NUTRITION AND RELATED ONTOGENETIC ASPECTS IN LARVAE OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS*

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NUTRITION AND RELATED ONTOGENETIC ASPECTS IN LARVAE OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS*

Johan Verreth

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The absence of adequate techniques for rearing fish larvae constitutes a bottleneck for sustainable aquacultural growth. Important constraints are the tiny size of the larvae, the dependance on live food organisms and the developmental stage of the fish larvae. The development of dependable rearing techniques requires knowledge of the critical aspects in nutrition in relation to the development of the digestive and metabolic system. In the present study, this relation between fish nutrition and development was investigated using the African catfish *Clarias gariepinus* (Burchell) as research model. The study was subdivided into three phases, dealing respectively with husbandry conditions, developmental aspects and with the lipid metabolism. During the first phase, in a comparative study with different dry diets, the dependance of African catfish larvae on live food organisms (e.g., *Artemia*) was demonstrated. A technique to estimate quantitative food rations was developed and used to elucidate the quantitative relations between food ration, water temperature and growth. African catfish larvae can be successfully weaned to a dry diet without growth loss provided that they receive live food organisms during the first four days of exogenous feeding. Durint the second phase of the project, the morpho-functional development of the digestive system and the activities of metabolic enzymes were investigated. The results indicated that at the onset of exogenous feeding, African catfish larvae can eat, digest, absorb and metabolize the absorbed nutrients. The changes in feeding habits were correlated with the development of stomach functions. Together with this functional development of the stomach, several other ontogenetic changes in the larval physiology occurr. This combined change of ontogenetic events occurs at a body size of approximately 10-20mg. Therefore this size is suggested as a marker for the end of the larval period in *C. gariepinus*. The different studies on the lipid metabolism, carried out during the third phase, showed that the eggs of African catfish contain moderate amounts of lipid of a high polar nature (70-75% of the lipid consist of phosphatidylcholine, PC). PC is an important energy source during the early life stages of this species. African catfish is capable to biosynthesize important *n*-3 highly unsaturated fatty acids (HUFA) from the yolk sac period onwards. Dietary enrichment with *n*-3 HUFA does affect liver ultrastructure. It does not influence growth and survival. Consequences of these results for defining an optimal feeding and rearing strategy for larval fish are discussed.
DSc thesis, Department of Fish Culture and Fisheries, Wageningen Agricultural University, Marijkeweg 40, 6709 PG Wageningen, The Netherlands.

NN08201, 1849

Stellingen

1. The absence or presence of stomach functions is not only crucial for the understanding of nutritional requirements but also for the life history of many larval fish species.

   This thesis

2. The rate of protein breakdown and absorption, together with the composition and the ratio of assimilated amino acids to these of the body free pool are critical for the growth and survival of larval fish.

   Dabrowski, K. and Culver, D. 1991. The physiology of Larval fish. Digestive tract and formulation of starter diets. Aquaculture Magazine: 49 - 61.
   This thesis.

3. In contrast to what may be suggested by the magnitude of the research effort spent on (*n*-3) fatty acids, their importance for the growth of larval fish is restricted to the development of different organs and the associated functions.

   This thesis

4. The paradigm "Enough but not too much" to describe the digestive and/or absorptive capacity of mammals (Diamond 1991) holds also for fish and explains why fish larvae survive and grow well on natural food and not on artificial diets.

   Diamond, J. 1991. Evolutionary design of intestinal nutrient absorption: Enough but not too much. News in Physiological Sciences 6: 92-96.
   This thesis.

5. In a world of increasing population pressure and increasing scarcity of resources, fisheries management should be recognized as a full-fledged part of a policy for sustainable and integral water management in terms of food supply, jobs and conservation of nature.

   See also: Weber, P. 1994. Net Loss: Fish, Jobs and the Marine Environment. Worldwatch paper 120, Worldwatch Institute, Washington DC, USA. 76 p.

6. The general focus by international donor agencies on "subsistence farming" and the "have-nots" neglects the lessons of the success stories in aquacultural development (salmon, catfish, shrimp) which created thousands of jobs and regional welfare wherever they occurred, both in the North ánd the South.

7. Integratie van visteelt met de teelt van rijst en/of landbouwhuisdieren staat al eeuwen garant voor duurzaamheid en voedselvoorziening van de mens.

8. De voortdurende reorganisaties ten aanzien van onderzoek (onderzoekinstituten) en onderwijs (vakgroepsfusies) wekken de verkeerde indruk dat kwaliteit te organiseren valt.

9. De disciplinaire indeling van het onderzoek binnen het onderzoekinstituut WIAS lijkt eerder ingegeven door het karakter van de Nederlandse veeteelt dan door het maatschappelijke fenomeen "het produceren van dierlijke producten".

10. De neiging om taalverbasteringen in het Noordnederlands als uitingen van een "levende taal" te zien en deze in het Zuidnederlands als "archaïsch" te omschrijven wijst op een gebrek aan goede kennis van de Nederlandse taal.

    Bron: Het Groot Nederlands Woordenboek van de Nederlandse taal, Van Dale.

11. Wat in Nederland als "vertrouwen in eigen kunnen" wordt gezien, wordt elders meer als "met het hoofd in de wolken" beschouwd.

    Bronnen: "zwarte maandag" (verdrag van Maastricht), internationale topfuncties (Lubbers), voetbal (Worldcup'94)

12. Het schrijven van een proefschrift in de heetste zomer van de laatste driehonderd jaar behoort tot de categorie "*Eéns maar nooit meer*".

Stellingen behorend bij het proefschrift "Nutrition and related ontogenetic aspects in larvae of the African catfish *Clarias gariepinus*" van Johan Verreth.
Wageningen, 26 oktober 1994.

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Voorwoord

Onlangs zag ik een proefschrift dat als de Nijmeegse vier"jaarse" werd aangekondigd. Dat kan van dit werk niet gezegd worden. Er is heel wat meer water door de Rijn gevloeid dan in een reguliere AIO-periode mogelijk zou zijn. Misschien moet ik het wel de Dendermondse Ommegang noemen, want die gaat zo eens in de 10 jaar uit. Alleen, Dendermonde is niet mijn streek en wie, behalve die van Dendermonde en Aalst, kent nu die Ommegang ? Laten we het voorlopig maar houden op WAGENINGS BITTER. In beide betekenissen van het woord. Als drankje is het aardig, maar het tintelt wel op de tong. Bij de eerste proef is het éven wennen en oogt het wat taai. En uiteraard, het gaat altijd een stuk makkelijker als je in gezelschap bent.

Zo is het dus ook gegaan met dit proefschrift. Terugblikkend op die beginperiode was het behoorlijk zwemmen in dik water, moet ik zeggen. Maar een mens went aan alles, zelfs aan Zootechniek in Wageningen. En naarmate de tijd verstreek werd het steeds gezelliger. Hartstikke leuk, veel geleerd, óók veel gedaan. Als docent lag er geen strakke tijdsplanning voor een proefschrift; het ging om de ontwikkeling van een onderzoekslijn. Het proefschrift is dan ook eerder de afronding van een meerjarig onderzoek dan van een "wetenschappelijke opleiding". Als docent is het nooit weg om met meerdere onderzoeken tegelijk bezig te zijn. Kwestie van ingraafgedrag en oogkleppen te vermijden. En vooral ook een kwestie om de voeling met het werkveld niet te verliezen. Is immers de opleiding die we bieden niet de reden van ons bestaan ? In ons geval is dat de visteelt en de visserij; niet de spijsverteringsfysiologie, of de immunologie of de aquatische oecologie. Visteelt is een integrerend en multi-disciplinair vak. Disciplines zijn hulpmiddelen, geen doel. In concreto betekent dit dat het onderwerp uit het werkveld moet komen en dat de relatie van het onderzoek met de toepassing niet uit het oog mag verloren worden. Tegelijkertijd vraagt de sector om structuréle oplossingen en dus moet er voldoende wetenschappelijke diepgang in het onderzoek worden gebracht. Het is precies deze hang naar evenwicht tussen diepgang en toepassing, tussen fundamenteel biologisch en meer zootechnisch onderzoek die de vakgroep Visteelt en Visserij zo boeiend maakt, een Wageningse verworvenheid die zou moeten gekoesterd worden, want binnen Europa is er haast geen vergelijk. Ofwel wordt er gewerkt vanuit een Zoologische of Zootechnische discipline ofwel wordt uitsluitend naar praktijkoplossingen gezocht. In het eerste geval heb je fysiologen of morfologen die aan vis werken, in het tweede geval had het onderzoek ook door het bedrijfsleven zelf kunnen uitgevoerd worden. In beide situaties ontbreekt de basis voor een gebalanceerde opleiding in de Visteelt en de Visserij, zoals deze in Wageningen bestaat.

