

The effect of dietary *n*-3 highly unsaturated fatty acids on growth, survival and biochemical composition of the coral reef damselfish *Acanthochromis polyacanthus*

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Abstract – Larvae of the coral reef damselfish *Acanthochromis polyacanthus* (Bleeker) were fed either unenriched *Artemia* nauplii or nauplii which had been enriched by pre-feeding with microcapsules containing either squid oil (SQO) or cod liver oil (CLO). Enriched nauplii contained elevated levels of the *n*-3 highly unsaturated fatty acids (HUFA) eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3) which made up 5.22 ± 0.34 and 2.62 ± 0.28 %, respectively, of total fatty acids in nauplii enriched with CLO, and 10.48 ± 0.36 and 3.43 ± 0.33 %, respectively, in nauplii enriched with SQO. In contrast, unenriched nauplii contained EPA (5.03 ± 1.04 %) but did not contain DHA. Survival differed significantly between treatments over the 18-d study; larvae receiving CLO enriched nauplii showed 100 % survival and those receiving SQO enriched nauplii showed 93.3 ± 6.6 % survival. In contrast, only 46 ± 6.7 % of larvae receiving unenriched nauplii survived to the end of the 18-d study. Wet weight, dry weight and proximate biochemical composition did not differ significantly between treatments at the end of the study. Mean standard length of larvae fed CLO enriched nauplii was significantly smaller than that of larvae fed SQO enriched nauplii; however, neither differed significantly from larvae fed unenriched nauplii. The fatty acid composition of *A. polyacanthus* larvae was significantly influenced by the fatty acid composition of the diet. The results indicate that *A. polyacanthus* larvae are unable to synthesise DHA from available dietary precursors and, as such, dietary DHA is required to maximise survival. Development of appropriate culture techniques for the larvae of coral reef fishes will allow controlled laboratory studies with these species and may eventually reduce pressure on wild populations exploited for the aquarium trade. © Ifremer/Elsevier, Paris

Damselfish / fatty acids / growth / larvae / survival / *Acanthochromis polyacanthus*

Résumé – Effets des acides gras polyinsaturés *n*-3 sur la croissance, la survie et la composition biochimique du poisson corallien *Acanthochromis polyacanthus*. Les larves du poisson corallien *Acanthochromis polyacanthus* ont été nourries soit de nauplii d'*Artemia* ou de nauplii qui ont été préalablement enrichies par l'alimentation de microcapsules contenant de l'huile de calmar (SQO) ou d'huile de foie de morue (CLO). Les « nauplii enrichies » contiennent des doses élevées d'acides gras insaturés *n*-3 (HUFA), d'acide eicosapenténoïque (EPA ; 20:5*n*-3) et d'acide docosahexénoïque (DHA ; 22:6*n*-3) qui représentent 5,22 ± 0,34 et 2,62 ± 0,28 %, respectivement, des acides gras totaux chez les nauplii non-enrichies avec CLO, et 10,48 ± 0,36 et 3,43 ± 0,33 %, respectivement, chez les nauplii enrichies avec SQO. En revanche, des nauplii enrichies contiennent de l'EPA (5,03 ± 1,04 %), mais ne contenaient pas de DHA. Le taux de survie diffère de façon significative entre les traitements après 18-j d'étude ; des larves recevant des nauplii enrichies en CLO ont 100 % de survie tandis que celles recevant des nauplii enrichies en SQO ont 93,3 ± 6,6 % de survie. Cependant, seuls 46 ± 6,7 % des larves recevant des nauplii non-enrichies ont survécu aux 18 j d'expérience. Le poids humide, le poids sec et la composition biochimique sont similaires entre les traitements à la fin de l'étude. La longueur standard moyenne des larves nourries de nauplii enrichies de CLO est significativement plus petite que chez celles nourries de nauplii enrichies de SQO ; cependant, aucune ne diffère significativement des larves nourries de nauplii non-enrichies. La composition d'acides gras des larves de *Acanthochromis polyacanthus* est influencée par celle de l'aliment. Les résultats indiquent que les larves de *Acanthochromis polyacanthus* ne sont pas capables de synthétiser le DHA à partir des précurseurs alimentaires disponibles, et ainsi le DHA dans l'aliment est nécessaire pour obtenir un maximum de survie. Le développement de ces techniques de culture appropriées aux larves de ce poisson corallien permettra des études contrôlées en laboratoire sur cette espèce et pourrait réduire la pression sur les populations sauvages exploitées et destinées aux aquariophiles. © Ifremer/Elsevier, Paris

