ADVANCES IN GROUPER AQUACULTURE

Editors: **M.A. Rimmer, S. McBride and K.C. Williams**

Australian Centre for International Agricultural Research
Canberra 2004

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

The Australian Centre for International Agricultural Research (ACIAR) was established in June 1982 by an Act of the Australian Parliament. Its primary mandate is to help identify agricultural problems in developing countries and to commission collaborative research between Australian and developing country researchers in fields where Australia has a special research competence.

Where trade names are used this does not constitute endorsement of nor discrimination against any product by the Centre.

ACIAR Monograph Series

This series contains the results of original research supported by ACIAR, or material deemed relevant to ACIAR's research and development objectives. The series is distributed internationally, with an emphasis on developing countries.

GPO Box 1571, Canberra, Australia 2601.
http://www.aciar.gov.au
email: aciar @aciar.gov.au

Rimmer, M.A., McBride, S. and Williams, K.C. 2004
Advances in Grouper Aquaculture
Canberra. ACIAR Monograph 110

ISBN 1 86320 438 5 (printed)
1 86320 439 3 (electronic)

Typeset, designed and edited by: Sun Photoset Pty Ltd, Brisbane, Australia
Printed by: BPA Print Group, Melbourne

Contents

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Abbreviations and acronyms

4-NPC	4-nitrophenyl caproate
AB-PAS	alcian blue–periodic acid Schiff
ACIAR	Australian Centre for International Agricultural Research
ACP	acid phosphatase
AD	apparent digestibility
ADMD	apparent dry matter digestibility
ALP	alkaline phosphatase
AMP	amino peptidase
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
A-P	Asia-Pacific
APD	apparent protein digestibility
APEC	Asia-Pacific Economic Cooperation
APMFAN	Asia-Pacific Marine Finfish Aquaculture Network
APNa	L-ascorbyl-2-monophosphate-Na-Ca
ARA	arachidonic acid
BF	body fat
bsd	bile salt dependent
BW	body weight
CM	circular muscle layer
CMC	carboxymethylcellulose
CP	crude protein
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DHA	docosahexaenoic acid
DM	dry matter
DO	dissolved oxygen
DOM	dissolved organic matter
DPH	days post hatch
DPI&F	Department of Primary Industries and Fisheries (Queensland, Australia)
DW	dry weight
E	energy
EA	environmental assessment
EAA	essential amino acid
EPA	eicosapentaenoic acid
FCR	food conversion ratio
GBRMPA	Great Barrier Reef Marine Park Authority
GC	gas chromatograph
GE	gross energy
GIS	Geographic Information System
GMO	genetically modified organism
HACCP	Hazard Analysis and Critical Control Point

HPLC	high pressure liquid chromatography
HSI	hepatosomatic index
HUFA	highly unsaturated fatty acid
LCFA	long chain fatty acids (C18+)
LIP	lipase
LM	longitudinal muscle layer
LP	lamina propria
lx	lux
MAL	maltase
MCFA	medium chain fatty acids (C10–C14)
n-	naupliar stage (copepods)
N	nitrogen
NACA	Network of Aquaculture Centres in Asia-Pacific
NEAA	non-essential amino acid
NFE	nitrogen free extract
NGO	non-governmental organisation
NHL	newly-hatched larvae
NL	neutral lipid
NSC	no significant change
NSE	non-specific esterase
PER	protein efficiency ratio
PL	polar lipid
POM	particulate organic matter
ppt	parts per thousand
PUFA	polyunsaturated fatty acid
PVC	polyvinylchloride
R&D	research and development
RDE	retention of digestible energy
RDN	retention of digestible nitrogen
RT-PCR	reverse transcriptase-polymerase chain reaction
S	serosa layer
S-	small (strain rotifers — *Brachionus rotundiformis*)
SD	standard deviation
SE	standard error
SEAFDEC AQD	Southeast Asian Fisheries Development Centre Aquaculture Department
SGR	specific growth rate
SM	submucosa
SPF	specific pathogen free
SS-	super small (strain rotifers — *Brachionus rotundiformis*)
STREAM	Support to Regional Aquatic Resources Management
SV	supranuclear vacuoles
TL	total length/total lipid
TRP	trypsin
UNSRAT	Sam Ratulangi University (Manado, Northern Sulawesi, Indonesia)
USA	United States of America
UV	ultra-violet
VNN	viral nervous necrosis

Contributors

Queensland Department of Primary Industries and Fisheries, Northern Fisheries Centre, Cairns, Queensland, Australia

Michael A. Rimmer
Richard M. Knuckey
Shannon McBride

Commonwealth Scientific and Industrial Research Organisation, Division of Marine Research, Cleveland, Queensland, Australia

Kevin C. Williams
David M. Smith
Ian H. Williams[1]
Simon Irvin
Margaret Barclay
Michelle Jones

Southeast Asian Fisheries Development Centre, Aquaculture Department, Iloilo, Tigbauan, Philippines

Joebert D. Toledo
Oseni Millamena
Gerald Quinitio
Perla Eusebio
Veronica Alava
R.M. Coloso
R.E.P. Mamauag
D. Chavez
J.C. Rodriguez, Jr
Nora B. Caberoy
Analyn S. Castor-Saan
Josefa D. Tan-Fermin
M.J.G. Bernas
F.M.P. Priolo
M.R. de la Peña
R.C. Caturao
M. Arnaiz

Research Institute for Mariculture, Gondol, Bali, Indonesia

Ketut Sugama
Nyoman Adiasmara Giri
Ketut Suwirya
Trijoko
Suko Ismi
Ketut Maha Setiawati
M. Marzuqi

Research Institute for Coastal Aquaculture, Maros, South Sulawesi, Indonesia

Taufik Ahmad
Muharijadi Atmomarsono
Rachmansyah
Asda Laining
Neltje N. Palinggi
Usman

Sam Ratulangi University, Manado, North Sulawesi, Indonesia

Inneke F.M. Rumengan
Stenly Wullur

Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand

Michael J. Phillips
Sih Yang Sim

[1] On sabbatical from Faculty of Natural and Agricultural Sciences, University of Western Australia, Crawley, Western Australia, Australia.

Foreword

Aquaculture of high-value finfish species, such as groupers, is an industry of increasing importance throughout the Asia-Pacific region, including Australia. The development of large and affluent markets for live reef fish in Hong Kong and southern China has increased pressure on wild stock resources.

During the late 1990s several regional workshops were held to look at the fisheries and aquaculture of grouper and reef fish. These workshops concluded that aquaculture of reef fish species will contribute to regional economies by providing product for domestic and export markets. However, the technologies for production of grouper were not yet commercially viable and a range of research issues were identified as high priority for the development of aquaculture production technology for groupers. It was also apparent that there were limited opportunities for interaction amongst many grouper researchers in the region and that improved communication and collaboration would reduce duplication and increase resource utilisation.

The ACIAR grouper project was designed to address some of the recommendations from these regional workshops by undertaking research in several critical areas of grouper aquaculture technology and by developing a collaborative network of grouper aquaculture researchers in the Asia-Pacific region.

The project had three major components and these are covered in this book; larval rearing to improve growth and survival of groupers during the hatchery phase; diet development to produce feeds with low environmental impact; and support for the NACA Grouper Aquaculture Research and Development Network.

The information gained during the project and reported here will be useful for further development and optimisation of grouper aquaculture.

Peter Core
Director
Australian Centre for International Agricultural Research

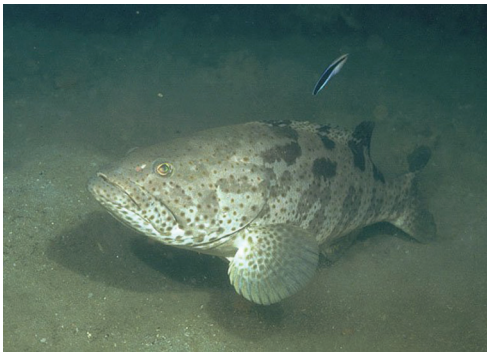
SECTION 1
INTRODUCTION

M.A. Rimmer

Aquaculture of high-value marine finfish species continues to develop rapidly in Southeast Asia. Many groupers (members of the Family Serranidae, Subfamily Epinephelinae) bring high prices (up to US$70/kg wholesale) in the live markets of Hong Kong and southern China (McGilvray and Chan 2001). Increasing market demand and the real or perceived profitability of the live reef food fish trade has led to many Southeast Asian and Pacific countries focussing on supplying this apparently lucrative trade through wild capture fisheries and aquaculture (Sadovy et al. 2003).

Worldwide, most grouper aquaculture production is from Southeast Asia. Based on FAO data, Taiwan and Indonesia are the major producers of farmed grouper, followed by Thailand and Malaysia (Table 1). However, unreported production may be substantial. Mainland China produced an estimated 8256 t of groupers in 1997 according to unofficial reports (NACA/TNC 1998), and production is likely to have increased substantially since then. Vietnam produced an estimated 2600 t of marine fish in 2001, of which a high proportion was cultured groupers (Le 2002). Based on these estimates, the regional total production of groupers through aquaculture in 2001 may have been more than 23,000 t and valued at around US$160 million.

Despite the continuing expansion of grouper aquaculture in the Asia-Pacific region, there remain several important constraints to the sustainable development of this industry sector. Foremost amongst these is the limited availability of fingerlings. Grouper aquaculture remains heavily dependent on the capture and grow-out of wild-caught juvenile fish; around 70–85% of cultured groupers are grown out from wild-caught fry (Sadovy et al. 2003). In addition, there is a recognised need to replace the widespread use of 'trash' fish as a feed source for groupers with compounded diets, as has been done for other marine finfish species such as barramundi/seabass (*Lates calcarifer*) and milkfish (*Chanos chanos*).



Epinephelus coioides is a mainstay of the live reef food fish trade and is now widely cultured throughout Southeast Asia. It is found from the Red Sea south to at least Durban and east to the western Pacific, where it ranges from the Ryukyu Islands to Australia and eastwards to Palau and Fiji. Other localities include the Persian Gulf, India, Reunion, Mauritius, Andaman Islands, Singapore, Hong Kong, Taiwan and the Philippines, and it has been reported from the Mediterranean coast of Israel. This species is frequently misidentified in the aquaculture literature as *E. tauvina* or *E. malabaricus* and is sometimes incorrectly named *E. suillis* (a synonym). *E. coioides* is widely known as green grouper, estuary cod in Australia, kerapu lumpur in Indonesia, and lapu-lapu in the Philippines.

Photo: David Cook

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Table 1. Aquaculture production (tonnes [t]) reported to FAO by country and total value (US$ million) of groupers, 1990–2001.

Country	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Brazil	—	—	—	—	—	—	—	—	1	2	3	~4
Hong Kong SAR	365	265	55	632	627	620	1110	1036	312	280	523	910
Indonesia	—	—	—	—	—	—	—	—	—	1759	1159	3818
Korea, Republic of	—	—	—	—	—	—	9	—	—	5	6	20
Kuwait	—	—	—	—	—	—	—	—	—	5	6	3
Malaysia	144	153	288	1006	931	834	857	799	465	948	1217	1101
Philippines	2363	6765	349	772	2129	715	595	654	135	151	167	136
Saudi Arabia	—	—	—	—	—	—	—	—	1	—	—	—
Singapore	185	198	233	147	133	101	93	82	97	94	111	157
Taiwan	2206	1229	1125	3942	1841	2104	1883	2525	3471	4122	5053	5386
Thailand	415	355	965	755	1078	674	74	795	1390	1143	1332	1442
Tunisia	—	—	2	~1	~1	<0.5	<0.5	<0.5	—	—	—	—
United Arab Emirates	—	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Total production (t)	5678	8965	3017	7255	6740	5048	5321	5891	5872	8509	9577	12,977
Total value (US$m)	28.5	64.4	29.4	59.5	81.1	67.5	65.5	73.0	45.7	63.9	67.2	89.9

Marine finfish aquaculture is an important contributor to the economies of coastal communities in the Asia-Pacific region, and aquaculture of high-value species such as groupers provides greater economic benefits to farmers than aquaculture of lower-value species such as milkfish (Yap 2002). Consequently, the development of sustainable grouper aquaculture can potentially provide widespread benefits to coastal communities in the Asia-Pacific region. The results described in this publication are the outcomes of a four-year research and development project funded by the Australian Centre for International Agricultural Research (ACIAR), involving collaborative research by institutions in Australia, the Philippines, Indonesia and the Asia-Pacific region. The project targeted two of the major production technology constraints for grouper aquaculture: increasing fingerling supply by improving hatchery production technology; and determining the requirements of juvenile groupers for critically-important nutrients, which is a prerequisite for the development of practical compounded diets to replace the feeding of 'trash' fish. In addition, the project provided a regional structure to coordinate and promote collaboration in grouper aquaculture research, development and extension activities to better utilise the existing institutional efforts in the Asia-Pacific region.

Larval rearing

As noted earlier, wild-caught groupers still make up the bulk of the seedstock supply in many parts of Southeast Asia, including Vietnam, Thailand and the Philippines. The trade in wild fry is associated with a number of resource management issues including overfishing, use of unsustainable harvesting techniques (including cyanide), high levels of mortality, and inadequate supply to support the demand of a developing aquaculture industry (Sadovy 2000). To meet aquaculture's demand for seedstock and to reduce pressure on wild fisheries, there is a recognised need to develop commercial marine finfish hatcheries throughout the Asia-Pacific region to supply hatchery-reared seedstock.

At the beginning of this project, despite at least a decade of research, hatchery production of groupers remained a limiting factor in grouper aquaculture. Average survival to fingerling stage (about 25 mm in length) was low, generally in the range 0–10% (Rimmer 1997, 1998, Rimmer et al. 2000). In addition, survival was highly irregular — one or two larval rearing 'runs' often resulted in reasonable (up to 10%) survival, while others had zero survival. Both of these factors (low average survival and unreliable hatchery production) were major constraints to the commercialisation of grouper larviculture technology.

In 1997 Taiwan was the only commercial producer of grouper fingerlings in the Asia-Pacific region (Rimmer 1998; Rimmer et al. 2000; Liao et al. 2001). Production of grouper fingerlings in Taiwan is largely limited to *Epinephelus coioides* and *E. malabaricus*, although there has been experimental production of several other

species, including *E. lanceolatus*, *Plectropomus* spp. and *Cromileptes altivelis* (Rimmer 1998; Liao et al. 2001). Larval rearing of groupers in Taiwan remains problematic, and many (wild caught) fingerlings are imported from other parts of Asia, particularly Thailand (Ruangpanit 1993; Sadovy 2000). Overall survival estimates are not available, but in 1997 Taiwanese farmers regarded the equivalent of 7% survival to 2.5–3 cm in length as excellent (Rimmer 1998).

The larval rearing component of the ACIAR project concentrated on a relatively broad range of research topics to assess which areas were likely to be limiting factors in successful grouper larviculture. These were:

- Environmental requirements to optimise survival in pre–feeding larvae.
- Nutritional (particularly fatty acid) requirements of larval groupers.
- Isolating and culturing SS–strain rotifers for use in grouper larviculture.
- Assessing the capacity of larval groupers to digest live and artificial prey, including documenting the development of the digestive tract and the ontogeny of digestive enzymes.
- Defining hatchery management techniques for larval rearing of groupers.

Cromileptes altivelis is found through the Western Pacific from southern Japan to Palau, Guam, New Caledonia, and southern Queensland (Australia); and in the eastern Indian Ocean from the Nicobars to Broome, Western Australia. It is a high-value species in the live reef fish trade, bringing up to US$70 per kg wholesale. Juvenile fish are in demand as aquarium inhabitants. Commonly known as humpback grouper or polkadot grouper, it is known as barramundi cod in Australia, kerapu tikus or kerapu bebek in Indonesia, and señorita in the Philippines.

Photo: GBRMPA

Research results were continually integrated into hatchery verification trials to assess the practical outcomes of the research. The results of the larval rearing component are detailed in Section 2 of this publication.

Development of grow–out diets

The need for compounded (pellet) feeds to replace the feeding of 'trash' fish is widespread throughout the Asia–Pacific region. Issues regarding the use of trash fish have been identified in detail in several publications (for example New 1996) and include: competition for fishery products with human nutritional requirements and with other agricultural sectors; relatively low efficiency of utilisation of 'trash' fish (dry matter FCRs typically range from 5:1 to 10:1, compared 1:1 with 2:1 for pellet diets); and localised pollution due to losses of feed material during feeding (Phillips 1998). Because using 'trash' fish for feed is not economic in Australia, marine finfish aquaculture relies on the development of suitable cost-effective pelleted feeds. In addition, Australia's strict environmental regulation of aquaculture requires feeds that minimise nutrient release to the environment.

The grow-out diet component of the project was addressed in a structured way, acquiring nutritional information on feeds available for diet manufacture, characterising the requirements of groupers for key nutrients and demonstrating the cost effectiveness of the compounded feeds. Major research areas within this overall structure were:

- Inventory and categorise the composition of feed ingredients in Indonesia.
- Determine the apparent digestibility of a range of feed ingredients available in Indonesia, the Philippines and Australia.
- Determine the requirements of groupers for critical nutrients, with emphasis on protein and energy requirements.
- Assess the capacity to replace fishmeal in grow-out diets for groupers with terrestrial protein sources.

Research results from the grow-out nutrition component were validated through trials using experimental pellet diets in comparison with 'trash' fish, and later through trials using

commercially produced diets. Results of this component of the project are detailed in Section 3 of this publication.

Epinephelus fuscoguttatus is widely distributed in the Indo-Pacific region, including the Red Sea, and occurs at most (probably all) of the tropical islands of the Indian and west-central Pacific oceans (east to Samoa and the Phoenix Islands) along the east coast of Africa to Mozambique, and it has also been reported from Madagascar, India, Thailand, Indonesia, the tropical coast of Australia, Japan, Philippines, New Guinea, and New Caledonia. Tiger grouper is a medium-priced species in the live reef fish trade and juveniles are in demand by farmers in Southeast Asia because this species survives well and grows rapidly in culture. Widely known as tiger grouper, this species is called flowery cod in Australia, kerapu macan in Indonesia, and lapu-lapu in the Philippines.

Research coordination and information dissemination

Although there has been considerable research and development effort extended on developing sustainable grouper aquaculture in the Asia-Pacific, at the start of the project it was apparent that this effort had been relatively fragmented and uncoordinated. Discussion with other grouper aquaculture researchers indicated that most felt that they were working in isolation. This lack of communication and coordination between research and development institutions in the region caused overlap, and in some cases outright duplication, of research effort, which diluted the overall research progress in this field.

The third component of the ACIAR project targeted this issue by improving communication between grouper aquaculture researchers worldwide, but with particular emphasis on the Asia-Pacific region. The primary mechanism for this component of the project was the development and maintenance of the Asia-Pacific Grouper Network, coordinated through the Network of Aquaculture Centres in Asia-Pacific (NACA). Participants at the Grouper Aquaculture Workshop held in Bangkok in April 1998 (Rimmer et al. 2000) committed to reducing duplication and overlap of research effort by participating in a Grouper Aquaculture Research and Development Program to be coordinated by NACA. This component of the project was augmented with an APEC Fisheries Working Group project *Collaborative APEC Research and Development Grouper Network*.

Section 4 of this publication outlines the activities of the Asia-Pacific Grouper Network and its successor, the Asia-Pacific Marine Finfish Aquaculture Network, and provides examples of how a networking approach can more widely spread research and development benefits in the region.

Further Information

This publication summarises the results of ACIAR project FIS/07/73 *Improved hatchery and grow-out technology for grouper aquaculture in the Asia-Pacific region*. Additional details of the research work carried out under this project can be found at www.enaca.org/aciar/. A list of project and Asia-Pacific Marine Finfish Network publications is provided in Appendix 2. Many of these publications are available for download from www.enaca.org/grouper/.

References

Le, T.L. 2002. Status of marine finfish aquaculture in Vietnam. In *Paper presented to the Workshop on Sustainable Marine Finfish Aquaculture in the Asia-Pacific Region, HaLong City, Vietnam*, 30 September–3 October 2002.

Liao, I.C., Su, H.M. and Chang, E.Y. 2001. Techniques in finfish larviculture in Taiwan. Aquaculture 200, 1–31.

McGilvray, F. and Chan, T.T.C. 2001. *The Trade in Live Reef Food Fish: A Hong Kong Perspective,* International Marine Life Alliance, Hong Kong.

NACA/TNC 1998. *Report of the Survey of Marine Fish Aquaculture and Live Marine Fish Markets in China.* Unpublished report to The Nature Conservancy from the Network of Aquaculture Centres in Asia-Pacific (NACA), Bangkok.

New, M.B. 1996. Responsible use of aquaculture feeds. Aquaculture Asia 1, 3–15.

Phillips, M.J. 1998. Chapter 2 — Tropical Mariculture and Coastal Environmental Integrity In *Tropical Mariculture* (De Silva, S.S. ed.), pp. 17–69. Academic Press, London.

Rimmer, M. 1997. Marine finfish aquaculture in Taiwan. Austasia Aquaculture 11, 37–41.

Rimmer, M. 1998. Grouper and snapper aquaculture in Taiwan. Austasia Aquaculture 12, 3–7.

Rimmer, M.A., Williams, K.C. and Phillips, M.J. 2000. *Proceedings of the Grouper Aquaculture Workshop held in Bangkok, Thailand, 7–8 April 1998,* Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.

Ruangpanit, N. 1993. *Technical manual for seed production of grouper (Epinephelus malabaricus),* National Institute of Coastal Aquaculture, Department of Fisheries, Ministry of Agriculture and Cooperative, and The Japan International Cooperation Agency.

Sadovy, Y. 2000. Regional survey of fry/fingerling supply and current practices for grouper mariculture: evaluating current status and long-term prospects for grouper mariculture in Southeast Asia, pp. 120. University of Hong Kong, Hong Kong SAR, China.

Sadovy, Y.J., Donaldson, T.J., Graham, T.R., McGilvray, F., Muldoon, G.J., Phillips, M.J., Rimmer, M.A., Smith, A. and Yeeting, B. 2003. *While Stocks Last: The Live Reef Food Fish Trade,* Asian Development Bank, Manila, Philippines.

Yap, W.G. 2002. Role of mariculture in securing food supply and reducing poverty in the Philippines. In *World Aquaculture 2002 International Aquaculture Conference and Exposition*, p. 864. World Aquaculture Society, Beijing, China.

SECTION 2
LARVAL REARING

Improvements in larval rearing techniques for groupers increased larval survival from around 3% to 20–40% for *E. coioides* and from <10% to 30–50% for *C. altivelis*. This has transformed grouper hatchery technology to commercial viability, as evidenced by the dramatic increase in grouper fingerling production from Indonesian hatcheries. Grouper hatchery technology has been disseminated more widely than the project participants through the activities of the Asia-Pacific Marine Finfish Aquaculture Network.

Summary

The larval rearing component of the project investigated a range of aspects of grouper larviculture to improve survival, as well as the consistency of production, in the hatchery phase. Grouper larvae are small and fragile with small reserves of endogenous nutrition and low initial feeding rates (Ordonio-Aguilar et al. 1995). This combination of factors is considered to be a fundamental cause of the high mortalities and delayed development observed during larviculture (Kohno et al. 1997).

An essential pre-requisite for successful marine finfish larviculture is to maximise survival and condition of the larvae prior to the commencement of exogenous feeding. Optimal environmental parameters during egg incubation and rearing of pre-feeding larvae were established for *E. coioides* and *C. altivelis* (Table 1).

The nutritional value of the live prey used to feed grouper larvae is a major determinant of larval growth and survival. *E. coioides* larvae conserve the fatty acids eicosapentaenoic acid (EPA, 20:5n-3), arachidonic acid (ARA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), indicating the essentiality of these fatty acids. These fatty acids are particularly conserved in the phospholipid fraction, whereas neutral lipids are primarily used as an energy source for developing larvae. The levels of EPA, ARA and DHA can be enhanced in rotifers and brine shrimp using various enrichment products and nutritional enhancement of live prey results in improved growth, survival and pigmentation of *E. coioides* larvae.

A better understanding of the nutritional physiology of grouper larvae was established through this project. The histological study of the digestive tract in *E. coioides* and descriptions of the ontogeny and of digestive enzymes in *E. coioides* and *C. altivelis* provide a basis for assessing the digestive capacities at different developmental stages. The low activities of digestive enzymes and the rudimentary structure of the digestive tract indicated that grouper larvae have a low digestive capacity prior to 10 days post hatch. As the structural complexity of the

Juvenile tiger and humpback grouper in an Indonesian hatchery. The outcomes of ACIAR-supported research have contributed substantially to overcoming the bottleneck of fingerling supply of high-value species such as these.

Table 1. Environmental conditions for incubation of eggs and early larvae of two grouper species for optimal survival.

Stage	Tank	Water exchange (%/day)	Aeration	Salinity (ppt)	Light Intensity
E. coioides					
Eggs (400/L)	4L	0	Moderate (100mL/min)	32–42	
Larvae, early stage	40L	0	Gentle (0.62–1.25 mL/min)	16–24	500–700 lx
C. altivelis					
Eggs (500/L)	Incubation	200[1]	High (600 mL/min)[2]	34–35	
Larvae, early stage	5 tonne	0	Gentle and evenly distributed	34–35	1000–1500 lx at water surface

[1] determined in a 100L tank.
[2] determined in a 4L tank.

stomach progressed between 10 and 16 days post hatch, fluctuations in trypsin and total protease activities were observed suggesting a change in digestive physiology. The appearance of gastric glands coincided with a general increase in enzyme activities indicating an increase in the digestive capacity of the larvae preceding metamorphosis. It is also apparent that there are differences between grouper species in the emergence of digestive enzymes implying there may be different capabilities between genera for digesting the major macronutrients.

The small mouth size and limited physical abilities of grouper larvae has limited the suitability of traditional live prey organisms for early feeding. Successful larval rearing relies on the use of smaller prey organisms, such as copepod nauplii and the 'super-small' (SS) — strain rotifer. Selection for smaller rotifers tends to select females that reproduce at a smaller size, but which still grow to a normal size. Larger proportions of smaller rotifers suitable for first-feeding grouper larvae are obtained when the rotifers are fed a microalgal diet of small particle size, such as *Stichococcus*.

Copepod nauplii show considerable potential as an alternative live prey for larval rearing of marine finfish because they are in many cases smaller than SS-strain rotifers and are of superior nutritional value to rotifers (McKinnon et al. 2003). Copepods are also a useful supplement to brine shrimp during the later stages of larval rearing, because of their better nutritional profile. The addition of copepods in the semi-intensive larval rearing of *E. coioides* also improved survival. The high total protease activity found in copepod nauplii in comparison to rotifers, suggests that they are more digestible by early stage grouper larvae and may partly explain this improved survival.