Het proefschrift dat voor u ligt volgt bovenstaande filosofie. In een snelgroeiende en dynamische sector zoals de visteelt is het onderscheid tussen ambachtelijk en industrieel, tussen kunde en kennis nog zeer gradueel en ondoorzichtig. Knelpunten en problemen zijn er te over en ééntje ervan is de opkweek van jongbroed tot jonge vis. Het probleem stelt zich bij vele vissoorten, en voor ieder van hen wordt vaak een praktische, en daarom ook vaak een casuistische, oplossing gezocht. Tijdens het onderzoek dat aan dit proefschrift ten gronde ligt, werd gepoogd een meer structurele benadering te volgen. "Wat zijn deze problemen bij de opkweek van jongbroed dan precies en waarom zijn ze zoals ze zijn" vormden de essentiële vragen. De Afrikaanse meerval werd daarbij uitgekozen als onderwerp van onderzoek. Niet omdat deze soort zo moeilijk is op te kweken (juist niet), maar omdat ervan uitgegaan werd dat we met deze soort de critische aspecten van de larvale visteelt op een gepaste wijze zouden kunnen analyseren en zodoende lering kunnen trekken voor vislarven in het algemeen. Een dergelijke analyse vergt een benadering vanuit diverse gezichtshoeken. Daarom komen in dit proefschrift ook verschillende disciplines aan bod: zootechniek, histologie, fysiologie, biochemie, enz. Zo een onderzoek kan niet door één persoon uitgevoerd worden, dat is evident. Er zijn dan ook vele "vaders" van dit proefschrift, en dat is maar goed ook. Modern onderzoek is vooral teamwork. En ook uit dit onderdeel blijkt hoe vaak universitaire bestuurders de relatie met *hun* werkveld uit het oog verliezen. Het is niet omdat er toevallig nog collega's zijn die aan vis werken of die over een gas chromatograaf beschikken dat daarom zinvolle samenwerkingsverbanden, laat staan functionele eenheden, kunnen gevormd worden. Teamwork (dus samenwerking) is iets ván en tussen mensen, en zonder dat ene kleine vonkje van gezamenlijk enthousiasme hebben alle reorganisaties en dito fusies geen zin, tenzij een bestaansreden bieden aan de uitvinders ervan. Tijdens het opbouwen van dit onderzoek is duidelijk geworden dat de "natuurlijke" partners voor larven-onderzoek zich in het buitenland bevonden. Het Europese program "Erasmus" is vervolgens een gouden hulpmiddel gebleken. Jaarlijks verzamelen zich in Wageningen een tiental "larven"-experts die tot de top van de wereld behoren om deel te nemen in het onderwijs van de Internationale Cursus "Fish Larvae Nutrition". Ik kan niet ontkennen dat veel van de concepten en ideeën die in dit proefschrift zijn uitgewerkt zijn ontstaan in de vele en informele gesprekken met deze mensen. Een "druppeltje" Bitter mocht daarbij wel eens van dienst zijn, al moet ik zeggen (*noblesse oblige*) dat een stevige Trappist, Brigand, Liefmans én Nelly's keuken meestal een betere bijdrage tot de ideevorming leverden...

Het punt is nu gezet. U dient het nog te lezen.

Johan Verreth

GENERAL INTRODUCTION

Critical Aspects of Fish Larval Rearing and Nutrition

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History and Importance of Fish Seed Production

Raising fish has a long history. The earliest records date back from 4000 years ago. Nevertheless, only during the last three decades fish culture is changing from an artisanal activity to a more rational exploitation by using animal production principles. During the past decade, the world aquacultural production showed continuous high annual growth rates (9%) (Born *et al.* 1994). In 1991, it attained a total volume of 12.7 million MT, out of which 69% consisted of finfish (FAO 1993). Also in the nearby future, a significant growth of the aquacultural production is expected, especially because the supply of fish from capture fisheries tends to stabilize (New 1991), while the continuous growth of the world population is boosting the demand.

Such a future growth of aquaculture must be supported by a reliable supply of fish seed to stock in grow-out tanks. To date, a significant part of the production relies entirely on the supply of wild sources of fingerlings. Examples are the cultures of milkfish (*Chanos chanos*), of yellowtail (*Seriola quinqueradiata*) and of eels (*Anguilla spp.*), together representing a production volume of about 600,000 MT per year. The seasonal fluctuations in seed supply makes these cultures fragile and limits their possibilities for expansion. Obviously, any future expansion of aquaculture must be supported by the supply from fingerlings raised in hatcheries.

The first step into this direction is the development of induced and out-of-the-season reproduction techniques. Major breakthroughs in this field were the development of techniques for hypophysation and for massive incubation of eggs in flow through funnels (Woynarovich 1961, Woynarovich and Horváth 1980). Additionally, significant research efforts were spent on unravelling the physiological basis of fish reproduction (Jalabert 1976; Scott and Sumpter 1983; Abraham 1988). Today for many farmed fish species it is possible to produce viable and qualified offspring at different moments throughout the year (Table 1), for instance in salmonids (Bromage *et al.* 1992), African catfish (Richter *et al.* 1994) or for European seabass and Gilthead seabream (Tandler 1993).

Another essential step in the production of fish seed consists of the culture of free swimming larvae until fingerling size. A very traditional way is to nurse larvae in ponds with abundant natural food supplies. The production of cyprinids (total production volume in 1991: 6.1 million MT) (FAO 1993) is entirely based on this system. When the yolk sac

Table 1. The availability of induced and/or out-of-the-season reproduction technology and different larval nursing techniques for the most important farmed fish species. P=larval nursing in prepared nursing ponds (natural food); LF=indoor nursing using cultured live food organisms, e.g. *Artemia*, FF=indoor nursing using formulated dry feeds. +/- = technology available/not available; ± = technology available but reduced growth and survival results when compared with live feeds; nn = not needed.

Species group	Hatchery technology available for:			
	Reproduction	Larval nursing		
		P	LF	FF
Cyprinids				
Common carp	+	+	+	±
Chinese carps (3 spp)	+	+	+	-
Indian carps (3 spp)	+	+	+	-
Salmonids				
Atlantic salmon	+	-	nn	+
Rainbow trout	+	-	nn	+
Tilapia (several species)	nn	+	nn	+
Milkfish (*Chanos chanos*)	-	+	-	-
Catfishes				
Channel catfish	nn	+	nn	+
Asian catfish (*Clarias spp.*)(3 spp)	+	+	+	-
African catfish (2-3 spp)	+	+	+	±
Yellowtail (*Seriola quinqueradiata*)	-	-	-	-
Eels (2-3 spp)	-	-	-	-
Flatfishes				
Japanese Flounder	+	-	+	-
Turbot	+	+	+	-
Halibut	+	+	+	-
Breams and Basses				
European Seabass	+	-	+	-
Asian Seabass	+	-	+	-
Golden Seabream	+	-	+	-
Red Bream	+	-	+	-
Groupers and Snappers				
Epinephelus spp	+	-	+	-
Other marine species				
Red Drum	+	-	+	-
Cod	+	+	+	-

Sources: Jones *et al.* 1993; Dhert 1992; Verreth 1987.

is absorbed, carp larvae are transported to specially prepared nursing ponds where plankton production is stimulated by fertilization. Predator infestations are reduced by short inundation times, pesticide control and mechanical sieving of the inlet water (Horváth *et al.*

1984, Zhang 1990). The larvae usually reach 2.5-3.0 cm in length after 25-30 days of rearing. During this period of nursery rearing, survival rates vary between 30 and 60% for common carp and between 30 and 80% for herbivorous carps, depending upon temperature fluctuations, the occurrence of predators and the "quality" of the plankton blooms present in the nursing ponds (Lei 1980; Horváth *et al.* 1984; Zhang 1990). For most other fish species, more dependable technologies for rearing larvae under controlled hatchery conditions are used, especially for the very first and delicate period of (larval) life.

Bottlenecks in larval rearing and nutrition

Development of dependable techniques for larval rearing faces several problems:

(1) the nutritional requirements for this specific stage of life are not known and (in the absence of a suitable rearing technology) are difficult to assess;

(2) the optimal environmental conditions and feeding behaviour during the early life stages are poorly understood;

(3) for most fish species, the covered rearing period, e.g. from hatched yolk sac larva to fingerling crosses several ontogenetic stages, and entails a weight increase of a thousand to several thousand times. It requests specific and changing husbandry and feeding practices;

(4) the tiny size of the organisms and the concomitant need for small food particle sizes poses specific strains on the feed technology.

