}	14.5±0.2^{d}	11.7±0.5^{ab}
C20:5 (n-3)	9.2±0.6^{c}	9.0±0.6^{c}	9.5±0.2^{c}	9.1±0.1^{c}	5.2±0.2^{ab}	4.6±0.3^{a}	5.9±0.5^{b}	4.6±0.1^{a}	8.8±0.6^{c}
C22:6 (n-3)	5.9±0.4	6.7±0.6	6.4±0.3	6.0±0.2	6.5±0.6	5.9±0.5	6.6±0.1	6.8±0.8	6.2±0.4
total (n-3) PUFA*	39.7±0.9^{bc}	38.0±3.9^{abc}	39.6±0.3^{bc}	38.2±0.6^{abc}	37.5±0.5^{abc}	35.4±0.9^{a}	37.2±0.1^{abc}	35.9±0.8^{ab}	40.4±0.2^{c}
DHA/EPA	0.6±0.0^{a}	0.7±0.1^{a}	0.7±0.0^{a}	0.7±0.0^{a}	1.2±0.2^{b}	1.3±0.1^{b}	1.1±0.1^{b}	1.5±0.2^{c}	0.7±0.0^{a}
ARA/DHA	0.3±0.0^{a}	0.4±0.0^{a}	0.5±0.0^{bc}	0.7±0.0^{e}	0.3±0.0^{a}	0.6±0.0^{d}	0.4±0.0^{ab}	0.6±0.1^{c}	0.3±0.0^{a}
ARA/EPA	0.2±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{bc}	0.4±0.0^{d}	0.3±0.0^{c}	0.7±0.0^{e}	0.5±0.0^{d}	0.8±0.0^{f}	0.2±0.0^{a}
(n-3)/(n-6)	3.4±0.0^{ef}	3.3±0.2^{de}	3.1±0.1^{de}	2.7±0.0^{ab}	3.1±0.0^{cd}	2.4±0.1^{a}	2.8±0.1^{bc}	2.5±0.1^{ab}	3.4±0.2^{ef}

Eye migration

At dah 11 eye migration was not initiated in any of the examined larvae (mean total length of 5.2 ± 0.20 mm ind^{-1}). At dah fifteen eye migration was initiated in about 6 percent of all 805 examined individuals, not significantly different between treatments (P=0.499). The mean larval size at dah 15 was 6.43 ± 0.40 mm ind^{-1}, while the size of larvae in which metamorphosis was initiated (S1-2) was 7.45 ± 0.40 mm ind^{-1}. Eye migration was significantly positively related to an increase in larval standard length (P<0.001, r^2=0.66). The eye migration was not related to differences in ARA tissue content (P=0.14). At dah nineteen metamorphosis was initiated (S ≥1) in 69 ± 6.9 percent of all examined larvae in all groups. At dah twenty two metamorphosis was initiated in 94 ± 2.0 percent and the percent larvae categorized as S3 or S4 was similar for all treatments. 19 to 47 percent were categorized as stage 4 having completed eye migration.

At dah 19 and 22 eye migration score was related to larval body length (P=0.006, r^2= 0.63), (P<0.001, r^2= 0.78) and not to larval ARA or EPA content.

At dah 43 a permanent abnormal incomplete eye migration was only observed in 0.3 percent (i.e 23 juveniles) of all examined fish (7580 individuals). 14 of these juveniles were albinos and 9 juveniles were highly malpigmented.

Pigmentation

Malpigmentation (i.e. hypomelanosis) was very low and not significantly different between treatments (P = 0.09) (table 4). An insignificant trend (P=0.06) was that an increase in dietary arachidonic acid from 0 to 8 percent seemed to result in a slightly elevated malpigmentation in larvae fed *Artemia* enriched with 8 percent ARA (+EPA) at dah 11-21.

The frequency of malpigmented juveniles with a malpigmented body was similar for all treatments (P = 0.08). Both the presence of larvae defined as albinos and larvae with a malpigmented tail was low and was not different between treatments.

Hypermelanosis / ambicolouration on the ventral side varied from a mean of 20 percent to 44 percent between treatment groups. However, there were no significant effect of treatments (P = 0.28) but large variations within replicates of the same treatment. This is examplified by the larvae fed *Artemia* not supplemented with ARA, but 20 percent EPA from dah 11; as hypermelanosis was observed in 2.8 percent, - 42.3 percent and 55.0 percent of the larvae in the 3 replicates.

In most juveniles hypermelanosis covered a part of the ventral side in very well defined „stamp“ like areas from beyond the tail area and further up the blind side, but some were completely pigmented on the ventral side. None of the ambicoloured fish showed both hypomelanosis on the ocular side and hypermelanosis on the blind side.

Prostaglandin PGE_2 levels

At dah 22 PGE_2 content was significantly higher ($P < 0.015$) in larvae fed *Artemia* enriched by 8 percent ARA from dah 3 to 21 than the remaining codes (except code 1) Table 4. Larval tissue PGE_2 content was not correlated to ARA tissue content ($P=0.45$, $r^2 =0.08$) (Fig 2), neither was hypermelanosis related to PGE_2 content ($P=0.77$).

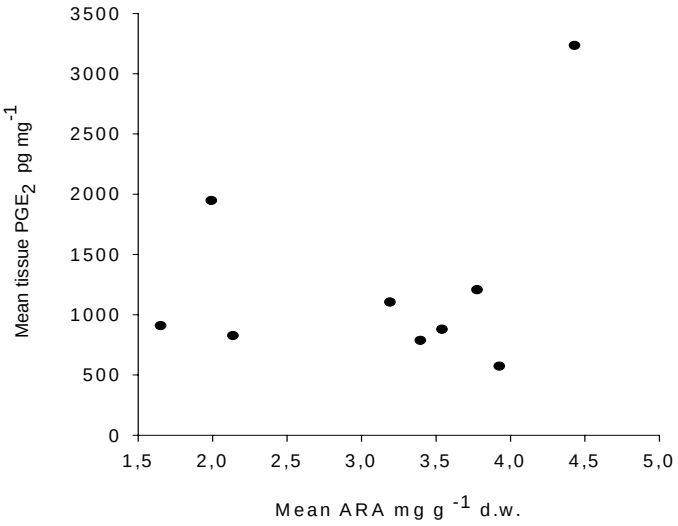
Table 4

Larval PGE_2 prostaglandin concentration at dah 22 given as a mean ± standard deviation. Juvenile malpigmentation at dah 43 given as a mean ± standard deviation for each treatment as well as the percent malpigmented individuals within each malpigmentation subgroup. Percent hyperpigmented fish were defined separately.

	Time interval for dietary manipulation (dah)								
	11 to 21							3 to 21	
	ARA supplementation								
	0	2	4	8	2	8	8 (dah 16-21)	8	8 (dah 3-10)
	EPA supplementation								
	20	20	20	20	0	0	20 (dah 11-15)	0	20 (dah 11-21)
PGE_2 (dah 22)	1948.2 ± 640.5[ab]	908.9 ± 16.8[a]	787.0 ± 167.9[a]	1105.1 ± 215.9[a]	572.2 ±51.9[a]	879.1 ± 27.0	N.A.	3234.1 ± 958.1[b]	827.0 ± 23.0[a]
Overall malpigmentation	2.0 ± 2.7	2.6 ± 1.9	6.7 ± 1.7	7.2 ± 3.2	1.2 ± 0.5	2.7 ± 1.7	2.0 ± 2.6	4.8 ± 5.4	1.6 ± 0.4
Albinos	0.3 ± 0.6	0.3 ± 0.2	1.1 ± 0.7	2.0 ± 1.1	0.5 ± 0.2	0.6 ± 0.5	0.8 ± 1.4	1.3 ± 1.5	0.7 ± 0.6
Malpig. body	1.6 ± 2.1	2.3 ± 2.0	5.3 ± 2.2	5.0 ± 2.3	0.7 ± 0.6	2.2 ± 1.3	1.2 ± 1.2	3.3 ± 3.8	0.9 ± 0.2
Malpig. tails	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.2 ± 0.2	0.0 ± 0.0	0.0± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
Hyperpigmented	33.3 ± 27.2	23.1 ± 11.1	20.9 ± 15.9	17.1 ± 7.7	21.3 ± 7.3	26.1 ± 4.8	43.6 ± 3.0	19.0 ± 10.4	35.8 ± 2.3

Figure 2.

The relation between mean larval ARA contents at dah 22 and larval PGE_2 prostaglandin contents.



Discussion

The overall larval growth rate was similar to previous studies on common sole larvae(Lund et al., 2007, Lund et al., in press, a). The relative differences in dietary ARA, and EPA contents had no significant influence on larval growth. The study could not confirm earlier findings that larval growth rate was higher during premetamorphosis defined at dah 3-10 than at metamorphosis (Parra, 1998, Lund et al., unpublished data, a). The mean growth rate was lower or similarly at dah 3-10 in comparison to dah 11-15, dah 15-19 and dah 19-22. Hence, this study indicates that the physiological process of metamorphosis and eye migration has no negative effect on feed intake, which is in agreement with other findings for this species (Lagardère et al., 1999).

Sole larvae reflected the dietary EPA and ARA compositions within a relatively few days of feeding as previously reported for fatty acids in other larval fishes (Bransden et al.,2005; Villalta et al.,2005 a, b). Initiation of eye migration was related to a larval size of about ≥ 7.5 mm and not to age or dietary ARA or EPA in support of similar results already reported (Amara and Galois, 2004, Lund et al., in press, a) but contrary to results on senegalese sole in which ARA slowed the degree of eye migration (Villalta et al., 2005 a)

Settling at dah 22 did not indicate a complete metamorphosis as only 15-47 percent of the larvae had completed eye migration as similar to previously reported (Lund et al., in press, a).