With improvements in larval nutrition and husbandry techniques, survival has now increased from around 3% to 20–40% for *E. coioides* and from <10% to 30–50% for *C. altivelis*. This has moved larval rearing technology for groupers into the realm of commercial viability. That this technology is commercially viable has been well demonstrated in Indonesia where, in 2003, there were an estimated 67 hatcheries (52 'backyard', six medium and nine large hatcheries) producing grouper fingerlings for the food fish and ornamental markets. Estimated production of grouper fingerlings from Indonesian hatcheries in 2002 was 3,350,200 fish (5–10 cm total length) comprising 2,656,200 *E. fuscoguttatus*, 697,800 *C. altivelis*, and 2200 *E. coioides* (Sugama 2003).

Despite this success, the viral disease viral nervous necrosis (VNN) continues to impact the survival of grouper larvae in hatcheries. VNN-related mortality in *E. coioides* was dramatically reduced using the protocols developed through this project, and particularly by increasing the levels of HUFAs in the live prey fed to grouper larvae. However, the prevalence and transmission mechanisms of the virus are poorly known in tropical marine finfish aquaculture and further research is necessary to better understand the disease and to develop methods of control.

Marine finfish hatcheries provide important socio-economic contributions to coastal communities. The socio-economic assessment of 'backyard' hatcheries in Bali, Indonesia, showed that hatcheries are important sources of employment and economic benefit in northern Bali. Economic features of these hatcheries include: high profitability ($6300–$100,000 per annum), high internal rates of return (>12%), positive benefit-cost ratios (1.3–3.1), and rapid payback of capital cost (often with one year). These hatcheries are important sources of employment for local people, including women, either directly or in associated industries such as fish brokerage. A feature of these hatcheries is that they may switch between different species as commodity prices fluctuate; thus, the industry as a whole is relatively robust to market fluctuations.

The impacts of this project were spread more widely than the participating countries by the development of the Asia-Pacific Grouper Network, and its successor, the Asia-Pacific Marine Finfish Aquaculture Network, coordinated by NACA. A regular training course at the Gondol Research Institute for Mariculture, Bali, Indonesia, has trained aquaculture researchers and industry practitioners from several countries in the Asia-Pacific region. This training has contributed directly to successful production of grouper fingerlings in Vietnam, Thailand and Malaysia as well as in Indonesia.

References

Kohno, H., Ordonio-Aguilar, R.S., Ohno, A. and Taki, Y. 1997. Why is grouper larval rearing difficult?: an approach from the development of the feeding apparatus in early stage larvae of the grouper, *Epinephelus coioides*. Ichthyological Research 44, 267–274.

McKinnon, A.D., Duggan, S., Nichols, P.D., Rimmer, M.A., Semmens, G. and Robino, B. 2003. The potential of tropical paracalanid copepods as live feeds in aquaculture. Aquaculture 223, 89–106.

Ordonio-Aguilar, R., Kohno, H., Ohno, A., Moteki, M. and Taki, Y. 1995. Development of grouper, *Epinephelus coioides*, larvae during changeover of energy sources. Journal of Tokyo University of Fisheries 82, 103–108.

Sugama, K. 2003. Indonesia focuses on groupers. Asian Aquaculture Magazine September–October 2003, 14–15.

Environmental Factors Affecting Embryonic Development, Hatching and Survival of Early Stage Larvae of the Grouper (*Epinephelus coioides*)

J.D. Toledo, N.B. Caberoy and G.F. Quinitio

Introduction

The orange spotted grouper, *Epinephelus coioides*, is known to spawn readily in tanks and floating net cages (Toledo et al. 1993). Despite recent developments in seed production techniques (Duray et al. 1997; Toledo et al. 1999), survival of early-stage larvae is still low. Low survival during the early larval stage is likely due to the effects of poor egg quality, the size and nutritional value of prey at the onset of feeding, and the environmental conditions during the incubation of fertilised eggs and early stage larvae. In this study, a series of experiments was conducted to determine the effects of density, salinity, aeration and light intensity on the survival of fertilised eggs and early stage larvae of *E. coioides.*

Methods

The effects of density, aeration, and salinity on embryonic development, hatching, and survival of the early larval stages of grouper were determined. Spontaneously spawned grouper eggs were collected 15–20 minutes (min) after spawning. Eggs at early cleavage (8–32 celled stage) were stocked in 4 L plastic containers at 200, 400, 800 or 1600 eggs/L. Fertilised grouper eggs at early cleavage stage were stocked in 4 L containers at a density of 400 eggs/L. Four levels of aeration were tested (0 ml/min, 100 ml/min, 500 ml/min, 1000 ml/min) to determine the effect of aeration intensity on egg and larval survival. To examine the sensitivity of grouper eggs to various salinities, eggs at early cleavage or 'eyed stage' were abruptly transferred to salinities of 8, 16, 24, 32 and 40 ppt. Survival rate was determined by the percentage of eggs that reached the eyed stage. There were three replicates for each treatment.

Newly hatched larvae (day of hatching = day-0) were randomly distributed in 15 units of 40 L aquaria containing 35 ppt seawater at a density of 1,500 larvae/aquarium. Five aeration levels were tested: 0 (no aeration); 0.62 ml/min/L; 1.25 ml/min/L; 2.50 ml/min/L and 3.75 ml/min/L. A single air-stone was positioned at the centre of the floor of each aquarium. Five salinity levels were tested: 8, 16, 24, 32 and 40 ppt. Five light intensities (0, 120, 230, 500 and 700 lx) were randomly assigned in five enclosed chambers. Three 40 L aquaria containing 22 ppt seawater were placed in each chamber. The desired light intensities were obtained by adjusting the number of 21 to 100 watt fluorescent bulbs in each chamber. Except for the dark chamber (0 lx), the photoperiod was maintained at a 12-hour light and 12-hour dark cycle. All aquaria were provided with aeration at 0.62 ml/min/L.

Twenty larvae in each aquarium were sampled daily at 1100 hrs. Total length (TL) and the diameter of the oil globule of each larva were measured using an Image Analysis System equipped with Image-Pro Plus Imaging Software for Windows. Larvae were then preserved in 5% formalin-seawater for gut content analysis.

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Results and Discussion

Embryonic survival, hatching rates and percentage of normal larvae were greatly affected by stocking density, intensity of aeration and salinity during egg incubation. The highest viability, hatching rates and percentage of normal larvae were observed at a stocking density of 400 eggs/L, aeration level of 100 ml/min and salinity of 32–42 ppt (Figs. 1–3). Low hatching rates were noted at salinities below 32 ppt. Almost all of the eggs exposed to a salinity of 8 ppt failed to hatch. Eggs at the eyed stage were more sensitive to salinity change than those at early cleavage. The most remarkable abnormalities were the presence of curvature in the body and some exceptionally small (shrunken) larvae.

Salinity, aeration and light intensity greatly influenced oil globule absorption, feeding incidence, growth and survival of *E. coioides* early stage larvae (Figs. 4–6). Under static conditions, early stage grouper larvae survived much better and with a bigger oil globule under gentle aeration of 0.62 and 1.25 ml/min/L than those under stronger aeration of 2.50–3.75 ml/min/L

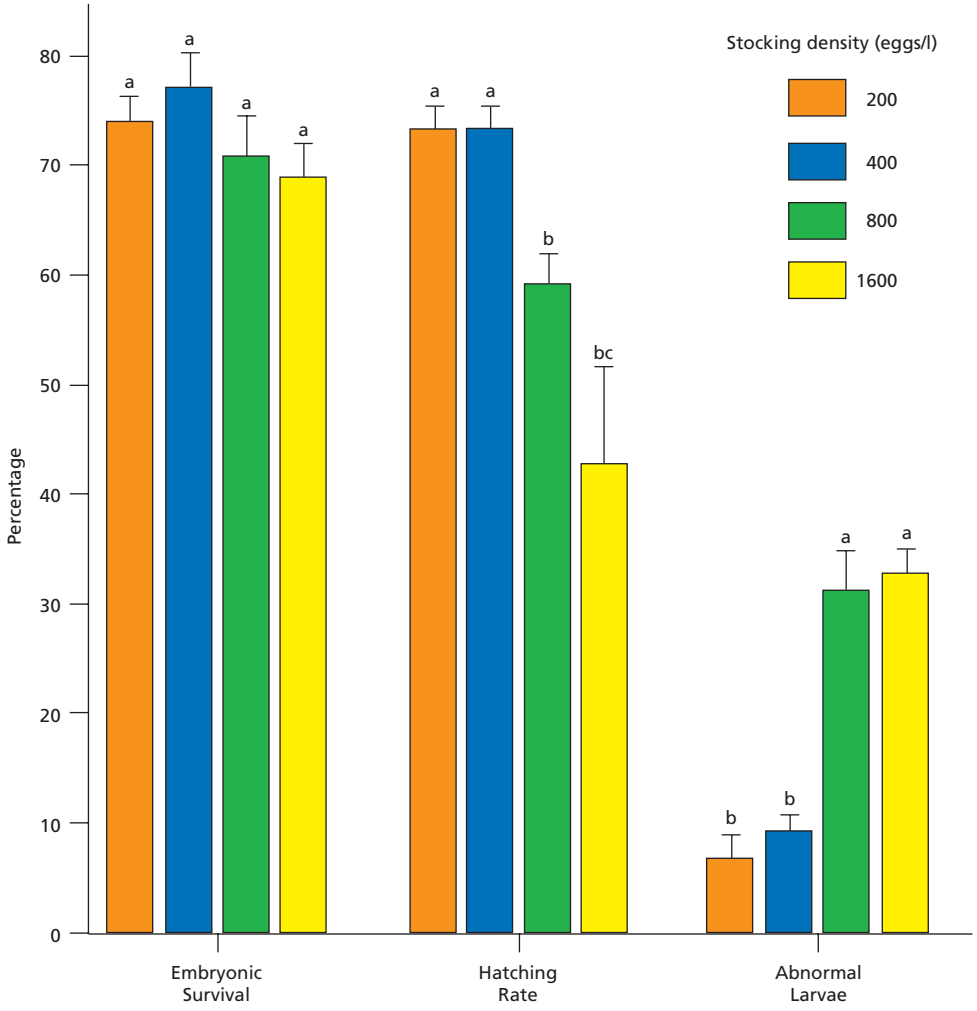


Figure 1. Effect of stocking density on embryonic survival, hatching and occurrence of abnormal larvae in the grouper *E. coioides*. Bars with the same letters are not significantly different (P > 0.05).

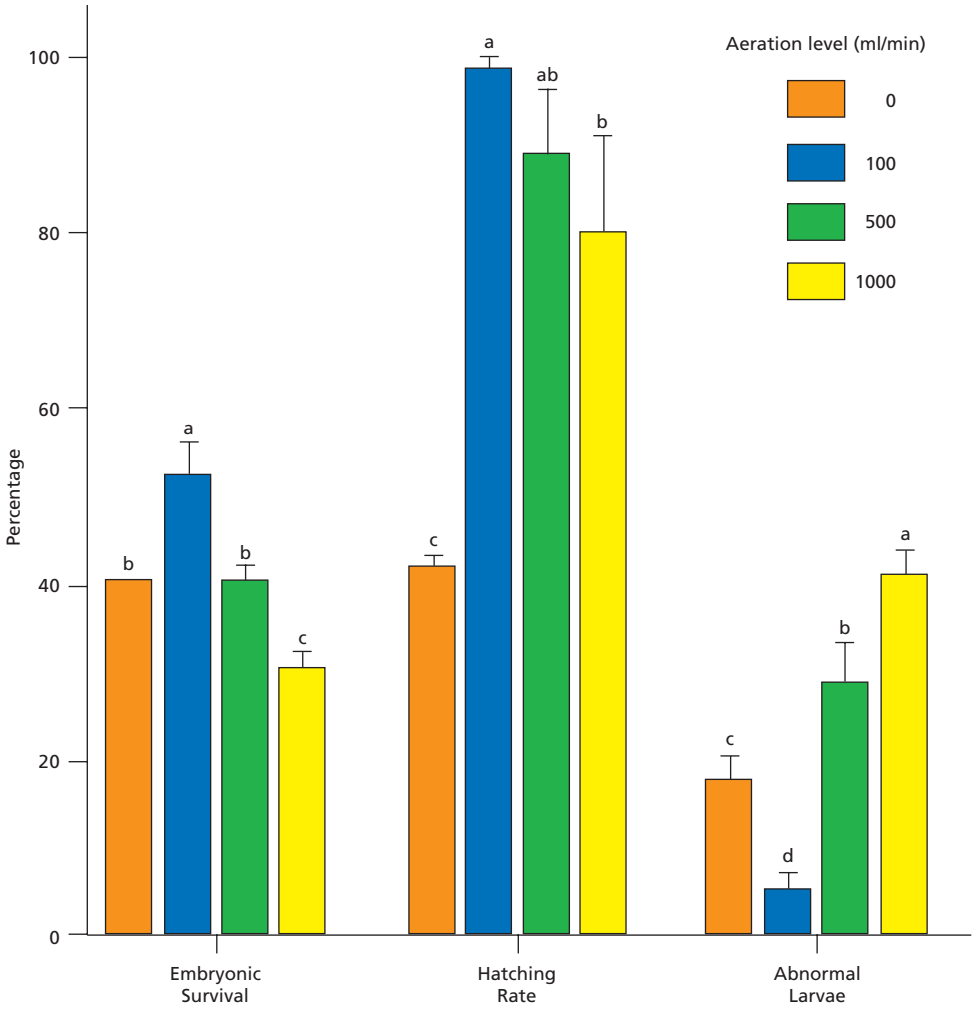


Figure 2. Effect of the intensity of aeration on embryonic survival, hatching and occurrence of abnormal larvae in the grouper *E. coioides*. Bars with the same letters are not significantly different (P > 0.05).

and under static conditions (Fig. 4). Larvae exposed to higher aeration levels most likely required more energy to maintain their swimming position. Low feeding incidence of larvae at higher aeration on day-5 also suggests that larvae experienced difficulty in catching prey.

Newly hatched larvae in this study survived an abrupt transfer from a spawning and hatching salinity of 35 ppt to salinity in the range of 8 to 40 ppt. This indicates that *E. coioides* larvae have a strong tolerance to salinity fluctuations. On day-1, larvae at salinities of 16 and 24 ppt swam to the middle or upper-portion of the water column while larvae at 8 ppt remained clustered at the bottom of the aquarium. They did not die and float as described by Yamaoka et al. (2000) for *E. akaara*.

Larval survival was significantly higher at the light intensities of 500 and 700 lx than the lower intensities tested. The lowest level of growth and biggest volume of remnant oil globules observed at 0 lx could have been caused by the lower activity of the larvae in total darkness.

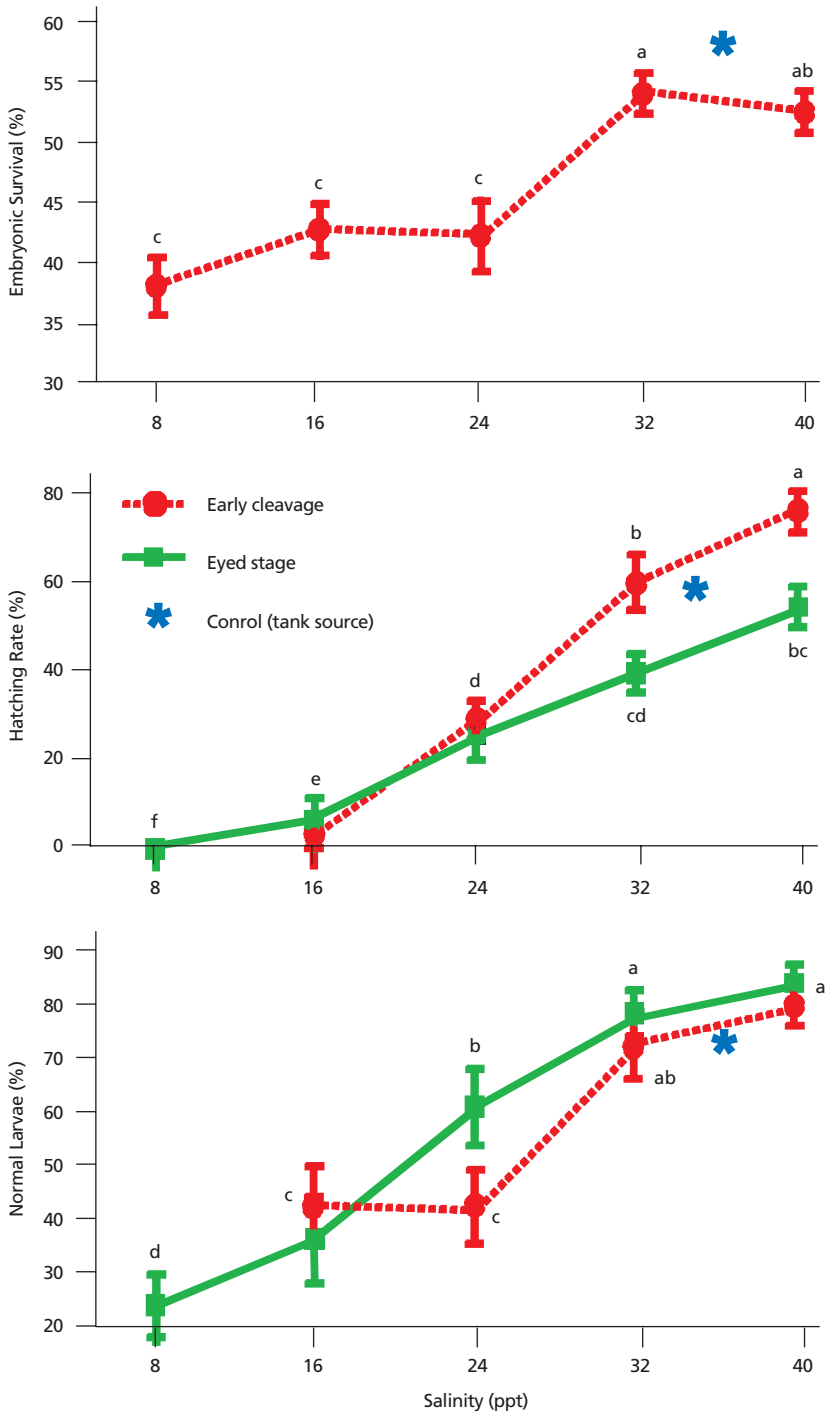


Figure 3. Embryonic survival, hatching and percentage of normal larvae of *E. coioides* abruptly transferred to different salinities at early cleavage (8–32 celled) and eyed stages. Means with the same superscripts are not significantly different ($P > 0.05$).

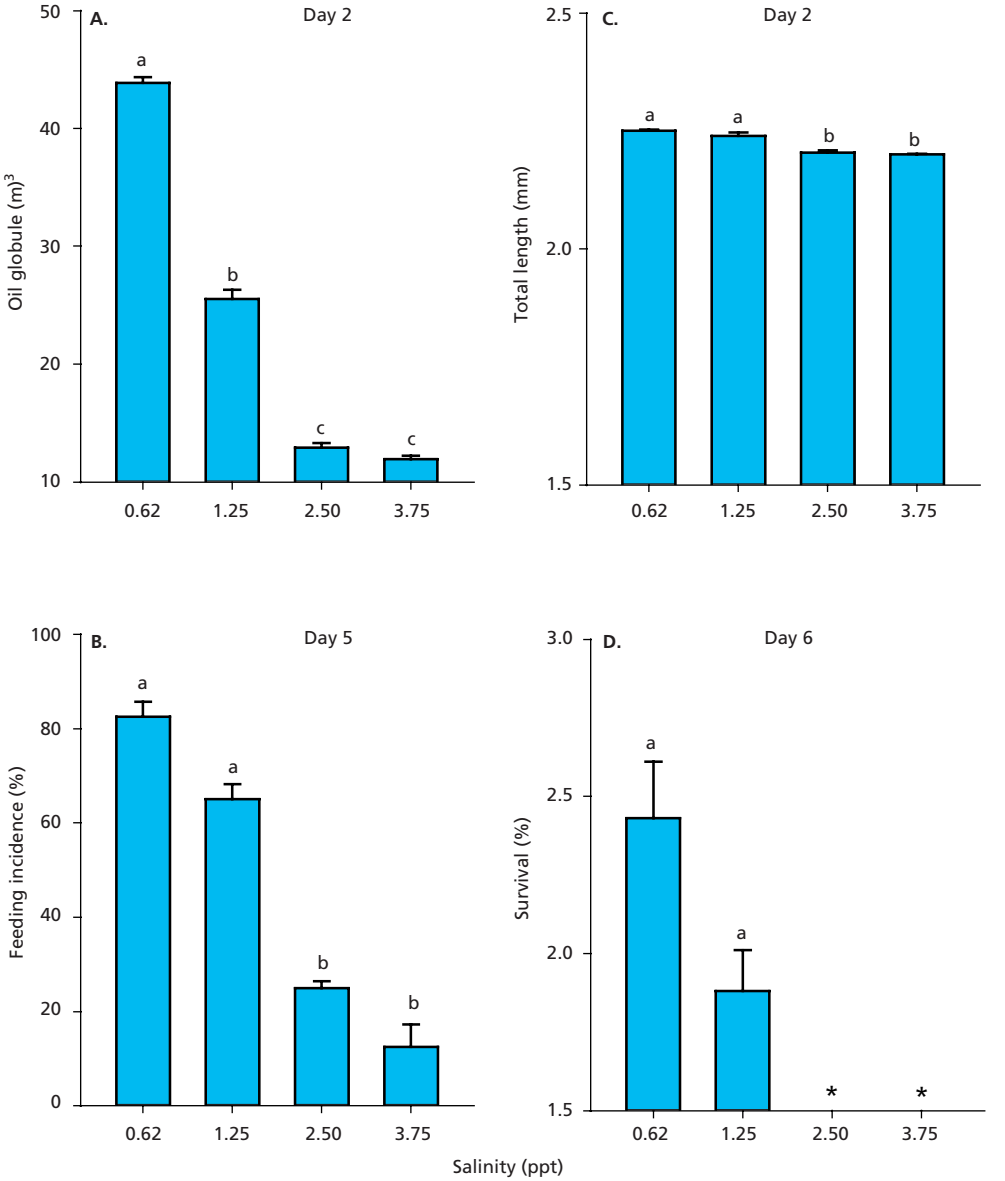


Figure 4. Effects of various aeration levels on A) oil globule absorption, B) feeding incidence, C) total length and D) survival of *E. coioides* larvae. Each bar represents mean ± SE of 3 replicates (n = 20). Bars with different letters are significantly different (P > 0.05). *All larvae died on Day 6.

No survival of larvae at light intensities 0 1x and 120 lx, and very low survival at 230 lx, suggests that the light intensity appropriate for first-feeding *E. coioides* larvae would be higher than 500 lx. Yamaoka et al. (2000) suggested that light intensity for seed production of *E. akaara* must be lower than 1000 lx at the water surface level of rearing tanks until 10 days after hatching.

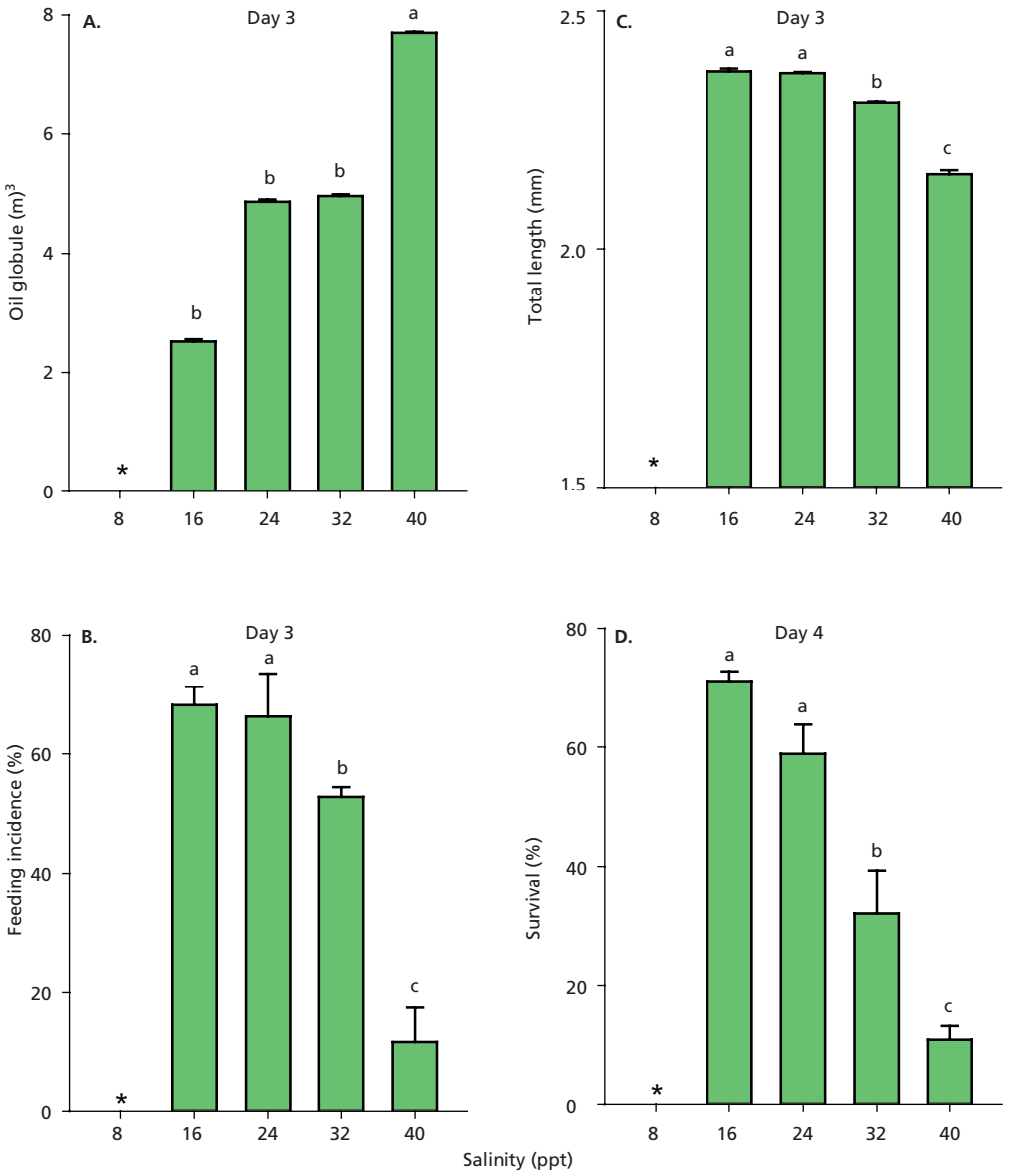


Figure 5. Effects of various salinity levels on A) oil globule absorption, B) feeding incidence, C) total length and D) survival of *E. coioides* larvae. Each bar represents mean ± SE of 3 replicates (n = 20). Bars with different letters are significantly different (P > 0.05). *All larvae died on Day 3.