Poisson corallien / acides gras / croissance / larves / survie / *Acanthochromis polyacanthus*

1. INTRODUCTION

Pressures on wild populations of coral reef fishes are increasing in some areas where heavy collecting occurs for the saltwater aquarium or restaurant trade [27, 34, 40]. This pressure has resulted in an increased interest in the development of culture techniques for these species [6, 42]. In addition to conservation and commercial benefits, production of hatchery-reared larvae would also benefit laboratory research into species-specific behaviour and ontogeny. The availability of known-age and/or live coral reef fish larvae currently restricts research into the important basic biology of these species because field-collected larvae are difficult to identify to species level, almost always of unknown age [20], and often in poor health. However, few species of coral reef fishes have been reared on a commercial scale and little is known of their culture requirements [41, 42]. The available literature suggests that the major constraint to rearing the larvae of these species is provision of an adequate live food source [41]. Most previous studies have used wild-caught zooplankton as a food source [6, 41], which is costly, labour intensive and restricted to facilities near the ocean. Therefore, a major factor in developing efficient culture methods for larvae of coral reef fishes will be the use of more readily available food sources. The major food source currently used for late-stage larvae of commercially-cultured fish species is brine shrimp, *Artemia* sp. [19].

The Indo-Pacific damselfish, *Acanthochromis polyacanthus* (Bleeker), is a common inhabitant of coral reefs [28]. It has an unusually long embryonic stage (10–18 d), and relatively large, well-developed hatchlings (5 mm) [15]. Because of these characteristics, *A. polyacanthus* was thought to be an excellent candidate for laboratory rearing. However, preliminary attempts to rear larvae of this species in the laboratory on a diet of newly-hatched *Artemia* nauplii were unsuccessful and resulted in heavy mortality approximately 10–30 d after hatching [15]. Although *A. polyacanthus* larvae readily ingested *Artemia* nauplii, larvae reared under these conditions showed an increased susceptibility to stress agents resulting in a shock response, or 'fainting', and subsequent mortality. This symptom has been recorded for the larvae of other species and has been associated with deficiencies in dietary *n*-3 highly unsaturated fatty acids (HUFA) [14, 24, 30].

It is now well established that the larvae of commonly cultured fish species have a dietary requirement for *n*-3 HUFA and a number of recent studies have shown eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3) to be essential dietary components [21, 29, 38]. These essential fatty acids have a critical role as membrane constituents and are particularly important in early developmental stages [26]; as such, they must be supplied in the diet to ensure optimal growth and survival. Many strains of *Artemia* are deficient in *n*-3 HUFA and therefore do

not provide adequate nutrition for marine fish larvae [37, 38]. To overcome this deficiency, *Artemia* nauplii can be 'enriched' by allowing them to ingest material rich in *n*-3 HUFA prior to feeding to the marine fish larvae [19]. A number of materials have been used successfully as HUFA enrichment diets including microalgae, HUFA-modified yeasts, compound diets, coated microparticles, oil-based emulsions and microencapsulated preparations [19, 32].

Many studies have reported on the HUFA requirements of marine fish larvae; however, the majority have focused on temperate species which are commercially cultured. In contrast, little is known about larval nutrition of tropical coral reef species and no prior study has reported on their HUFA requirements. The aims of this study were: (1) to establish whether *A. polyacanthus* larvae have a dietary requirement for *n*-3 HUFA; and (2) to assess the effect of dietary HUFA from various sources (cod liver oil or squid oil) on larval growth and survival.