Malpigmentation - hypomelanosis, was not significantly affected by ARA, even sole larvae fed 8 percent ARA oil at dah 3-21 or during premetamorphosis at dah 3-10 were not significantly affected despite increased sensitivity during this period (Lund et al., in press, a). Contrary to the present findings, in another study, malpigmentation was induced by means of live prey enriched

with 8 percent ARA at dah 11-21 (i.e. 10-13 percent of the indviduals were malpigmented in comparison to 3 percent when not given ARA (Lund et al., in press, a).The relative larval ARA tissue content in this study and the study performed by Lund et al. revealed almost identical levels of 4 percent of total fatty acids. It is interesting, that malpigmentation may be induced by a larval ARA contents of 4 percent (% TFA) which is similar to the native ARA contents in some batches of sole eggs and pre feeding larvae (Lund et al., in press, b).
This suggests, that experimental sensitivity to dietary ARA may differ between larval batches even when raised under apparently identical conditions and may be influenced by other factors than the ARA content.
McEvoy suggests that the balance of EPA and ARA in cells and tissues can influence an individual's ability to respond to stress during overcrowding, unsuitable illumination (McEvoy et al.,1998) eventually leading to an increase in the incidence of malpigmentation. In the present study it was not possible to demonstrate any positive influence of EPA on pigmentation as malpigmentation was low with or without supplementation of EPA. Similary the analysed PGE_2 concentrations were not related to dietary differences, and were lower than in a previous study, which reported a positive correlation between increasing tissue ARA content, malpigmentation and PGE_2 content (Lund et et al., in press, b). The similar and low malpigmentation in all larval groups, may be explained by the relative lower PGE_2 levels obtained in the present study as compared with earlier findings (Lund et al., in press, a).

Hypermelanosis (i.e. blind side pigmentation) was prevalent for all groups of larvae, which we previously have not observed in studies on common sole larvae (Lund et al., in press a,b). Hypermelanosis is one of the most important problems in production of Japanese flounder juveniles because it may reduce market price significantly (Iwata and Kikuchi, 1998). Hypermelanosis is not uncommon in flatfish as up to 95 percent may have black areas on the blind side (Tominaga and Watanabe,1998) and both nutritional and environmental factors are believed to be involved (Haga et al., 2004). Ambicoloured fish may have an equal number of melanophore pigment cells on each side (Venizelos and Benetti, 1999) contrary to normally pigmented flatfish with numerous melanophores on the ocular side only and albinic fish without (Bolker et al., 2005)
There are 2 main hypotheses about the development causes of malpigmentation, which are not necessarily mutual exclusive. One is that dietary deficiencies lead to abnormal retina development, which in turn derails hormonal events necessary to trigger melanophore differentiation (Kanazawa,

1993). The other involves the hypothesis that pigment cells on the ocular side follows a blind side differentiation (Seikai, 1992; Bolker and Hill, 2000, Bolker et al., 2005).

It is suggested that, at the onset of metamorphosis the adult melanophores on the ocular side become more diffuse than those on the blind side and move towards the surface of the skin. At mid – metamorphosis pigment cells can only be seen on the ocular side on normally developing flatfish, while the blind side contains chromatoblasts in various stages of cytolysis (Venizelos and Benetti, 1999). It is speculated if environmentally stress related factors during metamorphosis may have triggered – [1)] either an unbalanced migration of some of the larval melanophore cells to the blind side of the hyperpigmented larvae, - [2)] a differentiation of new adult cells, - [3)] or disrupted the mechanisms in pigment cell cytolysis on the blind side during metamorphosis. In summer flounder (*Paralichthys dentatus*) the dark areas on the blind side in a partial hyperpigmented fish looked like a normal ocular side and the light regions were not like a normal blind side, but contained mature melanophores. The blind side therefore seemed to follow a distinct pathway, not characteristic of any region of a fully pigmented fish (Bolker et al., 2005).

However, the etiology of hypermelanosis abnormality is not well understood and no evidence exists, whether defects of the ocular and blind – side represents distinct phenomena.

Ambicouloration is known to develop at the same time as normal ocular- side pigmentation around metamorphosis (Bolker et al.,2005). The melanin pigment formed by tyrosin kinase activity is believed to be the key enzyme in melanin synthesis within the melanophores (Seikai, 1992; Bolker and Hill, 2000) An ideal balance between tyrosin kinase synthesis and degradation is necessary for regulating pigmentation (Ando et al., 2004), in the present study this balance may somehow have been affected.

In the present study the form of hypermelanosis was permanent in contrast to „staining“, which is partially reversible in juveniles (Seikai, 1992) and normally develop later (Bolker et al.,2005).

Interestingly the large replicate variation in hypermelanosis of 3 to 55 percent of the individuals demonstrated that this abnormality was not treatment related or related to ARA, hence environmental conditions had an influence. In comparison to a similar study in which ARA induced malpigmentation, the rearing conditions were apparently identical with respect to density of larvae, illumination, water exchange rate, handling, feeding etc.so overall stressors suggested to be similar. Hence inducement of hypermelanosis may be related to local delicate environmental and physiological balances between replicates on a scale of the individual. Moreover genetics and strain

origin is likely to influence on pigmentation development and the sensitivity likely to be different between different batches of larvae, but is not well examined.
It is therefore suggested, that the present batch of larvae may have been predisposed to hypermelanosis, triggered or not by local environmental factors. Accordingly, this may have lowered larval sensitivity to ARA to induce hypomelanosis This suggests, that underlying physiological mechanisms of larval hypermelanosis and hypomelanosis may be similar.

Numerous studies have proposed both genetic, neurological, environmental and nutritional factors as influencing on pigmentation (Kanazawa, 1993, Venizelos and Benetti,1999, Bolker and Hill, 2000). From an evolutionary point normal pigmentation should be an extremely stable trait, considering that malpigmented individuals succomb quickly to predation (Ellis et al., 1997). Based on this and previous studies on sole (Lund et al., 2007, Lund et al., in press, a) the balance between normal pigmentation and malpigmentation is considered delicate and neurologically related. Apparently this balance can easily be upset in aquaculture by the somehow artificial environment and nutrition and illumination, tank colour, water quality, substrate, temperature, prey nutritional profile, polyunsaturated fatty acids, - ARA, vitamin A,D_3 have all previously been reported to affect pigmentation (Iwata and Kikuchi, 1998; Venizelos and Benetti, 1999; Bolker and Hill, 2000; Haga et al.,2004; Villalta et al., 2005 a, Bransden et al., 2005).
In continuation of the present study, future studies may focus on the importance of ARA and prostaglandin levels in relation to cortisol and tyrosin kinase activity during sole larval development, as the role of „stress levels“ in the disruption of the pigmentation process remains unclear (Venizelos and Benneti, 1999).

Conclusions

Pigmentation, eye migration, growth and survival in common sole were not affected by feeding *Artemia* enriched with dietary levels of ARA up to 8 percent inclusion and either 0 or 20 percent EPA. Environmental factors had an influence on hypermelanosis.

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VI

The influence of dietary concentrations of arachidonic acid and eicosapentaenoic acid at various stages of larval ontogeny on eye migration, pigmentation and prostaglandin content of common sole larvae (*Solea solea* L.).

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The influence of dietary concentrations of arachidonic acid and eicosapentaenoic acid at various stages of larval ontogeny on eye migration, pigmentation and prostaglandin content of common sole larvae (*Solea solea* L.)

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Abstract

Dietary manipulations of arachidonic acid, ARA and eicosapentaenoic acid, EPA may have an influence on pigmentation in common sole larvae (*Solea solea* L., Linnaeus 1758) which may be related to a "pigmentation window". This is a specific period in the larval ontogeny where nutritional factors determine pigmentation.

Malpigmentation was significantly related to elevated dietary and larval ARA contents, but not to EPA. The study reports evidence for a pigmentation window, as larval sensitivity to ARA or its derivatives was much higher during pre metamorphosis, than during metamorphosis.

Initiation of metamorphosis (i.e. start of eye migration) was related to the size of larvae and not related to ARA or EPA content. Dietary EPA or DHA did not retard the advance of eye migration.

More than 90 % of highly malpigmented juveniles, (i.e. "albinos") had a permanent aberrant eye migration, which was not related to dietary treatments. This malformation was not observed in normal or slightly malpigmented juveniles.

Mean larval ARA tissue contents, malpigmentation and prostaglandin, PGE_2 tissue contents were significantly positively correlated. PGE_2 levels and pigmentation were not affected by dietary or larval EPA contents. Consequently ARA induced prostaglandins are suggested to be involved in malpigmentation in common sole.

1. Introduction

Flat fish pigmentation development has been studied mainly in Japanese flounder (*Paralichthys olivaceus*) (Bolker and Hill, 2000). The beginning of metamorphosis and pigment cell

differentiation (differentiation of adult melanophores) starts when the larvae reach the stage, that marks the onset of metamorphosis (Estévez and Kanazawa, 1995). Ocular abnormalities appear around 3 weeks post hatch (Seikai et al., 1987). Studies in Japanese flounder and Atlantic halibut (*Hippoglossus hippoglossus*) have identified a specific premetamorphic stage, as a critical period for dietary supplementation to reduce later incidence of albinism (Seikai et al., 1987; Næss and Lie, 1998). In halibut the usage of copepods for only 7 days shortly before metamorphosis should be sufficient to prevent malpigmentation (Næss and Lie, 1998). In plaice (*Pleuronectes platessa*) the duration of feeding copepods rather than the exact timing may be the relevant factor for a normal pigmentation (Dickey-Collas, 1993). Little information exists for the underlying development processes for other flatfish species (Planas and Cunha, 1999, Bolker and Hill, 2000).

In a previous study pigmentation in common sole larvae at 3-21 days after hatching was negatively affected and highly correlated to dietary levels of arachidonic acid (Lund et al., 2007). In senegalese sole (*Solea senegalensis*), a close sister relative to common sole, it has been reported that ARA in addition to inducing malpigmentation slowed down eye migration (Villalta et al.,2005a). This suggests, that ARA is coupled to the underlying pigment cell differentiation, advocating for an influence of ARA during early larval ontogeny.

ARA is known to be a precursor of eicosanoids and hormone like substances (i.e. "the arachidonic cascade") for instance prostaglandins of the 2- series (PGE_2) while eicosapentaenoic acid, EPA is known to give rise to prostaglandins of the 3- series (PGE_3) (Bell et al.,1995a).

It is hypothesized, that PGE_2 is involved in the stress response of fish through modulation of cortisol release (Koven et al., 2003). Furthermore excess PGE_2 production has been suggested to cause biochemical stress and to be involved in inducing malpigmentation (Sargent et al., 1999, Bransden et al., 2005; Villalta et al., 2005a).