CRITERIA FOR DIET SELECTION

Nutritional aspects are apparent key factors for success in larval rearing. Larval diets may be selected according to different sets of criteria depending upon the viewpoint of the farm manager or of the biological requirements set by the growing larva (Figure 1) (Léger *et al.* 1987). In contrast to the situation in grow-out farms, in modern fish hatcheries, direct feed costs are of minor importance (12.2% of total production costs, Drs John and Elizabeth Sweetman, Cephalonian Fisheries, Greece, personal communication). Nevertheless, the indirect cost may be high, especially when different live food organisms have to be cultured. Such cultures often require high investments in specific installations and trained personnel to run them. Therefore, at present more emphasis is put on simplicity and versatility in use of the diets. Until today, survival is thereby considered more important than growth, because fingerlings are sold per piece and not per kg. Another important criterion is the

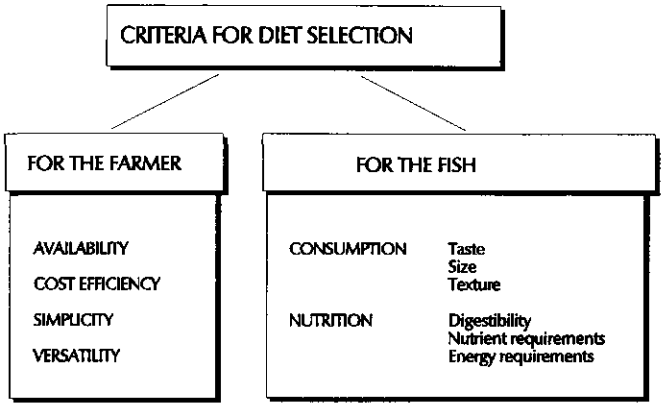

Figure 1 Selection criteria for larval diets from the viewpoint of the hatchery manager and of the cultured larva (after Léger et al. 1987).

constant availability of the diet. Collecting the natural food of the larvae, e.g., plankton organisms, is therefore not desirable. Especially for marine fish larvae which are usually much smaller than larvae of freshwater species, the collection of sufficient food organisms of the right size is almost impossible.

Criteria related to feed intake

When biological considerations are taken into consideration, the most important criteria for diet selection refer to the processes of feed intake and feed digestion (Figure 1). The ingestion of food by fish larvae is probably affected by its size and its palatability. Size is thereby considered as the most important aspect. Until now there is not much information on and not much attention paid to the palatability of larval diets. In nature, larvae of most fish species have a very selective food preference. Most fish species require tiny food organisms as first feed. Dabrowski and Bardega (1984) estimated that the initial food sizes of silver carp (*Hypophtalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*) and bighead carp (*Hypophtalmichthys nobilis*) ranged between 50-90, 90-150 and 150-270 µm respectively. Some marine fish species require even smaller organisms at the onset of exogenous feeding. In the case of groupers (*Epinephelus spp.*), eggs and trochophore larvae of bivalves may be used (Lim 1993) and for gilthead sea bream (*Sparus aurata*) ciliates and small rotifers have been tested (Chatain 1994). As food preference is closely related to the match between food and mouth size, the selected food changes with the growth of the fish. In pond nursery cultures, zooplankton succession should be synchronized with the changing

demands of the growing fish larvae (Geiger 1983, Opúszynski *et al.* 1984, 1985). In the early eighties the use of insecticides such as trichlorfon (00-dimethyl-2,2,2,-thrichlorohydroxyethyl phosphonate) was advocated to induce an effective zooplankton succession in cyprinid (Tamás and Horváth 1976, Horváth *et al.* 1984; Opúszynksi *et al.* 1984) and pike-perch (Verreth and Kleyn 1987) nursery ponds. Increased environmental awareness has brought this practice to a deadlock. In indoor hatcheries, food organisms of different sizes have to be cultured and provided successively. A typical feeding regime as it is currently used in marine larviculture is shown in Figure 2. It presents the periods during which the subsequent live foods are given to red seabream (*Pagrus major*) larvae in a mediterranean hatchery (Sweetman 1992). Time is expressed as day degrees, i.e. the product of average daily temperature with the number of days.

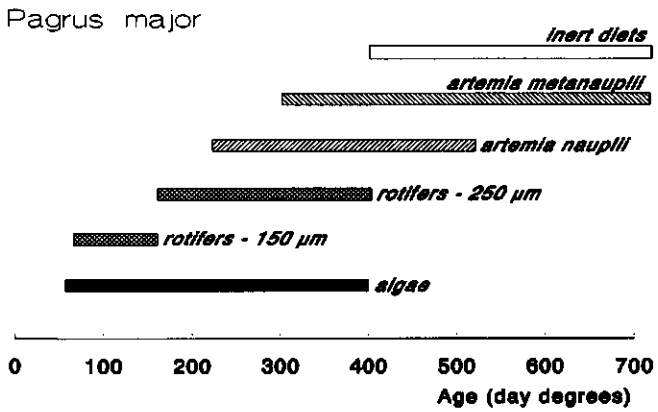

Figure 2 A typical feeding regime used for the cultivation of Red Seabream larvae (after Sweetman 1992).

Because of the small initial mouth size of the larvae, first a small variety of *Brachionus plicatilis* must be raised and given to the fish for about a week, followed by a larger variety during the next 2-3 weeks. Algae are added to the larval fish tanks to condition the within-tank light conditions (see also page 12) and to ensure a good and sustained level of essential fatty acids in the food organisms (Sweetman 1992). When the fish larvae have grown sufficiently, they receive the larger (400-500 µm) sized *Artemia* nauplii. In general, the food should have a size of around 2-3% of the larval length (Uys and Hecht 1985, Chatain 1994).

Criteria related to diet quality and feed digestion

Given a certain size, the most important feature of a larval diet is its nutritional quality. Unfortunately not much information is available on the nutritional requirements of larval fish (see also p. 5). Empirical evidence has shown that in larval feeds, dietary levels of essential fatty acids (mainly *n*-3 highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) play a crucial role. Especially in marine fish larvae, elevated dietary EPA and DHA levels improved survival rates (Izquierdo *et al.* 1989, 1992), stress resistance (Kraul *et al.* 1993), growth rate (Izquierdo *et al.* 1992; Koven *et al.* 1992) and skin pigmentation (Kanazawa 1993; Reitan *et al.* 1994). The importance of dietary fatty acids in larval feeds can be derived from the fact that at present about 75% of the studies published in the field of larviculture and larval nutrition deal in one or another way with the *n*-3/*n*-6 fatty acid requirements and/or dietary supplies of EPA and DHA to larval fish. Today research in larviculture starts to move its interest towards other nutritional compounds, such as free amino acids (Fyhn 1993), vitamins (especially vitamin C) (Merchie *et al.* 1994), and pigments (Sorgeloos and Léger 1992). Further, the development of dry off-the-shelf diets (Appelbaum 1979; Uys and Hecht 1985; Charlon *et al.* 1986; Kanazawa *et al.* 1989) and the addition of exogenous enzymes to these diets (Kolkovski *et al.* 1993) are important targets of research in larval nutrition.

Type of Diets

Cultured Live Food

During the seventies and the eighties, research was strongly oriented towards development of techniques for mass rearing of food organisms (Dhert 1992; Sorgeloos and Léger 1992). The most important live food organisms used today are micro-algae (especially for shrimp and bivalve larvae); rotifers (*Brachionus plicatilis*) and the brine shrimp *Artemia spp.* For many larval fish species the nutritional composition of these live feeds is not very appropriate (Watanabe *et al.* 1983; Léger *et al.* 1987). Especially the levels of essential fatty acids (DHA and EPA) are often insufficient to maintain good growth and survival. The so-called "bio-encapsulation" or "*Artemia* enrichment" technique was developed to augment the *n*-3 HUFA levels in *Artemia* and in *Brachionus* (Léger *et al.* 1986; Sorgeloos and Léger 1992). This technique is nowadays widely applied in marine fish and shrimp hatcheries and its development constituted a real break-through in the larviculture of marine fish species (Sorgeloos and Léger 1992).

Formulated Dry Diets

To date the most successful results with dry diets have been obtained in freshwater fish larvae. Successful attempts to raise larvae solely on dry diets have been reported for carp (*Cyprinus carpio*) (Charlon *et al.* 1986; Radunz Neto *et al.* 1993), coregonid species (*Coregonus spp*) (Bergot *et al.* 1986; Champigneuille 1988) and African catfish (*Clarias gariepinus*) (Uys and Hecht 1985; Appelbaum and Van Damme 1988; Van Damme *et al.* 1990). All the successful diets share the common feature that their major component (50-70%) consists of Single Cell Protein (SCP) (yeast cells belonging to the genera *Candida, Torula* and/or *Kluyveromyces*). However, reported growth rates are usually below the ones which are obtained with live food (see also Jones *et al.* 1993). Supplemented feeding with live food remains necessary (Hilge 1986; Hecht *et al.* 1988). Further, to date there exists no explanation why the use of dry diets is more successful in freshwater fish larvae than in marine species. Moreover it is not exactly known why SCP seems to be essential in successful dry diets.