2. MATERIALS AND METHODS

2.1. Microcapsule preparation

Gelatin-acacia microcapsules (GAM) were prepared as previously described [32]. Briefly, 2% (w/v) solutions of acacia (BDH) and gelatin (Merck) were made up in distilled water at 40 °C. Equal volumes (100 mL) of each solution were mixed and maintained at 40 °C. Cod-liver oil (CLO) or squid oil (SQO) (2.5 mL) was added to the gelatin-acacia solution (200 mL) and the mixture homogenised using a four-blade blender at maximum speed for 70 s. The resulting homogenate was poured into a glass beaker and stirred at 40 °C; the pH was then reduced to 3.7 by drop-wise addition of dilute HCl. Stirring was continued for a further 5 min before the pH was increased to 9.3 by drop-wise addition of 0.5 M NaOH solution. Stirring was continued for a further 5-min and the resulting microcapsule suspension was poured into an equal volume of cold (5 °C) distilled water and placed into a refrigerator for 2 h.

Microcapsules were washed free of gelatin-acacia solution and concentrated by repeated centrifugation and resuspension in distilled water. The dry weight of microcapsules per unit volume of stock suspension was determined by drying triplicate 250- μ L samples of the stock suspension at 50 °C. Stock suspensions of microcapsules were stored at 4 °C for the duration of the study and fresh batches of microcapsules were made weekly. Microcapsules prepared in this way had a mean diameter of 5.3 (\pm 1.2) μ m; previous research has shown GAM to contain approximately 93% oil [32].

2.2. Enrichment of *Artemia* nauplii

The *Artemia* used in this study originated from Great Salt Lake, Utah (Olympic Aqua Product).

Artemia cysts were first decapsulated [31] and incubated in vigorously aerated water for 36 h. The resulting nauplii were washed with seawater and poured into conical, vigorously aerated, plastic vessels at a density of approximately 220 per mL. Nauplii were either unfed or enriched with microcapsules containing either CLO or SQO. For enrichment, microcapsules were added to the vessels at a concentration of 1.3 g·L⁻¹. After 1 h, nauplii were retained on a nylon mesh, washed free of culture medium with 1 µm filtered seawater (FSW) and fed to *A. polyacanthus* larvae.

2.3. Larval rearing

Captive breeding pairs of *A. polyacanthus* were maintained in aquaria with a flow-through water supply, at James Cook University, where clutches of eggs (250–550) were attached to substrates provided in the aquaria. Eggs hatched approximately 15 d after fertilisation and newly hatched larvae had a standard length of approximately 5.7 mm [15]. Ten 2-d-old unfed larvae (mean standard length 6.0 ± 0.1 mm) were randomly assigned to each of nine aquaria containing 6 L of lightly-aerated FSW (35‰). Water temperature was maintained at 25 °C and 50 % of the water from each vessel was replaced every 3 d. Each of three replicate aquaria received either unenriched *Artemia* nauplii or nauplii enriched with either CLO or SQO. Nauplii were fed at a density of 5 mL⁻¹.

The experiment was terminated after 18 d when mortality in the treatment receiving unenriched *Artemia* exceeded 50 %. Remaining larvae were isolated on nylon mesh, washed first with FSW and then 0.9 % (w/v) ammonium formate to remove seawater [39]. Larvae from each treatment were measured (standard length) and weighed to determine wet weight. Larvae were then freeze-dried and reweighed to determine dry weight. Data were compared using ANOVA and significant differences between treatment means were determined using the Tukey test [43]. Larval survival data were arc sine transformed prior to statistical analysis and homoscedasticity was confirmed using Bartlett's test [43].

2.4. Biochemical analyses

Dried larvae were homogenised in distilled water and aliquots of the homogenate were taken for determination of lipid and carbohydrate content. Lipid was extracted with chloroform:methanol [11], charred with sulphuric acid and assayed colorimetrically using tripalmitin as the standard [22]. Carbohydrate was determined by the phenol-sulphuric acid method [10] using D-glucose as the standard. The nitrogen content of dried and powdered larvae was determined colorimetrically [2] by the salicylate-hypochlorite method following Kjeldahl digestion; protein content was estimated as N × 6.25 [4]. All analyses were conducted in triplicate and data were analysed as detailed above.

For fatty acid analysis, *Artemia* nauplii were washed free of culture medium and directly methylated for

fatty acid analysis [9]. Freeze-dried larvae from each treatment were powdered and prepared for fatty acid analysis in the same way. Fatty acid methyl esters (FAME) were separated using a Shimadzu GC 17A gas chromatograph and individual fatty acids were identified by comparing to FAME reference standards (Sigma) [32]. All fatty acid analyses were conducted in triplicate.