ARA and EPA compete for the same cell enzyme binding site, and the presence of EPA is reported to reduce the production of ARA derived 2-series prostaglandins (Bell et al., 1993). The PGE_3 series, however, have a lower biological activity as compared to the 2- series (Bell et al., 1995a, b). This suggests that the main effect of high dietary levels of EPA could be to reduce the presence and efficacy of the 2- series prostaglandins.

In a recent study, sole pigmentation was not improved by dietary supplementation of EPA oil (7.5-27.5 %) in larval *Artemia* diets in combination with a high dietary supplementation of ARA oil (20 %) (Lund et al., 2007). EPA, however, may be an inhibitor at lower dietary concentrations of ARA or at ratios of EPA: ARA higher than 1 as hypothesized on mice and recently in an in vitro study

(Whelan, 1996; Wada et al., 2007). Indirectly EPA could then be responsible for the pigmentation success. However, in the study on mice (Whelan, 1996), it was observed that when a dietary inclusion of EPA and ARA was given concomitantly, the effect of EPA on eicosanoid production was negated, which may be caused by a higher cell affinity for ARA.

The main objective of the present study was to examine if inducement of malpigmentation was related to a specific larval period of increased sensitivity to ARA, "a pigmentation window" and to investigate if PGE_2 prostaglandin contents were related to malpigmentation. Dietary EPA was supplemented to examine a possible inhibitory effect on PGE_2 prostaglandin production and therefore a positive influence on pigmentation.

To study these effects, live prey, *Artemia* were enriched by emulsions of fish oil supplemented with different concentrations of ARA and / or EPA oils and fed to sole larvae at various stages of larval ontogeny, either prior to initiation of metamorphosis, (i.e. defined as start of eye migration), during metamorphosis or during both periods.

2. Material and methods

2.1 Eggs and larvae

The common sole (*Solea solea*) larvae were obtained from Solea B.V. Ijmuiden, Holland. The eggs were kept at 8 º C in a 60 l incubator by a supply of borehole seawater and a slight aeration until hatching. After hatching, the temperature was slowly increased to 12º C and larvae were collected into oxygenated plastic bags (1/3 water), packed in polystyrene boxes with ice and transported to the rearing facility in Denmark.

The rearing facility consisted of 30 cylindrical-conical tanks holding 46 l each connected to a 1 µm filtrated flow through water system. Each tank had a separate inlet tap with a flowmeter, a 700 µm outlet filter and filtrated aeration

After temperature equilibration (15.5→17.1 º C) larvae were transferred to 30 rearing tanks by multiple subsampling from a 60 l bucket. An expected 768 larvae were added to each tank, equalling a density of 16 larvae l^{-1}. The water flow was 8 l h^{-1}. Water exchange was increased to 80 l h^{-1} one hour prior to feeding.

Oxygen saturation, temperature and pH were monitored daily by a hand- held Oxyguard / pH meter from Oxyguard, Birkerød, Denmark followed by measurements of NH_4^+, NO_2^-, NO_3^- by a Merckquant test kit from Merck, Darmstadt, Germany.

The temperature from dah 1 to 22 was 16.9 ± 0.35 º C. At dah 23 to 33 the temperature was 16.7 ± 0.12 º C. Levels of ammonia, nitrite and nitrate were below detection. Oxygen content was kept above 6.9 mg l^{-1} in all tanks. The photo period was 24 h light by the use of thirty 40 W light bulbs placed above the tanks. The illumination was measured to approximately 100 lux at the water surface.

2.2 Preparation of emulsions

The enrichment sources were freshly prepared high quality marine sprat fish oil from Fiskernes Fiskeindustri, FF, Skagen, Denmark which was substituted by natural arachidonic acid, ARA (VEVODAR) oil, produced by means of fermentation of a fungus (*Mortierella alpina*),from DSM Food Specialities BV, Holland and concentrated Incromega eicosapentaenoic acid, EPA oil from Croda Chemicals Europe, Snaith, UK. The emulgator was Soy lecithin (BioMar, Brande, Denmark) added as a growth promoter (7 %) (Macqueen et al., 2003) and an E- vitamin mixture (BioMar, Brande, Denmark) was added as an antioxidant (4 %). All ingredients were freshly prepared and stored at – 30 ° C until usage.

During preparation of the 6 emulsions the oils were heated to 25 ° C, the ingredients weighed and mixed and homogenised with a Büchi mixer B- 400 for 1 min. at 9000 rpm. The emulsions were flushed with nitrogen and each emulsion stored in four air free 20 ml sterile plastic syringes at – 80 ° C. At usage one syringe was thawed at a time. The daily amount of emulsion used for enrichment was weighed and mixed with 100 ml distilled water by homogenisation with a Büchi mixer B- 400 for 40 sec. at 9000 rpm. After usage the syringes were refrozen at - 25° C.

To test the influence of both ARA and EPA 4 diets were prepared with Vevodar, ARA oil concentrations of 0, 8, 16, 24 % and 20 % of Incromega, EPA oil and 2 diets were prepared with 8 and 24 % ARA oil without EPA oil. Table 1 illustrates the dietary formulations and the fatty acid contents.

2.3 Live prey feed and larval rearing

Artemia IH cysts were incubated for 18 h at 28 -30 ° C. IH nauplii (second stage) were harvested 8 h post hatch, washed with 0.2 µm seawater followed by freshwater. *Artemia* were divided into 10 buckets of 5 l each and either left unenriched or enriched with 0.6 g l^{-1} of each of the 6 emulsions for 17-18 h at 20-22 º C. EG grade *Artemia* were enriched similarly apart from a cyst incubation –

and enrichment period of 22-24 h. Feed was harvested once a day in the morning. Feed for the second daily feeding was kept refrigerated with aeration.

To investigate the effect of altered dietary levels of ARA and EPA during different time intervals the following feeding regimes were formed, illustrated in Table 2:

High EPA and altered ARA content during dah 11-21. In this feeding regime, groups of sole larvae were fed un-enriched IH *Artemia* at dah 3-10, whereupon the diet was changed to EG *Artemia,* supplemented with 0, 8, 16 or 24 % ARA and 20 % EPA.

Low EPA and altered ARA content during dah 11-21. In this feeding regime, groups of sole larvae were fed un-enriched IH *Artemia* during dah 3-10, whereupon the diet was changed to EG *Artemia* enriched with 8 or 24 % ARA oil.

High EPA content during dah 11-15 and high ARA content during dah 16-21. In this feeding regime larvae were fed un-enriched IH-*Artemia* during 3-10 dah, whereupon the feed was changed to EG *Artemia* enriched with 24 % ARA, which was given during 11-15 dah. After this the larvae were fed EG *Artemia* enriched with 20 % EPA (dah 16-21).

Altered ARA and EPA levels during dah 3-21. In this feeding regime two groups of sole larvae were fed enriched IH *Artemia* (dah 3-10) and EG *Artemia* (dah 11-16). One group received IH *and* EG *Artemia* enriched with 24 % ARA, and the other group was given IH and EG *Artemia* enriched with 16 % ARA and 20 % EPA.

High ARA content during 3-10 dah and high EPA content during 11-21 dah. In this feeding regime larvae were fed IH *Artemia* enriched with 24 % ARA during 3-10 dah, upon which the feed was changed to EG *Artemia* enriched with 20 % EPA.

Artemia were administered 2 times a day; at 1000 and 2000 h at a prey concentration of 2 ml $^{-1}$. Juvenile pigmentation and eye translocation were evaluated at dah 33. All groups of settled larvae were treated with the same diet; EG *Artemia* enriched by a commercial Easy DHA Selco emulsion from INVE, Belgium at dah 22 until dah 32.

2.4 Estimation of larval size, pigmentation rate and eye migration

Standard length of larvae was measured by a digital high resolution DFC 320 Leica camera connected to a dissecting microscope (Leica MZ6).

The evaluation of pigmentation was carried out by evaluating all juvenile fish individually. Overall malpigmentation was defined as hypomelanosis. Overall malpigmentation was classified into 4 subgroups; 1) normal pigmented juveniles, 2) juveniles with malpigmented tails, 3) juveniles with a

malpigmented part of the body, or 4) “albinos” (> 95 percent body malpigmentation). Juveniles with excess pigment on the blind side; hyperpigmented fish were registered but categorized as normal. The overall malpigmentation rate was calculated as the number of malpigmented fish / total surviving number of fish x 100.

The degree of metamorphosis was characterized by the position of the migrating left eye modified according to a traditional index (Fernández-Diaz et al., 2001). It was closely followed and was evaluated on sampled larvae at dah 11, 16, 21: The 5 sub stages were: 0, symmetrical left and right eye position, 1, an asymmetrical position of the left eye and right eye; the left eye starts to migrate, 2, the migrating eye reaches at maximum the midline of the dorsal surface, 3, the migrating eye can be seen from the right ocular side or migrates within the dorsal side, 4, eye translocation is completed and the orbital arch is visible.

Juvenile eye migration was evaluated as complete or incomplete at dah 33. An incomplete abnormal position of the eyes was defined as when the migrating eye was in position 1- 3 or when both the left and right eyes were in other ways abnormal positioned.

2.5 Samples for dry weight (d.w.)

Whatman Ø 25 0.7 µm GF/F glass fibre filters were precombusted at 450 ° C for 24 hrs and weighed on a Mettler Toledo MT5 d = 0.1 µg. After filtration of larvae and prey, 5 ml of 0.5 M v/v ammonium formate was used to remove seawater from the filter samples. The filter samples were dried in an oven at 80 ° C for 24 hrs and then reweighed.

2.6 Samples for fatty acids and prostaglandins

Prey subsamples of IH *Artemia* were collected at dah 10, and at dah 15 and dah 21 for EG *Artemia* (i.e. 50-100 pcs. sample^{-1}). Pre-feeding larvae were sampled at dah 3, as well as larvae from each rearing tank at dah 11, 16, 21 (i.e. 8-10 pcs sample^{-1}). Samples were washed gently on a 60 µm filter net with 0.2 µm seawater to remove eventual dirt or excess emulsion and collected on Whatman Ø 25 0.7 µm GF/F glass fibre filters in duplicate prior to feeding in the morning. Filters were covered with nitrogen and frozen at – 80 ° C in sterile 5 ml cryovials until extraction.

Larval samples for prostaglandin analyses were stored in 5 ml sterile cryo-vials in 4 ml of Hanks solution containing 0.6 ml of absolute alcohol and 0.2 ml of 2M formic acid and immediately frozen in liquid nitrogen (-196 ° C).