Present status of the larval rearing of *Clarias spp.*

The importance of CLARIAS in aquaculture

Catfishes of the genus *Clarias* are the second most important group of farmed catfish in the world (FAO 1993). In 1991, the world production of clariid catfish totalled more than 67,000 MT and increased since 1985 with about 7% per year (FAO 1993). Recent estimates suggest even a total production of 125,000 MT (Csavas 1994). The farm output relates principally to *Clarias batrachus* in Thailand, Indonesia and India; *C. fuscus* in Hong Kong and The Philippines; *C. macrocephalus* in The Philippines, Malaysia and Thailand and *C. gariepinus* in Africa (mainly South Africa and Nigeria) (Hecht 1994) and Europe (The Netherlands, Germany, Belgium) (Verreth and Eding 1993). *C. gariepinus* has an endemic geographic distribution from the Middle East in the North to the Orange River in the South (Teugels 1984). During the last decade, *C. gariepinus* has also been introduced into Europe, Asia (Vietnam, Indonesia) and Latin America (Brazil). The two most important *Clarias* species in aquaculture are *C. batrachus* and *C. gariepinus*. However, the majority of the present *Clarias* production consists of a hybrid of either *C. gariepinus* and *C. batrachus* or of *C. gariepinus* and *C. macrocephalus* (Karnasuta 1993; Lawonyawut *et al.* 1993, Csavas 1994).

Table 2. Feeding strategies applied in hatchery rearing of larvae of *Clarias gariepinus* and *Clarias batrachus*. A distinction is made between the Indian and Indonesian variety of the latter species. Data are obtained from literature, and personal observations by Dr. G. Rao, CIFA, Bhubaneswar, India (Magur) and ir. F. Huskens, Nusantara Aquafarm, Solo, Indonesia.

Clarias gariepinus	*Clarias batrachus*
A. Semi-extensive	
Janssen 1985, 1987.	Knud-Hansen *et al.* 1990
day 1-15: *Artemia* ad lib. Fish size at harvest: 100-300 mg	day 1-16: *Artemia* ad lib. Fish size at harvest: 40 mg
15-30: nursing ponds suppl. feed: wheat bran	16-56: nursing ponds suppl. feed: fish meal
Fish size at harvest: 1 g	Fish size at harvest: 3.9 g (7.2 cm)
B. Intensive/live food	
Verreth *et al.* 1987	Rao *et al.* (in press) (per 1000 larvae^{-1}.day^{-1})
day 1-10: *Artemia* restricted Fish size at day 10: 100-150 mg	day 1-3: 2 ml zooplankton
10-14: gradual weaning	4-10: 4 ml zooplankton + 1 g egg white
	11-16: 6 ml zooplankton+ 2 g egg (whole) + 2 g mollusc paste
15-30: commercial trout pellet	17-26: 8 ml zooplankton + egg + mollusc paste + fish meat (2 % BW)
Fish size at harvest : 1 g	Fish size at harvest: 450 mg
C. Intensive/dry diets	
Britz & Hecht 1988; Hecht & Oellermann 1994	Huskens (pers.comm)
day 1-14: SCP diet + *Artemia* (3-5 days, 3 times/day)	day 1-3: *Artemia* ad lib. (15 h.day^{-1})
	4-8: *Artemia*+Nippai ad lib. (15 h.day^{-1})
	9-16: Nippai ad lib. (day-time) + dough (36 % protein) (at night)
Fish size at day 14: 16-25 mm	Fish size at day 13: 52 mg
14-35/42: nursing ponds	17-29: dough (24 h.day^{-1})
Fish size at harvest : 3-4 cm	Fish size at day 27: 228 mg; at day 44: 1.3 g

Source: Verreth *et al.* 1993

Feeding practices in larval rearing

The strong increase in production of farmed *Clarias* species has led to a wide array of both extensive and intensive techniques for fingerling production (Janssen 1985, 1987, Polling *et al.* 1988, Britz and Hecht 1988, Knud-Hansen *et al.* 1990, Rao *et al.* (in press), de Graaf *et al.* 1995) (Table 2). All this procedures recognize the existence of an "early" and an "advanced" larval stage, each of which requires a specific feeding regimen. During

the early stage, roughly coinciding with the first two weeks after the start of exogenous feeding, a specific larval diet (mostly live food) seems to be required. After this period, the larvae are less dependent upon live food, and the additional administration of inert wet or dry diets is quite common during this period. Clariid larvae are reared from hatching to fingerling size, either completely in extensive pond culture systems (de Graaf *et al.* 1995), solely in the hatchery (Verreth *et al.* 1993, Haylor 1993) or for a 10 - 16 day period in the hatchery, followed by a nursery phase in ponds (Polling *et al.* 1988, Britz and Hecht 1988, Knud-Hansen *et al.* 1990 (Table 2). When reaching the "advanced" stage, larvae are gradually weaned to inert diets or when raised in ponds they receive wheat bran or fish meal in addition to the natural food. For *C. batrachus* in India, the first adult-like feed (molluscan meat paste) is given 11-16 days after the start of exogenous feeding (Rao *et al.* in press). At that age, the same species in Indonesia is already weaned to a dry diet, but a specific dry larval diet is still administered in parallel (*Nippai artificial plankton C1*). Weaning to the more "adult-like" dough diet is completed at day 20 after the start of exogenous feeding. For the African species *C. gariepinus*, weaning starts after 10 (Verreth *et al.* 1987), 12 (Polling *et al.* 1988) or 14 (Janssen 1987) days. Before that period, a specific larval diet is required, either in the form of live food (Verreth *et al.* 1987; Verreth and van Tongeren 1989; Polling *et al.* 1988) or in the form of a Single Cell Protein based diet (Uys and Hecht 1985, Hecht 1994). Especially in South Africa (Uys and Hecht 1985), Israel (Appelbaum and Van Damme 1988) and recently in Belgium (Van Damme *et al.* 1990), major progress has been made in rearing *Clarias* larvae with dry diets based on yeast. The best results are still obtained when live food is given as supplement to the SCP-diet during the very first days of exogenous feeding (Britz and Hecht 1988; Van Damme *et al.* 1990, Hecht 1994).

The biological basis of larval rearing and feeding

With exception of a few species, teleosts are oviparous. According to Balon (1975) the embryonal period lasts from fertilization to complete yolk absorption, e.g., including both the egg stage and the free swimming yolk sac (eleuthero-embryo) stage. Only after complete resorption of the yolk sac, the larval period starts. Fish larvae are thus dependant upon exogenous food sources. It means that the larvae must be capable to detect, capture, ingest, digest and metabolize the food at the moment that exogenous feeding starts.

Aspects related to feed intake

Most fish larvae are visual predators and have large eyes in comparison to their juvenile and adult conspecifics. Salmonids hatch with pigmented eyes and subsequently a duplex retina develops during the yolk sac period (Noakes and Godin 1988). In coincidence with this development, also the tectum opticum changes, in particular with regard to the synaptic differentiation (Noakes and Godin 1988). Many marine fish species have no pigmented eyes at hatching and have a pure cone retina at the start of exogenous feeding. Rods are developed during the larval period. It is very probable that in these species also brain structures change after the onset of exogenous feeding, similar to the ones found in salmonids during the yolk sac stage (Blaxter 1986). Some species (e.g., European seabass *Dicentrarchus labrax*) have few large cones (Chatain 1994), others have many small ones. As a consequence, at first feeding many fish larvae have a reasonable visual acuity, but cannot adapt their vision to changes in light intensities. Moving prey will be essential for early larvae of *D. labrax*. Maybe the contrast between food particles and background illumination is more important for larvae with many small cones. Howell (1979) demonstrated that white tanks were less suitable than black ones for rearing of turbot larvae. This may be related to reflection of light from the white sides of the tank thereby reducing the contrast between prey items and background illumination (Støttrup 1993). Feeding and visual capabilities seem also to be related to the perception distance at which larvae do recognize food. For most fish, perception distance increases with growth, but remains shorter than body length (Blaxter 1986). This means that, depending upon the size of the fish, food particles must be within a distance of 0.5 to 2 cm of the fish. As a consequence, the distribution of the prey over the available volume might be more important than the absolute amount of food. The same applies to the fish larvae. Many fish larvae (e.g., *D. labrax*, *S. aurata*, *C. gariepinus*) tend to clump at the bottom and the walls of the rearing tank, and as a consequence prey items in the "open water" area of the tank remain unavailable to the larvae. Also this might be an effect of within-tank light conditions, because in turbot larvicultures this behaviour can be reduced by covering the tanks or by adding algae to the tanks (Støttrup 1993).

Besides the ability to detect a food particle, larvae must also capture and ingest it. Drost and van den Boogaart (1986) and Osse and van den Boogaart (1994) have presented extensive overviews of the constraints which are faced by the young larvae in capturing food and how they manage to cope with it. The smaller the larvae, the greater the relative importance of viscosity forces compared to the forces of inertia. Apparently, larvae experience the water properties as those of a kind of a syrup. Under such conditions it seems

to be advantageous for the larvae to use an anguilliform mode of locomotion because each body section can then contribute to an effective production of locomotor power by pushing against the water. Continuous swimming is then energetically efficient and therefore used by the young larvae (Batty 1984; Blaxter 1986, 1988). This mode of swimming facilitates also the uptake of oxygen by skin diffusion, the primary mode of respiration in early larvae since gills are not yet ready for gas exchange (El-Fiky *et al.* 1987; Osse 1989). Skin respiration is further facilitated by the presence of a so-called superficial red muscle layer which surrounds the body in early larval stages and by an inner embryonic white muscle mass which contains a relative large amount of mitochondria and in which an aerobic metabolism prevails (Hinterleitner *et al.* 1987; El-Fiky *et al.* 1987; Akster *et al.* 1994).