Conclusions

- Under static incubation conditions, the highest egg viability, hatching rate and percentage of normal larvae were obtained at a stocking density of 400 eggs/L with moderate aeration (100 ml/min), and salinity of 32–42 ppt.
- Gentle aeration at 0.62 to 1.25 ml/min/L, rearing water of 16 to 24 ppt and a light intensity of 500 to 700 lx maximised the survival of early stage *E. coioides* larvae in the hatchery.

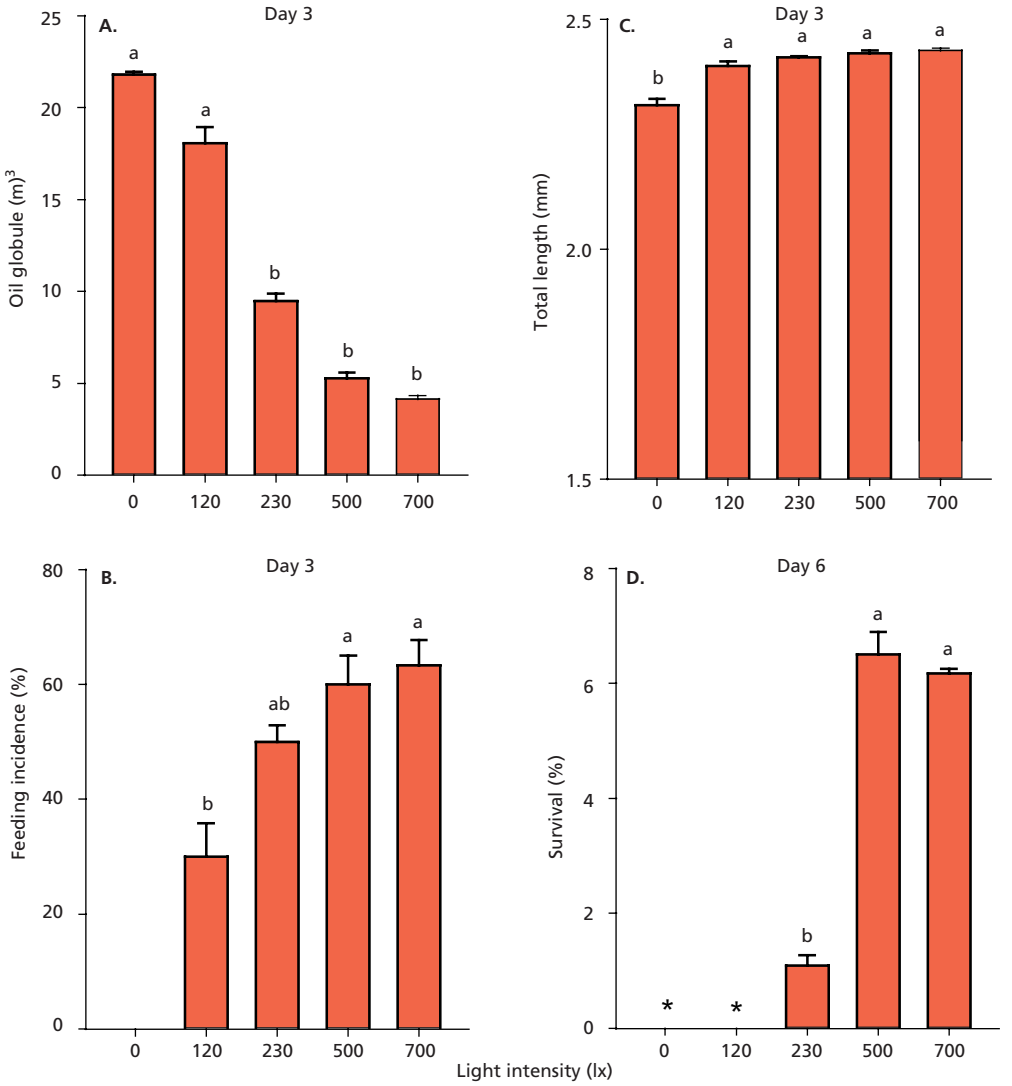


Figure 6. Effects of various light intensities on A) oil globule absorption, B) feeding incidence, C) total length and D) survival of *E. coioides* larvae. Each bar represents mean ± SE of 3 replicates (n = 20). Bars with different letters are significantly different (P > 0.05). *All larvae died on Days 4 and 6, respectively.

References

Duray, M.N., Estudillo, C.B. and Alpasan, L.G. 1997. Larval rearing of the grouper *Epinephelus suillus* under laboratory conditions. Aquaculture 150, 63–76.

Toledo, J.D., Nagai, A. and Javellana, D. 1993. Successive spawning of grouper *Epinephelus suillus* (Valenciennes), in a tank and a floating net cage. Aquaculture 115, 361–367.

Toledo, J.D., Golez, M.S.N., Doi, M. and Ohno, A. 1999. Use of copepod nauplii as food for the early larval stages of grouper *Epinephelus coioides*. Fisheries Science 65, 390–397.

Yamaoka, K., Nabu, T., Miyagawa, M., Isshiki, T. and Kusaka, A. 2000. Water surface tension-related deaths in pre-larval red-spotted grouper. Aquaculture 189, 165–176.

Environmental Factors Affecting Embryonic Development and Hatching of Humpback Grouper (*Cromileptes altivelis*) Larvae

K. Sugama, Trijoko, S. Ismi and K. Maha Setiawati

Introduction

Larvae of humpback grouper show drastic changes in morphology as they develop from hatched larvae to juvenile stage (Mishima and Gonzares 1994). Until larvae complete metamorphosis to juvenile stage, they are very sensitive to environmental conditions and are prone to stress and high mortality rates.

Egg handling should be done very carefully to prevent mechanical shocks. Sensitivity of humpback grouper eggs may be different at each stage of egg development. Eggs are very sensitive before the embryonic stage and just before hatching. Salinity, temperature and egg density are important factors during the incubation of marine fish eggs (Holliday 1988).

The purpose of the present studies was to determine the effect of egg density, water exchange and aeration rate on embryonic development and hatching rate of humpback grouper, *Cromileptes altivelis,* eggs.

Material and Methods

Spawners and Eggs

Grouper spawners weighing 1.06–3.16 kg each, total length 41.5–57.0 cm were held in round concrete tanks (75 m^3) with a density of 24 fish (16 female and 8 male). Broodfish were fed trash fish and squid that were supplemented with a vitamin mix and vitamin C. Water exchange in the broodstock tanks was 300–500% per day using flow through system.

Humpback grouper spawn at night, mostly around 22:00–24:00 hours. Since fertilised grouper eggs float, spawned eggs flowed directly into the egg collector (300–400 μm mesh size) that was connected to the tank overflow pipe. Collected eggs were then harvested on the following morning and transferred into a transparent 30-litre polycarbonate tank filled with filtered sea water. Only floating eggs were transferred into the polycarbonate tank for the experiment.

Experiment 1: Effect of Egg Density

Six treatments of egg density were studied in this experiment: 500, 1000, 1500, 2000, 2500 and 3000 eggs/litre. The experiment was conducted using three litre transparent bottles each filled with two litres of sea water. The experiment was a completely randomised design with three replicates for each treatment. Samples of eggs were collected every hour after stocking for observation of embryonic development.

Experiment 2: Effect of Water Exchange

This experiment used 12 transparent tanks, 100 litres in volume and filled with sea water (34 ppt). In each tank were stocked newly spawned eggs at a density of 500 eggs/litre. Treatments for this experiment were: without water exchange; 100% water exchange; and 200% water exchange per day. The experiment was a completely randomised design with three

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

treatments and three replicates per treatment. Samples were taken every hour after stocking for embryonic development and hatching observation.

Experiment 3: Effect of Aeration Rate

This experiment used 12 transparent bottles, three litres in volume filled with sea water (34 ppt). In each bottle was stocked newly spawned eggs at a density of 500 eggs/litre. The treatments in this experiment were: (A) aeration rate of 0 ml/min, (B) 200 ml/min, (C) 400 ml/min, and (D) 600 ml/min. The experiment was a completely randomised design with four treatments and three replicates per treatment. Samples were taken every hour after stocking for observation of embryonic development and hatching.

Embryonic Development

Time from spawning to various embryonic stages was recorded for the different treatments. Ten to 30 eggs were analysed each hour and their development was examined by microscope.

Result

Experiment 1

Egg development from spawning to various embryonic stages at different egg density is summarised in Table 1. Total time required from spawning to hatching ranged from 18 hours and 41 minutes to 20 hours and 29 minutes. The hatching rate was significantly higher for eggs incubated at lower density (Table 2). However, eggs incubated at the density of 2000, 2500 and 3000 eggs per litre resulted in the same hatching rate.

Table 2. Average value of hatching time and hatching rate of humpback grouper eggs incubated at different densities.

Treatment (eggs per litre)	Hatching time (hrs)	Hatching rate (%)
A (500)	18.68 ± 0.55^{a}	77.0 ± 12.3^{a}
B (1000)	18.76 ± 0.47^{ab}	71.3 ± 6.6^{b}
C (1500)	18.85 ± 0.38^{ab}	65.0 ± 0.2^{c}
D (2000)	19.18 ± 0.02^{ab}	59.7 ± 5.2^{d}
E (2500)	19.43 ± 0.02^{b}	56.7 ± 8.2^{d}
F (3000)	20.48 ± 1.28^{c}	59.0 ± 5.8^{d}

Values followed by the same letter within a column are not significantly different (P > 0.05).

Experiment 2

Table 3 shows that embryonic development from many cells to hatching was faster at higher water exchange. Almost 70% of eggs had hatched at 17:00 for eggs incubated at a water exchange of 200%/day. On the other hand, eggs incubated at 100%/day water exchange and without water exchange had a hatching rate of

Table 1. Time observation on the development of humpback grouper eggs from spawning to hatching during the incubation at different egg densities.

Developmental stages	Egg density (per litre)					
	A (500)	B (1000)	C (1500)	D (2000)	E (2500)	F (3000)
Spawning	23:19	23:19	23:19	23:19	23:19	23:19
1-cell	23:30	23:35	23:37	23:37	23:40	23:40
2-cells	23:45	23:50	23:50	23:50	23:55	23:55
4-cells	23:46	23:46	23:55	23:55	23:55	23:57
16-cells	23:54	00:00	00:00	00:00	23:05	23:05
64-cells	00:00	00:10	00:10	00:15	00:17	00:17
Many cells	00:10	00:15	00:20	00:20	00:22	00:22
Morula	02:15	02:20	02:25	02:25	02:25	02:25
Blastula	02:46	02:50	02:50	02:55	02:57	02:57
Gastrula	03:00	03:02	03:02	03:02	03:10	03:10
Head and tail bud	06:56	06:58	06:58	06:58	06:58	06:58
Optic bud	07:00	07:05	07:05	07:10	07:15	07:15
Digestive system	08:40	08:50	08:50	08:55	09:00	09:00
Form fin	11:05	11:15	11:20	11:25	11:30	11:30
Heart beating	11:10	11:25	11:25	11:30	11:35	11:35
Pre-hatching	14:20	14:30	14:30	14:35	14:45	14:45
Hatching	18:00	18:05	18:10	18:30	18:45	19:10

Table 3. Observations of embryonic development of humpback grouper eggs at different water exchange-rates during incubation.

Embryonic development	Water exchange (% per day)					
	200		100		0	
	Time	%	Time	%	Time	%
1-4 cell	21:10	80	21:15	80	21:17	80
Many cells	22:30	80	22:35	80	22:37	80
Morola stage	23:00	—	—	—	—	—
Blastula stage	02:10	70	02:15	65	02:20	50
Gastrula stage	03:37	—	—	—	—	—
Neorula stage	05:29	70	05:35	62	05:40	50
Digestive system	09:55	—	08:30	60	—	—
Hatching	17:00	70	17:30	60	18:00	50

only 60% and 50% at 17:30 and 18:00, respectively. The hatching rate of eggs incubated at 200%/day (71.6%) and 100%/day (57.7%) water exchange was not significantly different, but significantly higher compared with no water exchange (48.3%).

Experiment 3

Hatching time of eggs incubated at different aeration rates are shown in Table 4. Hatching time is significantly different ($P < 0.05$). All eggs hatched at around 18:00 in the afternoon. Dissolved oxygen increased and ammonia concentration decreased in incubation tanks with increasing aeration rate (Table 4). Hatching rate and survival of 3-day old larvae increased significantly ($P < 0.05$) with increasing aeration rate (Table 5).

Table 4. Hatching time of humpback grouper eggs incubated at different aeration rates, dissolved oxygen (DO) and ammonia concentration in the incubation water.

Aeration rate (ml/min)	Hatching time (hour)	Dissolved Oxygen (ppm)	Ammonia (ppm)
A (0)	18.63 ± 0.39^{a}	2.50–3.40	0.445–0.892
B (200)	18.20 ± 0.03^{ab}	4.50–5.00	0.380–0.646
C (400)	18.13 ± 0.03^{ab}	4.80–5.40	0.241–0.553
D (600)	18.00 ± 0.23^{b}	4.90–5.50	0.221–0.443

Values in column followed by same letter are not significantly different ($P > 0.05$).

Table 5. Hatching rate of humpback grouper eggs and survival of 3-day old larvae after the eggs were incubated at different aeration rates.

Aeration rate (ml/min)	Hatching rate (%)	Survival (%)
A (0)	21.0 ± 3.6^{a}	31.7 ± 2.9^{a}
B (200)	38.3 ± 2.9^{b}	40.3 ± 2.5^{b}
C (400)	69.3 ± 1.2^{c}	52.3 ± 2.5^{c}
D (600)	78.7 ± 3.2^{d}	62.3 ± 2.5^{d}

Values within a column followed by the same letter are not significantly different ($P > 0.01$).

Discussion

After fertilisation, humpback grouper eggs hatched within 18.41–19.51 hours at an egg density of 500–3000/litre; 18–18.38 hours at aeration rate of 0–600 ml/min; 19.50–20.50 hours at water exchange of 0–200%/day at 29–32°C water temperature and salinity 34 ppt. Hussain et al. (1975) (cited in Kawahara et al. 1997) reported that grouper *Epinephelus tauvina* eggs hatched within 26–35 hours after fertilisation at 27–30°C, much longer than the present observation. Hatching time of *E. fuscoguttatus* eggs was 18–19 hours at 28–30°C water temperature (Chao et al. 1993 cited in Kawahara et al. 1997). The hatching time of artificially-fertilised egg of *E. striatus* was 21.1–22.3 hours for the first-hatching and 23–25.5 hours for the complete hatching at 28°C (Watanabe et al. 1995). However, other environmental conditions, such as

light intensity, aeration and salinity could influence the incubation time of grouper eggs.

The average hatching rate success of *Cromileptes altivelis* eggs was 77%, with an egg density of 500/litre, 71.6% with a water exchange 200%/day, and 78.67% with an aeration rate of 600 ml/minute. Watanabe et al. (1995) reported that hatching success of *E. striatus* eggs was as high as 82.5% at the water temperature of 26–30°C, but the mortality of yolksac larvae was accelerated at higher temperatures. The result of survival rate of pre-feeding stage of *C. altivelis* larvae (day-3) in present study was 62.3% with an aeration rate of 600ml/min. Biochemical quality of eggs that related to the condition of the broodstock can influence larval survival (Watanabe et al. 1984). Other environmental factors such as aeration (water circulation) and light intensity may also influence metabolism and survival of yolksac larvae.

Conclusion

- For optimum hatching rates of *C. altivelis* eggs, an egg density of 500 eggs/litre, water exchange of 200%/day and an aeration rate of 600ml/minute are recommended.

References

Holliday, F.G.T. 1988. The effects of salinity on the eggs and larvae of teleosts. In: W.S. Hoar and D.J. Randall (editors), Fish Physiology. Volume I. Academic Press. London, pp. 293–311.

Mishima, M. and Gonzares. 1994. Some biological aspects on *Cromileptes altivelis* around Palawan Island, Philippines. Suisanzoshoku, 42(2): 354–349 (in Japanese).

Kawahara, S., Shams, A.J., Al-Bosta, A.A., Mansoor, M.M. and Al-Baqqal. 1997. Effects of incubation and spawning water temperature and salinity on egg development of the orange-spotted Grouper *Epinephelus coioides, Serranidae*. Asian Fisheries Science 9: 239–250.

Tridjoko, Slamet, B., Aslianti, T., Wardoyo, Ismi, S., Hutapea, J.H., Setiawati, K.M., Rusdi, I., Makatutu, D., Prijoni, A., Setiadharma, T., Matsuda, H. and Kumagai, S. 1999. The seed production technique of humpback grouper, *Cromileptes altivelis*. Japan International Cooperation Agency (JICA) and Gondol Research Station for Coastal Fisheries, 56 pp.

Watanabe, T., Arakawa, C., Kitajima, C. and Fujita, S. 1984. Effect of nutritional quality of broodstock diets on reproduction of red sea bream. Bull. Jpn. Soc. Scientific Fish., 50: 495–501

Watanabe, W.O., Lee, C.S., Ellis, S.C. and Ellis, E.P. 1995. Hatchery study of the effects of temperature on eggs and yolksac larvae of the Nassau grouper *Epinephelus striatus.* Aquaculture 136: 141–147.

SS-strain Rotifer Culture for Finfish Larvae with Small Mouth Gape

Richard M. Knuckey, Inneke Rumengan and Stenly Wullur

Introduction

In the aquaculture of temperate marine finfish, the rotifer *Brachionus plicatilis* (L-strain) has been the primary live food for early larval stages. As aquaculture has diversified to include tropical marine species the small strain rotifer, *B. rotundiformis,* is now routinely cultured. Many marine finfish have larvae with a small mouth gape that has required the selection of a super small (SS) strain of *B. rotundiformis.* However, even this SS-strain of rotifer is not an optimal size and a smaller size would be ideal for first-feeding finfish larvae.

The objective of this work was to develop culture techniques that would select for smaller sized rotifers and reduce the average size of rotifers in a population. A typical population of rotifers contains reproductive females of varying sizes with the distribution skewed toward the smaller animals. The aim was to apply selection pressures that would exaggerate this skewed distribution and result in a higher proportion of smaller rotifers.

Methods

Small rotifer body size, as a heritable trait related to the size of amictic and resting eggs, was determined by: measuring amictic eggs attached to rotifers; measuring harvested eggs (amictic and resting); isolating individual eggs to form clonal colonies; and measuring adult rotifers of resulting populations. Initial rotifer populations and clonal lines were maintained at 28°C, 30 ppt salinity and fed *Nannochloropsis oculata* at 3×10^6 cell/ml.

Effects of the environmental factors salinity and diet on rotifer body-size were determined. To examine the effect of salinity on the development rate and final size of rotifers, 30 eggs from a clonal culture were placed in each well of a 24-well plate at three salinities (5, 20 and 30 ppt). Over a 24-hour period following hatching, rotifers were removed hourly from a well (n = 30), measured and the time of appearance of reproductive females was noted.

The effect of diet on rotifer body size was determined by feeding rotifers an equal ash-free, dry-weight ration (equivalent to 3×10^6 cells/ml of *N. oculata*) of algae of different cell mass. Algal mass ranged from 1 pg/cell for *Stichococcus* to 10 pg/cell for *N. oculata,* 170 pg/cell for *Tetraselmis* and 572 pg/cell for *Heterocapsa niei*.

In the first experiment, first laid eggs from a clonal culture were distributed among wells of 24-well plates containing each of the algal diets. Three-day old, F1 rotifers were collected from the resulting populations and measured. In the second experiment, 16×1 L rotifer cultures (20 rotifers/ml) were fed with four different algae (4 replicates/algal diet).

Replicates were fed daily and the population adjusted to 20 rotifers/ml. A sample (~40) of harvested, egg-bearing rotifers was measured every second day. After 14 days, the size distribution of the replicate populations was compared to one fed the control species, *N. oculata*.

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Results and Discussion

The SS-strain rotifer was isolated from Centenary Lakes, Cairns. At the start of the program, reproductive females had an average lorica length of 151 ± 15 µm and width of 111 ± 10 µm. The distribution was skewed toward smaller sizes with the length of the smallest reproductive female measured being 96 µm (Fig. 1). A poor relationship was found between the length of the parent and the length of its egg (r^2 = 0.09); and between the area of the parent rotifer and the area of its egg (r^2 = 0.24). The maximum width of amictic eggs averaged 96 ± 11 µm of which 14% were smaller than 85 µm (average –1 SD). Offspring hatched from this sub-group of eggs had an average size of 147 µm and a distribution similar to the initial population. The optimal salinity for hatching and resting eggs was 5 ppt seawater. Resting eggs, collected and sorted into small resting eggs (77 ± 6 µm), hatched to produce females with an average length of 135 ± 9 µm at commencement of egg production. However, selection and culture of the two smallest females (100–120 µm) produced populations with an average body length of 140 and 148 µm (Fig. 2).

Salinity affected the rate of rotifer development. Development was fastest at the lowest tested salinity of 5 ppt and slowed as salinity rose to 20 ppt and 30 ppt (Fig. 3). Rotifers also became reproductive earlier at lower salinity and egg-bearing rotifers appeared before maximal size was attained.

Diet also affected the development of rotifers. After feeding on four equal ration diets of varying particle size (1 pg/cell to 572 pg/cell) for 14 days, the average length and width of the rotifer populations was not significantly different (Fig. 4). However, the distribution of sizes within the populations was different. Rotifers raised on the control diet of *N. oculata* had an average body dimension of 179 µm in length and 140 µm in width. Fifty-six per cent of the population had a body length less than the average and 46% had a body width greater than the average. Rotifers fed *Stichococcus*-like algae, (the smallest diet at 1 pg/cell) had 72% of the population with a body length less than the average length of the control rotifers fed *N. oculata*. Rotifers fed *Tetraselmis* had a larger proportion of wide rotifers with 64% being larger than the average width of those fed the

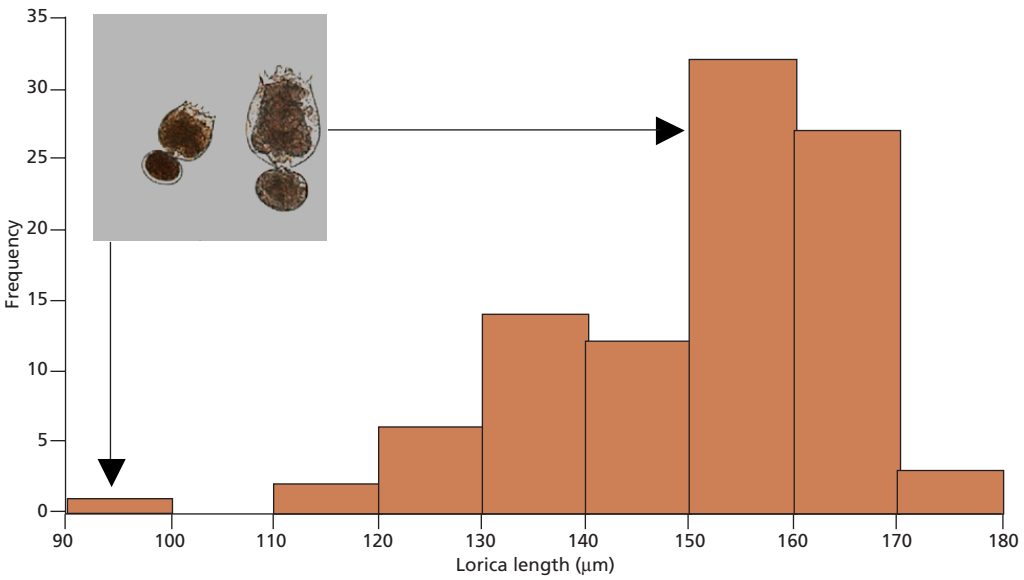


Figure 1. Size distribution of the Cairns isolate of the SS-strain rotifer. Average lorica length = 151 ± 15 µm.

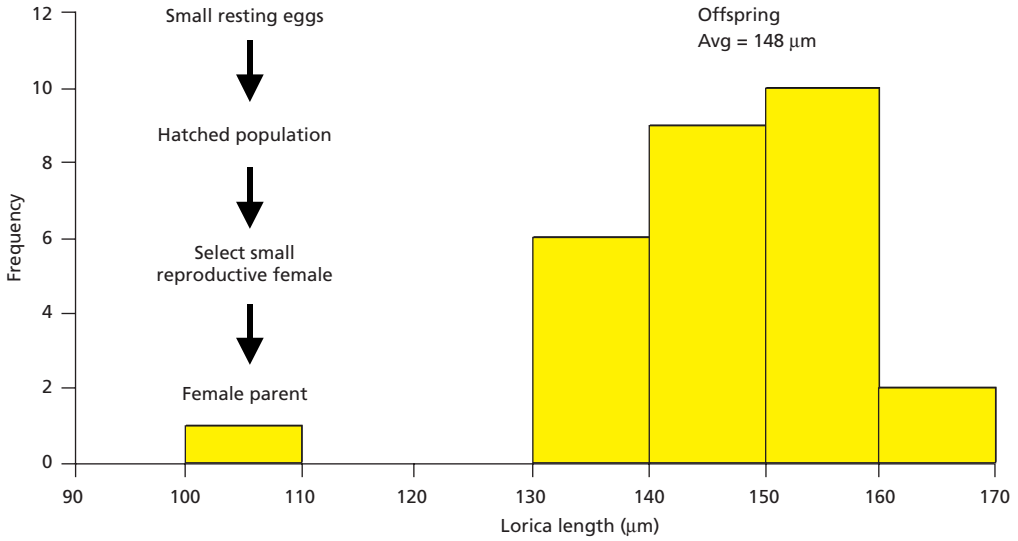


Figure 2. Example of mean size (lorica length) of resulting population of rotifers bred from a single, small parent isolated from a population hatched from smallest resting eggs.