2.7 Lipid extraction and fatty acid analysis

The fatty acid composition was determined by extraction of the lipids by a chloroform / methanol mixture, (Folch et al., 1957) and either sonicated in a ultrasound cleaner, model Branson, 2510, or homogenised (i.e larger larvae) by a tissue tearor probe diameter 4.5 mm, Biospec Products, Inc. Then followed trans esterification of the lipids by acetyl chloride in methanol. The fatty acid methyl esters were analyzed by gas chromatography - mass spectrometry (GC-MS). Peaks on a given chromatogram were identified by comparison with the retention time of a commercial mix of a known FAME standard, SUPELCO 18919 (4:0-24:0), from SIGMA. Peaks were quantified by means of the target response factor of the fatty acids to a 23:0 internal standard. Fatty acid concentrations were calculated (Chem. Station programme) based on the quantified peaks of the standard series and the samples as well of dry weight of prey and larvae and expressed as ng sample^{-1}. A total of 34 fatty acids were analysed. For larval samples only the n-6 PUFA; 20:4n-6 and the n-3 PUFAs; 20:5n-3 and 22:6n-3 are presented in the tables for clearness.

2.8 Sample preparation and prostaglandin analysis

Prostaglandin E_2 content was analysed by Cayman Chemicals**,** Michigan, USA according to a Cayman monoclonal Competitive Enzyme Immunoassay (EIA) kit; Cat. No 514010, (pp. 1-17). The PGE_2 EIA assay converts all major PGE_2 metabolites into a single stable derivative, which is easily measured by EIA. The assay is based on the competition between PGE_2 and a PGE_2-acetylcholinesterase (ACh) conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody. The amount of PGE_2 tracer is held constant and the amount that is bound to the PGE_2 monoclonal antibody is inversely proportional to the concentration of PGE_2 in the well. The monoclonal antibody binds to goat polyclonal anti-mouse IgG that is attached to the well. The 96 – well plate is washed to remove unbound reagents and Ellmans reagent (+ ACh) added to the well. The product of the enzymatic reaction is determined spectrophotometrically at 412 nm, the intensity is inversely proportional to the amount of free PGE_2 in the well.

The combined weighed mass (mg) of each sample of larvae was placed in 300 µl of 50 mM Phosphate buffer (pH 7.4) and homogenized using a Polytron tissue tearor. The homogenates were spun for 15 min. at 8000 rpm, and the supernatant was transferred to a clean tube. The supernatants were assayed at dilutions (in EIA buffer) ranging from 1:2 to 1:50. Standard curves were constructed by serial dilution as described in the EIA kit protocol. The concentration of the samples were calculated from a log-regression line fit to a curve of standard concentrations versus the logit

(i.e., ln(x/1-x)) transformed B/B_0 values. Protein concentrations were also determined for each sample supernatant, using the Bradford method, to standardize the PGE_2 concentrations between samples.

2.9 Survival and growth

Survival was estimated by counting all individuals at dah 21, including larvae sampled for analyses. Growth rate was calculated as Specific Growth Rate (SGR) based on body lengths according to the formula; $SGR = (\ln L_f - \ln L_i \times 100)/ t$, Where $\ln L_{f\,i}$ = the natural logarithm of the final and initial length, t = time (days) between ln L_f and ln L_i.

2.10 Statistics

Comparison of larval growth and the FA composition between treatments were compared by a one-way ANOVA and a pairwise multiple comparison of means using Tukey`s test $P< 0.05$). Percent data were arcsine transformed prior to analysis. Comparison of pigmentation was carried out by a Kruskal- Wallis test

Linear regression analysis was used to identify the relationship between diet, larval FA contents and PGE_2 contents. The variance of data is given as standard deviation (sd), (n=3) unless otherwise stated. Contingency analyses (Chi^2 tests) were used to assess dependency of eye migration, larval size and dietary treatment. A Pearson Product Moment Correlation Analysis was used to determine a possible dependent relationship.

3. Results

3.1 FA content in emulsions and Artemia

The relative contents of ARA and EPA in the emulsions and *Artemia* are presented in Table 1 and 2. The six fatty acids 16:0, 18:1n-9, 18:2n-6, 20:5n-3, 20:4n-6 and 22:6n-3 constituted 70-80 % of the fatty acids analysed. The ARA: EPA in the emulsions varied from 0.1 – to 2.3 and the n-6: n-3 ratio from 3.9 to 0.8.

Table 1. Analysed TFA content (mg g d.w. $^{-1}$) and FA content (percent of TFA) of the 6 different emulsions and EG Artemia.
**Incl. minor fatty acids omitted. For Artemia ; a different superscript in a horizontal rows defines a significant difference (P < 0.05). No letter defines no significance*

	ARA supplementation											
	0	8	16	24	8	24	0	8	16	24	8	24
	EPA supplementation											
	20	20	20	20	0	0	20	20	20	20	0	0
	Emulsion						*Artemia*					
Total analysed FA	833.0	943.9	752.7	965.5	846.9	988.6	295±19	217±29	195±13	198±23	208±12	241±45
FA (% TFA)												
C14:0	4.1	3.9	2.6	4.0	4.9	3.7	0.6±0.3	1.2±0.4	1.5±0.3	1.2±0.2	1.4±0.1	0.9±0.4
C16:0	12.7	11.1	12.2	11.9	13.9	13.5	10.2±0.2	10.7±1.8	12.2±0.8	10.7±1.2	12.1±0.7	12.1±1.4
C18:0	2.2	3.2	3.6	4.3	3.6	5.2	4.5±0.6	5.9±0.7	6.6±0.2	6.2±0.5	6.3±0.5	6.8±1.3
total SFA*	20.4	19.9	20.0	22.5	24.5	24.7	17.8±0.8	20.2±3.3	23.8±0.9	21.1±1.7	23.8±0.0	24.0±2.9
C16:1 (n-7)	4.9	4.4	3.0	2.5	5.9	4.3	2.2±1.0	3.1±0.3	2.4±0.1	2.2±0.9	4.3±0.4	3.2±0.4
C18:1 (n-9) cis	16.8	15.4	14.9	14.4	19.0	17.6	20.9±1.1^{c}	16.2±0.4^{a}	18.8±0.5^{b}	15.6±0.4^{a}	25.6±0.3^{d}	22.9±0.2^{c}
C20:1 (n-9)	2.1	2.0	1.5	1.0	2.5	1.9	1.3±0.1	1.5±0.0	1.2±0.1	1.1±0.0	2.1±0.1	1.6±0.2
C24:1 (n-9)	1.3	1.2	0.8	1.2	1.7	1.2	0.2±0.3	0.9±0.0	0.5±0.1	0.5±0.3	1.3±0.0	0.8±0.1
total MUFA*	26.1	23.9	22.2	20.1	30.2	25.9	25.1±1.9^{b}	22.2±0.0^{b}	23.6±0.8^{b}	19.7±1.1^{a}	34.2±0.5^{d}	29.2±1.0^{c}
C18:2 (n-6) c	8.6	8.9	7.6	7.6	7.9	8.5	8.6±2.4	6.1±0.4	6.6±0.2	6.1±0.3	6.5±0.1	7.9±0.7
C18:3 (n-6)	0.2	0.5	0.8	1.2	0.5	1.1	0.4±0.0	0.4±0.0	0.7±0.0	0.8±0.1	0.5±0.0	0.9±0.1
C20:2 (n-6)	0.5	0.5	0.5	0.6	0.7	0.6	0.5±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.0
C20:3 (n-6)	0.1	0.7	0.9	1.4	0.5	1.3	0.2±0.0^{a}	0.4±0.0^{b}	0.5±0.0^{b}	0.7±0.1^{c}	0.3±0.0^{b}	0.8±0.1^{c}
C20:4 (n-6), ARA	1.6	7.2	11.0	15.2	6.4	15.9	1.4±0.1^{a}	4.6±0.1^{b}	7.3±0.3^{c}	10.9±0.5^{d}	4.9±0.2^{b}	10.5±0.4^{d}
total (n-6) PUFA*	11.0	17.9	20.8	26.1	15.9	27.5	11.1±2.5^{a}	12.0±0.6^{ab}	15.4±0.2^{bc}	18.9±2.5^{cd}	12.8±0.3^{ab}	20.6±1.3^{d}
C18:3 (n-3) c	3.2	3.0	2.1	2.5	3.4	2.5	16.4±2.5^{b}	16.7±0.1^{b}	13.3±1.1^{ab}	12.5±0.6^{ab}	10.1±0.3^{a}	11.9±0.7^{ab}
C20:3 (n-3) c	0.2	0.2	0.1	0.2	0.2	0.2	0.8±0.0^{b}	0.3±0.1^{a}	0.6±0.1^{ab}	0.6±0.0^{ab}	0.5±0.0^{ab}	0.7±0.2^{b}
C20:5 (n-3), EPA	23.6	21.8	23.2	19.2	9.6	7.0	23.3±2.2^{b}	20.5±1.9^{b}	19.4±1.0^{b}	23.3±1.3^{b}	10.9±0.4^{a}	8.3±0.9^{a}
C22:6 (n-3), DHA	15.6	13.5	11.6	9.5	16.1	12.3	5.5±0.5^{a}	8.2±0.8^{b}	3.9±0.1^{a}	4.0±0.2^{a}	7.6±0.1^{b}	5.4±0.6^{a}
total (n-3) PUFA*	42.6	38.4	37.0	22.4	29.4	21.9	46.0±0.3^{c}	45.6±2.8^{c}	37.2±1.8^{b}	40.3±1.6^{b}	29.2±0.1^{a}	26.2±0.7^{a}
DHA:EPA	0.7	0.6	0.5	0.5	1.7	1.8	0.2±0.0^{b}	0.4±0.0^{c}	0.2±0.0^{ab}	0.2±0.0^{a}	0.7±0.0^{e}	0.7±0.0^{d}
ARA:DHA	0.1	0.5	1.0	1.6	0.4	1.3	0.3±0.0^{a}	0.6±0.0^{b}	1.9±0.1^{c}	2.7±0.0^{d}	0.6±0.0^{b}	2.0±0.1^{c}
ARA:EPA	0.1	0.3	0.5	0.8	0.7	2.3	0.1±0.0^{a}	0.2±0.0^{b}	0.4±0.0^{c}	0.5±0.1^{c}	0.5±0.0^{c}	1.3±0.1^{d}
(n-3):(n-6)	3.9	2.2	1.8	1.2	1.8	0.8	4.2±0.9^{c}	3.8±0.1^{bc}	2.4±0.1^{ab}	2.1±0.1^{ab}	2.3±0.1^{ab}	1.3±0.1^{a}

Native unenriched IH *Artemia* were poor in total fatty acid, (TFA) content (mg g^{-1}d.w.) and TFA was much lower than in enriched IH *Artemia* (data not shown). The EPA content in unenriched IH *Artemia* was 5 % and these *Artemia* contained almost no DHA. In enriched IH and EG *Artemia* strains the relative ARA and EPA contents were significantly different between treatments ($P < 0.001$) and reflected the profile of the emulsions, but at lower relative contents (Table 1). ARA calculated as mg g^{-1} d.w. varied from 4.1 to 19.2 in non ARA enriched and highly ARA enriched EG *Artemia* groups, respectively. Similarly, EPA content varied from 15.9 – 67.1 mg g^{-1} d.w.