3. RESULTS

The fatty acid compositions of unenriched *Artemia*, and those enriched with GAM containing either CLO or SQO are shown in table I. Unenriched nauplii contained 5.03 ± 1.04 % EPA but contained no DHA. Nauplii enriched with microcapsules containing either CLO or SQO contained 5.23 ± 0.34 % and 10.48 ± 0.38 % EPA, respectively and, in contrast to unenriched nauplii, contained DHA at levels of 2.62 ± 0.28 % and 3.42 ± 0.33 % of their total fatty acids, respectively.

Table I. Major fatty acid composition (mean % total fatty acids ± SD, *n* = 3) of unenriched *Artemia* nauplii and those enriched with microcapsules containing either cod liver oil or squid oil. nd = Not detected; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

Fatty acid	Diet		
	Unenriched	Cod liver oil	Squid oil
16:0	10.84 ± 0.11	9.68 ± 0.62	8.91 ± 0.89
16:1 <i>n</i> -7	2.81 ± 0.14	3.75 ± 0.24	2.22 ± 0.51
18:0	6.25 ± 0.13	4.76 ± 0.71	4.72 ± 0.20
18:1 <i>n</i> -9	19.77 ± 0.11	17.89 ± 1.02	16.06 ± 2.13
18:2 <i>n</i> -6	4.89 ± 0.16	3.96 ± 0.04	4.14 ± 0.24
18:3 <i>n</i> -3	22.47 ± 0.52	16.66 ± 0.96	23.61 ± 1.12
18:4 <i>n</i> -3	0.67 ± 0.01	3.77 ± 0.61	1.14 ± 0.06
20:5 <i>n</i> -3 (EPA)	5.03 ± 1.04	5.23 ± 0.34	10.48 ± 0.38
22:6 <i>n</i> -3 (DHA)	nd	2.62 ± 0.28	3.42 ± 0.33
EPA + DHA	5.03	7.85	13.90

Growth of *A. polyacanthus* larvae was rapid and mean wet weight increased from approximately 5.7 mg at the start of the experiment to between 44.4 and 46.5 mg at the end of the study (table II). Wet weight and dry weight did not differ significantly between treatments at the end of the study (*P* > 0.05). However, differences between treatments were detected in standard length (*P* < 0.01). A post hoc Tukey's multiple comparisons test indicated that fish from the CLO treatment were shorter than those from SQO, but neither differed significantly from the unenriched treatment (table II).

Despite apparently normal development, mortality of *A. polyacanthus* larvae receiving unenriched *Artemia* nauplii began after 10 d and survival of these larvae at the end of the 18-d study was significantly lower (*P* < 0.001) than those receiving enriched nauplii (table II). Only 46.7 ± 6.7 % of larvae receiving unenriched nauplii survived to the end of the experiment. In contrast, a mean survival of 100 and

Table II. Mean \pm SE survival, standard length, wet weight and dry weight of *A. polyacanthus* larvae fed unenriched *Artemia* nauplii or nauplii enriched with microcapsules containing either cod liver oil or squid oil at the end of the 18-d feeding trial. Means in columns with the same superscript are not significantly different ($P > 0.05$).

Diet	Survival (%)	Standard length (mm)	Wet weight (mg)	Dry weight (mg)
Unenriched	46.7 ^a \pm 6.7	12.86 ^{ab} \pm 0.01	45.88 ^a \pm 1.50	16.46 ^a \pm 0.48
Cod liver oil	100 ^b	12.79 ^a \pm 0.08	44.43 ^a \pm 0.96	16.03 ^a \pm 0.28
Squid oil	93.3 ^b \pm 6.6	13.06 ^b \pm 0.08	46.46 ^a \pm 1.11	16.63 ^a \pm 0.42

93.3 \pm 6.6 % was shown by larvae fed CLO or SQO enriched nauplii, respectively.