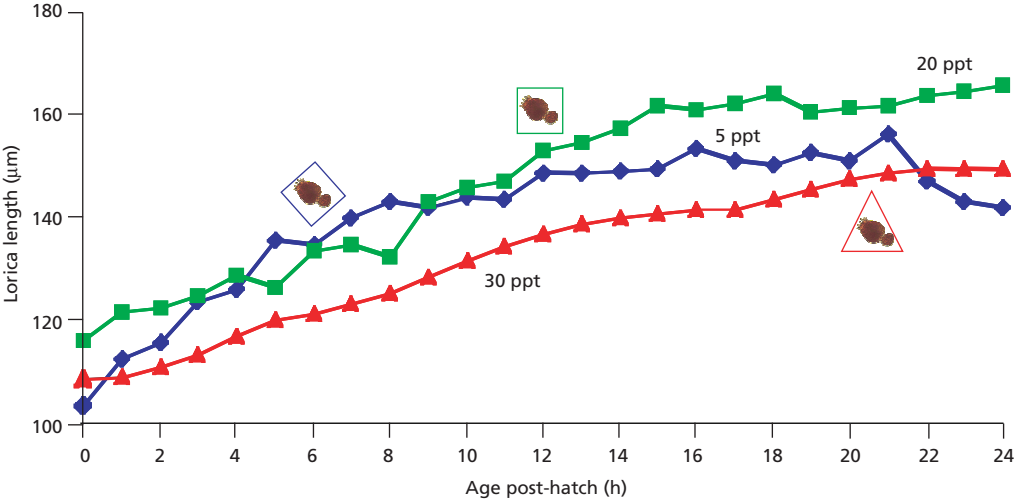


Figure 3. Effect of salinity on the rate of rotifer development (increase in lorica length). Rotifer symbols indicate the time of the first appearance of egg production. All sample points n = 30 rotifers.

control diet *N. oculata*. Rotifers fed the largest size alga, *H. niei,* were similar to those fed the control. This indicates that this alga could be too large for rotifers to ingest so they are feeding on algal cell debris and bacteria.

The results confirm the plasticity of the rotifer lorica and the polymorphism that occurs in populations. Rotifer size may vary by more than 100% between habitats (Ruttner-Kolisko 1977). Increase in rotifer lorica size when a fed diet of large-celled algae (*Tetraselmis*) has been reported (Rumengan et al. 1998). However, Reitan et al. (1997) found differences in lorica length due to different diets that were not large enough to affect their availability to fish larvae. We found diet had no significant effect on the

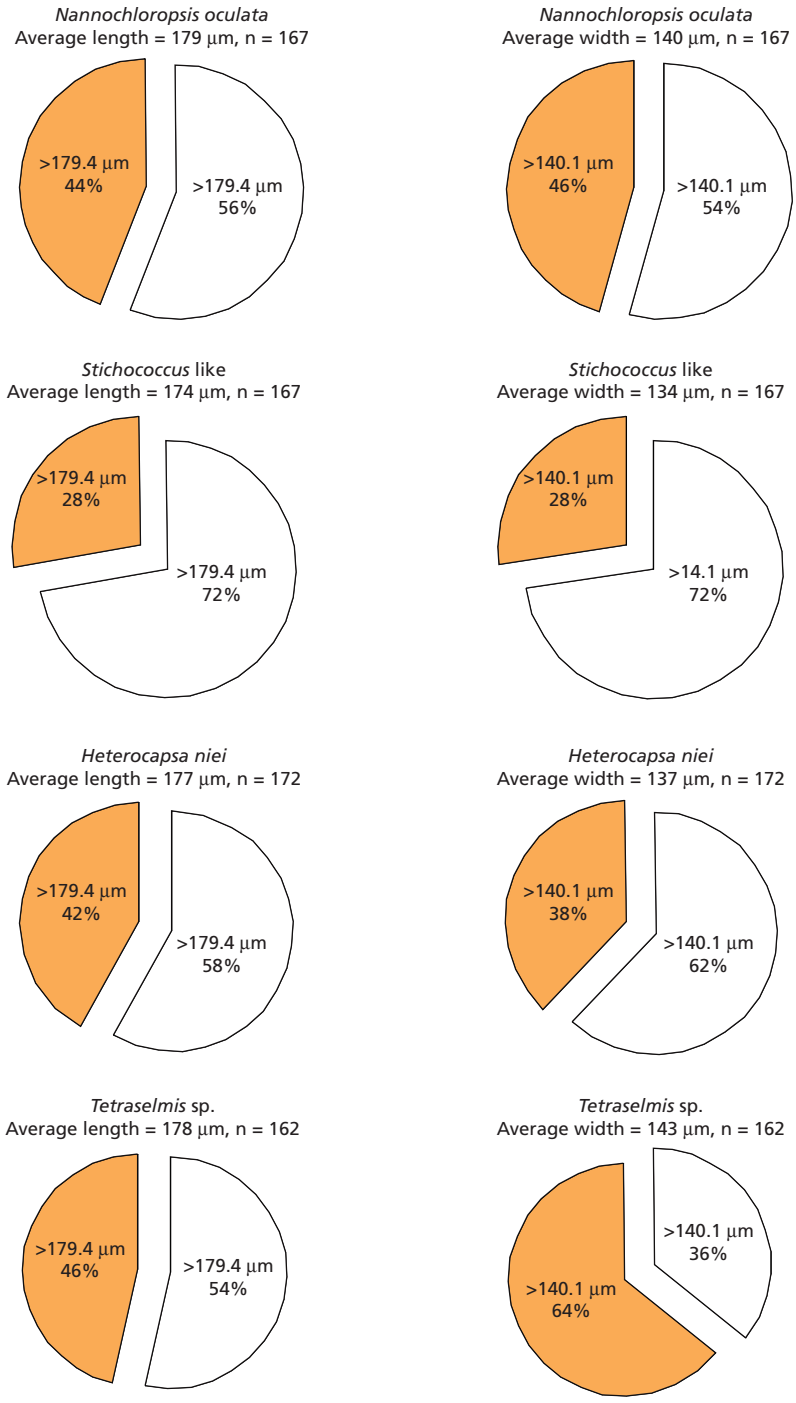


Figure 4. The average size (left: body length; right: body width) and size distribution of populations of rotifers raised for 14 days on equal ration diets of four microalgae of varying particle size (cell mass). Algal cell mass (ash-free, dry-weight/cell) for the control diet *N. oculata* = 10 pg/cell. For the test diets, *Stichococcus*-like = 1 pg/cell, *Tetraselmis* sp. 170 pg/cell and *H. niei* = 572 pg/cell. Size distributions are in relation to the average size (length 179.4 µm and width 140.1 µm) of the control rotifers fed *N. oculata*.

average size of rotifers but feeding with very small algae did increase the percentage of smaller rotifers within a population. This is beneficial when rotifers are used to feed fish larvae with a small mouth gape.

Conclusions

- Higher percentages of smaller rotifers suitable for first feeding larvae are obtained when rotifers are raised on ultra-small algae such as *Stichococcus*.
- Selection of small resting eggs is more successful than amictic eggs in producing a rotifer population of reduced size.
- Much of the skewness toward smaller sized reproductive rotifers in a population is a result of rotifers reproducing before achieving maximal size. This reduces the chance of successfully reducing the size of rotifers by selecting for small reproductive females since most of these rotifers will continue to increase in size as they mature. Synchronous rotifer cultures are required for this purpose, in which all rotifers have reached maximal size, before the smallest individuals are selected.

References

Rumengan, I.F.M., Warouw, V. and Hagiwara, A. 1998. Morphometry and resting egg production potential of the tropical ultra-minute rotifer *Brachionus rotundiformis* (Manado strain) fed different algae. Bulletin of the Faculty of Fisheries, Nagasaki University, 79, 31–36.

Reitan, K.I., Rainuzzo, J.R., Øie, G. and Olsen, Y. 1997. A review of the nutritional effects of algae in marine fish larvae. Aquaculture 155, 207–221.

Ruttner-Kolisko, A. 1977. Suggestions for biomass calculation of planktonic rotifers. Archiv für Hydrobiologie Beihefte Ergebnisse der Limnologie 8, 71–76.

DPI&F researcher Dr Richard Knuckey with UNSRAT staff and students.

Changes in the Gastrointestinal Tract and Associated Organs During Early Development of the Grouper (*Epinephelus coioides*)

G.F. Quinitio, A.C. Sa-an, J.D. Toledo and J.D. Tan-Fermin

Introduction

Production of grouper, *Epinephelus coioides,* juveniles for stocking in grow-out ponds and floating net cages is hindered by problems encountered during larval rearing. Although some advances in grouper larval rearing have been made using live food organisms (Duray et al. 1997; Toledo et al. 1999), there is still a need to further improve survival. One approach is to understand the nutritional physiology of the larvae. The development of the gastrointestinal tract during growth may be related to habitat, body form, and diet of the fish (Ferraris et al. 1987). Knowledge of the changes directly associated with the process of food assimilation is important for understanding the nutritional physiology of the larvae (Segner et al. 1993) and thereby improve survival under laboratory rearing conditions. This paper describes the morpho-histological changes in the gastrointestinal tract of *E. coioides* and associated organs during its early development.

Methods

Larvae of *E. coioides* were reared in five-tonne rectangular concrete tanks using the semi-intensive culture system described by Toledo et al. (1999). Copepods were propagated by adding nauplii, adults, and copepodids of *Acartia* and *Pseudodiaptomus* to the larval rearing tank 2–3 days before stocking with newly hatched larvae. *Brachionus rotundiformis* were added to the tank at increasing density (2–10 individual/ml) from day 2 to day 18. *Artemia* nauplii and metanauplii were given to satiation starting on day 16 until they could feed on 'trash fish' or pellets. Weaning to trash fish or an artificial diet started at day 30 to day 35. Larval samples were collected at day 0 (newly hatched), day 2, day 4, day 6, day 8, day 10, day 12, day 14, day 16, day 20, day 25, day 30, day 35, day 40, day 45, and day 60. Total length (TL) of about 10–20 larvae per sampling was measured. Water temperature and salinity during the rearing period were 26–28°C and 20–30 ppt, respectively.

Larvae were preserved in Bouin's solution, dehydrated through a graded alcohol series, and embedded in paraffin. Prior to embedding in paraffin, a portion of the head and tail were cut-off in large larvae to reduce the size of the sample for sectioning. About 6–8 µm longitudinal sections were stained with Harris' hematoxylin and eosin and alcian blue-periodic acid-Schiff (AB-PAS) (Cook 1990). At least three samples were examined from each stage for longitudinal sections using light microscopy. Depending on the number of samples available (1–3 larvae), 6–8 µm cross-sections were also processed and examined to counter-check the observations made in the longitudinal sections.

Results and Discussion

Newly hatched (day 0) grouper larvae (TL: 1.42–1.80 mm, n = 20) have a large yolk that contains a single oil globule. The primordial digestive tract was a straight tube located above the yolk sac and below the notochord. At day 2 (TL: 2.00–2.70 mm, n = 20), the mouth and anus opened. The intestine and rectum were already distinct from each other due to the presence of the intestinal-rectal valve between them. Moreover, the liver was observed with the gall bladder next to it while the pancreas was located at the dorsal side of the mid-portion of the intestine and posterior to the swim bladder.

At day 4 (TL: 2.37–2.66 mm, n = 20), the yolk sac and oil globule were resorbed. The oesophagus was differentiated and the anterior part of the intestine had clearly formed into the primordial stomach. Coiling of the intestine was observed in day 6 larvae (TL: 2.50–3.21 mm, n = 10) and the spleen was located posterior to the swim bladder. At day 10 (TL: 2.33–3.60 mm, n = 20), the primordial stomach had broadened into a voluminous pouch and increased in size by day 12 (TL: 2.55–4.23 mm, n = 20) and transformed into a stomach.

At day 30 (TL: 11.38–22.6 mm, n = 20), the pancreas continued to enlarge and extend posteriorly along the intestine. The pyloric caeca was already prominent at this age. A prominent blind sac was observed at day 35. From day 40 (TL: 14.20–22.4 mm, n = 20) to day 60 (TL: 31.61–66.95 mm, n = 20), the pancreas and liver continued to enlarge. These changes are very similar to the description by Trijuno (2001) of coral trout, another grouper species.

The histological changes in the digestive tract of *E. coioides* from day 0 to day 60 are summarised in Table 1. The straight, undifferentiated digestive tract of day 0 larvae was composed of simple cuboidal cells. In day 2 larvae, the pharynx, oesophagus, primordial stomach, and intestine showed cellular differentiation. Mucosal folds with villi at the apical region were already apparent at day 4. Moreover, supranuclear vacuoles with eosinophilic granules were seen in the rectum, which increased in number as the larvae grew. These vacuoles have also been observed in many marine fish larvae (Tanaka 1971, 1972 a, b; Walford and Lam 1993; Kaji et al. 1996, 1999; Trijuno 2001). The function of these supranuclear vacuoles is for protein absorption before differentiation of the digestive glands before the stomach is fully functional. These vacuoles gradually disappeared as the stomach became fully functional.

Mucus cells were observed in the pharynx and oesophagus at day 8 and were AB-PAS positive. In the intestine, goblet cells were seen from day 12 and were also AB-PAS positive. At day 20, gastric glands were first observed in the stomach and few goblet cells were observed in the pyloric caeca. Tanaka (1973) described these occurrences as an indication of transformation from larva to juvenile.

Proliferation of gastric glands was observed at day 30 which may be an indication that the larvae were developing the ability to feed on fish as reported for Pacific bluefin tuna (Kaji et al. 1996) and yellowfin tuna (Kaji et al. 1999). At day 35, the lamina propria and submucosa in the stomach were very distinct while the blind sac had become prominent. The tissue layers in the intestine had also become well developed. Therefore, feeding minced fish to *E. coioides* larvae at day 35, when reared using the semi-intensive system as developed by Toledo et al. (1999), appears to be appropriate. There is minimal morpho-histological change from day 40 to day 60.

Conclusion

- The digestive tract of day 0 larvae was a straight, undifferentiated tube composed of simple cuboidal cells.
- At day 2, cellular differentiation was observed in the pharynx, oesophagus, primordial stomach, and intestine.
- The primordial stomach broadened into a voluminous pouch at day 10.
- The gastric gland was observed in the stomach from day 20.
- Day 35 seems to be the proper time to feed fish larvae fish flesh when using the semi-intensive rearing system.
- There were no significant morpho-histological changes in the metamorphosing grouper larvae from day 40 to day 60.

Table 1. Summary of histological changes in the digestive tract of the grouper, *E. coioides,* larvae.

Age (post-hatching)	Region of Digestive Tract				
	Pharynx	Esophagus	Stomach	Pyloric Caeca	Intestine
0	Straight, undifferentiated tube made up of simple cuboidal cells				
2	Stratified squamous cells	Stratified squamous cells, +CM	Cuboidal cells, +CM	Absent	Tall columnar cells, +CM
4	Sac-like widening, muscle layer	Mucosal folds	Tall columnar cells, mucosal folds	Absent	Mucosal folds, wider lumen, apical region with villi, SV with eosinophilic granules in rectum
6	+LP and +SM but not distinct from each other	+LP and +SM but not distinct from each other	NSC	Absent	Increased no. of SV in rectum
8	Pharyngeal teeth, mucus cells, taste buds	Mucus cells, developing LM, thicker CM than stomach	NSC	Absent	NSC
10	NSC	NSC	NSC	Absent	NSC
12	NSC	LP/SM, CM and +S distinct from each other	Distinct CM, hollow space below mucosal epithelium	Absent	+Goblet cells, hollow space below mucosal epithelium
14	NSC	Increased no. of mucus cells, CM continued thickening	Developed mucosal folds, +LP and +SM not distinct, +S	Absent	Few goblet cells, thin strand along hollow space, decreased no. of SV in rectum
16	NSC	NSC	NSC	Absent	+S
20	Moderate no. of mucus cells	+LM below LP and SM which are now distinct from each other, numerous mucus cells	2 mucosal cell layers (outermost mucosal epithelium and below are gastric glands), thicker CM in pyloric region than cardiac region	Present with goblet cells	Moderate no. of goblet cells, taller mucosal folds, LP and SM not distinct, no SV in rectum
25	NSC	Reduced SM by the invasion of LM	NSC	NSC	NSC, abundant SV with eosinophilic granules in rectum
30	Abundant mucus cells, developed LP and SM	Developed 4 tissue layers (taller mucosal folds with numerous mucus cells, SM with LM, thicker CM and S)	Gastric glands proliferate, taller mucosal folds and deeper pits	Present with abundant goblet cells	Abundant goblet cells, distinct LP, mucosal folds branching, no SV in rectum
35	LP and SM very distinct	LP and SM very distinct	LP and SM very distinct, blind sac distinct	Prominent	Well-developed tissue layers, abundant SV in rectum
40	NSC	Well-developed LP	NSC	Branching	Further height increase of mucosal folds and more branching, 1 fish with SV in rectum
45	NSC	Well-developed tissue layers	Well-developed tissue layers	Pronounced branching	Distinct SM
60	NSC	NSC	NSC	NSC	NSC

CM = circular muscle layer; NSC = no significant change; LM = longitudinal muscle layer; LP = lamina propria; S = serosa layer; SM = submucosa; SV = supranuclear vacuoles; + = present.

References

Cook, H.C. 1990. Carbohydrates. In: Bancroft J.D., Stevens A., Turner D.R. (Eds), Theory and Practice of Histology Techniques. Third Edition. Churchill Livingstone, U.K. 177–213 pp.

Duray, M.N., Estudillo, C.B. and Alpasan, L.G. 1997. Larval rearing of the grouper *Epinephelus suillus* under laboratory conditions. Aquaculture 150, 63–76.

Ferraris, R.P., Tan J.D. and de la Cruz, M.C. 1987. Development of the digestive tract of milkfish, *Chanos chanos* (Forsskal): Histology and histochemistry. Aquaculture 61, 241–257.

Kaji, T., Tanaka, M., Takahashi, Y., Oka, M. and Ishibashi, N. 1996. Preliminary observations on development of Pacific bluefin tuna *Thunnus thynnus* (Scrombridae) larvae reared in the laboratory, with special reference to the digestive system. Marine and Freshwater Research 47, 261–269.

Kaji, T., Tanaka, M., Oka, M., Takeuchi, H., Ohsumi, S., Teruya, K. and Hirokawa, J. 1999. Growth and morphological development of laboratory-reared yellowfin tuna *Thunnus albacores* larvae and early juveniles, with special emphasis on the digestive system. Fisheries Science 65, 700–707.

Segner, H., Rösch, R., Verreth, J. and Witt, U. 1993. Larval nutrition physiology: Studies on *Clarias gariepinus*, *Coregonus lavaretus* and *Scophthalmus maximus*. Journal of the World Aquaculture Society 24, 121–134.

Tanaka, M. 1971. Studies on the structure and function of the digestive system in teleost larvae-III. Development of the digestive system during postlarval stage. Japanese Journal of Ichthyology 18, 164–174.

Tanaka, M. 1972a. Studies on the structure and function of the digestive system in teleost larvae-IV. Changes in the epithelium related to fat absorption in the anteromedian part of the intestine after feeding. Japanese Journal of Ichthyology 19, 15–25.

Tanaka, M. 1972b. Studies on the structure and function of the digestive system in teleost larvae-V. Epithelial changes in the posterior-gut and protein ingestion. Japanese Journal of Ichthyology 19, 172–180.

Tanaka, M. 1973. Studies on the structure and function of the digestive system of teleost larvae. PhD Thesis, Department of Fisheries, Faculty of Agriculture, Kyoto University, Japan. 136 pp.

Toledo, J.D., Golez, S.N., Doi, M. and Ohno, A. 1999. Use of copepod nauplii during early feeding stage of grouper *Epinephelus coioides*. Fisheries Science 65, 390–397.

Trijuno, D.D. 2001. Studies on the development and metamorphosis of coral trout *Plectropomus leopardus* under rearing experiments. PhD thesis. Graduate School of Agriculture, Kyoto University, Japan. 124 pp.

Walford, J. and Lam, T.J. 1993. Development of digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juvenile. Aquaculture 109, 187–205.

Localisation of Enzymes in the Digestive System During Early Development of the Grouper (*Epinephelus coioides*)

G.F. Quinitio, A.C. Sa-an, J.D. Toledo and J.D. Tan-Fermin

Introduction

With growth, the morphological structure of the digestive tract of fish larvae becomes more complex and is accompanied by periods of sharp increases in enzyme activity (Timeyko and Novokov 1987). Understanding this process would be helpful in improving growth and survival of fish larvae. Several studies have been done on the localisation of digestive enzymes in larval fishes, including those by Ferraris et al. (1987), Minjoyo (1990), Sarasquete et al. (1995), Gawlicka et al. (1995), and Trijuno (2001).

This paper describes the occurrence of some digestive enzymes in the gastrointestinal tract during early development. The work was conducted to provide information on formulating an appropriate feeding scheme and an artificial diet for the early development of the grouper *Epinephelus coioides*.

Methods

Larvae of *E. coioides* were reared in five-tonne rectangular concrete tanks using the semi-intensive culture system described by Toledo et al. (1999). Copepods were propagated by adding nauplii, adults and copepodids of *Acartia* and *Pseudodiaptomus* to the larval rearing tank two to three days before stocking with newly hatched larvae. *Brachionus rotundiformis* were added to the tank at increasing density (2–10 individual/ml) from day 2 to day 18. *Artemia* nauplii and metanauplii were given to satiation starting on day 16 until they began to feed on 'trash fish' or pellets. Weaning to trash fish or an artificial diet started at day 30 to day 35.

Larval samples were collected at day 0 (newly-hatched), day 2, day 4, day 6, day 8, day 10, day 12, day 14, day 16, day 20, day 25, day 30, day 35, day 40, day 45, and day 60.

Water temperature and salinity during the rearing period were 26-28°C and 20-30 ppt respectively.

Larvae were collected and fixed in formal calcium at 4°C for 18 hours, washed in tap water, blotted dry, placed in gum sucrose at 4°C for 18 hours, blotted dry again and then embedded in an embedding medium for frozen specimens (Bancroft 1990 — with slight modification). Samples were kept in a freezer at –80°C until cryostat sectioning. Longitudinal sections (8–10 µm) were cut using a Minotome cryostat at about –12°C. Sections were collected on a glass slide. The digestive enzymes localised were acid phosphatase (ACP), alkaline phosphatase (ALP), non-specific esterase (NSE), aminopeptidase (AMP), trypsin, (TRP), maltase (MAL), and lipase (LIP).

Techniques used were based on those of Cousin et al. (1987), Bancroft (1990), Cook (1990), Gawlicka et al. (1995), and Goodsell et al. (1995). About three to four larvae were used per enzyme.

Results and Discussion

The distributions of enzyme activity in the digestive system of grouper larvae at different ages are summarised in Tables 1 and 2. Most of the digestive enzymes were not observed at day 0 in *E. coioides* larvae, except for weak activity of the ACP and MAL enzymes in the yolk sac and weak staining of NSE in the oil globule.

Enzymes were localised in the pharynx, esophagus, intestine, and liver of day 2 larvae. NSE was found in all of these organs at this age with the intestine having the most enzymes. Among the enzymes observed in the intestine at day 2 (ACP, ALP, NSE, TRP, and MAL), ALP and NSE were stained intensely whereas ACP and MAL were moderate.

In general, weak activity of the enzymes occurs during the yolk sac stage because the larvae are still dependent on endogenous nutrition for metabolism as observed in other fishes (Buddington and Christofferson 1985; Ferraris et al. 1987; Minjoyo 1990). However we observed strong staining of ALP and NSE in the intestine from day 2 in the grouper larvae. Stroband et al. (1979) suggested that the presence of ALP during the early larval stage is necessary for nutrient transport in the intestine when the larvae start exogenous feeding. High levels of ALP are usually associated with absorptive cells (Troyer 1980).

NSE was already strong in the intestine from day 2 while AMP was weak at day 2 but its activity slowly became strong as the larvae grew. Ferraris et al. (1987) also made this observation in milkfish and suggested that esterases may be more essential than aminopeptidases since grouper and milkfish larvae feed on rotifers and copepods as their initial food. The late occurrence of high levels of AMP in grouper larvae (day 14) happened several days prior to feeding on brine shrimp (Toledo et al. 1999). Minjoyo (1990) correlated a high level of AMP in day 20 sea bass larvae, *Lates calcarifer,* to its carnivorous habit.

TRP in the intestine of *E. coioides* larvae exhibited weak staining during initial feeding, then stronger staining during active feeding, weak staining again at the start of brine shrimp feeding and strong again thereafter. Such fluctuations were also seen in the grouper *Plectropomus leopardus* (Trijuno 2001) and sea bass *Dicentrachus labrax* (Cahu and Zambonino-Infante 1994). It is also interesting to note that this enzyme was very weak in all the digestive organs at day 16, but was again strong in most organs at day 20 in *E. coioides.* The latter coincided with the appearance of the gastric glands. In the sea bass, Walford and Lam (1993) observed there is sharp decline in TRP activity after the stomach has become functional.

MAL was also always present in the intestine from day 2 and was strong from day 14 onwards indicating that grouper larvae have the capacity to digest carbohydrates. A similar trend was seen in flatfishes by Martinez et al. (1999). Several other carnivorous larvae have also been shown to have this capacity, particularly during the first half of larval development (Oozeki and Bailey 1995; Moyano et al. 1996; Kim, 2001; Divakaran et al. 1999).

LIP activity in the intestine was weak from day 14, moderate at day 20, and stronger from day 25. The increase in enzyme activity coincided with the occurrence of gastric glands at day 20 and increased in number thereafter.

Conclusions

- Weak enzyme activity occurs during the yolk sac stage when the grouper larvae are still dependent on endogenous nutrition for metabolism.
- High AMP activity started at day 14 prior to *Artemia* feeding at day 16.
- Fluctuations in TRP activity may be related to stomach formation.
- Occurrence of MAL during early development demonstrates a capacity to digest carbohydrates.
- An increase in LIP activity coincides with the occurrence of gastric glands.
- No significant changes in digestive enzymes were observed in the metamorphosing grouper larvae from day 40 to day 60.

Table 1. Distribution of enzyme activity in the digestive system of day 0 to day 14 grouper, *E. coioides,* larvae.