3.2 Larval fatty acid composition

11 days after hatching

ARA content was significantly higher in larval groups fed IH *Artemia* enriched by 24 % ARA or 16 % ARA and 20 % EPA than in larvae fed unenriched IH *Artemia* ($P < 0.001$), (Table 2). Furthermore EPA content was significantly elevated in larvae fed IH *Artemia* enriched with EPA during dah 3-11 ($P < 0.001$).

16 days after hatching

ARA increased proportionally when fed 8, 16 and 24 % ARA enriched EG *Artemia* from dah 11. Larvae fed *Artemia* supplemented with 24 % ARA from dah 3 had the significantly highest content of all groups ($P \leq 0.04$). ARA tissue content decreased significantly ($P < 0.001$) when the diet was shifted from *Artemia* enriched with 24 % ARA at dah 11, to *Artemia* enriched with 20 % EPA. Larval EPA content reflected the dietary compositions and was significantly lowest for larvae fed *Artemia* not enriched with EPA ($P < 0.001$).

22 d*ays after hatching*.

As for dah 16 the relative ARA tissue content was significantly different between groups ($P < 0.001$) and closely related to the dietary content. ARA was highest in larvae fed *Artemia* supplemented with 24 % ARA from dah 3 followed by larval groups fed *Artemia* enriched with 24 % ARA or 24 % ARA and 20 % EPA during dah 11-21. Similar and insignificantly different ARA contents ($P > 0.97$) were observed for larvae fed *Artemia* enriched with 16 % ARA and 20 % EPA either during dah 3-21 or during dah 11-21 as well as the group fed 24 % ARA at dah 16-21. Moreover low and similar ARA contents ($P = 0.98$) were observed in larvae fed *Artemia* enriched

with 20 % EPA at dah 3-21 or at dah 11-21 in which the diet was changed from *Artemia* enriched with 24 % ARA at dah 3-10.

The relative larval EPA content reflected the dietary treatments and differed significantly ($P < 0.001$). The tissue EPA content varied from 7.2 - 9.4 % when fed *Artemia* enriched with EPA and from 3.5 - 4.8 % without EPA enrichment. Larval EPA tissue content decreased significantly by an increase in tissue ARA content ($r^2 = 0.824$, $P = 0.033$), when EPA was not supplemented. However, EPA tissue content was not correlated to an increase in ARA tissue content when fed diets supplemented by EPA ($r^2 = 0.27$, $P > 0.19$).

Larval DHA content was significantly different between treatments ($P < 0.001$) despite relative differences of less than 1.6 %. A decrease in DHA tissue content was related to an increase in ARA tissue content ($r^2 = 0.63$, $P < 0.001$).

Larval tissue EPA: DHA was significantly highest for the groups of larvae fed EPA enriched *Artemia*. ARA: EPA reflected the dietary inclusion levels of both ARA and EPA ($P < 0.001$). A 10 fold difference in larval ARA: EPA was observed between treatment groups, hence significantly highest for larval groups fed *Artemia* enriched with 24 % ARA and lowest for the larval group fed *Artemia* supplemented with 20 % EPA ($P <0.001$)

Table 2.

Total fatty acid content (TFA mg g^{-1} d.w.) and composition (% of TFA) given as means ± standard deviation in larvae at 3, 11, 16 and 22 days after hatching. N =3 unless otherwise indicated

31 fatty acids omitted at each test day for clearness. A different superscript in a horizontal rows defines a significant difference (P < 0.05). No letter defines no significance (P > 0.05).

		Time interval for dietary manipulation (dah)									
		11 to 21							3 to 21		
		ARA supplementation									
		0	8	16	24	8	24	24 (dah 16-21)	24	16	24 (dah 3-10)
		EPA supplementation									
		20	20	20	20	0	0	20 (dah 11-15)	0	20	20 (dah 11-21)
	Dah 3	Dah 11									
	n=2	n=2	n=2	n=2		n=2					n=2
TFA	71.5± 26.8	80.3	70.8±43.8	42.8±7.1	59.6±6.1	71.3±12.1	72.6±4.2	83.5±40.4	97.8±0.5	104.7±5.9	92.3±11.8
FA (% TFA)*											
Total SFA	26.2±1.9	37.6±2.1	36.6±4.1	32.1±3.7	30.3±1.2	29.7±3.5	34.6±0.6	33.1±4.5	30.4±2.2	29.0±5.9	26.7±1.8
Total MUFA	26.6±1.4	27.1±0.3	26.3±0.2	28.0±1.2	28.7±0.9	28.2±1.1	27.3±0.5	27.5±1.0	28.3±0.6	27.3±1.5	28.5±1.0
C20:4 (n-6) ARA	3.0±0.7	1.3±0.1^{a}	1.5±0.2^{a}	1.4±0.2^{a}	1.6±0.3^{a}	1.7±0.1^{a}	1.7±0.2^{a}	1.8±0.5^{a}	3.7±0.3^{b}	3.8±0.4^{b}	3.6±0.0^{b}
Total (n-6) PUFA	6.8±2.1	9.2±0.4^{a}	9.7±0.5^{a}	10.2±0.3^{a}	10.3±0.1^{a}	10.3±0.2^{a}	10.2±0.3^{a}	10.7±0.6^{a}	12.6±0.3^{b}	12.8±0.8^{b}	12.9±0.5^{b}
C20:5 (n-3) EPA	5.1±0.1	1.5±0.0^{a}	1.3±0.3^{a}	1.5±0.0^{a}	1.7±0.1^{a}	1.9±0.1^{a}	1.7±0.2^{a}	1.6±0.2^{a}	1.8±0.1^{a}	4.6±0.8^{b}	1.9±0.2^{a}
C22:6 (n-3) DHA	34.8±5.5	2.5±0.1^{a}	3.0±1.0^{ab}	1.8±0.3^{a}	1.9±0.2^{a}	3.7±0.9^{ab}	2.6±0.4^{a}	3.0±1.4^{ab}	4.0±0.5^{ab}	5.0±0.4^{b}	3.9±0.3^{ab}
Total (n-3) PUFA	40.4±5.3	26.1±1.6	27.4±3.7	29.7±2.7	30.7±0.5	31.8±2.2	27.9±0.9	28.8±3.2	28.7±1.8	30.8±3.6	31.9±0.3
DHA:EPA	6.9±1.2	1.7±0.2	2.2±0.3	1.2±0.2	1.1±0.1	2.0±0.6	1.5±0.3	1.9±0.8	2.2±0.2	1.1±0.1	2.1±0.1
ARA:DHA	0.1±0.0	0.5±0.0^{a}	0.5±0.1^{a}	0.8±0.0^{ab}	0.8±0.1^{b}	0.5±0.1^{a}	0.6±0.0^{ab}	0.7±0.3^{ab}	0.9±0.0^{b}	0.8±0.0^{ab}	0.9±0.1^{b}
ARA:EPA	0.6±0.1	0.9±0.0^{a}	1.1±0.0^{a}	1.0±0.1^{a}	0.9±0.1^{a}	0.9±0.1^{a}	1.0±0.2^{a}	1.1±0.2^{a}	2.0±0.1^{b}	0.8±0.1^{a}	1.9±0.2^{b}
(n-3):(n-6)	6.4±2.7	2.8±0.1^{b}	2.8±0.2^{b}	2.9±0.4^{b}	3.0±0.1^{b}	3.1±0.2^{b}	2.7±0.0^{ab}	2.7±0.3^{ab}	2.3±0.2^{a}	2.4±0.1^{a}	2.5±0.1^{a}