Aspects related to digestion

Ingested food must be digested, absorbed and metabolized. The implications of ontogenetic changes on the digestive physiology in fish have been reviewed by Stroband and Dabrowski (1982), Dabrowski (1984; 1986) and Govoni *et al.* (1986). Larval fish can be divided into three groups according to the morphology and physiology of the digestive tract (Dabrowski 1984). Salmonids have a functional stomach at the start of exogenous feeding, while some other species (e.g. all cyprinids) remain stomachless throughout their life. A third group consists of those species (e.g., *Coregonus spp.*, *Clarias gariepinus* and most marine fish species important to aquaculture) which have no functional stomach at the start of exogenous feeding but which develop it later on (Stroband and Kroon 1981; Dabrowski 1984; Pedersen and Falk-Petersen 1992). At the onset of exogenous feeding, most fish larvae have a relative short and straight digestive tract, which reduces the gut passage time. In the absence of a functional stomach, an alkaline pH prevails and pepsin digestion will not be possible. Trypsin, chymotrypsin and aminopeptidase activity have been detected in the intestine of first feeding larvae (Dabrowski 1984; Cousin *et al.* 1987). Some authors suggested that a poor proteolytic activity is counterbalanced by pinocytotic absorption of small peptides in the hindgut (Stroband and Dabrowski 1982; Govoni *et al.* 1986). Others believe that this pinocytosis serves for reabsorption of digestive enzymes (Pedersen *et al.* 1987) or for absorption of immunoglobulins (Rombout and van den Berg 1989). Dabrowski and Glogowski (1977), Lauff and Hofer (1984) and Munilla-Moran *et al.* (1990) provided evidence for an active role of prey enzymes in the digestion of food in fish larvae. This hypothesis is further substantiated by the results of Kolkovski *et al.* (1993) who realized in cultures of *Sparus aurata* larvae similar growth and survival rates when pancreatin was added to a dry diet as in the live food controls. Dabrowski and Poczyczyński (1988) suggested the inhibition of fish enzymes by proteinase inhibitors present in dry diets as an

explanation for the consistent lower growth rates found in larvae fed with formulated feeds. In a study on larval herring, Pedersen and Hjelmeland (1988) and Pedersen and Andersen (1992) demonstrated that the problems in rearing fish larvae on dry diets may be more related to an insufficient stimulation of endogenous enzyme release than to a low digestive capacity.

Aspects related to metabolism

It might be obvious that the specific morpho-functional organization of the larval organism must have its consequences on the energy and nutrient requirements of the fish larvae. Because aerobic energy fuelling prevails, in first feeding larvae the major energy substrates must be amino acids and/or fatty acids (Forstner *et al.* 1983). Their oxidation for energy fuelling competes with their use for tissue formation. This trade-off situation between growth and activity has considerable consequences for the early life history of fish (Wieser *et al.* 1988) and for the rearing conditions of fish larvae. Further, the dominance of aerobic pathways to fuel energy in larval fish means that glycolysis and thus also the activity of glycolytic enzymes will be low. This hypothesis was supported by low glycolytic activities found in coregonid larvae (Hinterleitner *et al.* 1987). Together with the switch from the superficial red muscle layer to gills as the major respiratory organ and the concomitant differentiation from embryonic to adult muscle types, the glycolytic capacity of the white muscle mass will increase (Hinterleitner and Wieser 1988). Fauconneau *et al.* (1986) found that in coregonid larvae of 90 mg, carbohydrates became an important energy source. Hence, differences in the intermediary metabolism of larvae and juveniles/adults are probably quantitative rather than qualitative.

Concepts in larval nutrition studies and in this thesis

The success of a larval rearing protocol depends to a large extent on the quality of the larval diets. This has induced an empirical and technical *Feed oriented approach* as opposed to a more biological *Fish oriented approach* in research on larval nutrition. To date the former approach has been very successful in eliminating bottlenecks for the commercial upscaling of larviculture. Increasing survival rates was and is thereby the major target (Chatain 1994). However, in the long run the quality of the produced fingerlings will be as important as the quantity. To produce strong and healthy fingerlings, not only survival but also growth must be fostered. This means that there must be a sound understanding of the physiology of nutrient utilization and growth in the larval fish, hence a shift towards the

second approach. It is envisaged that elucidating the mechanisms behind larval growth and nutrient utilization will also provide cues for the development of suitable alternatives for the live foods which are presently used in fish hatcheries. However, one may assume that the biological processes governing nutrition, survival and growth would not be affected qualitatively by the age of the fish. In that case it would be questionable whether there is a need for separate nutrition studies in larval fish. However, in previous paragraphs of this chapter sufficient evidence has been given that in practice, fish larvae do perform differently from juvenile and adult fish: they do not consume the same food, and when forced to, they do not utilize this food as juveniles and adults do. So, the question is whether the observed differences are just a matter of scaling or of qualitative differences.

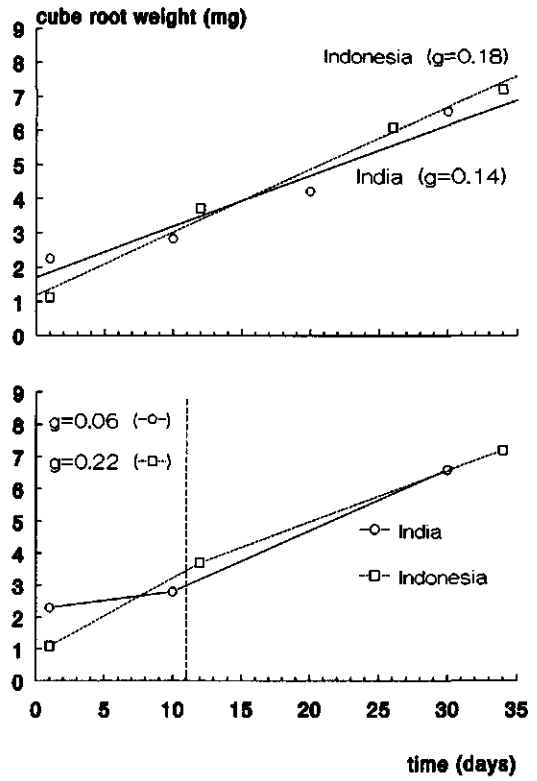

Figure 3 Growth of *Clarias batrachus* larvae obtained in India and Indonesia. Top: Data from the whole rearing period are used to estimate -g-. Bottom: The rearing period is subdivided in two periods (see text) and growth rate calculated for the respective periods. The mentioned -g- values refer only to the first period of 10, resp. 12 days. (Source: Verreth et al. 1993)

Only a fish oriented approach which is complementary to the present "feed oriented" and technical approach and which puts more emphasis on the relation between husbandry, diet and the capacity of the animal to cope with it, can answer this question. The fish larva should not be an instrument but the subject of research. Basic questions in this respect are: to what extent are larvae able to ingest, digest, absorb, transport and metabolize nutrients and why can the larvae cope with nutrients in live food and (mostly) not with those in dry food? In the present paragraph, this concept is further substantiated by some examples which demonstrate the necessity of combining practical with basic biological data.

The importance of staging and delineation of the experimental period

Based upon the empirical results presented earlier, the larval period of *Clarias* species can be divided in an early stage, lasting 10-15 days, and an advanced stage lasting until fingerling size, e.g. until about 30-45 days after start feeding. It is questionable however, whether this second period should still be regarded as belonging to the larval period. The real nutritional problems which relate *sensu stricto* to larval feeding (dependence on live food) occur during the first 10-14 days. The importance of this conclusion was shown by Verreth *et al.* (1993) by comparing the average growth rates obtained during the larval rearing of *Clarias batrachus* in India and Indonesia (Figure 3). If growth rates are plotted for the entire feeding period (30-35 days) (Fig. 3A), differences are insignificant (g = 0.18 and 0.14 for the Indian and Indonesian example respectively). Making the subdivision for early and advanced larvae (Fig. 3B), a different picture emerges. During the first 10 days of feeding, the growth rate obtained in India was very small (g= 0.06) compared to the growth rate obtained with the same species in Indonesia (g = 0.22). It is very probable that the restricted feeding levels applied in India during this period using only zooplankton and boiled egg as food, were the major reason for the lower growth rate compared to Indonesia where *Artemia* was fed *ad libitum*. Better growth rates of *C. batrachus* larvae in India could be expected if *ad libitum* feeding of zooplankton would be applied. Obviously, a clear definition of the larval period is needed in studies of larval nutrition.