Age of Larvae (post-hatch)	Digestive Organs								
	Pharynx	Esophagus	Stomach		Pyloric caeca	Intestine	Liver	Pancreas	Spleen
			Columnar cells	Gastric glands					
0						++ALP ++NSE			
2	+ACP +NSE +MAL	+ACP +NSE				++ACP +++ALP +++NSE +TRP ++MAL	+ALP ++NSE +MAL		
4	+ACP ALP* ++NSE +TRP +MAL	+ACP ALP* ++NSE +TRP +MAL	+ACP +MAL			+++ACP +++ALP +++NSE +AMP +TRP ++MAL	+++ACP ++ALP +++NSE +MAL	ACP* ALP* TRP* +MAL	
6	+ACP ALP* ++NSE +TRP +MAL	+ACP ALP* ++NSE +TRP +MAL	+ACP ++NSE +MAL			+++ACP +++ALP +++NSE +AMP +TRP ++MAL	+++ACP ++ALP +++NSE +TRP +MAL	ACP* ALP* NSE* TRP* +MAL	++ACP ALP* +NSE TRP* MAL*
8	+ACP ++ALP ++NSE +TRP +MAL	+ACP +ALP ++NSE +TRP +MAL	+ACP ++NSE +TRP +MAL			+++ACP +++ALP +++NSE +AMP +++TRP ++MAL	+++ACP ++ALP +++NSE ++TRP +MAL	ACP* ALP* +TRP +MAL	++ACP ALP* +NSE +TRP +MAL
10	+ACP ++ALP ++NSE ++TRP +MAL	+ACP ++ALP ++NSE ++TRP +MAL	+ACP ++NSE +++TRP +MAL			+++ACP +++ALP +++NSE +AMP +++TRP ++MAL	+++ACP ++ALP +++NSE ++TRP +MAL	+ACP ALP* ++TRP +MAL	++ACP ALP* +NSE ++TRP +MAL
12	+ACP ++ALP ++NSE ++TRP +MAL	+ACP ++ALP ++NSE ++TRP +MAL	+ACP ++NSE +++TRP +MAL			+++ACP +++ALP +++NSE ++AMP +++TRP ++MAL	+++ACP ++ALP +++NSE ++TRP +MAL	+ACP +ALP ++TRP +MAL	++ACP ALP* +NSE ++TRP +MAL
14	++ACP ++ALP ++NSE ++TRP +MAL	++ACP ++ALP ++NSE ++TRP +MAL	+ACP ++NSE ++TRP +MAL			+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +LIP	+++ACP ++ALP +++NSE ++TRP +MAL	+ACP ++ALP +++TRP +MAL	+++ACP ALP* +NSE +++TRP +MAL

– = negative; + = weak.; ++ = moderate; +++ = intense; ACP = acid phosphatase; ALP = alkaline phosphatase; NSE = non-specific esterase; AMP = aminopeptidase; TRP = trypsin; MAL = maltase; LIP = lipase. * = organ not observed in samples.

Table 2. Distribution of enzyme activity in the digestive system of day 16 to day 60 grouper, *E. coioides,* larvae.

Age of Larvae (post-hatch)	Digestive Organs								
	Pharynx	Esophagus	Stomach		Pyloric caeca	Intestine	Liver	Pancreas	Spleen
			Columnar cells	Gastric glands					
16	++ACP ++ALP ++NSE +TRP +MAL	++ACP ++ALP ++NSE +TRP +MAL	+ACP ++NSE +TRP +MAL			+++ACP +++ALP +++NSE +++AMP +TRP +++MAL +LIP	+++ACP ++ALP +++NSE +TRP +MAL	+ACP ++ALP +TRP +MAL	+++ACP +ALP +NSE +TRP +MAL
20	++ACP ++ALP ++NSE +AMP ++TRP +MAL	++ACP ++ALP ++NSE +AMP ++TRP +MAL	+ACP ++NSE ++TRP +MAL	+ACP		+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL ++LIP	+++ACP ++ALP +++NSE ++TRP +MAL	+ACP ++ALP +++TRP +MAL	+++ACP +ALP ++NSE +++TRP +MAL
25	++ACP ++ALP ++NSE +AMP +TRP	++ACP ++ALP ++NSE +AMP +TRP	++ACP ++NSE +TRP	++ACP ++NSE	+++ACP +++ALP +++NSE +++AMP TRP* +++MAL +++LIP	+++ACP +++ALP +++NSE +++AMP +TRP +++MAL +++LIP	+++ACP ++ALP +++NSE +TRP	++ACP ++ALP +NSE +TRP	+++ACP +ALP +NSE +TRP
30	++ACP ++ALP ++NSE +AMP +TRP +LIP	++ACP ++ALP ++NSE +AMP +TRP +LIP	++ACP ++NSE +TRP +LIP	++ACP ++NSE	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL ++LIP	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +++LIP	+++ACP ++ALP +++NSE +TRP +LIP	++ACP ++ALP +NSE +++TRP 	+++ACP +ALP +NSE +TRP
35	++ACP ++ALP ++NSE +AMP +TRP +LIP	++ACP ++ALP ++NSE +AMP +TRP +LIP	++ACP ++NSE +TRP +LIP	++ACP ++NSE	+++ACP +++ALP +++NSE +++AMP ++TRP +++MAL +++LIP	+++ACP +++ALP +++NSE +++AMP ++TRP +++MAL +++LIP	+++ACP ++ALP +++NSE –TRP +LIP	++ACP ++ALP +NSE ++TRP 	+++ACP +ALP +NSE –TRP
40	++ACP ++ALP ++NSE +AMP +TRP LIP*	++ACP ++ALP ++NSE +AMP +TRP LIP*	++ACP ++NSE +++TRP +LIP	++ACP ++NSE	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +++LIP	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +++LIP	+++ACP ++ALP +++NSE ++TRP +LIP	++ACP ++ALP +NSE ++TRP 	+++ACP +ALP +NSE ++TRP
45	++ACP ++ALP ++NSE +AMP –TRP +LIP	++ACP ++ALP ++NSE +AMP –TRP +LIP	++ACP ++NSE +++TRP ++LIP	++ACP ++NSE	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +++LIP	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +++LIP	+++ACP ++ALP +++NSE –TRP +LIP	+++ACP ++ALP +NSE ++TRP 	+++ACP +ALP +NSE +TRP
60	++ACP ++ALP ++NSE +AMP +TRP +LIP	++ACP ++ALP ++NSE +AMP +TRP +LIP	++ACP ++NSE +AMP +++TRP ++LIP	++ACP ++NSE +AMP	+++ACP +++ALP +++NSE AMP* +++TRP +++MAL +++LIP	+++ACP +++ALP +++NSE +++AMP +++TRP +++MAL +++LIP	+++ACP ++ALP +++NSE AMP* ++TRP ++LIP	+++ACP ++ALP ++NSE AMP* ++TRP ++LIP	+++ACP ALP* NSE* AMP* TRP*

– = negative; + = weak.; ++ = moderate; +++ = intense; ACP = acid phosphatase; ALP = alkaline phosphatase; NSE = non-specific esterase; AMP = aminopeptidase; TRP = trypsin; MAL = maltase; LIP = lipase. * = organ not observed in samples.

References

Bancroft, J.D. 1990. Enzyme histochemistry. In: Bancroft J.D., Stevens A., Turner D.R. (Eds.), Theory and Practice of Histology Techniques. Third Edition. Churchill Livingstone, U.K. 379–399.

Buddington, R.K. and Christofferson, J.P. 1985. Digestive and feeding characteristics of chondrosteans. Environmental Biology of Fishes 14, 31–41.

Cahu, C.L. and Zambonino-Infante, J.L. 1994. Early weaning of sea bass *Dicentrachus labrax* larvae with compound diet: effect on digestive enzymes. Comparative Biochemistry and Physiology 109, 213–222.

Cook, H.C. 1990. Carbohydrates. In: Bancroft J.D., Stevens A., Turner D.R. (Eds.). Theory and Practice of Histology Techniques. Third Edition. Churchill Livingstone, U.K. 177–213.

Cousin, J.C.B., Baudin-Laurencin, F. and Gabaudan, J. 1987. Ontogeny of enzymatic activities in fed and fasting turbot, *Scophthalmus maximus* L. Journal of Fish Biology 30, 15–33.

Divakaran, K., Kim, B.G. and Ostrowski, A.C. 1999. Digestive enzymes present in Pacific threadfin *Polydactylus sexfilis* (Bloch and Schnieder 1801) and bluefin travelly *Caranx melampygus* (Cuvier 1833). Aquaculture Research 30, 781–187.

Ferraris, R.P., Tan J.D. and de la Cruz, M.C. 1987. Development of the digestive tract of milkfish, *Chanos chanos* (Forsskal): Histology and histochemistry. Aquaculture 61, 241–257.

Gawlicka, A., Teh, S.J., Hung, S.S.O., Hinton, D.E. and Noüe, J. de la. 1995. Histological and histochemical changes in the digestive tract of white sturgeon larvae during ontogeny. Fish Physiology and Biochemistry 14, 357–371.

Goodsell, A., Wikeley, D. and Searle, L. 1995. Histological, histochemical and morphological development of striped trumpeter larvae diet. Final report to the Fisheries Research and Development Corporation. Project 92/139.

Kim, B.G., Divakaran, S., Brown, C.L. and Ostrowki, A.C. 2001. Comparative digestive enzyme ontogeny in two marine larval fishes: Pacific Threadfin (*Polydactylus sexfilis*) and bluefin trevally (*Caranx melampygus*). Fish Physiology and Biochemistry 24, 225–241.

Martinez, L., Moyano, F.L., Fernandez-Diaz, C. and Yufera, M. 1999. Digestive enzymes activity during larval development of the Senegal sole (*Solea senegalensis*). Fish Physiology and Biochemistry 21, 317–323.

Minjoyo, H. 1990. Histochemical studies on the early stages of devlopment of the digestive tract of sea bass *Lates calcarifer* (Bloch). Masters Thesis, Marine Science Institute, College of Science, University of the Philippines. 56 pp.

Moyano, F.J., Diaz, M., Alarcon, F.J. and Saraquete, M.C. 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). Fish Physiology and Biochemistry 15, 121–130.

Oozeki, Y. and Bailey, K.M. 1995. Ontogenetic development of digestive enzyme activities in larval walleye Pollack, *Theragta chalcogramma*. Marine Biology 122, 177–186.

Sarasquete, M.C., Polo, A. and Yúfera, M. 1995. Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L. Aquaculture 130, 79–92.

Stroband, H.W.J., van der Meer, H. and Timmermans, L.P.M. 1979. Regional functional differentiation in the gut of the grass carp, *Ctenopharyngodon idella* (Val.). Histochemistry 64, 235–249.

Timeyko, V.N. and Novikov, G.G. 1987. Proteolytic activity in the digestive tract of Atlantic salmon, *Salmo salar*, during larval development. Journal of Ichthyology 27, 27–33.

Toledo, J.D., Golez, S.N., Doi, M. and Ohno, A. 1999. Use of copepod nauplii during early feeding stage of grouper *Epinephelus coioides*. Fisheries Science 65, 390–397.

Trijuno, D.D. 2001. Studies on the development and metamorphosis of coral trout *Plectropomus leopardus* under rearing experiments. PhD Thesis. School of Agriculture, Kyoto University, Japan. 124 pp.

Troyer, H. 1980. Principles and techniques of histochemistry. Little Brown and Co., Boston. 433 pp.

Walford, J. and Lam, T.J. 1993. Development of digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juvenile. Aquaculture 109, 187–205.

Digestive Enzyme Activity in Developing Grouper (*Epinephelus coioides*) Larvae

P.S. Eusebio, J.D. Toledo, R.E.P. Mamauag and M.J.G. Bernas

Introduction

Different fish species have their own unique digestion and food assimilation properties due to differences in the structure of their digestive tracts and in their way of feeding. Knowledge of the functional changes that are taking place in the digestive tract during food ingestion, digestion and assimilation is necessary to determine the ability of fish larvae to utilise a given diet (Segner et al. 1994). Timeyko and Novokov (1987) found that the complexity of the morphological structure of the digestive tract is accompanied by periods of sharp increases in enzyme activity. The variations in digestive enzyme activity during larval development are indicative of the type and level of macronutrients that should be included in artificial feeds (Cahu and Zambonino-Infante 1995). This study was undertaken to determine the activity of alkaline and acid type proteases, α-amylase, lipase, trypsin, chymotrypsin, leucine aminopeptidase, and alkaline and acid phosphatases during larval development of the grouper *Epinephelus coioides*.

Methods

Samples for measuring digestive enzyme activity were collected (sampling time: 8:00–11:00 hours) at different larval stages: day 0, day 2, day 4, day 8, day 12, day 16, day 20, day 25, day 30, day 35, day 40, day 45, day 50, day 55 and day 60. Whole larvae were used in the preparation of a crude enzyme extract from day 0 to day 30 and the mid-portion of the larvae was used from day 35 to day 60. Samples were freeze-dried, weighed and stored in a bio-freezer at–68°C prior to the preparation of crude enzyme extracts. Freeze-dried larvae (70 mg/3.5 ml) were homogenised in 50 mM Tris-HCl buffer, pH 7.5, centrifuged (12,500 × G, 30 min at 4°C), filtered through a Sephadex G-25 M column (1 × 10 cm.), centrifuged (2000 × G, 5 min at 4°C) and then decanted. The supernatant (crude enzyme extract) was used for total protein and different enzyme assays.

Total protein was determined using the method of Lowry et al. (1951). Alkaline type protease activity was measured using 1% casein as substrate; and one unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 μg of tyrosine per minute (modified method of Walter (1984)). Acid type protease (pepsin) activity was determined using haemoglobin as a substrate; and one unit of pepsin activity expressed in tyrosine was equal to 0.001 of TCA soluble hydrolysis products per minute under standard conditions (Worthington Biochemical Corporation 1993). α-Amylase activity was quantified using soluble starch as a substrate; and one unit was defined as the amount of enzyme able to produce one micromole of reducing groups (calculated as maltose) per minute at 25°C (Worthington Biochemical Corporation 1993).

Lipase activity was measured as the rate of hydrolysis of an olive oil emulsion that was determined by titration using a pH meter (Worthington Biochemical Corporation 1993). One unit of activity was equal to one micromole

of acid produced per minute at 25°C under specified conditions. The activity of trypsin, chymotrypsin and leucine aminopeptidase was quantified according to methods described by Worthington Biochemical Corporation (1993). Trypsin activity was equivalent to one micromole of Nα-p-Tosyl-L-arginine Methyl Ester (TAME) that was hydrolysed per minute (25°C; pH 8.1), chymotrypsin activity was equivalent to one micromole of N-Benzoyl-2-monophosphate-Na-Ca (BTEE) that was hydrolysed per minute (25°C; pH 7.8), and the activity of leucine aminopeptidase was equal to one micromole of leucinamide hydrolysed per minute (25°C; pH 8.5). The activities of acid and alkaline phosphatases were determined at pH 4.8 and pH 9.8 respectively with nitrophenyl phosphate as the substrate (Bergmeyer 1974). The amount of 4-nitrophenol liberated per unit time in acidic solution was a measure of acid phosphatase activity, while the amount of 4-nitrophenol liberated per unit time in alkaline solution was a measure of alkaline phosphatase activity.

Results and Discussion

The total protein concentration of the newly hatched larvae (day 0) to day 2 was negligible (0.04 μg/larva). The concentration gradually increased with age of the larvae from 0.3 μg/larva at day 12 to 84.8 μg/larva at day 60 (Fig. 1). Both alkaline and acid type protease activities (Fig. 2) were detected at early stages of development in grouper larvae. Alkaline type protease activity was identified in the newly hatched larvae (0.01 mU/larva) and gradually increased to a peak at day 50 (7334.9 mU/larva). In contrast, acid type protease (pepsin) activity was not detected in the newly hatched larvae, but was detected at day 2 (2.2 U/larva). Its activity started to progress from day 12 (53.2 U/larva), which can be associated with the formation of the stomach. A two-fold increase in pepsin activity was observed from day 16 (53.2 U/larva) and every five days thereafter until day 40 (2706.7 U/larva). The decrease in the activity of alkaline type protease from day 50 to day 60 can be linked to metamorphosis. The relationship between a marked decrease in the specific activity of alkaline type protease and metamorphosis was reported by Tanaka et al. (1996) in Japanese flounder, *Paralichthys olivaceus,* and also by Alliot et al. (1980) in Senegal sole.

Figure 3 shows that α-amylase activity was detected in day 2 larvae (0.03 U/larvae) and a progressive increase was observed from day 16 (0.7 U/larva) until day 60 (141.9 U/larva). Early detection of α-amylase activity has also been reported for other marine fish larvae and in all cases, the activity increased with age (Munilla-Moran et al. 1990). Moyano et al. (1996) observed that a marked increase in the activity of α-amylase in seabream was closely related to

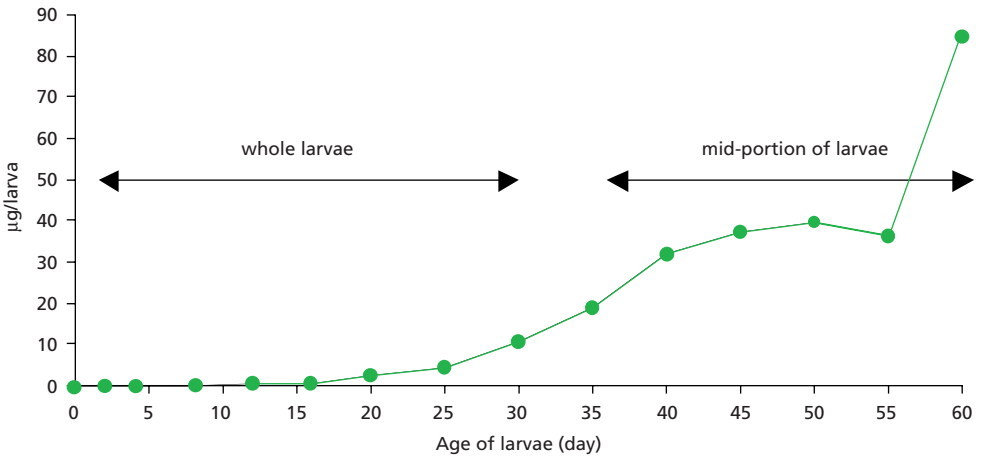


Figure 1. Total protein concentration in crude enzyme extracts from grouper larvae.

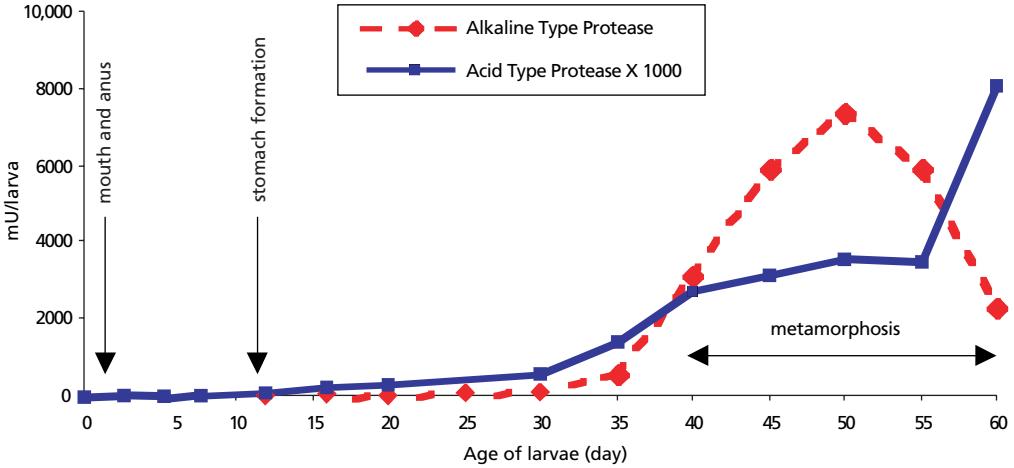


Figure 2. Protease activity in crude enzyme extracts from grouper larvae.

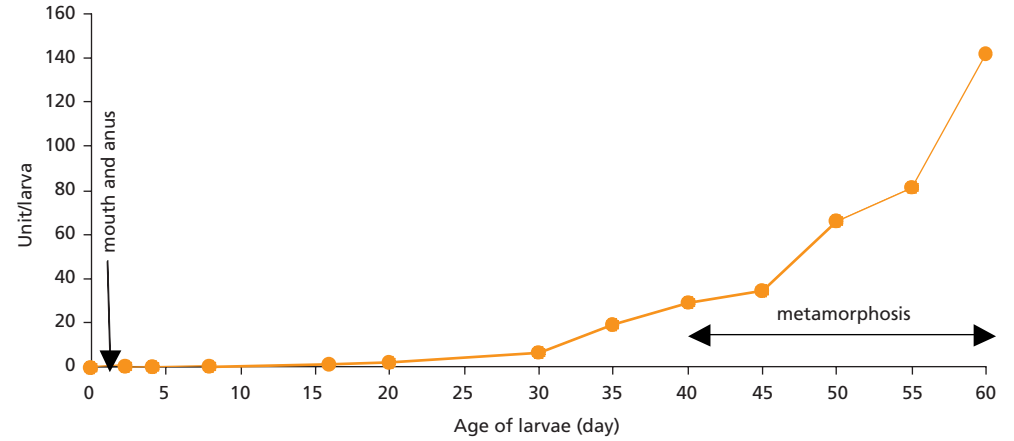


Figure 3. α-amylase activity in crude enzyme extracts from grouper larvae.

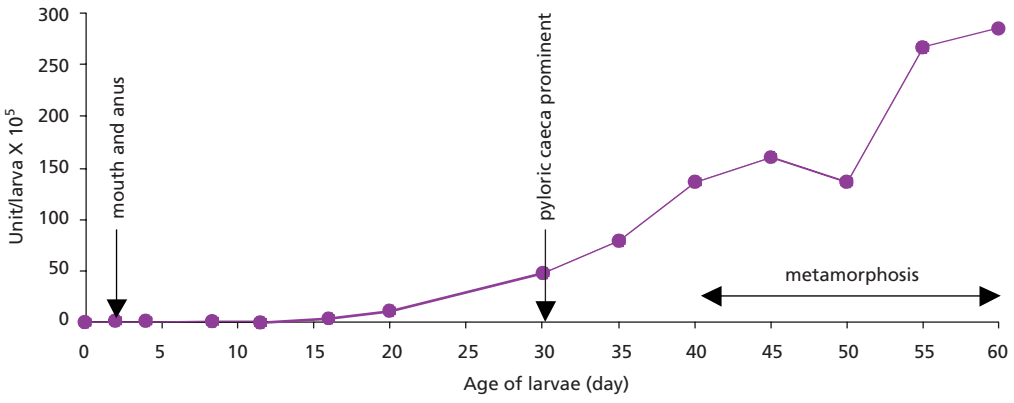


Figure 4. Lipase activity in crude enzyme extracts from grouper larvae.

its feeding habits. In this study, α-amylase activity in grouper larvae increased with age and they may be capable of digesting carbohydrates at day 16. Also, the activity of lipase increased with age of grouper larvae (Fig. 4). The gradual increase in lipase activity (0.04–285 × 10^{-5} U/larva) can be related to the development of the pyloric caeca and intestine, which were fully developed at day 30.

Leucine aminopeptidase activity started to increase to an appreciable amount from day 16 (5.4 U/larva) until day 60 (447.3 U/larva) but was highest at day 40 (601.5 U/larva), which was the onset of metamorphosis in the grouper larvae (Fig. 5). The trypsin and chymotrypsin activity patterns are shown in Figure 6. Trypsin activity (9.0–16,407.5 mU/larva) seemed to be higher than chymotrypsin activity (1.3–10,368.7 mU/larva) from day 8 until day 60. However, there was a change in the pattern when chymotrypsin activity increased from 1964.6 mU/larva at day 40 to 2932.3 mU/larva at day 45 while trypsin activity decreased from 3130.6 mU/larva at day 40 to 709.5 mU/larva at day 50. As shown in Figure 7, the activity of acid phosphatase was increasing, which started from day 12 until day 60 (0.1–46.5 mU/larva), whereas alkaline phosphatase activity started from newly hatched

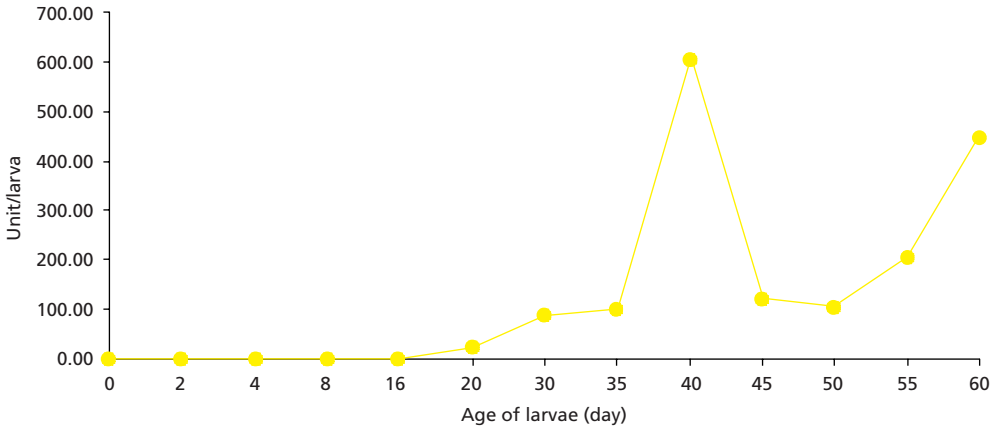


Figure 5. Leucine aminopeptidase activity in crude enzyme extracts from grouper larvae.

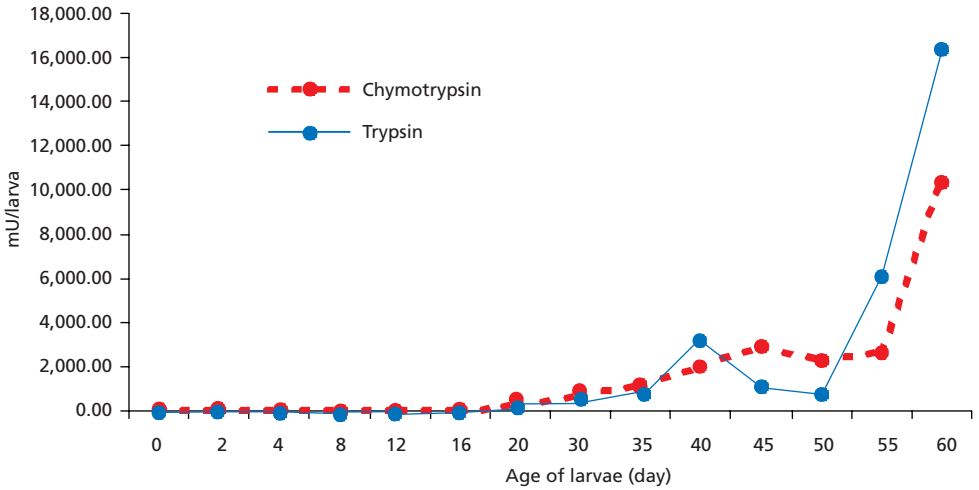


Figure 6. Trypsin and chymotrypsin activity in crude enzyme extracts from grouper larvae.