The biochemical composition of *A. polyacanthus* larvae at the end of the study is shown in table III. Protein corresponded to between 57.6 and 60.4 % of the dry weight of *A. polyacanthus* larvae. No significant differences were detected between the protein contents of larvae fed different diets ($P > 0.05$). Lipid composed between 10.9 and 11.5 % of larval dry weight and was similar among treatments ($P > 0.05$). The carbohydrate content of *A. polyacanthus* larvae was low (3.4–3.6 % of dry weight) and did not differ significantly between treatments ($P > 0.05$).

Table III. Proximate biochemical composition (mean \pm SE, $n = 3$) of *A. polyacanthus* larvae at the end of an 18-d study when fed either unenriched *Artemia* nauplii or nauplii enriched with microcapsules containing either cod liver oil or squid oil. Means in columns with the same superscript are not significantly different ($P > 0.05$).

Diet	Biochemical composition (% dry weight)		
	Protein	Lipid	Carbohydrate
Unenriched	57.85 ^a \pm 3.36	11.50 ^a \pm 0.43	3.62 ^a \pm 0.23
Cod liver oil	60.41 ^a \pm 4.05	11.44 ^a \pm 0.85	3.43 ^a \pm 0.29
Squid oil	57.65 ^a \pm 2.38	10.96 ^a \pm 1.47	3.40 ^a \pm 0.21

Table IV. Major fatty acids (mean % total fatty acids \pm SD, $n = 3$) of *A. polyacanthus* larvae at the end of an 18-d study when fed either unenriched *Artemia* nauplii or nauplii enriched with microcapsules containing either cod liver oil or squid oil. Tr, Trace (< 0.1 %); EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

Fatty acid	Diet		
	Unenriched	Cod liver oil	Squid oil
16:0	24.08 \pm 1.57	17.80 \pm 2.51	16.40 \pm 2.04
16:1 n -7	5.52 \pm 0.36	6.65 \pm 0.73	5.01 \pm 0.18
18:0	9.64 \pm 0.85	7.64 \pm 0.87	6.95 \pm 0.93
18:1 n -9	24.77 \pm 2.31	20.26 \pm 1.49	19.17 \pm 2.34
18:2 n -6	4.22 \pm 0.54	5.18 \pm 0.58	4.69 \pm 0.88
18:3 n -3	11.66 \pm 0.72	11.60 \pm 2.61	14.74 \pm 0.83
18:4 n -3	0.92 \pm 1.05	1.12 \pm 0.22	1.26 \pm 0.40
20:5 n -3 (EPA)	1.99 \pm 1.42	3.20 \pm 0.15	3.41 \pm 0.65
22:6 n -3 (DHA)	Tr	1.16 \pm 0.34	1.67 \pm 0.38
EPA + DHA	1.99	4.36	5.08

The fatty acid compositions of *A. polyacanthus* larvae at the end of the study are shown in table IV. The DHA content of the larvae reflected that of their diet. DHA was not present at a quantifiable level in larvae receiving unenriched *Artemia* nauplii which, them-

selves, lacked DHA. The fatty acid composition of larvae fed nauplii enriched with either CLO or SQO, which contained 2.62 and 3.42 % DHA, respectively, contained 1.16 \pm 0.34 and 1.67 \pm 0.38 % DHA, respectively.

4. DISCUSSION

This study has shown that enrichment of *Artemia* nauplii with GAM containing marine oils produced elevated levels of n -3 HUFA and resulted in the significantly improved survival of *A. polyacanthus* larvae. These results support those found for other species which demonstrate the beneficial effects of improved dietary n -3 HUFA content on larval growth or survival [5, 7, 13, 21, 36]. The importance of HUFA for normal growth and development in marine fish results from their limited ability to bioconvert dietary C₁₈ PUFA precursors to longer chain C₂₀ and C₂₂ fatty acids which have roles in the structure and functioning of biological membranes [3].