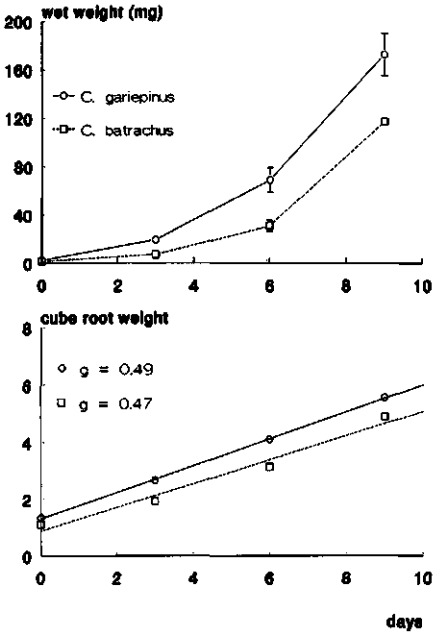

Figure 4 Growth of *C. batrachus* and *C. gariepinus*. Top: Growth expressed as wet weight increase. Bottom: Growth expressed as a rate according to the cube root model (Hogendoorn 1980). The slope of the regression lines (g) is a measure for the growth rate. (Source: Verreth et al. 1993)

The importance of appropriate growth parameters

The importance of choosing the most appropriate parameter in comparing different species and different feeding strategies is further evidenced by Figure 4. *Clarias batrachus*

was regarded as a slow grower because it reaches much smaller sizes than the African species in the same period of time (Table 2). In a comparative growth study (Herath 1988) the African species attained a final weight of 190 (± 5.4) mg after 10 days of feeding, against 119.6 (± 3.5) mg for the Asian species (Figure 4A). However, when the growth rate *g* was calculated (according to Hogendoorn (1980) growth rate can be presented by the slope of the regression line between the cube root of weight and time) both species grew equally fast (g = 0.49 and 0.47 for *C. gariepinus* and *C. batrachus* respectively). Apparently, size differences at the onset of exogenous feeding, often directly related to the egg size, have a strong impact on growth. Therefore, by comparing relative growth rates instead of absolute growth, the dynamics of growth are better described. Further, reference to the growth potential (maximum growth rates) of the species under consideration would be advisable. The latter calls for detailed studies on the growth performance under different regimes of temperature, feeding level, feeding frequency, etc., providing a reference set of growth values for different environmental circumstances.

The Present Research

Hypothesis and Objectives

At the Department of Fish Culture and Fisheries of the Wageningen Agricultural University research on larval rearing was initiated back in 1981. The aim was to develop a suitable protocol for producing pike-perch (*Stizostedion lucioperca*) fingerlings in the Netherlands. At that time (1983-1984), most larviculture studies focused on development and comparison of different larval diets and/or on description of the larval ontogeny. Dietary performance was reported in terms of larval survival and growth (either in length or in weight) in comparison to a control treatment (natural plankton or *Artemia*). A reference for evaluating the results in the control group was usually lacking. As a consequence, when in 1986/1987 the research focus shifted from (nursery) pond management to a larval fish nutrition study on *C. gariepinus*, it was decided to develop first standardized research conditions and a reference set of feeding and growth relations, e.g., the "husbandry-system". In the second phase of the project, this approach was further elaborated by describing the "fish-system", e.g., the ontogenetic development of the hardware (morpho-functional aspects) and of the software (physiology) of the digestive system in *C. gariepinus*. The third phase of the project focused on the nutritional (protein, energy and lipid) requirements in early life stages of this catfish species.

The project started from the assumption that adequate larval rearing and feeding procedures can only be developed when the biological properties of the developing young animal are taken into account. The basic hypothesis underneath the research project was that changes in nutritional requirements and the concomitant growth and survival of fish larvae are associated with ontogenetic changes. In other words, those aspects which request specific larval rearing and feeding strategies are related to the ontogenetic differences between larvae and juveniles. To our view, nutritional differences between larvae and juveniles are primarily a matter of scaling because of size differences. At the same time, these quantitative (scaling) effect is superimposed by qualitative differences set by ontogenetic effects, e.g., incomplete development of organ functions. Therefore, the relation between larval nutrition and associated ontogenetic effects was selected as subject of the present research project.

In this study, the African catfish *Clarias gariepinus* was used as research model because (a)it is representative for the large group of fish species which have an incomplete developed digestive tract at the onset of exogenous feeding (Dabrowski 1984); (b)it is relatively easy to culture during the larval period and this enables to perform nutritional studies under optimal conditions of growth and survival. The latter aspect is considered of utmost importance. In many marine fish species however, these optimal conditions are not easy to attain. Often, survival rates are not higher than 10-50%, and also high percentages of deformities are noticed.

The aims of the study were (a)to define the optimal conditions for growth and survival in larval *C. gariepinus*. Optimal conditions with regard to feed type, feeding level, temperature, and minimal experimental duration are considered as essential for standardized experimental conditions in research on larval nutrition; (b)to describe the ontogenetic changes in structure and functions of the larval digestive and metabolic capacities; (c)to analyze the relation between ontogeny and lipid, energy and protein metabolism. The latter objective was approached by studying the metabolism in embryos (eggs and yolk sac larvae) and in starving larvae of *C. gariepinus*. The rationale of this approach was that these developmental stages provide a simple system, without interference of external feeds, to study growth and nutrient dynamics in early life stages of fish while valuable information can still be collected. Similar experiments in feeding larvae were done as well.

OUTLINE OF THE THESIS

The eight publications which form the basis of this thesis cover the first two phases (description of the reference "husbandry" and "fish" system) and the majority of the studies dealing with lipid metabolism from the third phase of the project. The remaining parts of the third project phase are treated in separate PhD studies of Abdurrahman Polat (Çukurova University, Adana, Turkey) and of Luis Conceição (Portugal). The present thesis is structured according to the project organization: Part 1 deals with the general rearing and feeding conditions, Part 2 deals with the ontogeny of the digestive system in larval *C. gariepinus* and Part 3 refers to the Lipid metabolism in early life stages of this species.

Part 1. The three studies (chapters) carried out during phase one of the research project answer the questions: what reference diet should be used (Chapter 1), what is the potential (reference) growth and growth performance under different conditions of feeding and temperature (Chapter 2), and when is the larval *C. gariepinus* ready to cope with a common dry food (Chapter 3).

Part 2. The two studies which constitute part 2 of the thesis deal with the morpho-functional development of the digestive system (Chapter 4) and the development of the activities of some key enzymes of the intermediary metabolism (Chapter 5).

Part 3. Three studies on lipid metabolism in early life stages of *C. gariepinus* were included in this part. Chapter 6 describes the conversion and dynamics of lipid classes from yolk to body tissue, chapter 7 the dynamics of fatty acids during the same period. Chapter 8 reports the results of a pair growth experiment with feeding larvae of *C. gariepinus* which received diets with either high or low contents of HUFA, in an attempt to study the importance of dietary *n*-3 HUFA for this larval freshwater species.

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Part 1

REARING OF *CLARIAS GARIEPINUS* LARVAE

CHAPTER 1

A Comparative Study on the Nutritional Quality of Decapsulated Artemia Cysts, Micro-Encapsulated Egg Diets and Enriched Dry Feeds for *Clarias gariepinus* (Burchell) Larvae

Johan Verreth, Volker Storch and Helmut Segner

Abstract

The nutritional value of several dry test diets for larvae of the African catfish, *Clarias gariepinus*, was determined. Dried and decapsulated cysts of *Artemia* gave consistently the best growth and survival rate (over 100 mg and 96%, respectively, after 2 weeks). Dry larval feeds enriched with an acetone extract of *Artemia* resulted in low growth and extremely low survival rates (less than 20%). The hepatocyte ultrastructure indicated a nutritional deficiency in this diet. Micro-encapsulated egg diets resulted in high survival rates (varying between 64 and 93 %) but low and varying growth rates. The best growth was attained after addition of casein and a vitamin/mineral mix to the egg diet. The hepatocyte ultrastructure revealed a shift from a glycogen to a lipid-based metabolism, which is related to the high fat content of the egg diets. According to the hepatocyte structure, the egg diets provide all essential nutrients. It is therefore hypothesized that the low growth is caused by a decreased feed intake which again may be related to the high fat content of the diets.

Key-words: Clarias gariepinus, Larval Nutrition, Dry Diets, Artemia

Introduction

The use of dry compound feed for larval fishes is not yet widespread. The major constraint is the poor survival and/or growth obtained with commercial dry feeds in the past (Van Der Wind 1979; Msiska 1981). To explain the inferiority of dry feeds, several hypotheses have been proposed which generally emphasize the role of food intake and/or food digestion. The intake of food depends upon chemical attractants (Appelbaum 1979 1980) and physical characteristics of the food such as particle size, elasticity, texture, etc. (Van Limborgh 1979; Tacon and Cowey 1982; Dabrowski and Bardega 1984). Further, leaching or oxidation of nutrients may impair the nutritional quality of the feed (Tacon and

Cowey 1982). According to Van Limborgh (1979) and Meyers (1979), these problems can be solved technologically. The digestion of larval feed depends upon the ontogenetic development of the digestive system. In fish larvae the latter is often underdeveloped at the onset of exogenous feeding (Stroband and Kroon 1981; Stroband and Dabrowski 1982). It has been stated that enzymes of the prey may stimulate proteolytic activity in the larvae (Dabrowski 1979; Lauff and Hofer 1984). To formulate a suitable dry starter feed, more information on the nutritional requirements of fish larvae is needed.