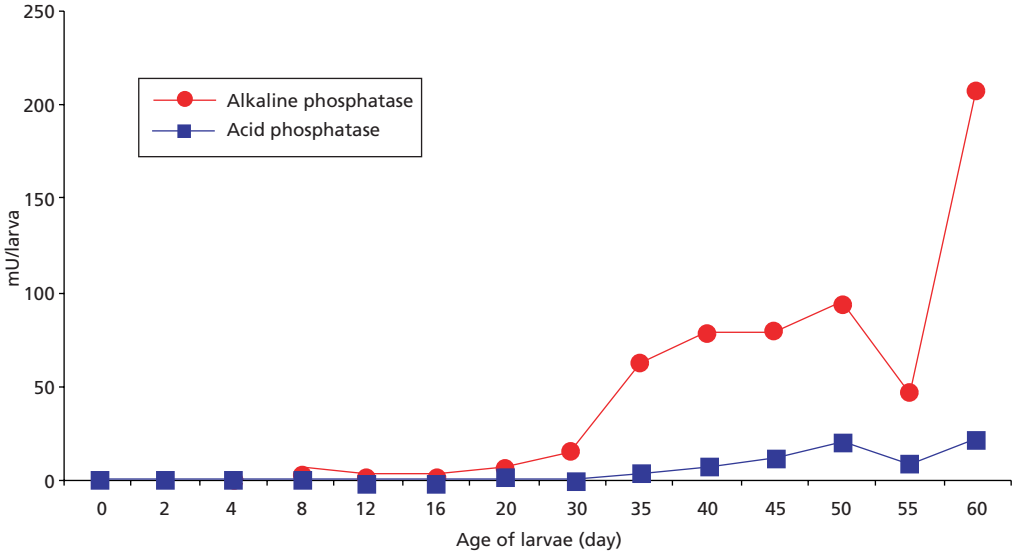


Figure 7. Acid and alkaline phosphatase activity in crude enzyme extracts from grouper larvae.

larvae (day 0) to day 60 (0.03–207.2 mU/larva). Both enzymes showed similar profiles, with higher values for alkaline phosphatase activity during metamorphosis. Moyano et al. (1996) also found that the activity of alkaline phosphatase was higher than that of acid phosphatase in gilthead seabream larvae.

Conclusion

- The maximum variation in specific activities of alkaline and acid type proteases, α-amylase, lipase, trypsin, chymotrypsin, leucine aminopeptidase, and acid and alkaline phosphatases in the digestive tract of grouper larvae are mostly related to the onset or the end of metamorphosis during larval development.

References

Alliot, E., Pastoureaud, A. and Trellu, J., 1980. Evolution des activites enzymatiques dans le tractus digestif au cours de la vic larvaire de la sole. Variations des proteinogrammes et des zymogrammes. Biochemical Systematics and Ecology 8, 441–445.

Bergmeyer, H.U. 1974. Methods of Enzymatic Analysis. Vol 2. Phosphatases. Academic Press, New York. 856–860.

Cahu, C.L. and Zambonino-Infante, J.L. 1995. Effect of molecular form of dietary nitrogen supply in sea bass larvae: response of pancreatic enzyme and intestinal peptidases. Fish Physiology and Biochemistry 14, 209–214.

Lowry, O.H., Roserbrough, N.J., Farr, A.L. and Randall, N.J. 1951. Estimation of total protein. Journal of Biological Chemistry 193, 265.

Moyano, F.J., Diaz, M., Alarcon, F. J. and Sarasquete, M.C. 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). Fish Physiology and Biochemistry 15, 121–130.

Munilla-Moran, R., Stark, J.R. and Barbour, A. 1990. The role of exogenous enzymes in digestion in culture of turbot larvae (*Scopthalmus maximus* L.). Aquaculture 88, 337– 350.

Segner, H., Storch, V., Reinecke, M., Kloas, W. and Hanke, W. 1994. The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus.* Marine Biology 119, 471–486.

Tanaka, M., Kawai, S., Seikai, T. and Burke, J.S. 1996. Development of the digestive organ system in Japanese flounder in relation to metamorphosis and settlement. Marine and Freshwater Behaviour and Physiology 28, 19–31.

Timeyko, V.N. and Novikov, G.G. 1987. Proteolytic activity in the digestive tract of Atlantic salmon, *Salmo salar,* during larval development. Voprosssy Ikhtiologii 2, 300–306.

Walter, H.E. 1984. Proteinases: Methods with hemoglobin, casein and azocoll as substrates. In: Methods of Enzymatic Analysis. Bergmeyer, H.J. (Ed.) Vol. V. Verlag Chemie, Weinham. 270–277.

Worthington Biochemical Corporation, 1993. Worthington, V. (Ed.). Worthington Enzyme Manual: Enzymes, and related biochemicals. Lakewood, New Jersey, USA. 411 pp.

The Activity of Digestive Enzymes in Larval Grouper and Live Feed

S. McBride

Introduction

Low and inconsistent survival in the larval rearing of groupers is a major production bottleneck (Hussain and Higuchi 1980; Kohno et al. 1997; Toledo et al. 1999). The digestive tract of first feeding grouper larvae is rudimentary (G. Quinitio unpublished data; McBride unpublished data) and there is a short window of opportunity for the successful transition from endogenous to exogenous nutrition in comparison with many other marine species (Kohno et al. 1990; Ordonio-Aguilar et al. 1995). A better understanding of the digestive physiology of grouper larvae could assist in improving the quality and accessibility of nutrients from the diet. The aim of these studies was to investigate the ontogeny of digestive enzymes in larval groupers and assess the suitability of different live feeds.

Methods

Larvae of *Epinephelus coioides* were reared in a green-water semi-intensive system in five-tonne tanks at the Southeast Asian Fisheries Development Center (SEAFDEC) facility in Tigbauan (Iloilo, Philippines) as described by Toledo et al. (1999). Larvae of *Cromileptes altivelis* were reared at the Gondol Research Institute for Mariculture (Bali, Indonesia) in five-tonne concrete tanks in a green-water culture system as described by Sugama et al. (2001). Larvae of *E. fuscoguttatus* were reared in 300 L tanks in a clear-water recirculation system at the Northern Fisheries Centre (Cairns, Australia). Samples of live prey organisms (SS-strain rotifers *Brachionus rotundiformis* and the copepod *Acartia sinjiensis*) were collected from standard cultures at the Northern Fisheries Centre.

Three to five replicates of pooled larvae (5–30 depending on age) were collected each sampling day except for *C. altivelis* where one or two replicates were collected. Samples of *E. fuscoguttatus* were only collected from three to six days post hatch (DPH) after which there was total mortality. A known number of live prey organisms were collected in triplicate. Samples were homogenised in a 10 mM Tris-HCl (pH 7.5) buffer, and centrifuged before the supernatants were collected for enzyme and protein analysis.

Concentration of soluble protein was determined using BioRad Protein Assay (Bradford) reagents (USA). Total protease and α-amylase activity were measured by sensitive fluorescent assays using casein and starch substrates respectively (Molecular Probes, USA). The activity of bile salt-dependent (bsd) lipase was estimated by an absorbance assay using the substrate 4-nitrophenyl caproate (4-NPC) (Gjellesvik et al. 1992). All enzyme assays were performed at 30°C. One unit of total protease activity was defined as the percentage change in fluorescence units from a negative control per hour. One unit of amylase activity was defined as the amount of enzyme required to liberate one milligram of maltose from starch in three minutes. One unit of bsd lipase was defined as nmoles 4-NPC hydrolysed per hour.

Differences in the emergence of digestive enzyme activity between *E. coioides* and *C. altivelis* were investigated using non-linear regression. A generalised logistic model was found the most appropriate with the enzyme activity modelled against age and grouped into species.

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Results and Discussion

Generally the emergence of digestive enzyme activity in grouper larvae was characterised by three phases.

1. Low activities were detected in the three grouper species prior to nine DPH. An exception was bsd lipase activity, which was not detected in *E. coioides* or *E. fuscoguttatus* over this period.
2. The second phase occurred between 10 and 18 DPH in *E. coioides* and *C. altivelis*. Modulations in digestive enzyme activity were observed and corresponded with key developmental changes of the gastro-intestinal tract in *E. coioides* (G. Quinitio unpublished data) and *C. altivelis* (McBride, unpublished data).
3. From 20 DPH, enzyme activity generally increased with age in both *E. coioides* and *C. altivelis* (Figs. 1 and 2).

The emergence of total protease and amylase activity with age in *E. coioides* was significantly different to the activities in *C. altivelis* ($P < 0.001$; adjusted $R^2 = 0.892$ and 0.960 respectively). The emergence of bsd lipase activity with age was similar between the two species ($P = 0.238$). These findings suggest the two species may have different abilities to digest proteins and carbohydrates at the larval stage and this is likely to have implications for the development of artificial diets for larvae and juveniles.

Total protease activity in early feeding *E. coioides* larvae increased in response to initial feeding incidence (Fig. 3). In contrast, amylase activity was not correlated with feeding incidence (Fig. 3). Live food organisms may stimulate enzyme activity in the gut of early stage larvae either by their physical presence (Hjelmeland et al. 1988; Pedersen et al. 1987), the release of hormonal factors (Hjelmeland et al. 1988; Kamisaka et al. 2001; Srivastava et al. 2002) or by contributing an exogenous source of digestive enzymes (Dabrowski and Glogowski 1977; Lauff and Hofer 1984; Munilla-Moran et al. 1990; Oozeki and Bailey 1995; Pedersen et al. 1987).

Significant differences in digestive enzyme activities were observed between the live feed organisms (Fig. 4). The potential contribution from the live feed to the enzyme activity measured in a larva was estimated by multiplying the activity per individual prey item by the total number of prey items observed in the

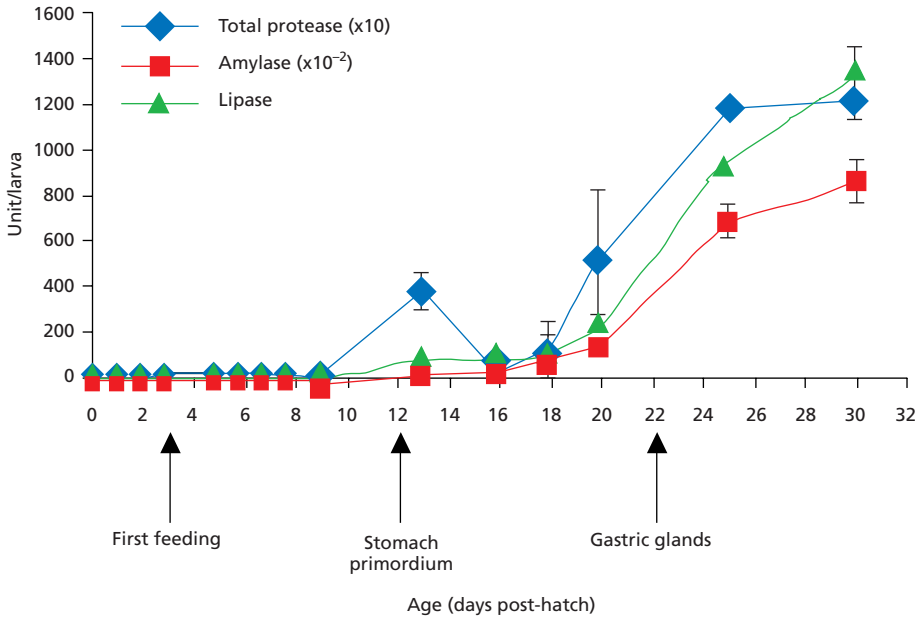


Figure 1. Emergence of digestive enzyme activities in *E. coioides* larvae with age. Arrows indicate major morphological changes in the gut development of larval *E. coioides*.

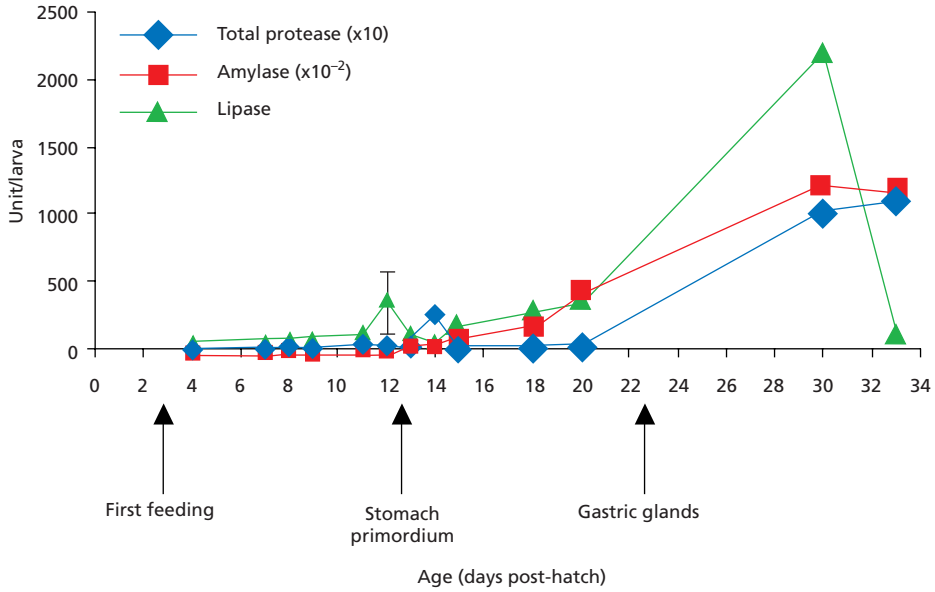


Figure 2. Emergence of digestive enzyme activities in *C. altivelis* larvae with age. Arrows indicate major morphological changes in the gut development of larval *C. altivelis*.

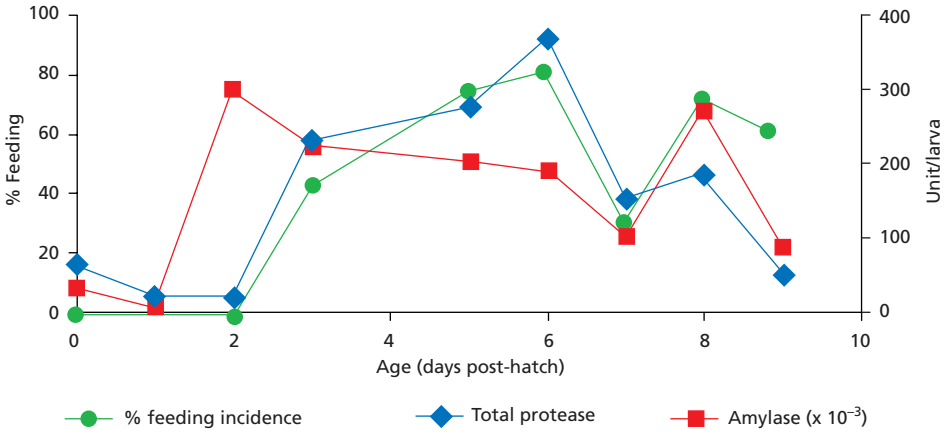


Figure 3. Correlation between feeding incidence in *E. coioides* larvae and the activity of total protease (r = 0.791, P = 0.011) and amylase (r = 0.468, P = 0.204).

gut for each age reported by Toledo et al. (1999). Rotifers contributed only 0.7% of total protease so it is unlikely that they make a significant contribution to larval digestion by providing exogenous protease enzymes. Non-feeding naupliar stages of *Arcartia* (n1–n2) contributed less than 2.5% of total protease activity and the feeding stages (n3–n4) contributed up to 35.6% of total protease activity. These results indicate that n3–n4 copepod nauplii are potentially a significant source of exogenous proteases for the larvae. Surprisingly, the potential contribution of amylase from rotifers and copepod nauplii was relatively high (Fig. 5).

Copepod nauplii contained approximately twice the amount of soluble protein than

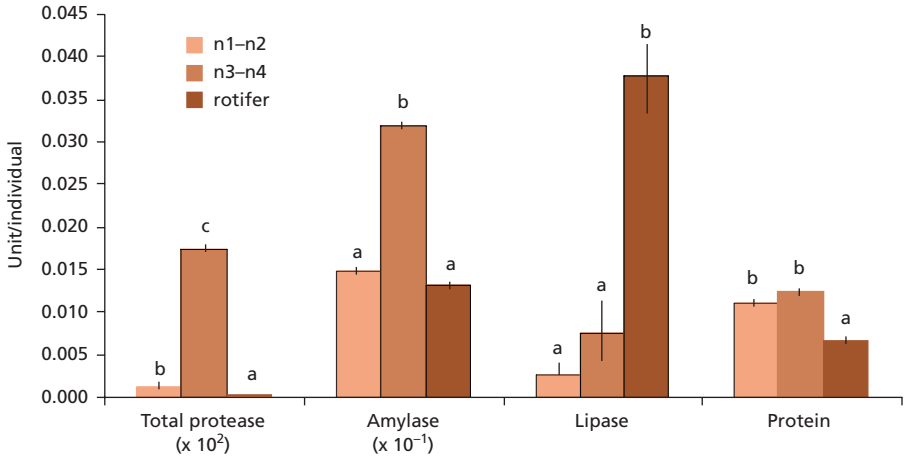


Figure 4. The digestive enzyme activities and protein content in rotifers, and n1–n2 and n3–n4 copepod nauplii. A unit/individual is a unit of enzyme activity/individual (total protease, amylase and lipase) or one µg of protein/individual (protein). Means within a category that are not significantly different share common superscripts (ANOVA; $P > 0.01$).

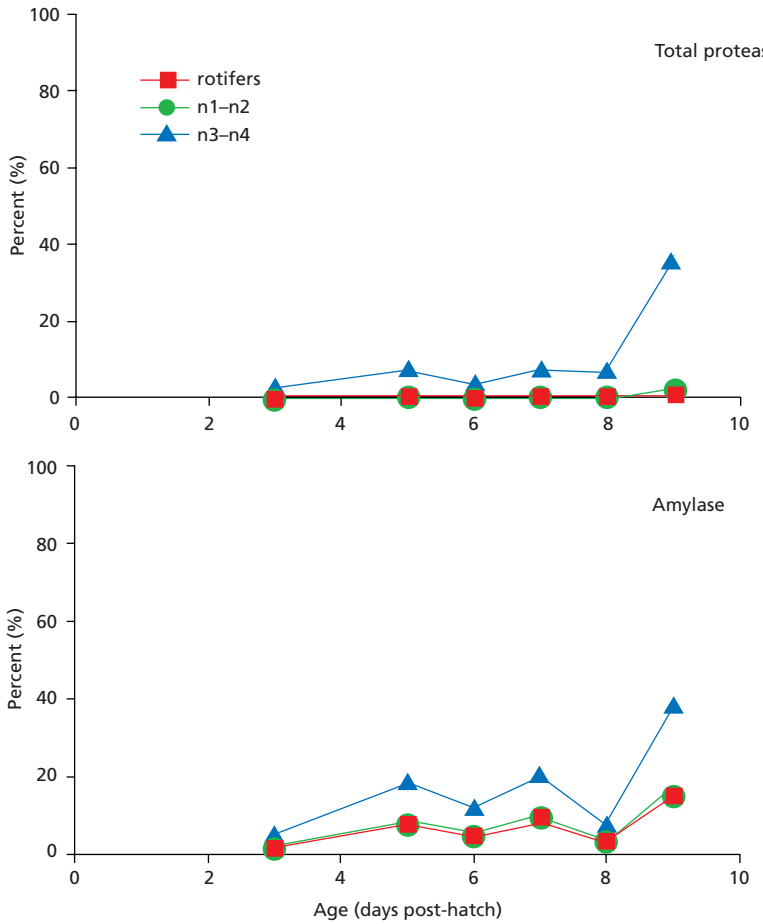


Figure 5. The estimated percent contribution of total protease and amylase activity from live feed to the respective activities measured in *E. coioides* larvae.

rotifers. The greater amount of soluble protein and protease enzymes in copepod nauplii indicates that they may provide a greater opportunity for access to protein than rotifers. This may have implications for the successful transition to exogenous feeding in grouper larvae (Ordonio-Aguilar et al. 1995). Improving nutrition during the initial feeding stages (3 to 9 DPH) may be a key to improving the quality of larvae, which are then able to undergo the major morphological changes between 10 to 20 DPH, faster and more successfully.

Conclusions

- Generally, digestive enzyme activities in larval *E. coioides* and *C. altivelis* were low prior to 18 DPH and then increased with age.
- Changes in the activity of digestive enzymes were associated with the morphological development of the digestive system.
- Total protease activity increased with feeding incidence in early feeding (3 to 9 DPH) *E. coioides* larvae.
- The emergence of total protease and amylase activity was different between *E. coioides* and *C. altivelis* larvae.
- n3–n4 copepod nauplii contained high total protease and amylase activities in comparison to n1–n2 nauplii and rotifers.

References

Dabrowski, K. and Glogowski, J., 1977. Studies on the role of exogenous proteolytic enzymes in digestion processes in fish. Hydrobiology 54, 129–134.

Gjellesvik, D.R., Lombardo, D. and Walther, B.T., 1992. Pancreatic bile salt-dependent lipase from cod (*Gadus morhua*): purification and properties. Biochimica et Biophysica Acta 1124, 123–134.

Hjelmeland, K., Pedersen, B.H. and Nilssen, E.M., 1988. Trypsin content in intestines of herring larvae, *Clupea harengus*, ingesting inert polystyrene spheres or live crustacea prey. Marine Biology 98, 331–335.

Hussain, N.A. and Higuchi, M., 1980. Larval rearing and development of the brown-spotted grouper, *Epinephelus tauvina* (Forskål). Aquaculture 19, 339–350.

Kamisaka, Y., Totland, G.K., Tagawa, M., Kurokawa, T., Suzuki, T., Tanaka, M. and Rønnestad, I., 2001. Ontogeny of cholecystokinin-immunoreactive cells in the digestive tract of Atlantic Halibut, *Hippoglossus hippoglossus*, larvae. General and Comparative Endocrinology 123, 31–37.

Kohno, H., Diani, S., Sunyoto, P., Slamet, B. and Imanto, P.T., 1990. Early developmental events associated with changeover of nutrient sources in the grouper, *Epinephelus fuscoguttatus*, larvae. Bulletin Penelitian Perikanan, Special Edition 1, 51–64.

Kohno, H., Ordonio-Aguilar, R.S., Ohno, A. and Taki, Y., 1997. Why is grouper larval rearing difficult?: an approach from the development of the feeding apparatus in early stage larvae of the grouper, *Epinephelus coioides*. Ichthyological Research 44, 267–274.

Lauff, M. and Hofer, R., 1984. Proteolytic enzymes in fish development and the importance of dietary enzymes. Aquaculture 37, 335–346.

Munilla-Moran, R., Stark, J.R. and Barbour, A., 1990. The role of exogenous enzymes in digestion in cultured turbot larvae (*Scophthalmus maximus* L.). Aquaculture 88, 337–350.

Oozeki, Y. and Bailey, K.M., 1995. Ontogenetic development of digestive enzyme activities in larval walleye pollock, *Theragra chalcogramma*. Marine Biology 122, 177–186.

Ordonio-Aguilar, R., Kohno, H., Ohno, A., Moteki, M. and Taki, Y., 1995. Development of grouper, *Epinephelus coioides*, larvae during changeover of energy sources. Journal of Tokyo University of Fisheries 82, 103–118.

Pedersen, B.H., Nilssen, E.M. and Hjelmeland, K., 1987. Variations in the content of trypsin and trypsinogen in larval herring (*Clupea harengus*) digesting copepod nauplii. Marine Biology 94, 171–181.

Srivastava, A.S., Kurokawa, T. and Suzuki, T., 2002. mRNA expression of pancreatic enzyme precursors and estimation of protein digestibility in first feeding larvae of the Japanese flounder,

Paralichthys olivaceus. Comparative Biochemistry and Physiology 132A, 629–635.

Sugama, K., Tridjoko, Slamet, B. Ismi, S., Setiadi, E. and Kawahara, S., 2001. Manual for the seed production of humpback grouper, *Cromileptes altivelis*. Gondol Research Institute for Mariculture and Japan International Cooperation Agency, Indonesia, 37 pp.

Toledo, J.D., Golez, M.S., Doi, M. and Ohno, A., 1999. Use of copepod nauplii during early feeding stage of grouper *Epinephelus coioides*. Fisheries Science 65, 390–397.

Lipid Nutrition Studies on Grouper (*Epinephelus coioides*) Larvae

V.R. Alava, F.M.P. Priolo, J.D. Toledo, J.C. Rodriguez, G.F. Quinitio, A.C. Sa-an, M.R. de la Peña and R.C. Caturao

Introduction

In marine fish, n-3 highly unsaturated fatty acids (HUFA), such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are important constituents of cell membranes, especially in the brain and retina; and are needed during early life stages to assure normal visual and neural development. Since arachidonic acid (20:4n-6, ARA) has an important function in producing eicosanoids, it is also an essential fatty acid (EFA) for marine fish larvae.

There were three main objectives of this project. The first was to study the lipid chain transfer from the egg stage through hatching and the patterns of lipid conservation or loss during starvation and feeding of larvae in order to elucidate the lipid metabolism of grouper (Phase 1).

The second objective was to determine the fatty acid composition of HUFA boosters and enriched live food organisms to make it possible to choose food organisms that provide various dietary levels and ratios of DHA: EPA: ARA (Phase 2).

The third objective was to determine the effect of *Brachionus* and *Artemia,* containing different levels and ratios of DHA: EPA: ARA, on the growth and survival of grouper larvae (Phase 3).

Methods

Total lipids (TL) of samples were extracted (Folch et al. 1957), separated into neutral (NL) and polar lipids (PL) in silica cartridges (Juaneda and Rocquelin 1985), the fatty acids methyl esters were prepared (AOAC 1996) and analysed on a gas chromatograph (Shimadzu GC-17A). Three to six replicate samples and analyses were done. Results were compared using ANOVA and Duncan's multiple range test at $P < 0.05$.