Table 2 - continued

	Time interval for dietary manipulation (dah)									
	11 to 21							3 to 21		
	ARA supplementation									
	0	8	16	24	8	24	24 (dah 16-21)	24	16	24 (dah 3-10)
	EPA supplementation									
	20	20	20	20	0	0	20 (dah 11-15)	0	20	20 (dah 11-21)
	Dah 16									
	n=2	n =2	n =2	n=2				n=2		n=2
TFA	149.5	136.6±30.6	116.5±30.5	121.7±10.4	118.3±	105.0±15.0	147.6±50.1	131.3±10.9	134.3±30.7	128.5±10.6
FA (% TFA)*										
Total SFA	26.2±1.3	30.6±2.6	29.0±1.1	30.3±1.0	31.9±4.0	40.0±5.9	29.1±3.3	31.6±0.7	30.3±4.5	27.1±2.8
Total MUFA	24.7±0.9^{ab}	24.4±1.9^{ab}	23.6±0.2^{ab}	22.7±0.0^{ab}	25.1±1.6^{b}	21.7±2.0^{a}	24.2±0.8^{ab}	24.1±0.2^{ab}	22.2±0.5^{a}	25.8±0.7^{b}
C20:4 (n-6) ARA	1.5±0.0^{ab}	2.5±0.1^{ab}	3.7±0.4^{bc}	4.4±0.1^{c}	2.3±0.1^{ab}	4.4±0.6^{cd}	1.5±0.1^{a}	5.5±0.5^{e}	4.1±0.6^{d}	2.3±0.1^{ab}
Total (n-6) PUFA	11.1±0.3^{a}	12.3±0.5^{a}	14.0±1.1^{ab}	14.3±0.1^{ab}	12.1±0.4^{a}	13.7±1.4^{a}	11.6±0.6^{a}	16.0±0.4^{b}	14.1±0.9^{ab}	11.9±0.5^{a}
C20:5 (n-3) EPA	6.8±0.1^{b}	5.7±0.2^{b}	5.6±0.3^{b}	5.5±0.1^{b}	3.3±0.1^{a}	2.2±0.4^{a}	6.4±0.3^{b}	2.5±0.4^{a}	6.0±0.8^{b}	6.7±0.4^{b}
C22:6 (n-3) DHA	5.6±0.2^{ab}	4.7±0.5^{ab}	4.1±0.4^{ab}	3.4±0.1^{a}	4.2±0.4^{ab}	3.3±0.9^{a}	4.9±0.2^{ab}	3.9±0.1^{ab}	5.0±1.7^{ab}	6.0±0.3^{b}
Total (n-3) PUFA	37.9±0.9^{bc}	32.7±0.2^{bc}	33.4±0.2^{bc}	32.7±1.1^{bc}	30.9±2.2^{abc}	24.7±2.7^{a}	35.1±2.0^{c}	28.3±0.5^{ab}	33.4±3.3^{bc}	35.3±3.0^{c}
DHA:EPA	0.8±0.0^{ab}	0.8±0.1^{ab}	0.7±0.0^{a}	0.6±0.0^{a}	1.3±0.1^{bc}	1.5±0.4^{bc}	0.8±0.0^{a}	1.5±0.2^{c}	0.8±0.2^{a}	0.9±0.0^{ab}
ARA:DHA	0.3±0.0^{a}	0.5±0.0^{ab}	0.9±0.0^{bc}	1.3±0.1^{cd}	0.6±0.0^{ab}	1.4±0.3^{d}	0.3±0.0^{a}	1.4±0.1^{d}	0.9±0.2^{bc}	0.4±0.0^{a}
ARA:EPA	0.2±0.0^{a}	0.4±0.0^{a}	0.7±0.0^{b}	0.8±0.0^{b}	0.7±0.0^{b}	2.0±0.1^{c}	0.2±0.0^{a}	2.2±0.1^{c}	0.7±0.0^{b}	0.3±0.0^{a}
(n-3):(n-6)	3.4±0.1^{d}	2.7±0.1^{bc}	2.4±0.2^{b}	2.3±0.1^{b}	2.5±0.1^{b}	1.8±0.1^{a}	3.0±0.0^{d}	1.8±0.0^{a}	2.4±0.1^{b}	3.0±0.1^{cd}

Table 2- continued

	Time interval for dietary manipulation (dah)									
	11 to 21							3 to 21		
	ARA supplementation									
	0	8	16	24	8	24	24 (dah 16-21)	24	16	24 (dah 3-10)
	EPA supplementation									
	20	20	20	20	0	0	20 (dah 11-15)	0	20	20 (dah 11-21)
	Dah 22									
	n=2				n=2					
TFA	182.4±63.4	160.1±39.9	189.3±61.5	189.2±61.6	241.4±8.0	206.9±98.6	227.3±53.3	236.2±4.3	228.8±129.6	187.1±36.1
FA (% TFA)*										
Total SFA	22.9±0.1	22.9±0.3	22.5±1.2	22.6±0.7	23.7±0.7	23.5±0.3	23.0±0.7	22.0±0.1	23.0±0.1	23.0±0.4
Total MUFA	25.2±0.5^{bc}	24.9±0.3^{bc}	23.3±0.2^{a}	23.0±0.0^{a}	25.8±0.2^{c}	25.5±0.5^{c}	25.8±0.2^{c}	25.7±0.6^{c}	24.1±0.6^{ab}	25.6±0.2^{c}
C20:4 (n-6) ARA	1.8±0.0^{a}	3.5±0.1^{b}	5.6±0.1^{c}	7.1±0.1^{d}	3.6±0.1^{b}	7.3±0.6^{d}	5.7±0.1^{c}	8.2±0.5^{e}	5.4±0.2^{c}	2.1±0.1^{a}
Total (n-6) PUFA	12.0±0.1^{a}	14.4±0.1^{b}	17.0±0.2^{c}	18.3±0.1^{d}	15.3±0.1^{b}	19.6±0.4^{e}	18.0±0.3^{d}	21.2±0.6^{f}	16.8±0.3^{c}	12.6±0.3^{a}
C20:5 (n-3) EPA	9.4±0.1^{d}	8.0±0.3^{c}	7.9±0.2^{c}	7.7±0.3^{c}	4.5±0.2^{b}	3.5±0.2^{a}	4.8±0.5^{b}	3.6±0.1^{a}	7.3±0.2^{c}	8.8±0.4^{d}
C22:6 (n-3) DHA	5.9±0.5^{c}	5.4±0.4^{abc}	5.4±0.4^{abc}	4.7±0.0^{a}	6.0±0.1^{bc}	4.6±0.5^{a}	5.0±0.6^{ab}	5.4±0.3^{abc}	5.0±0.2^{ab}	6.3±0.3^{c}
Total (n-3) PUFA	40.0±0.4^{e}	37.8±0.1^{d}	37.2±1.1^{d}	36.1±0.8^{c}	35.1±1.0^{c}	31.4±0.4^{ab}	33.2±0.8^{b}	31.2±0.4^{a}	36.1±0.4^{cd}	38.8±0.4^{de}
DHA:EPA	0.6±0.0^{a}	0.7±0.0^{a}	0.7±0.1^{a}	0.6±0.0^{a}	1.3±0.0^{c}	1.3±0.1^{c}	1.0±0.1^{b}	1.5±0.1^{d}	0.7±0.0^{a}	0.7±0.0^{a}
ARA:DHA	0.3±0.0^{a}	0.6±0.0^{b}	1.0±0.1^{c}	1.5±0.0^{d}	0.6±0.0^{b}	1.6±0.0^{d}	1.1±0.1^{c}	1.5±0.1^{d}	1.1±0.0^{c}	0.3±0.0^{a}
ARA:EPA	0.2±0.0^{a}	0.4±0.0^{a}	0.7±0.0^{b}	0.9±0.1^{b}	0.8±0.1^{b}	2.1±0.0^{d}	1.2±0.1^{c}	2.3±0.1^{d}	0.7±0.0^{b}	0.2±0.1^{a}
(n-3):(n-6)	3.3±0.0^{f}	2.6±0.0^{d}	2.2±0.0^{c}	2.0±0.1^{b}	2.3±0.1^{c}	1.6±0.0^{a}	1.8±0.1^{b}	1.5±0.1^{a}	2.1±0.0^{c}	3.1±0.1^{e}

3.3 Larval survival and growth performance

Initial larval mortality was elevated until dah 5, after which the mortality abated rapidly. Survival was not significantly different between any of the groups ($P > 0.8$) (Table 3).

Table 3.

Larval survival (%), individual dry weight (d.w. mg ind. $^{-1}$), total length (mm ind. $^{-1}$) and length growth calculated as specific growth rate (SGR) until dah 21 as a mean ± standard deviation for all treatments

A different superscript in a horizontal row denotes a significant difference $P<0.05$. No value indicates no significance ($P>0.05$)

	Time interval for dietary manipulation (dah)									
	11 to 21							3 to 21		
	ARA supplementation									
	0	8	16	24	8	24	24 (dah 16-21)	24	16	24 (dah 3-10)
	EPA supplementation									
	20	20	20	20	0	0	20 (dah 11-15)	0	20	20 (dah 11-21)
Survival dah 3-21	23.5 ± 18.6	20.5 ± 4.0	21.2 ± 5.9	22.4 ± 7.8	26.1 ± 0.5	23.4 ± 5.4	28.0 ± 6.4	31.4 ± 6.2	28.7 ± 4.4	24.1 ± 7.7
d.w. (dah 11)	0.46 ±0.0^{ab}	0.29±0.10^{ab}	0.51±0.12^{b}	0.39±0.16^{ab}	0.35±0.06^{ab}	0.29±0.05^{a}	0.28±0.09^{a}	0.33±0.01^{ab}	0.31±0.04^{ab}	0.38±0.05^{ab}
d.w. (dah 16)	0.82± 0.09	0.57±0.15	0.81±0.10	0.90±0.29	0.77±0.19	0.64±0.09	0.66±0.14	0.77±0.10	0.80±0.06	0.79±0.15
d.w. (dah 21)	1.80±0.66	1.95±0.48	1.89±0.27	1.94±0.54	1.28±0.1	1.66±0.33	1.49±0.16	2.02±0.37	1.84±0.16	1.89±0.24
length (dah 11)	6.32±0.57^{a}	6.22±0.52^{a}	6.86±0.45^{b}	6.51±0.52^{ab}	6.47±0.43^{ab}	6.42±0.44^{a}	6.36±0.60^{a}	6.43±0.49^{a}	6.37±0.50^{a}	6.44±0.63^{ab}
length (dah 16)	8.06±0.89	7.65±0.78	8.01±0.78	8.25±0.78	7.91±0.87	7.53±0.73	7.58±0.84	8.16±0.91	8.32±0.77	7.89±0.80
length (dah 21)	10.25±1.52	10.40±1.23	10.62±1.15	10.85±1.53	9.87±1.17	10.24±1.25	9.99±1.17	10.69±1.24	11.12±1.46	10.49±1.64
SGR (length) (dah 3-21)	5.13±0.5	5.21±0.5	5.35±0.2	5.47±0.3	4.92±0.1	5.13±0.4	4.99±0.4	5.39±0.2	5.64±0.2	5.28±0.1

Specific Growth Rate calculated as the increase in larval length from dah 3-21 did not reveal any significant differences between treatments ($P = 0.241$). Specific growth rate was higher during pre metamorphosis (6.5 percent d^{-1}), than during metamorphosis (4.5 percent d^{-1}) calculated as a mean for all groups.

3.4 Eye migration

In all groups initiation of eye migration (S1) was significantly positively correlated with increases in larval standard length. Hence, at dah 11, eye migration (S1) was initiated in 4 to 10 % of all the analysed larvae in all groups, at a mean standard length of 7.13 ± 0.33 mm ind^{-1}, while larvae in a stage of non initiated eye migration (S0) were 6.45 ± 0.17 mm ind.$^{-1}$ ($P<0.001$).