Various approaches have been used to study larval nutrition. Chow (1981) reported good results with the so-called "micro-encapsulated egg diet" (MED), which is basically identical to chicken eggs to which some vitamins and/or minerals are added. Flüchter (1982) eliminated the nutritional deficiency of a dry starter feed by enriching it with an acetone extract of *Artemia*, indicating that the deficient factor in the dry feed is lipophilic and can be obtained by extraction of *Artemia*. Recently, major breakthroughs in the development of artificial larval feeds have been realised by using yeast (Appelbaum 1979; Dabrowski *et al.* 1984 1985; Uys and Hecht 1985). Verreth and Den Bieman (1987) successfully used decapsulated and dried Artemia cysts for rearing *Clarias gariepinus* (Burchell 1822) larvae. However, successful application of yeast or dried and decapsulated Artemia cysts does not give additional information on the specific nutritional requirements of the fish larvae. One of the problems encountered with the empirical approach of testing various diet formulations is the interpretation of quantitative survival and growth data into qualitative diet composition data. Additional information may be obtained from the ultrastructure of the liver cells (Storch *et al.* 1983, 1984).

In this study, experimental diets based on the MED of Chow (1981) and the procedure of Flüchter (1982) were compared with reference diets using processed Artemia. Larvae of the African catfish *(Clarias gariepinus)* were used as test animals. Growth and survival and the morphological structure of the hepatocytes were studied and used as parameters.

Materials and methods

All the experiments were carried out in rectangular 2 L aquaria, which were placed in a large tray, filled with water. The tray and the aquaria were part of a recirculation system, including a 100 L biological filter (lava-rock) and an oxygenation tower. Heating occurred in the filter. Oxygenated and heated water (30°C) from the filter was distributed over all the

aquaria. The water flow through each aquarium was about 8 $L.h^{-1}$. The aquaria overflow drained into the tray from where the water returned to the filter unit. The fry of *C. gariepinus* were obtained by artificial reproduction (Hogendoorn and Vismans 1980). On the final day of yolk absorption, each aquarium was stocked with 300 larvae. Feeding started the next morning when all yolk was absorbed. At this stage, the average weight of the larvae was 2.2 mg (± 0.2 mg). All experiments were carried out with two replicates per treatment.

Experimental diets

The following types of diets were tested: (a) Diets based on *Artemia* and (b) MED

(a) Diets based on *Artemia*

- Dry decapsulated cysts of *Artemia* (strain Burliu, U.S.S.R.) which were dried in a fluidised bed dryer (Vanhaecke and Sorgeloos 1982; Sorgeloos *et al.* 1983) and produced by the *Artemia* Reference Centre, Gent, Belgium.
- Shock-frozen *Artemia* nauplii were prepared by immersing freshly hatched nauplii in liquid nitrogen.
- Flüchter's (1982) diets (FD) were prepared as follows: An acetone extract of 25 g shock-frozen *Artemia* nauplii was added to 30 g dry starter feed Larvit A (Trouw and Co., Putten, The Netherlands). The suspension was dried in a rotary evaporator at 25-30°C and ground until a small particle size was obtained (varying between 150 and 300, µm). To prevent oxidation, the antioxidant BHT (butylhydroxytoluene) was incorporated in the diet. The level of BHT varied between the experiments (Table 1).

(b) MED

Yolk and albumin of fresh chicken eggs were separated and weighed. Depending upon the desired diet composition, different products were added to the yolk (vitamin mix, mineral mix, calcium, casein, lipid mix). The composition of the mixes of vitamins and minerals is given in Table 2. The lipid mix contained fish oil, soy-bean oil, lecithin and BHT in a ratio of 100:100:70:1. Casein and lipid mix were added in a concentration of respectively 2.44 g and 2.15 g/50 g egg weight, i.e. 50% of the average protein or lipid content of chicken eggs (Chow 1981). The other ingredients were added in the recommended doses (Table 1). In order to prevent fungal infection, Gentian Violet (5 ppm) was added to all the egg diets. The ingredients were mixed and cooked for 2 min in a steam bath. After deep freezing (-27°C), freeze drying and grinding, a dry and fine granulate product was obtained.

TABLE 1. The composition of the experimental test diets used in experiments 1, 2 and 3 (identical to 2): the quantities of the MED additives correspond to the amount added per 50 g of egg weight

	Vitamin mix (g)	Mineral mix (g)	$CaCO_3$ (g)	Lipid mix (g)	Casein (g)	Other	Protein (% dry weight)	Fat (% dry weight)
EXPERIM. 1								
Decapsulated								
Artemia cysts	-	-	-	-	-	-	53.2	10.9
MED[1]	-	-	-	-	-	-	47.6	21.8
MED+	0.26	-	-	-	-	-	46.6	34.7
MED+	0.44	0.44	0.88	-	-	-	34.4	34.2
MED+	0.53	0.53	1.07	2.15	-	-	36.6	40.8
MED+	0.55	0.55	1.10	-	2.44	-	50.9	23.7
MED+	0.64	0.64	1.29	2.15	2.44	-	43.9	35.5
FD+[2]	-	-	-	-	-	5000 ppm BHT	54.2	-[3]
Shock-frozen								
Artemia(SFA)	-	-	-	-	-	-	58.6	-[3]
MED+	0.44	0.44	0.80	-	-	1 g SFA	42.8	31.8
EXPERIM. 2								
Decapsulated								
Artemia cysts	-	-	-	-	-	-	-	-
MED	-	-	-	-	-	-	-	-
MED+	0.27	0.27	0.20	-	2.44	-	-	-
FD+	-	-	-	-	-	125 ppm BHT		

[1] MED = micro-encapsulated egg diet (Chow 1981).
[2] FD = dry started feed enriched with an acetone extract of *Artemia* (Flüchter 198882).
[3] Data were discarded because of unreliable analysis.

TABLE 2. The composition of the vitamin and mineral mixes: quantities are expressed per kg of dry feed

Vitamins		Minerals	
Vitamin A	6000 IU	FE	50 mg
Vitamin D_3	1000 IU	Cu	3 mg
Vitamin E	60 IU	Mn	20 mg
Vitamin K	12 mg	Zn	30 mg
$Vitamin_{12}$	2.5 mg		
Choline chloride	550 mg		
Niacin	220 mg		
Riboflavin	25 mg		
Pyridoxine	25 mg		
Thiamine	25 mg		
D-calcium pantothenate	50 mg		
Biotin	0.2 mg		
Folacin	5 mg		
Ascorbic acid	200 mg		

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Experiments

The first experiment was set up to screen a series of test diets as listed in Table 1. Feed was given in excess of satiation, four times a day (between 08.00 h and 20.00 h). Before feeding, the aquaria were cleaned, dead fishes counted and removed and the water circulation interrupted. Fifteen minutes after feeding, the water circulation was re-established. In experiment 2 a few diets from the first experiment were retested (Table 1) after reducing the concentration of vitamins, minerals and calcium in MED as well as the concentration of the anti-oxidant BHT in FD. On days 4, 7, 10 and 14, a sample of 10 fishes was taken from each aquarium, fried on tissue paper and individually weighed to the nearest 0.01 mg. At the end of the experiment (day 14), all fishes were counted. In order to obtain the exact survival rate, the final number of fishes was corrected for the number of sampled fishes to which a mortality rate was attributed according to the measured mortality distribution.

Experiment 3 was designed to evaluate the effect of the diets from experiment 2 on the hepatocyte ultrastructure of the fish larvae. After yolk absorption the larvae were fed for 6 days with decapsulated and dried cysts of *Artemia*, whereafter they were starved for 5 days. To avoid cannibalism during the period, dead or dying larvae were removed four times a day. After the starvation period the larvae were re-fed for 2 days with the different test diets (Table 1). From each test group a few fish livers were sampled, fixed and processed according to the procedure of Storch *et al.* (1984) and investigated using a transmission electron microscope.

Analysis of data

For the statistical analysis only average values per aquarium were used since aquaria represented the experimental units. Homogeneity of variances was tested by Bartlett's test (Sokal and Rohlf 1969). Individual weight data were linearized by a third root transformation (Hogendoorn 1980). The survival data were normalized by an arsine (arcsin $\sqrt{\%}$) transformation. The effect of diet type on average final weight in exp. 1 and the survival rates in experiments 1 and 2 were tested by a one-way analysis of variance. Duncan's multiple range test ($P < 0.05$) (Steel and Torrie 1960) was used to determine differences between means. The growth results of experiment 2 were analyzed by a single regression analysis of larval weight ($y^{0.33}$) on culture period (days). In order to correct for differences in culture period, treatment effects were analyzed by a test on the equality of slopes of the regression lines (Sokal and Rohlf 1969). The analysis of variance and multiple range test were performed using SAS General Linear Models procedure (SAS Institute Inc. 1985). The regression analysis was done by the BMDP 1R-program (Dixon *et al.* 1983).