In Phase 1, the samples collected were: floating neurula eggs, newly hatched (NHL) and unfed 4-day old larvae (Table 1); larvae fed with live food organisms for 25 and 35 days or starved for three days (day 28 and 38, Table 2); and wild-sourced larvae starved for a week (Table 3).

In Phase 2, the samples collected were: phytoplankton, *Brachionus* cultured in phytoplankton for four days; the cladoceran *Diaphanosoma celebensis*; and the copepod *Pseudodiaptomus annandalei* (Table 4 A, B, C and D). Other samples collected in this phase were: enrichment products and *Brachionus* enriched with products (HUFA boosters) at 320 mg/million for 14 hours (Table 5A and B); *Brachionus* starved for 3, 6 and 12 hours, or one hour enriched with an emulsion (Table 6); and *Artemia* nauplii and pre-adults enriched with different HUFA boosters at 200 mg/L of seawater/100,000 nauplii for 14 hours (Table 7A and B).

In Phase 3, early feeding larvae (day 2) stocked at 20/L were fed *Brachionus* (Table 5B) until day 14 in 300 L of water with a salinity of 22–24 ppt, while at early metamorphic stage (day 25) larvae were stocked at 1/L and fed *Artemia* (Table 7B) for 10 days in 200 L of seawater. A completely randomised design with three replicates per treatment was followed and results were compared using ANOVA and Duncan's multiple range test at $P < 0.05$.

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Table 1. Neutral (NL) and polar (PL) fatty acids in *E. coioides* eggs, newly hatched larvae (NHL), and unfed day 4 larvae (μg ind^{-1} DW)[1,2].

	NL			PL			NL % loss[3]		PL % loss[3]	
	Egg	NHL	Day 4	Egg	NHL	Day 4	NHL	Day 4	NHL	Day 4
NL or PL	5.15^a	3.04^b	0.61^c	1.77^a	1.49^b	1.25^c	40.9	88.16	15.8	29.37
Fatty acids:										
Saturates	1.75^a	1.10^b	0.16^c	0.84^a	0.65^b	0.60^c	37.1	90.86	22.6	28.57
Monoenes	1.52^a	0.96^b	0.15^c	0.27^a	0.24^b	0.19^c	36.8	90.13	11.1	29.63
PUFA	1.88^a	0.99^b	0.29^c	0.66^a	0.60^b	0.47^c	47.3	84.57	9.1	28.79
20:4n-6	0.16^a	0.11^b	0.06^c	0.10^a	0.10^a	0.05^b	31.2	62.50	0.0	50.00
20:5n-3	0.22^a	0.12^b	0.00	0.07^a	0.04^b	0.02^c	45.4	100.00	42.7	71.43
22:6n-3	0.58^a	0.21^b	0.06^c	0.18^a	0.16^b	0.14^c	63.7	89.66	11.1	22.22
Ratio:			—							
DHA:EPA	2.63^a	1.75^b	1.00^c	2.57^a	4.00^b	7.00^c				
DHA:ARA	3.63^a	1.91^b	—	1.80^a	1.60^b	2.80^c				
EPA:ARA	1.37^a	1.09^b	0.61^c	0.70^a	0.40^b	0.40^b				

[1] Egg and larval dry weight (μg ind^{-1} DW): neurula eggs, 26.82; NHL, 24.04; and unfed day 4 larvae, 7.36.
[2] Treatment means in rows under NL or PL with the same superscripts are not significantly different ($P > 0.05$).
[3] Percent loss from egg stage.

Table 2. Larval dry weight (DW), total (TL), polar (PL), and neutral (NL) lipids and fatty acids of *E. coioides* larvae fed live food organisms for 25 and 35 days then starved for three days.

	Fed (day)		3 day starving		% gain	% loss	
	25	35	28	38	25–35	25–28	35–38
Larval DW (mg ind^{-1})	3.2^a	51.0^b	1.7^a	44.8^b	1622.0	46.2	12.2
TL (μg ind^{-1})	172.7^a	2664.3^b	76.0^a	1905.4^b	1542.2	56.0	28.5
PL (μg ind^{-1})	37.2^a	867.0^b	21.3^a	608.4^b	1325.7	42.6	27.8
NL (μg ind^{-1})	135.6^a	1797.3^b	54.7^a	1297.0^b	2331.8	59.7	29.8
NL FA (μg ind^{-1}):							
Saturates	47.7^a	530.3^b	18.3^a	404.3^b	1111.8	61.6	23.8
Monoenes	39.2^a	605.3^b	15.8^a	495.2^b	1542.5	59.8	18.2
PUFAs	48.6^a	661.8^b	20.6^a	397.5^b	1360.5	57.7	39.9
20:4n-6	4.9^a	67.6^b	2.3^a	42.4^b	1369.9	52.7	37.3
20:5n-3	7.4^a	114.4^b	1.6^a	75.6^b	1538.6	78.3	33.9
22:6n-3	18.3^a	173.1^b	4.0^a	131.7^b	947.7	77.9	23.9
Ratio:							
DHA:EPA	2.5^a	1.5^b	2.5^a	1.7^b			
DHA:ARA	3.7^a	2.6^b	1.7^a	3.1^b			
EPA:ARA	1.5^a	1.7^b	0.7^a	1.8^b			

[1] Treatment means in rows under 'fed' or '3 day starving' with the same superscripts are not significantly different ($P > 0.05$).

Table 3. Neutral (NL) and polar (PL) lipids and fatty acids in wild-sourced starved *E. coioides* larvae.

	NL		PL		% loss	
	Initial	day 7	Initial	day 7	NL	PL
NL or PL (mg ind^{-1})	1.3^a	0.5^b	1.5^a	1.3^b	64.8	19.4
FA (μg ind^{-1}):						
Saturates	572.4^a	100.8^b	560.6^a	152.6^b	82.4	72.8
Monoenes	318.9^a	103.5^b	368.8^a	324.1^b	67.5	12.1
n-3 FA	337.1^a	134.7^b	300.3^a	489.4^b	60.0	
n-3 HUFA	304.9^a	131.9^b	268.7^a	466.4^b	56.7	
20:4n6		34. 9		39.2		
20:5n3	56.6^a	27.1^b	57.2^a	134.4^b	52.2	
22:6n3	170.0^a	0.0	211.5^a	15.0^b	100.0	92.9
Ratio:						
DHA:EPA	3.0		3.7^a	0.1^b		
DHA:ARA	—		—	0.4		
EPA:ARA	—	0.8	—	3.4		

[1] Dry weight (mg ind^{-1}) of initial larvae and starved for seven days were 116.1 ± 3.6 and 96.5 ± 2.3.
[2] Treatment means in rows under NL or PL with the same superscripts are not significantly different ($P > 0.05$).

Table 4. Total lipids (TL), HUFA levels and ratios in phytoplankton, *Brachionus*, *Diaphanosoma*, and *Pseudodiaptomus* cultured in various feeds.

	TL % DM	% of total fatty acids			Ratio		
		20:4n-6	20:5n-3	22:6n-3	DHA:EPA	DHA:ARA	EPA:ARA
A. *Phytoplankton*:							
Chlorella vulgaris	0.3[e]	—	—	2.9[b]	—	—	—
Isochrysis galbana	3.8[a]	—	—	12.1[a]	—	—	—
Nannochloropsis oculata	3.3[b]	—	—	—	—	—	—
Tetraselmis tetrahele	2.2[c]	—	3.5[c]	—	—	—	—
Chaetoceros calcitrans	1.8[d]	—	9.4[b]	—	—	—	—
Thalassiosira pseudonana	0.1[f]	—	16.2[a]	1.8[c]	0.1	—	—
B. *Brachionus* cultured in phytoplankton:							
Chlorella vulgaris	13.6[a]	5.0[a]	13.5[a]	8.4[a]	0.6[a]	1.7	2.7[b]
Tetraselmis tetrahele	9.7[c]	1.2[c]	1.3[d]	—	—	—	1.1[c]
Chaetoceros calcitrans	12.1[b]	2.8[b]	10.0[b]	—	—	—	3.6[a]
Isochrysis galbana	8.2[d]	—	—	1.3[b]	—	—	
Nannochloropsis oculata	6.1[e]	1.2[c]	2.5[c]	—	—	—	2.1[b]
Thalassiosira pseudonana		—	9.7[b]	1.7[b]	0.2[b]	—	—
C. *Diaphanosoma celebensis* cultured in:							
Rice bran	15.3[a]	0.1	—	—	—	—	—
Cow dung	8.7[c]	—	—	—	—	—	—
Tetraselmis tetrahele	10.1[b]	—	0.4	0.1	0.2	—	—
D. *Pseudodiaptomus annandalei* cultured in:							
Chlorella vulgaris		3.9[b]	7.6[a]	28.6[c]	3.8[b]	7.3[c]	1.9[b]
Chaetoceros calcitrans		3.8[b]	7.3[a]	30.4[a]	4.2[a]	8.0[b]	1.9[b]
Isochrysis galbana		3.3[c]	7.5[a]	29.2[b]	3.9[b]	8.7[a]	2.3[a]
Tetraselmis		5.6[a]	6.5$_b$	10.0[d]	1.5[c]	1.8[d]	1.2[c]

[1]Treatment means in columns under each subheading with the same superscripts are not significantly different ($P > 0.05$).

Table 5. Total lipids (TL), HUFA levels and ratios in HUFA boosters; and in *Brachionus* fed these HUFA boosters.

	TL % DM	% of total fatty acids			Ratio		
		20:4n–6	20:5n–3	22:6n-3	DHA:EPA	DHA:ARA	EPA:ARA
A. HUFA boosters:							
Algamac 2000 (Alg2000)	27.0[e]	—	7.0[c]	20.0[g]	2.9[b]	—	—
Algamac 3050 (Alg3050)	34.8[d]	—	—	38.2[d]	—	—	—
Aquagrow Advantage (AgAdv)	10.8[h]	—	—	58.6[a]	—	—	—
Aquagrow *Chlorella* (AqChl)	20.5[g]	—	—	45.6[c]	—	—	—
Aquagrow Feed 15 (AqF15)	15.7[g]	—	—	54.1[b]	—	—	—
Aquagrow AA (AqAA)	25.2[f]	45.4[a]	0.4[e]	0.3[j]	1.0[d]	0.01[e]	0.01[e]
HUFA Enrich	64.3[a]	0.8[c]	14.3[b]	13.3[i]	0.9[d]	16.2[d]	17.4[b]
Ratio HUFA	61.4[b]	0.5[d]	5.7[d]	22.7[f]	4.0[a]	49.2[a]	12.3[d]
Super HUFA	60.8[c]	1.2[b]	23.8[a]	28.5[e]	1.2[c]	24.4[c]	20.3[a]
DHA Protein Selco	27.3[e]	0.5[d]	7.0[c]	17.0[h]	2.4[b]	37.7[b]	15.5[c]
B. *Brachionus* fed HUFA boosters:							
Initial (*Chlorella*-fed)	10.1[f]	0.8[c]	9.4[c]	1.5[g]	0.2[f]	1.8[h]	11.4[a]
Alg2000	16.0[d]	1.3[b]	3.9[f]	18.2[c]	4.6[c]	14.5[d]	3.1[d]
Alg3050	15.8[d]	1.1[b]	3.4[f]	29.0[a]	8.6[b]	26.4[c]	3.1[d]
AqAdv	17.2[c]	0.7[c]	3.1[f]	28.6[a]	9.3[a]	39.2[a]	4.2[c]
AqChl	12.5[e]	1.2[b]	5.8[d]	11.7[d]	2.0[d]	9.8[e]	4.8[c]
AqF15	13.0[e]	0.6[c]	4.8[e]	21.2[b]	4.4[c]	34.3[b]	7.7[b]
HUFA Enrich	20.4[b]	1.5[b]	10.9[b]	4.8[e]	0.4[f]	3.2[f]	7.3[b]
Ratio HUFA	21.8[a]	1.1[b]	12.7[a]	1.6[g]	0.1[f]	1.5[h]	11.5[a]
Super HUFA	21.6[a]	1.1[b]	3.6[f]	2.5[f]	0.7[e]	2.3[g]	3.4[d]
DHA Protein Selco	8.1[a]	3.2[a]	4.4[e]	2.8[f]	0.6[e]	0.9[i]	1.4[e]

[1]Treatment means in columns under each subheading with the same superscripts are not significantly different ($P > 0.05$).

Table 6. Total (TL), neutral (NL), and polar (PL) fatty acids of starved and emulsion-enriched rotifers (% of dry weight)[1,2].

	NL									
	Initial	Starved			Enriched[1]	Inital	Starved (hrs)			Enriched[1]
		3	6	12			3	6	12	
NL or PL, % DW	4.5[a]	4.3[b]	3.9[c]	3.6[d]	7.1	3.7[a]	3.5[b]	2.6[c]	2.3[d]	4.3
Fatty acids (% DW):										
Saturates	1.0[a]	0.8[b]	1.0[a]	0.7[c]	1.8	0.1[a]	0.5[b]	1.1[c]	1.1[c]	2.3
Monoenes	2.0[a]	1.8[b]	1.6[c]	0.8[d]	2.6	2.2[a]	1.7[b]	0.6[c]	0.9[d]	1.3
n-3 HUFAs	1.2[a]	1.2[a]	1.2[a]	0.6b	1.7	0.3[a]	0.4[b]	0.6[b]	0.5[d]	0.4
20:4n6	0.1[a]	0.1[a]	—	—	0.3	0.1[a]	0.1[a]	0.1[a]	0.1[a]	0.1
20:5n3	0.8[a]	0.7[b]	0.7[b]	0.3[c]	1.0	0.3[a]	0.3[a]	0.3[a]	0.2[b]	0.3
22:6n3	0.1[a]	0.1[a]	—	—	0.2	0.1[a]	0.1[a]	0.1[a]	—	0.1
Ratio:				—					—	
DHA:EPA	0.1[a]	0.1[a]	—	—	0.2	0.2[a]	0.2[a]	0.2[a]	—	0.2
DHA:ARA	1.4[a]	1.4[a]	—	—	0.6	0.6[a]	0.5[b]	2.2[c]	0.5	
EPA:ARA	10.7[a]	14.0[b]	—	—	3.6	4.0[a]	3.8[b]	3.1[c]	—	2.9

[1] Dripping emulsion of cod liver oil, egg yolk, vitamins, and water for one hour.

[2] Treatment means in rows under NL or PL with the same superscripts are not significantly different ($P > 0.05$).

Table 7. Total lipids (TL), HUFA levels and ratios in *Artemia* nauplii and pre-adults enriched with HUFA, and grouper larvae after feeding with pre-adult *Artemia.*

	TL % DM	% of total fatty acids			Ratio		
		20:4n-6	20:5n-3	22:6n-3	DHA:EPA	DHA:ARA	EPA:ARA
A. *Artemia* nauplii fed HUFA boosters:							
Initial	12.6	2.4	—	—	—	—	—
AqAdv	17.0[d]	2.0[a]	0.3[c]	13.6[a]	50.4[a]	6.7[a]	0.1[c]
AqChl	16.2[e]	2.4[a]	0.2[c]	1.3[f]	6.1[c]	0.6[d]	0.1[c]
AqF15	18.1[c]	2.4[a]	0.3[c]	6.5[b]	20.9[b]	2.7[c]	0.1[c]
Alg2000	17.0[d]	2.4[a]	0.3[c]	1.7[f]	5.8[c]	0.7[d]	0.1[c]
Alg3050	15.6[e]	2.7[a]	1.5[a]	5.7[c]	3.8[d]	2.2[c]	0.6[b]
HUFA Enrich	21.9[a]	0.8[b]	0.8[b]	2.8[e]	3.5[d]	3.5[b]	1.0[a]
Ratio HUFA	22.0[a]	0.8[b]	0.8[b]	2.9[e]	3.4[d]	3.8[b]	1.1[a]
Super HUFA	20.8[b]	0.8[b]	0.8[b]	3.1[d]	3.8[d]	3.9[b]	1.0[a]
B. Pre-adult *Artemia* fed HUFA boosters:							
Initial	13.0	2.7	3.5	—	—	—	1.3[e]
Rice bran extract (RB, control)	11.2[f]	1.3[d]	1.7[e]	—	—	—	1.3[e]
Ratio HUFA	24.3[a]	2.1[b]	5.3[c]	7.4[b]	1.4[a]	0.5[a]	2.6[c]
HUFA Enrich	23.3[b]	1.7[c]	7.7[b]	3.6[e]	0.5[b]	2.1[c]	44.5[a]
Super HUFA	18.9[e]	2.0[b]	8.1[a]	4.1[d]	0.5[b]	2.1[c]	4.1[b]
Alg2000	18.7[e]	2.3[b]	4.3[d]	6.4[c]	1.5[a]	2.8[b]	1.9[d]
Alg3050	21.2[c]	2.9[a]	4.8[d]	8.6[a]	1.8[a]	2.9[b]	1.8[d]
Mixed[2]	20.8[d]	3.0[a]	5.1[c]	7.6[b]	1.5[a]	2.5[b]	1.7[d]
C. Grouper larvae fed pre-adult *Artemia* fed HUFA boosters:							
Rice bran extract (RB, control)	5.1[e]	1.6[c]	1.7[f]	1.0[f]	0.6[d]	0.6[d]	1.1[d]
Ratio HUFA	14.8[b]	2.2[b]	3.9[d]	6.8[c]	1.8[b]	3.1[a]	1.8[c]
HUFA Enrich	16.1[a]	1.7[c]	5.9[b]	5.2[d]	0.9[c]	3.0[a]	3.5[a]
Super HUFA	13.6[c]	2.3[b]	6.5[a]	3.2[e]	0.5[d]	1.4[c]	2.8[b]
Alg2000	12.3[d]	3.1[a]	3.0[e]	7.8[b]	2.6[a]	2.5[b]	1.0[e]
Alg3050	14.0[c]	3.7[a]	3.7[d]	9.8[a]	2.6[a]	2.7[b]	1.0[d]
Mixed[2]	15.1b	3.8[a]	4.4[c]	8.0[b]	1.8[b]	2.1[b]	1.2[d]

[1] Treatment means in columns under each subheading with the same superscripts are not significantly different ($P > 0.05$).

[2] Mixed: AqAA, Alg3010, HUFA Enrich at 1:1:2 ratio.

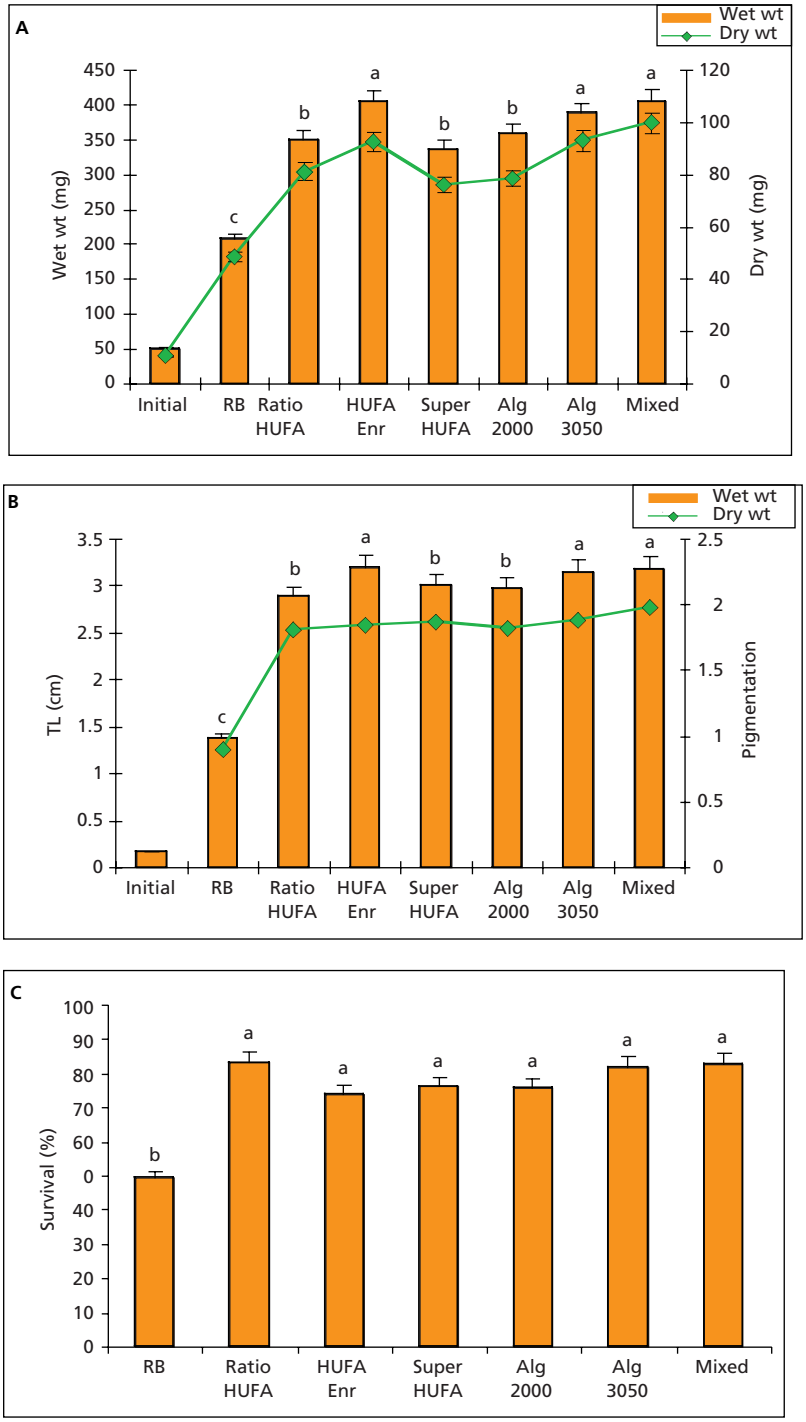


Figure 1. Wet and dry weight (A), total length and pigmentation (B), and survival (C) of grouper larvae (day 25 to –35) fed unenriched or enriched *Artemia*. Initial weight and TL = 51.1 ± 3.1 mg, 16.2 ± 0.3 mm. RB = rice bran extract; Ratio HUFA; HUFA Enrich; Super HUFA; Algamac 2000 (Alg 2000); Algamac 3050 (Alg 3050) and Mixed: Aquagrow AA, Algamac 3050 and HUFA Enrich at 1:1:2 ratio. Bars with the same letters are not significantly different (P > 0.05).

Table 8. Growth and survival of grouper larvae fed unenriched and HUFA-enriched rotifers (day 2–14).

Treatment	Weight mg ind^{-1}	Standard length mm	Total length mm	Survival %
Control	0.2 ± 0.2^{d}	1.5 ± 0.1^{c}	1.8 ± 0.1^{c}	1.3 ± 0.7^{b}
Alg 2000	0.8 ± 0.1^{b}	3.3 ± 0.2^{b}	3.6 ± 0.3^{b}	3.1 ± 0.2^{a}
Alg 3050	1.0 ± 0.3^{a}	3.5 ± 0.3^{a}	3.9 ± 0.4^{a}	3.2 ± 0.1^{a}
AqAdv	1.1 ± 0.2^{a}	3.6 ± 0.1^{a}	3.9 ± 0.1^{a}	3.1 ± 0.1^{a}
AqChl	0.9 ± 0.1^{b}	3.3 ± 0.1^{b}	3.6 ± 0.2^{b}	2.9 ± 0.3^{a}
AqF15	0.8 ± 0.1^{b}	3.4 ± 0.1^{b}	3.7 ± 0.2^{b}	2.8 ± 0.7^{a}

[1] Treatment means in columns with the same superscripts are not significantly different (P > 0.05).

Results and Discussion

Polar lipids (PL) were generally conserved while NL was primarily spent as energy in eggs, newly hatched larvae and unfed day-4 larvae (Table 1). In eggs, neutral and polar DHA:EPA ratios were similar, whereas neutral DHA:ARA and EPA:ARA ratios were twice those of PL. In day 4 unfed larvae, neutral and polar DHA and ARA were retained but EPA was low in PL and depleted in NL. Hatchery-bred larvae contained higher NL than PL; their EFA increased with feeding but three days of starvation decreased these (Table 2). Wild larvae had higher levels of PL than NL. One week of starvation totally spent the neutral DHA while some polar DHA was retained (Table 3).

DHA was present only in *Chlorella*, *Isochrysis* and *Thalassiosira* (Table 4A) and in *Brachionus* cultured in phytoplankton (Table 4B). *Diaphanosoma* grown in *Tetraselmis* contained only a little DHA and EPA indicating that HUFA enrichment is necessary to improve its nutritional value (Table 4C). *Pseudodiaptomus* reared in *Chlorella, Chaetoceros,* or *Isochrysis* had better HUFA ratios than it did cultured in *Tetraselmis* (Table 4D). Except for AqAA that contained high ARA, all boosters provided DHA, particularly high in AqAdv, AqChl, AqF15, and Alg3050 (Table 5A). *Brachionus* enriched with AqAdv and Alg3050 contained the highest DHA (Table 5B).

In starved *Brachionus*, lipids declined with time (Table 6) and to ensure optimal essential fatty acids content, *Brachionus* should be fed to larvae right after harvest or within the next three hours. Supplements of AqAdv, AqF15, and Alg3050 improved the DHA in *Artemia* nauplii (Table 7A), while in pre-adult *Artemia*, Alg3050, mixed HUFA, Ratio-HUFA and Alg2000 enhanced DHA levels and HUFA-Enrich and Super-HUFA increased EPA levels (Table 7B).

HUFA-enriched *Brachionus* and *Artemia* enhanced better growth, survival or pigmentation in early feeding (Table 8) and metamorphic larvae (Fig. 1) than un-enriched live food. Dietary HUFAs were reflected in the larvae (Table 7C).

Conclusions

- *Epinephelus coioides* eggs contained high DHA, EPA and ARA demonstrating their importance in larval development; larvae primarily spent NL as energy while PL was generally conserved.
- Wild grouper larvae had higher levels of PL than NL, whereas hatchery-sourced eggs and larvae contained higher levels of NL than PL. Based on the lipid content of wild larvae, high phospholipid diets are essential for larval survival and normal development.
- A variety of enrichment products were effective in enriching the HUFA content (particularly dietary levels and ratios of DHA, EPA and ARA) of live food organisms.
- HUFA-enriched live food organisms enhanced growth, survival and pigmentation in grouper larvae.

References

Association of Official Analytical Chemists (AOAC), 1996. Official Methods of Analysis. Fatty acids in oils and fats. Preparation of methyl esters boron trifluoride method. AOAC 41, 18–21.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226, 497–509.