At dah 16, eye migration ($S \geq 1$) was initiated in 66 to 90 % of the sole larvae in all groups not significantly different between groups ($P = 0.24$). Of these a mean of 52 ± 9.5 percent of larvae in all groups ($P = 0.37$) was categorized as S1, (body length 7.89 ± 0.20 mm ind.$^{-1}$). At dah twenty two eye migration was initiated in 96 to100 % of all examined larvae in all groups.

The eye migration score at dah 16 and 22 was not different between treatments (P>0.06), but there was a significant positive interaction between a higher eye score and larval length (P > 0.0001). Similarly, a correlation test significantly correlated eye score to increases in mean larval standard length both at dah 16 (P < 0.001, r^2 =0.62) and at dah 22 (P < 0.001, r^2 =0.46). Dietary treatment did not interact with eye score (P > 0.09). The mean eye score was not correlated to mean tissue ARA or EPA contents (P > 0.1). At dah 22 completed eye translocation (S4) was observed in 43 to 58 % of all individuals in all groups. At dah 22 it was estimated that more than 95 % of all larvae in all groups had settled.

About 90 % of all examined juveniles which were characterised by an abnormal incomplete position of the migrating eye were albinos, but the trait was not significantly related to dietary treatment (P ≥ 0.07). Highly malpigmented individuals, within the subgroup of larvae with a malpigmented body, made up the remaining 10 %. Normal pigmented larvae, - or larvae with malpigmented tails did not show this characteristic.

3.5 Juvenile pigmentation

Malpigmentation was significantly highest for the larval groups fed *Artemia* enriched with 24 % ARA, - or 16 % ARA and 20 % EPA during dah 3-21 (table 4) (P < 0.001). Malpigmentation was significantly different in larval groups fed *Artemia* enriched with 24 % ARA during different stages of larval ontogeny (P ≤ 0.015); Of these the highest malpigmentation was observed in larvae fed *Artemia* enriched by 24 % ARA during pre metamorphosis at dah 3-10, - then followed larvae fed *Artemia* enriched by 24 % ARA during metamorphosis, at dah 11-21, and finally the lowest malpigmentation in larvae fed *Artemia* enriched by 24 % ARA at dah 16-21.

Malpigmentation was significantly different in larval groups fed *Artemia* enriched by EPA and either 0, - 2, - 4 or 8 % ARA content during dah 11 -21 (P < 0.001). It was significantly related to an increase in ARA concentration in both diets and tissue (P ≤ 0.002, r^2 ≥ 0.92). .

Malpigmentation was relatively low and similar in the larval groups fed *Artemia* enriched with 8 % ARA (tissue EPA: ARA 1.2 at dah 22) or 8 % ARA and 20 % EPA (tissue EPA: ARA 2.3) at dah 11-21 (P = 0.21). Malpigmentation was relatively high and similar in larvae fed *Artemia* enriched with 24 % ARA (tissue EPA: ARA 0.47) or 24 % ARA and 20 % EPA (tissue EPA: ARA 1.1) at dah 11-21 (P = 0.2).

Table 4.

Larval content of PGE_2 (pg mg^{-1}) prostaglandins at dah 22 given as a mean ± standard deviation. n=3

Overall malpigmentation rate (%) and malpigmentation classified into subgroups (% overall malpigmentation rate) at dah 37 given as a mean ± standard deviation. n=3

A different superscript following the values indicate significance (P< 0.05). No value indicates no significant difference.

	Time interval for dietary manipulation (dah)									
	11 to 21							3 to 21		
	ARA supplementation									
	0	8	16	24	8	24	24 (dah 16-21)	24	16	24 (dah 3-10)
	EPA supplementation									
	20	20	20	20	0	0	20 (dah 11-15)	0	20	20 (dah 11-21)
PGE_2 (dah 22)	1553.5 ± 1082.6	1935.4 ± 885.1	1434.1 ± 385.5	2200.8 ± 565.9	1214.2 ± 51.0	3281.1 ± 611.0	2574.6 ± 914.8	2592.1 ± 166.9	3040.6 ± 99.7	916.4 ± 171.4
Overall Malpigmentation	3.0 ± 2.7^{a}	12.7 ± 3.3^{ab}	21.5 ± 5.1^{c}	28.1 ± 2.0^{cd}	9.6 ± 1.5^{ab}	37.3 ± 10.4^{d}	16.7 ± 4.9^{b}	90.4 ± 1.3^{f}	92.1 ± 1.5^{f}	57.0 ± 4.1^{e}
Albinos	1.9 ± 1.7^{ab}	3.9 ± 3.6^{ab}	4.8 ± 1.3^{ab}	9.1 ± 2.5^{b}	3.3 ± 2.1^{ab}	18.9± 7.9^{bc}	3.5± 3.5	42.9± 8.1^{d}	44.4± 3.4^{d}	23.3± 3.5^{c}
Malpigment. body	0.9 ± 0.8^{a}	5.9 ± 2.7^{ab}	13.1± 4.7^{abc}	16.3± 4.0^{bc}	5.4 ± 1.1^{ab}	15.5± 7.5^{bc}	8.6± 5.1^{abc}	40.4± 6.0^{d}	41.1± 5.0^{d}	20.8± 5.8^{c}
Malpigment. tails	0.2 ± 0.3^{a}	3.0 ± 1.6^{ab}	3.6± 2.6^{ab}	2.6± 2.3^{ab}	0.9 ± 0.8^{a}	2.9± 0.6^{ab}	4.6± 3.5^{ab}	7.2± 1.1^{b}	6.7± 2.4^{b}	14.9± 1.1^{c}
Hyperpigment	1.9 ± 2.0	1.5 ± 1.9	0.0 ± 0.0	0.8 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	1.3± 1.2	0.2 ± 0.4	0.2 ± 0.3	2.0 ± 2.9

The percentage of juveniles defined as “albinos” and the percentage of juveniles with body, - or tail malpigmentation were significantly different between groups ($P < 0.001$), but the relative frequency of each subgroup in percent of malpigmented juveniles was not different between treatments ($P = 0.17$-0.42).

3.6 Prostaglandins (PGE_2)

An insignificant trend was that mean larval PGE_2 content at dah 22 was highest in larvae fed *Artemia* enriched with 24 % ARA (table 4) ($P = 0.26$). However, this trend was not seen at dah 11 for the larval group fed *Artemia* enriched with 24 % ARA at dah 3-10 changing to *Artemia* enriched with 20 % EPA at dah 11-21. Hence, in this group the PGE_2 concentrations were 1135.5 + 343.8 pg mg $^{-1}$ at dah 11 and 916.4 + 171.4 pg mg $^{-1}$ at dah 22 ($P = 0.51$).

A significant treatment related correlation was observed between percent mean larval tissue ARA content at dah 22 and the mean tissue PGE_2 content, but no such correlation between EPA and PGE_2 (Fig.1a, b). Moreover, in comparable groups, juvenile malpigmentation and larval tissue PGE_2 contents at dah 22 were significantly related (Fig.2).

Figure 1 a-b.

Mean prostaglandin PGE_2 larval tissue content pg mg $^{-1}$ at dah 22 as a function of the relative larval ARA (a) and EPA tissue content (b) (% TFA).

a) *b)*

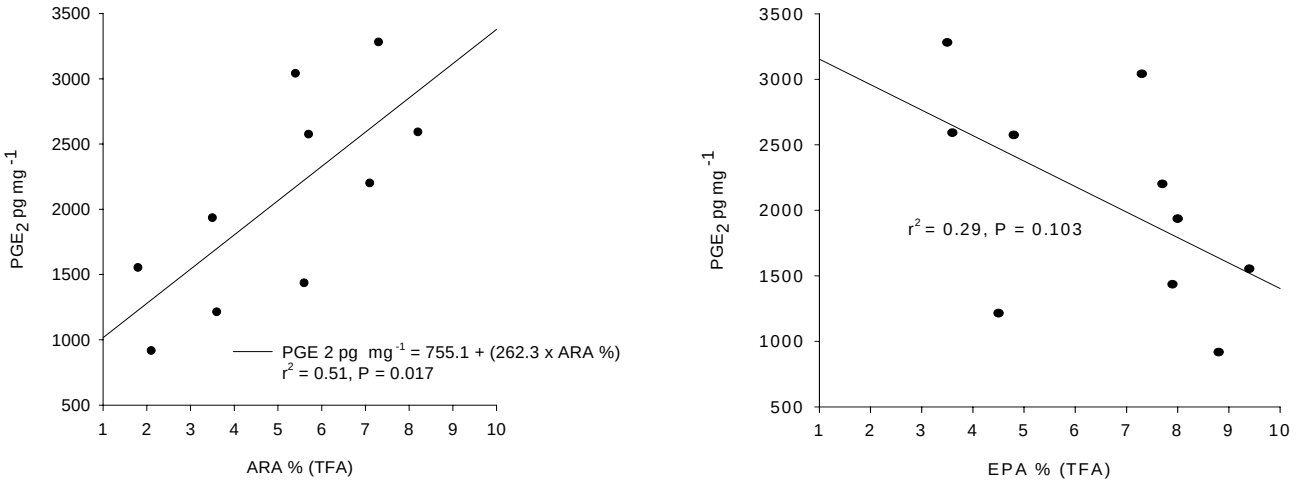
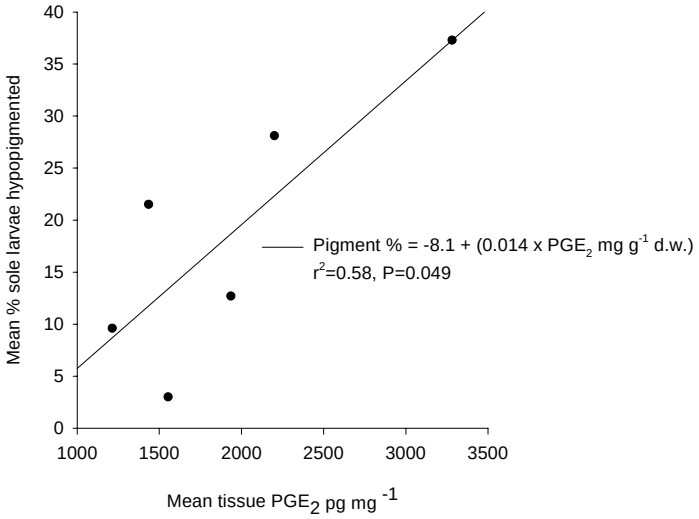


Figure 2.