Contrasting results have been reported for the influence of dietary n -3 HUFA on growth and survival of marine fish larvae. In this study, higher levels of dietary n -3 HUFA improved survival of *A. polyacanthus* larvae but did not improve growth. Larvae fed enriched *Artemia* were not significantly larger than those fed unenriched nauplii and mortality was clearly not related to the size of the larvae. In contrast to these results, elevated dietary n -3 HUFA content has been shown to improve both growth and survival in a number of species including striped bass, *Morone saxatilis* [21] and Japanese flounder, *Paralichthys olivaceus* [13]. In similar studies, increased dietary n -3 HUFA content was shown to improve growth of gilthead sea bream, *Sparus aurata*, larvae [17, 23]; however, survival was not significantly correlated with dietary n -3 HUFA content [17]. Other studies have shown no significant improvement in growth or survival of larvae resulting from improved dietary n -3 HUFA content, but have reported improved stress resistance in sea bass, *Lates calcarifer* [8] and striped mullet, *Mugil cephalus* [1].

A symptom of dietary HUFA deficiency in fish larvae is a reduced ability to tolerate stress factors such as physical handling and rapid environmental changes. Under these conditions, *A. polyacanthus* larvae were observed to 'faint' and die. Similar 'fainting' behaviour has been described for *L. calcarifer* larvae [30] and a number of other species [14, 24]. Reduced toler-

ance to physical stressors is now considered to be a major symptom of dietary HUFA deficiency for marine fish larvae [8, 36]. As a result, stress tests have become useful tools in assessing the suitability of experimental diets and a number of studies have shown a correlation between increased dietary *n*-3 HUFA and increased stress resistance [1, 8, 35, 36].

The fatty acid composition of pre-fed *A. polyacanthus* was not determined in this study and, as such, changes in the fatty acid composition of larvae fed unenriched *Artemia* cannot be determined. However, the eggs and first-feeding larvae of marine fish have been shown to contain high levels of *n*-3 HUFA, particularly DHA [33] and, in a number of species, selective retention of DHA by larvae under nutritive stress has been reported [3, 12, 16, 25]. The very low level of DHA in *A. polyacanthus* larvae fed unenriched *Artemia* for 18 d is surprising and suggests that any reserves of DHA in pre-feeding larvae were exhausted by day 18. These data indicate that *A. polyacanthus* larvae are unable to synthesise DHA from available dietary precursors such as linolenic acid (18:3*n*-3) and EPA. Similar results have been reported for hybrid bass (*Morone saxatilis* × *M. chrysops*) larvae which, when fed unenriched *Artemia* nauplii for 23 d, contained only trace amounts of DHA in their lipids [5].

Recent studies have suggested that the physiological roles of EPA and DHA are distinct [18, 23, 36]. DHA is thought to have greater importance as an essential fatty acid for fish and has been shown to be of greater importance than EPA in reducing stress-related mortality in larval red sea bream, *Pagrus major* [36]. In the present study, the lipids of unenriched *Artemia* nauplii contained 5.03 % EPA but did not contain

DHA. Nauplii enriched with CLO contained a similar level of EPA (5.23 %) to unenriched nauplii but also contained 2.64 % DHA. Mean survival of larvae fed unenriched nauplii was only 46.7 ± 6.7 % at the end of the 18-d trial compared to 100 % survival of larvae fed CLO enriched nauplii. These results indicate that EPA alone may not satisfy the *n*-3 HUFA requirement of *A. polyacanthus* larvae and dietary DHA is required to maximise survival. Mortality in larval yellowtail, *Seriola quinqueradiata*, fed *Artemia* enriched with EPA but lacking DHA has been associated with an imbalance of EPA and DHA in membrane phospholipids similar to that shown by larvae fed diets deficient in both EPA and DHA [36]. It has been suggested that the correct functioning of biomembranes depends on the presence of EPA and DHA in the correct proportions [36].

Despite growing interest in the development of culture methods for the larvae of coral reef fishes [6, 34, 41], very little is known of their nutritional requirements. Major research interest in this field has focused on the larvae of temperate species and those of high commercial value or with aquaculture potential. A major step towards developing suitable culture techniques for the larvae of coral reef fishes will be to establish suitable feeding regimes which optimise larval growth and survival. This study has shown that *Artemia* are a suitable food for *A. polyacanthus* larvae under culture conditions, if previously enriched with a material high in *n*-3 HUFA. The results indicate that DHA must be present in the diet to maximise survival of *A. polyacanthus* larvae. These results are an important contribution towards the development of appropriate larval culture techniques for coral reef fishes.

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