Juaneda, P. and Rocquelin, G., 1985. Rapid and convenient separation of phospholipids and non-phosphorus lipids from rat heart using silica cartridges. Lipids 20, 40–41.

Amino and Fatty Acid Profiles of Wild-Sourced Grouper (*Epinephelus coioides*) Broodstock and Larvae

V.R. Alava, F.M.P. Priolo, M. Arnaiz and J.D. Toledo

Introduction

There is a lack of information on the amino acid content of grouper broodstock tissues, eggs and newly hatched larvae. This study was carried out to provide information on levels of amino acids in muscle, liver, and gonad of wild-sourced broodstock and larvae, as well as on neurula eggs and day 35 larvae from a hatchery. The fatty acid compositions of grouper broodstock tissues were also determined. This information can be used as to guide the development of a high quality diet for grouper broodstock and larvae since their dietary nutrient requirements will be closely related to their nutrient profiles.

Methods

Samples analysed for crude protein and amino acids were: (1) abdominal muscle, liver and gonad tissues dissected out from a broodfish collected from a trap at Tigbauan Bay, Panay Gulf, Philippines; (2) wild-sourced larvae from Samar, Leyte, Philippines; (3) neurula eggs obtained from broodstock groupers in a tank fed raw fish; and (4) larvae reared in a hatchery and fed live food organisms for 35 days.

The broodfish tissues were also analysed for total lipids and fatty acid content. Crude protein (CP) was analysed using the micro-kjeldahl method (AOAC 1980). Samples were hydrolysed with trichloroacetic acid and analysed for amino acid contents using the HPLC (Shimadzu LC-10AT). Total lipids (TL) were extracted based on the method of Folch et al. (1957), fatty acid methyl esters were prepared by trans-esterification with BF_3 methanol (AOAC 1996) and analysed using a gas chromatograph (Shimadzu GC-17A). Three replicate analyses were done per sample.

Results

Table 1 shows the CP and amino acid contents of early maturing grouper broodstock tissues, eggs and larvae. Muscle contained higher levels of crude protein (dry matter basis) and amino acids than ovary and liver. CP and amino acid contents in wild-sourced larvae were higher than in the hatchery-sourced eggs and larvae. Among the ten essential amino acids (EAA), leucine and lysine were dominant in all samples analysed.

Of the non-essential amino acids (NEAA), glutamine and asparagine were the highest. TL and fatty acid content in grouper broodstock is given in Table 2. Total lipids content was highest in liver, followed by ovary then muscle. The levels of highly unsaturated fatty acids (HUFA) in these three tissues were: 22:6n3 (DHA) > 20:4n-6 (ARA) > 20:5n-3 (EPA). In the ovary, a DHA:EPA ratio of 6.8 and a DHA:ARA ratio of 2.5 was obtained.

Conclusions

- At the early maturing stage, the grouper ovarian protein was 73.3% and lipid was 19.3% indicating high dietary requirements of these nutrients for ovarian development.
- Crude protein and amino acids in wild-sourced larvae were higher than the eggs and larvae sourced from a hatchery.

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

Table 1. Crude protein and polymerised amino acids (% of protein, DW) of wild *E. coioides* broodstock tissues and larvae, eggs and day 35 larvae.

	Wild grouper			Wild	Hatchery	
	Ovary[1]	Liver[1]	Muscle	larvae	Eggs	larvae
Crude protein, % DW	73.33	26.62	94.34	72.22	69.14	69.53
Larval DW, mg ind^{-1}				116.21	0.03	51.04
EAA[2]:						
Arg	3.66	1.41	4.20	4.67	4.31	4.23
His	1.80	0.78	2.46	1.62	1.83	1.62
Ile	3.54	1.31	4.91	3.47	3.35	3.58
Leu	6.15	2.45	9.34	6.16	6.19	5.88
Lys	5.88	2.21	8.01	6.49	4.35	6.40
Met	2.34	0.77	3.14	2.11	1.34	2.09
Phe	3.03	1.42	4.27	3.19	3.50	3.27
Thr	3.66	1.40	4.71	3.55	3.83	3.44
Val	4.43	1.55	4.96	3.83	4.99	3.88
Sum EAA	34.49	13.29	46.04	35.11	33.70	34.39
NEAA:						
Asp	9.30	2.94	8.81	7.85	5.75	7.41
Ser	3.24	1.28	4.71	3.17	3.01	2.95
Glu	14.02	3.78	14.31	11.75	10.24	11.10
Pro	1.98	1.15	5.05	3.02	5.07	2.97
Gly	2.93	1.41	3.83	3.95	2.80	3.85
Ala	4.68	1.87	6.94	4.54	4.66	4.30
Tyr	2.62	0.88	4.62	2.80	3.90	2.56
Sum NEAA	38.76	13.30	48.28	37.07	35.44	35.14

[1] The wild broodstock (2.90 kg) had a gonadosomatic index (GSI) of 0.73 and hepatosomatic index (HSI) of 1.24.
[2] Tryptophan was undetected and might have been destroyed during sample hydrolysis.

Table 2. Total lipids and fatty acids in wild *E. coioides* tissues.

	Ovary[1]	Liver[1]	Muscle
Total lipids (% DM)	19.28	40.13	4.73
Fatty acids (% of TL, DM):			
14:0	0.64	0.82	0.11
16:0	4.46	13.31	1.51
16:1n-7	2.32	7.62	0.02
18:0	1.10	1.43	0.35
18:1n-9	4.31	9.40	0.98
18:2n-6	0.09	0.05	0.03
20:1n-9	0.19	0.88	0.05
20:4n-6	0.99	1.25	0.36
20:5n-3	0.37	0.63	0.13
20:4n-3	0.16	0.04	0.03
22:4n-6	0.46	0.03	0.12
22:4n-3	0.30	0.23	0.09
22:5n-3	0.58	0.51	0.09
22:6n-3	2.53	1.85	0.59
Total:			
Saturates	6.31	15.75	2.01
Monoenes	6.89	17.90	1.08
n-3 FA	4.09	3.72	1.02
n-6 FA	1.53	1.24	0.50
n-3 HUFA	3.92	3.22	0.93
Ratio:			
n-3: n-6	2.67	3.00	2.04
DHA: EPA	6.84	2.94	4.54
DHA: ARA	2.55	1.48	1.64
EPA: ARA	0.37	0.50	0.36

[1] See Table 1.

References

Association of Official Analytical Chemists (AOAC), Official Methods of Analysis. Fatty acids in oils and fats. Preparation of methyl esters boron trifluoride method. AOAC 41, 18–21.

Association of Official Analytical Chemists (AOAC), 1980. Official Methods of Analysis (13th edition). AOAC, Washington. 1108 pp.

Folch J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226, 497–509.

Studies on Semi-Intensive Seed Production of Grouper (*Epinephelus coioides*)

J.D. Toledo, D. Chavez and J. Rodriguez Jr.

Introduction

Previous studies have demonstrated that the use of copepod nauplii, alone or in combination with rotifers, increase the growth and survival of early larval stages of the grouper *Epinephelus coioides.* An average survival rate of 3% was obtained after metamorphosis in a pilot-scale production using 10-tonne tanks (Toledo et al. 1999). Other studies in this project, performed at a laboratory-scale, reported higher survival rates when early larval stages were reared under appropriate salinity, aeration and light intensities. Higher growth and survival were also observed when live prey organisms were enriched with highly unsaturated fatty acids (HUFA). The main objective of this study was to improve hatchery survival by verifying and incorporating laboratory-scale experimental results to a hatchery-scale operation.

Materials and Methods

Nine units of 200–metre square ponds were used to verify mass culture techniques for zooplankton. Three fertilisation schemes were tested with three replicate ponds per fertilisation treatment. Incoming water was screened with a 0.8–1.0 mm mesh net to minimise entry of predators but allow entry of natural populations of copepods. The initial water depth was one metre. The culture period was 45 days. The initial quantity and quality of copepods and other zooplankton in each pond were monitored a day after filling, then every three to four days thereafter. Zooplankton samples were collected, preserved and identified as described by Ohno et al. (1996).

To test copepod production in tanks, adults and copepodids were transferred into six one-tonne oval fibreglass production tanks at a starting density of 60 individuals/L. Zooplankton in three of the tanks were fed daily with a mixture of *Nannochloropsis* sp., *Tetraselmis* sp., and *Chaetoceros* sp. at a final density of 300,000 cells/ml. Zooplankton in the remaining three tanks were fed daily with half the amount of a mixture of the same algae (150,000 cells/ml) and bread yeast (0.5 gm/100,000 individuals). A moderate airlift system kept the algae and bread yeast suspended in the water column.

Larval rearing runs using five-tonne tanks were performed from 2000 to 2002 to verify earlier experimental results. The protocol of Toledo et al. (1999) was tested in 2000. To propagate copepod naulpii in larval tanks, *Acartia* copepodids and adults were inoculated in four 10-tonne larval rearing tanks at 60–80 individuals/L, two to three days before stocking of grouper eggs or newly hatched larvae. *Brachionus* were added daily from day 2 to day 18 at increasing densities of 2–10 individuals/ml. *Artemia* nauplii and metanauplii were fed to satiation from day 15 until metamorphosis. Pond-grown zooplankton was added in separate tanks from day 15 onwards as a supplement to *Artemia.* In 2001, environmental conditions (20–25 ppt, moderate aeration, ca. 700 lux light) found appropriate for early larval stages were tested. To further improve larval survival, live prey organisms were 'enriched' in 2002 with

Advances in Grouper Acquaculture
Edited by M.A. Rimmer, S. McBride and K.C. Williams
ACIAR Monograph 110 (printed version published in 2004)

commercial enrichment products or homemade oil emulsions made of fish oil, bread yeast, egg yolk and vitamin mix.

Results and Discussion

The population of copepods in ponds fertilised with various combinations of organic and inorganic fertilisers increased a week after flooding of ponds and fertilisation (Figs. 1–3). In all treatments, the density of copepods rapidly increased from 86–148 individuals/L a day after flooding to 1524-3186 individuals/L 9–12 days thereafter. Zooplankton compositions were: *Apocyclops* and *Oithona* sp. in Treatment I; *Apocyclops*, *Brachionus rotundiformis*, *Oithona,* and *Penilia* in Treatment II; and *Apocyclops*, *Pseudodiaptomus* sp. and *Penilia* in Treatment III. Salinity, temperature and water transparency during the experiment varied from 26–31 ppt, 29–31°C and 36–80 cm, respectively.

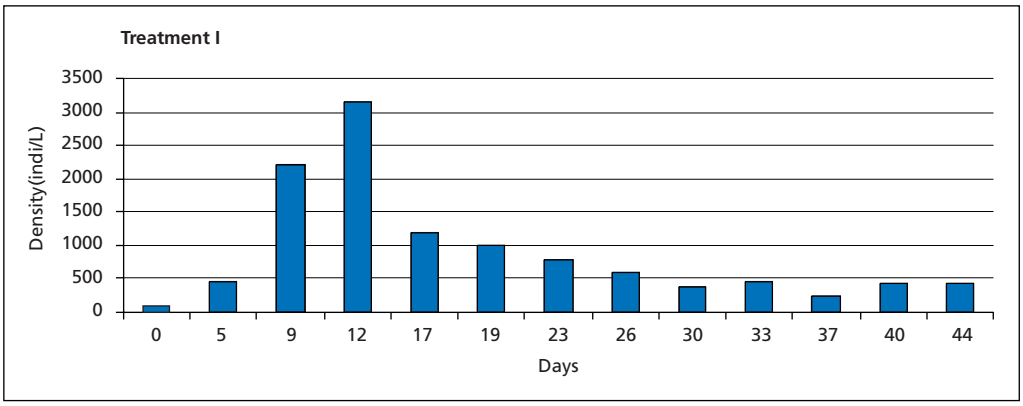


Figure 1. Density of zooplankton in earthen ponds fertilised following the methods of Ohno and Okamoto (1988). Chicken manure was applied evenly as a basal fertiliser at 500 kg/ha. After filling the pond with water, urea, ammonium sulfate and ammonium phosphate were added and then every three days thereafter at a rate of 2.7, 4 and 6.0 kg/ha, respectively.

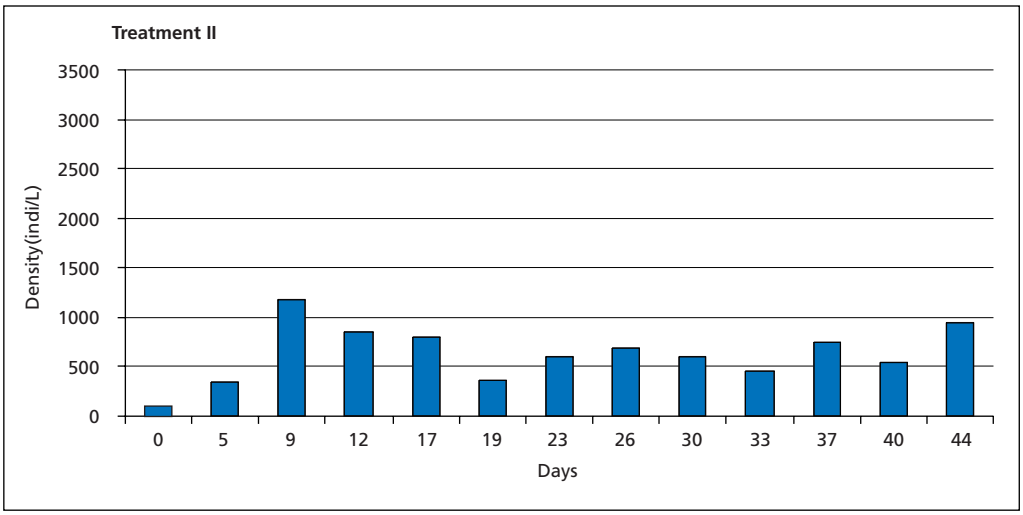


Figure 2. Density of zooplankton cultured in ponds fertilised following SEAFDEC AQD. Basal fertiliser composed of two-tonnes of chicken manure, 25 kg urea, and 50 kg ammonium phosphate per hectare.

Copepodids and adults of *Acartia tsuensis* fed a mixture of algae alone, or in combination with bread yeast, seemed to propagate well in tank conditions (Fig. 4). Density of *Acartia* rapidly increased from 60 individuals/L to about 900 individuals/L (including various naupliar stages) three days after stocking. A decrease in density was observed 5–6 days after stocking, probably due to cannibalism and contamination of rotifers. The present results suggest that bread yeast could be used in combination with algae for nauplii production of *Acartia* in tanks.

Verification runs indicate that pond-grown copepods (*Oithona, Pseudodiaptomus* and *Acartia*) can be used as a supplement to *Artemia.* Larvae fed copepods and *Artemia* starting at day 15 showed similar survival rates to those fed *Artemia* only (Fig. 5). Larval survival from days 5–15 was higher in larvae reared in 20–25 ppt (36.6–73%) to those reared in normal seawater (21.8–41.7%) (Fig. 6). However, survival at harvest appeared similar (4.9–6.4%) (Figs. 5–6). From day 20 onwards, moribund larvae swimming listlessly near the water surface with abrupt swirling movements were commonly observed.

Thirty-nine out of 71 tanks were discarded in 2000, while three of the 12 and eight out of 26 production runs were aborted in 2001 and 2002 (Table 1), respectively. Mean survival at harvest

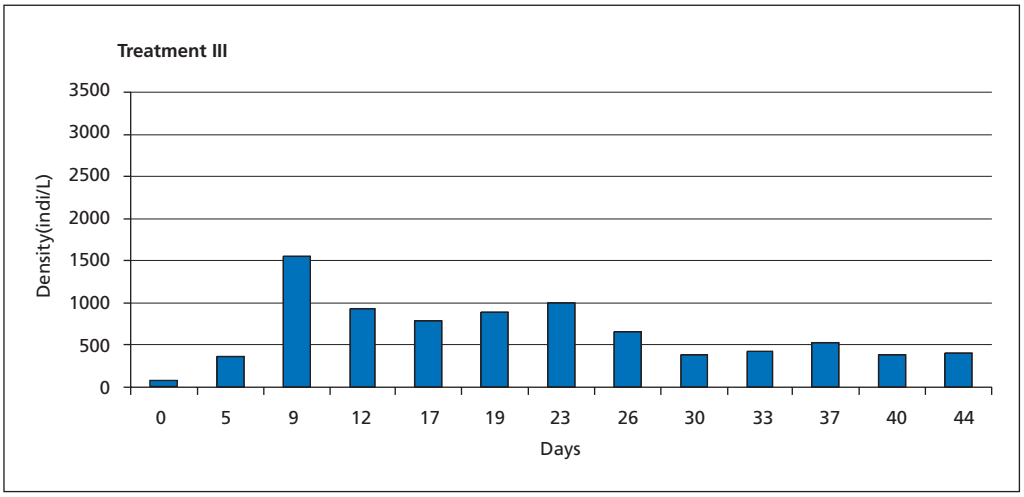


Figure 3. Density of zooplankton cultured in ponds (modifying Geiger et al. (1983)). Rice bran and liquid inorganic fertiliser were added weekly, at a rate of 300 kg and 50 L per hectare, respectively.

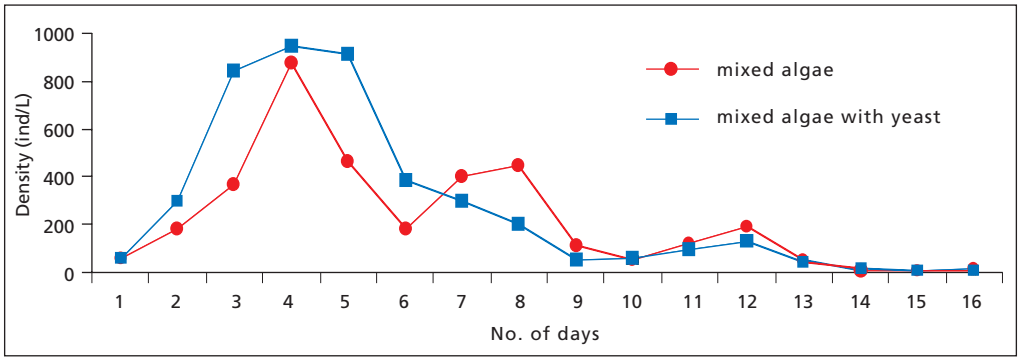


Figure 4. Density of *Acartia tsuensis* cultured in a one-tonne tank fed with mixed algae alone or in combination with bread yeast.

(day 35) increased from 3.06% (0.62–10.2%) in 2000 to 5.33% (1.2–12.1%) in 2001 and 10.39% (1.1–49.4%) in 2002. Larvae in discarded or aborted tanks had high cumulative mortalities with clinical signs of viral nervous necrosis (VNN). Mortalities were associated with VNN (Maeno et al. 2002). Fertilised eggs as well as larvae at various ages were shown to be positive for VNN by cell culture and RT-PCR. Histopathological observations revealed vacuoles in the retina and brain of moribund larvae.

Table 1. Summary of larval rearing runs in five-tonne tanks from 2000–2002.

	2000	2001	2002
Total number of tanks	71	12	20
Aborted/discarded	39	3	8
Mean survival (%)	3.1	5.3	10.4
Range (%)	0.6–10.2	1.2–12.1	1.1–49.4

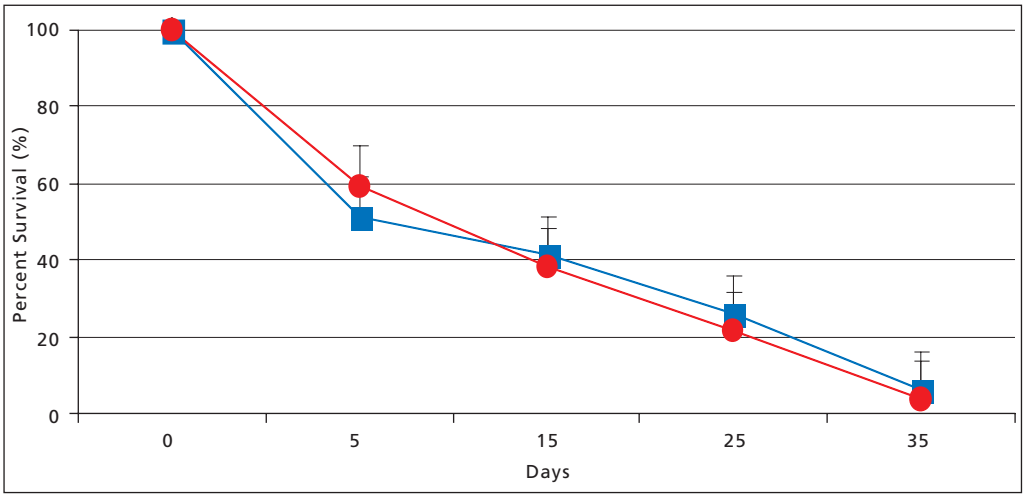


Figure 5. Percentage survival of grouper larvae fed with *Artemia* alone (circles) or in combination with copepods (squares). Figures are mean ± SEM of four replicates.

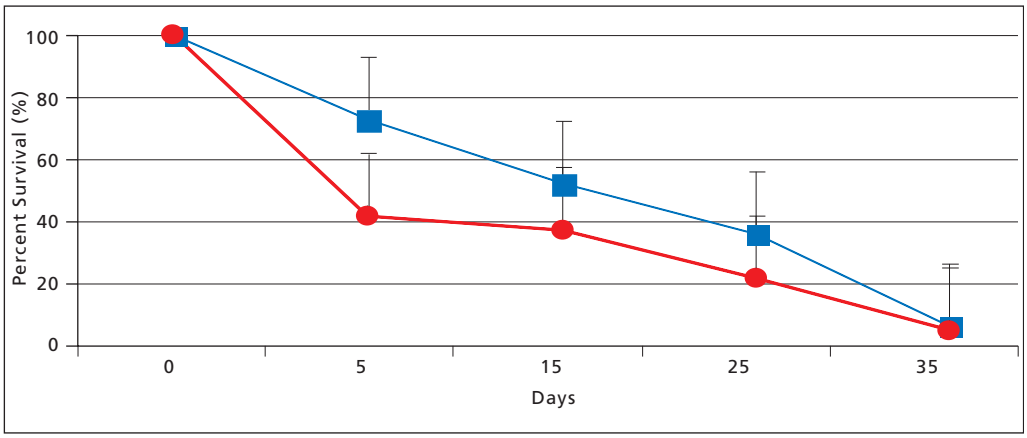


Figure 6. Percentage survival of grouper larvae reared in salinities of 20–25 ppt (squares) or 34–35 ppt (circles). Figures are means ± SEM of six replicates.

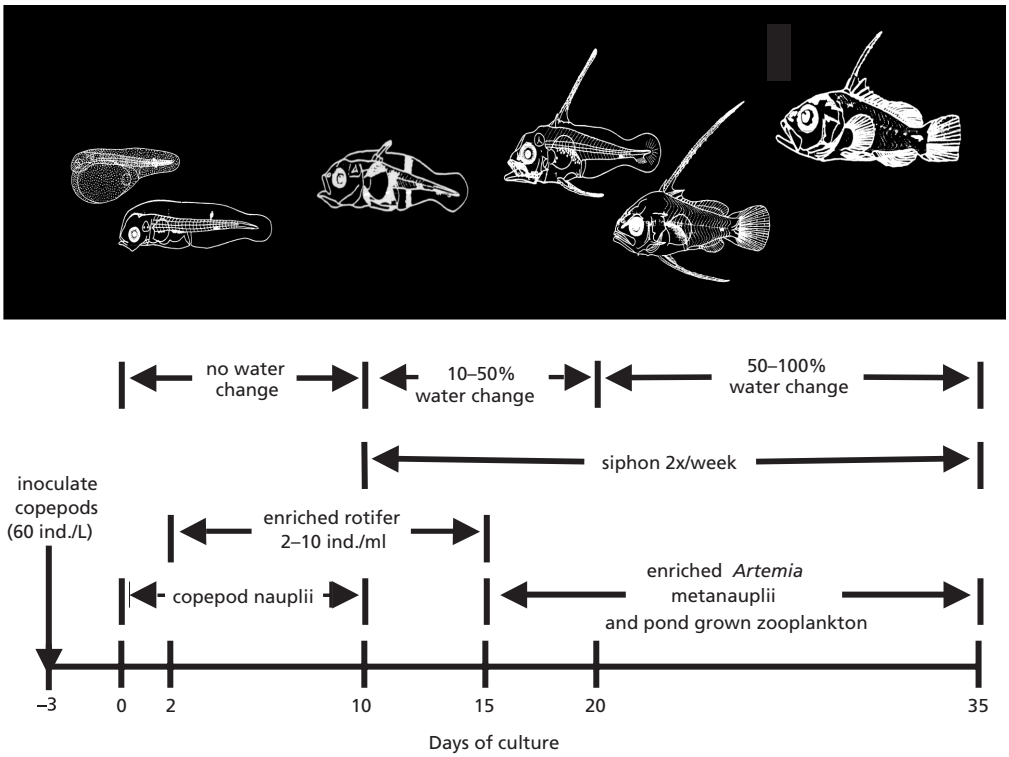


Figure 7. Feeding and water management scheme for semi-intensive rearing of grouper larvae.

Sampling larval *Epinephelus coioides* from larval rearing tanks at the Southeast Asian Fisheries Development Centre Aquaculture Department, Iloilo, Philippines.

Conclusions

- Brackish water (20–25 ppt) could increase survival at early larval stages.
- Pond-grown copepods can be used as a supplement to *Artemia.*
- Up to 40% survival at harvest may be obtained following the protocol shown in Figure 7.
- Larval survival in the hatchery was highly affected by VNN infection.

References

Geiger, J.G. 1983. A review of pond zooplankton production and fertilization in striped bass rearing ponds. Aquaculture, 35, 353–369.

Maeno, Y., de la Pena, L.D. and Cruz-Lacierda, E.R. 2002. Nodavirus infection in hatchery-reared orange-spotted grouper *Epinephelus coioides*: First record of viral nervous necrosis in the Philippines. Fish Pathology, 37, 87–89.

Ohno, A. and Okamoto, Y. 1988. Propagation of calanoid copepod, *Acartia tsuensis*, in outdoor tanks. Aquaculture 70, 39–51.

Ohno, A. 1996. Guide for the identification of copepods and their life cycle. Tokyo University of Fisheries. 28 pp.

Toledo, J.D., Golez, M.S.N., Doi, M. and Ohno, A. 1999. Use of copepod nauplii during the early feeding stage of grouper *Epinephelus coioides*. Fisheries Science, 65, 390–397.