Juvenile malpigmentation (hypopigmentation) illustrated as a function of prostaglandin, PGE_2 tissue concentration in larvae at dah 22 for comparable groups of sole fed enriched Artemia at dah 11-21.



4. Discussion

Larval fatty acid profiles of fatty acids were reflected in the dietary profiles as observed in previous studies (Mourente et al., 1993; Ghioni et al., 1997; Koven et al., 2003; Bransden et al., 2005; Villalta et al., 2005a, b; Lund et al., 2007). Shifts of diets showed that FA profiles were altered in favour of the new dietary composition. A dietary treatment for 5-6 days made larval FA profiles resemble larvae fed a similar diet throughout the study. The fast fatty acid turn -over, was most likely related to the relative fast growth rate.

The initial larval mortality observed may partly be caused by the long transportation of larvae (10 hrs) and acclimatization to the new rearing system. Elevated dietary ARA has been found to increase overall survival in gilthead seabream larvae (*Sparus aurata*) (Bessonart et al., 1999), but this was not observed in the present study.

A high inclusion of ARA, did not affect larval growth rate. This is consistent with findings in other species (Willey et al., 2003; Bransden et al., 2005).

The growth rate calculated as increase in d.w was lower (16-19 % d^{-1}) than previously observed with common sole larvae, (20-27 % d^{-1}) (Lund et al., 2007), which may reflect strain related differences.

Growth rate was higher during pre metamorphosis at dah 3-10 than during metamorphosis at dah 11-21. This supports similar findings for senegalese sole (Parra, 1998). The growth rate, however, was not different within defined periods of early and late metamorphosis, dah 11-15 and dah 16-21 respectively. Hence, common sole larvae may not diminish feed intake in relation to physiological processes during eye migration and metamorphosis.

Initiation or advance of eye migration was strongly dependent on the body length of the sole larvae ($P<0.001$) -and not influenced by dietary or tissue related differences of ARA and EPA. In some specimens, eye migration was initiated (S1) 11 days after hatching at a body length of 7.13 mm, much less than 8.5 mm reported by Amara on common sole (Amara and Galois, 2004). However, the mean larval size of larvae categorized as S1 was 7.89 mm at dah 16.

A high EPA: ARA tissue ratio of 5 had no positive effect on the advance of eye migration, neither did a relative high ARA: EPA tissue ratio of 2 have a negative effect. Contrary to this, in studies on senegalese sole increasing dietary levels of ARA as well as DHA slowed the degree of eye migration at 15 and 20 days after hatching (Villalta et al., 2005a, b). Moreover in yellow tail flounder (*Limanda ferruginea*), a diet high in DHA and EPA but low in ARA improved eye migration as compared to a diet high in DHA only (Copeman et al., 2002). Contrary to findings in studies on senegalese sole (Villalta et al., 2005a, b), the statistical contingency analyses indicated that eye migration (eye score) in common sole was a process not linked to the dietary EFAs, but highly related to the body length. Caution should be taken not to confuse a possible linkage with minor growth effects of EFAs as due to the highly significant correlation between eye score and larval length.

An estimated more than 95 % larval settlement at dah 22 did not indicate complete end of metamorphosis as only approximately 50 % of all larvae examined were scored as having completed eye migration (i.e. 81 % incl. S3). Benthic settlement behaviour is probably influenced by the morphological transformation from a bilaterally symmetrical to an asymmetrical body shape (Inui et al., 1995), which appears at eye position 2-3 according to the index presented by Fernandez Diaz et al.(2001). Eye migration rather than progress of settlement should therefore characterise the metamorphosis.

A permanent and abnormal eye migration in juveniles was correlated with “albinism” or with a high degree of malpigmentation as observed in a previous study by Lund (Lund et al., 2007). In the present study, this seemed to be caused by an incomplete metamorphosis in highly malpigmented individuals rather than deleterious effects caused by ARA, as 85 % of the albinos (only few) also had this characteristic when not exposed to supplemented ARA. This is consistent with previous observations (Lund et al., 2007). Based on the present results eye migration is suggested similar until final transformation (S3) and not related to ARA or EPA. The observed differences at juvenility may be related to disturbances in the final eye transformation rather than related to the speed of eye migration. The conclusion is, that ARA or its derivatives induce hypomelanosis, but

the trait “albinism” itself is characterised by independent morphological and physiological appearances in sole.

Sole larvae fed increasing dietary ARA levels until 21 days after hatching caused increased hypomelanosis in juveniles at dah 33 as previously observed (Lund et al., 2007). It was hypothesised, that supplementation of EPA, i.e an increased ratio of EPA: ARA may negate the negative effect of exogenous ARA on malpigmentation, (Whelan, 1996; McEvoy et al., 1998). However, malpigmentation was similar in comparable larval groups with a tissue EPA: ARA of 2.2 or 1.1, or in groups with tissue EPA: ARA of 1.1 or 0.42

Previous findings on Japanese flounder (Estévez et al., 2001; Bell et al., 2003), as well as halibut, turbot, yellow tail flounder and senegal sole have revealed evidence that increasing ratios of ARA: EPA increased malpigmentation dramatically (Hamre et al., 2005, review). Based on the present study and previous observations (Lund et al, 2007), it is most likely the actual concentration of dietary ARA combined with larval stage of metamorphosis that is responsible for malpigmentation, so the ARA: EPA may be misleading.

A high juvenile malpigmentation of 59 ± 4 % was observed for larvae fed *Artemia* enriched by 24 % ARA during pre metamorphosis at dah 3-10. Malpigmentation was significantly related to the relatively high ARA tissue content at premetamorphosis, dah 11, and not to the much lower relative contents at dah 16 and dah 22. Metamorphosis was initiated in only 6.5 % of the larvae in this group at dah 11 when the diet changed and the larvae were fed *Artemia* enriched with 20 % EPA. At dah 21, larval ARA content was similar to larvae fed *Artemia* enriched with 20 % EPA.

In comparison only 17 % of the individuals were malpigmented in larvae fed *Artemia* enriched with 24 % ARA at late metamorphosis from dah 16. This in spite that 36 % of the larvae were categorized as having not initiated metamorphosis and 86 % was scored as either stage 0 or 1. The duration of exposure, of both groups to *Artemia* enriched with 24 % ARA, was almost similar, 7 (5) and 5 days respectively; - (5) when taken into consideration that larval intake at dah 4-5 is very limited (Lund et al, unpublished data). Larval sensitivity to ARA or its derivatives is therefore considered much higher during pre metamorphosis than during metamorphosis. In support of a pre metamorphic “pigmentation window”, larvae fed diets high in ARA (i.e. 16 and 24 % ARA) at dah 3-21 were almost all malpigmented (> 90 %) contrary to larvae fed similar diets from dah 11 (i.e 21-37 % malpigmented).

For larval Japanese flounder and halibut, it is hypothesized that a sensitive pigmentation period is only a few days just before metamorphosis. Based on these studies, therefore an even higher

frequency of malpigmentation might have been expected for larvae treated with ARA during pre metamorphosis and an even lower frequency of malpigmentation for larvae fed ARA during metamorphosis in the present study. However, individual variations related to larval size, feed intake and ARA tissue uptake may be the explanation.

In a previous study, growth was significantly reduced when larvae were fed inadequate diets of rotifers or *Tisbe* (*Tisbe holothuriae*) in comparison to larvae fed *Artemia* during pre metamorphosis at dah 3-9, but pigmentation was normal in more than 95 % of the individuals when later evaluated (Lund et al., unpublished data). Besides possible differences in batch related pigmentation sensitivity, those results may indicate that pigmentation is not directly related to the physical condition of the larvae, but instead may be under hormonal and neural control as suggested by Estévez (1996). In support of this hypothesis, significant correlations were observed between an increase in tissue content of ARA, an increase in malpigmentation and increasing PGE_2 tissue contents (Fig 1a, Fig.2.) in the present study. This has not been reported previously for common sole. ARA and its hormonal derivatives may therefore play a major role in the pigmentation process.

DHA, which is considered important for the normal neural development and function (Bell and Tocher, 1989), however, seem to have no effect on sole pigmentation (Lund et al., 2007). EPA and ARA derived eicosanoids are potent regulators of metabolism (Hamre et al.,2005) and as PGE_2 and PGE_3- series have opposite effects (Bell et al., 2003) it is important to have a balance in the synthesis of the 2- series, which is believed to be dependent on a balanced dietary intake of both fatty acids (Hamre et al., 2005). Previous findings in common sole larvae have reported that low dietary n-3 content and therefore a higher ratio of ARA: EPA significantly increased total PGE_{2-3} production relatively to a high ratio of n-3: n-6 (Logue et al, 2000). In the current study, however, dietary EPA apparently did not cause a modification of PGE_2 tissue levels and its opposite effect in the synthesis of the PGE_2-series in common sole is therefore questioned.

Recently, Villalta et al.(2007) observed a positive correlation between malpigmentation and prostaglandin production in Senegal sole. The biochemical mechanisms linking eicosanoids and pigmentation has not been substantiated (Bransden et al., 2005), but prostanoids produced by ARA may modify the subsequent production of tyrosinase, which is one of the initial enzymes involved in the complex pathway from L- tyrosine to melanin (Bransden et al.,2005).

The exact mechanisms of colour anomaly in relation to internal development processes are unknown (Planas and Cunha, 1999; Bolker and Hill, 2000) and further studies on common sole

larvae may focus on the pre metamorphic importance of ARA and its hormonal derivatives in relation to light intensity and retinal and pigment cell formation.

5. Conclusion

The study revealed that ARA induced malpigmention in common sole larvae, and sensitivity was highest during pre metamorphosis. Hence, confirming the hypothesis of the presence of an early pigmentation window. The initiation or advance of eye migration is not related to dietary ARA, EPA, but is related to the size of the individuals. An abnormal incomplete eye migration is a trait related to highly malpigmented sole individuals. It indicates aberrations in final eye translocation (S4) more than relations to the progress of eye migration. Increased concentrations of prostaglandins of the 2- series may be related to an increased tissue content of ARA and seem to be involved in malpigmentation. EPA had no effect on sole larval pigmentation or PGE_2 concentrations.

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