The results of the electron microscope analysis were evaluated according to the following criteria: cell compartmentalization, size and structure of mitochondria, size and distribution of the endoplasmatic reticulum (ER), and the quantity of glycogen and fat reserves.

Results

Growth and survival

All diets, especially the MED diets, were eagerly taken up, except for the FD diet which was poorly accepted. After a few days, consumption of the FD diet decreased and the larvae exhibited an apathetic behaviour. The one-way analysis of variance of the final weight data of experiment 1 indicated a highly significant diet effect ($F = 185.3$; $P = 0.0001$). Decapsulated cysts of *Artemia* gave the highest average final weight (101.8 mg), followed by shock-frozen *Artemia* (57.1 mg) and a micro-encapsulated egg diet enriched with vitamins, minerals and casein (15.8 mg) (Table 3). All the other MED diets resulted in significantly lower average weights. The FD diet did not differ significantly from the MED diets in which casein was incorporated. These results were partly substantiated by the regression analysis of weight ($y^{0.33}$) on culture period in experiment 2 (Table 4). Decapsulated cysts of *Artemia* again yielded a significantly better growth rate than the other test diets ($P < 0.001$). The latter were not significantly different from each other ($P = 0.458$). According to the slope of the regression lines, larvae fed with FD showed the lowest growth rate.

In experiment 1, the best survival rate (96.9%) was attained with decapsulated cysts of *Artemia* (Table 3). The survival rates of larvae receiving shock-frozen *Artemia*, MED with casein and MED with casein and lipid mix were not significantly different from those fed decapsulated *Artemia*. Pure MED and MED with lipid mix addition resulted in significantly lower survival rates than MED with casein addition. The FD diet gave a significantly lower survival rate than all the other diets. Generally, the survival data of experiment 2 confirmed the previous conclusions (Table 4) but showed increased differences between treatments.

Hepatocyte ultrastructure

The hepatocytes of the larvae fed with decapsulated cysts of *Artemia* (Fig. 1) had an average cell diameter of 16-20 µm. They showed a well-developed internal compartmentalization and, as with all other diets, the nucleus was small and lobulated. Stacks of rough

TABLE 3. Final average weight and survival rate of C. gariepinus larvae after 14 days of feeding with different test diets (experiment 1).

Test diet	Final average weight (mg)[1]	Survival rate (%)[1]
Decapsulated *Artemia* cysts	101.8^{a}	96.9^{a}
MED	6.7^{fg}	77.2^{e}
MED + vitamins	5.8^{g}	86.2^{bcde}
MED + vitamins + minerals + Ca^{2+}	8.8^{ef}	81.2^{de}
MED + vit. + min. + Ca^{2+} + lipid mix	7.0^{fg}	78.0^{e}
MED + vit. + min. + Ca^{2+} + casein	15.8^{c}	92.0^{abcd}
MED + vit. + min. + Ca^{2+} + cas. + lipid	10.5^{de}	93.4^{abc}
FD	13.2^{cd}	20.6^{f}
Shock-frozen *Artemia* nauplii	57.1^{b}	94.6^{ab}
MED + vit. + min. + Ca^{2+} + *Art.* extract	10.0^{def}	82.7^{cde}

[1] Results not followed by a letter in common differ significant ($P < 0.05$) when compared on the basis of Duncan's multiple range test.

TABLE 4. Growth[1] and survival rate of C. gariepinus larvae after 9 days of feeding with four selected and modified test diets from the first experiment (experiment 2)

Test diet	g*	Survival rate (%)
Decapsulated *Artemia* cysts	0.25^{a}	75.3^{a}
MED	0.04^{b}	63.7^{b}
Med + vit. + min. + Ca^{2+} + casein	0.05^{b}	79.9^{a}
FD	0.02^{b}	0.0[2]

* Results not followed by a letter in common differ significantly ($P < 0.05$) when compared on the basis of Duncan's multiple range test.

[1] As expressed by the regression coefficient g of the regression of weight ($y^{0.33}$) on time (days).

[2] Since no fishes survived at the end of the experiment, this diet was excluded from Duncan's test.

endoplasmic reticulum (rER) could be observed around the nucleus and partially along the periphery of the cell. The mitochondria were closely associated with the rER or with lipid inclusions. They were of a roundish or cylindrical shape, and possessed electron-lucent matrices. The regularly arranged cristae were long, with a small lumen. The number of peroxisomes was moderate; they were generally located in the perinuclear layer of organelles. The lysosomal number was relatively low, while autophagic vacuoles were numerous. The amount of stored products was higher than with all other diets examined. Although the main storage product was glycogen, lipid droplets of varying size were also deposited in considerable numbers.

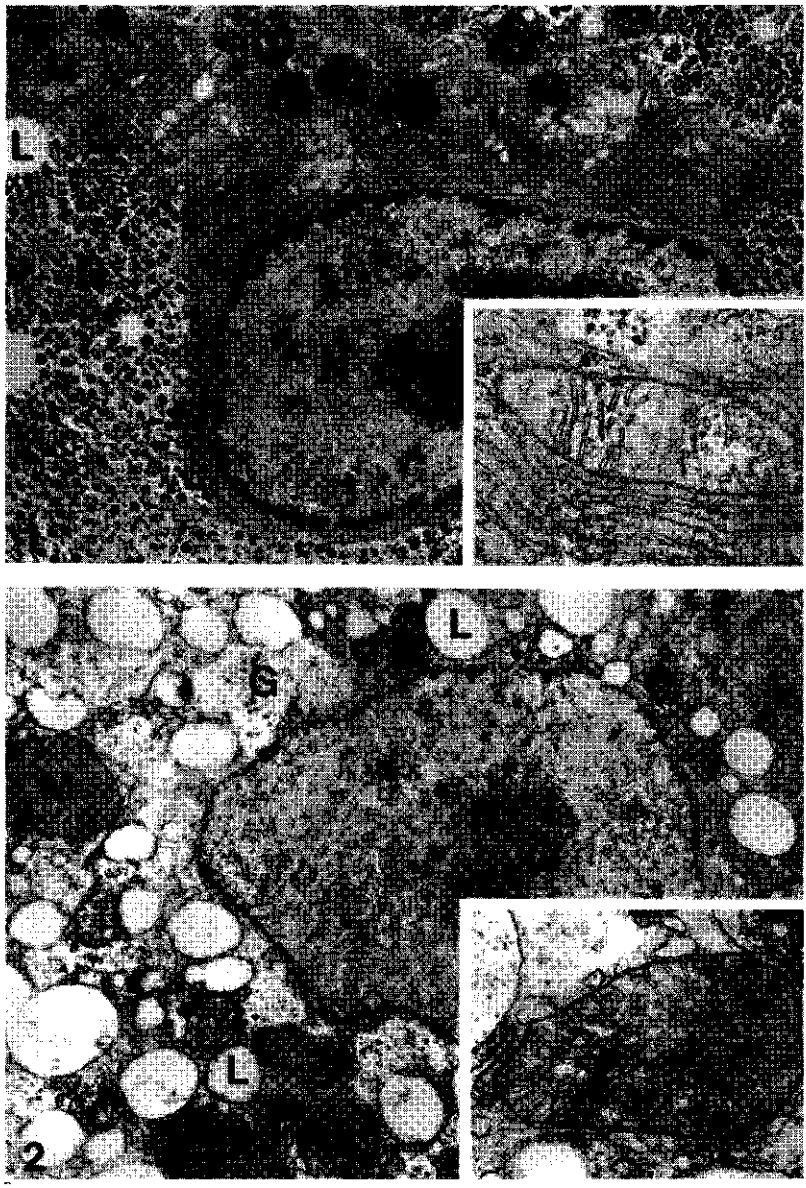

FIGURE 1. Electron micrograph of hepatocytes of *Clarias gariepinus* larvae fed with decapsulated *Artemia* cysts. They are characterized by a well-developed perinuclear layer of organelles and glycogen as the main storage product. 9220 X. Inset: normal mitochondrion. 27 120 X.

FIGURE 2. Electron micrograph of hepatocytes of *Clarias gariepinus* larvae fed with the FD diet. There is no clear spatial separation of areas containing organelles and areas containing storage deposits. Glycogen and lipid are stored, but in moderate amounts. Mitochondria display aberrant structural features. 10 200 X. Inset: mitochondrion with pathologically altered morphology. 27 120 X.

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The hepatocytes of larvae fed with FD (Fig. 2) had a somewhat irregularly shaped nucleus, surrounded by a broad layer of organelles containing short cisternae of rER, some mitochondria, lysosomes, peroxisomes and an enhanced number of dictyosomes. The cell diameter was 14-17 μm. The mitochondria were enlarged and exhibited electron-dense matrices. The arrangement of the cristae was aberrant (Fig. 2, inset). Often a longitudinal orientation of cristae could be observed. The rER was concentrated in the few short cisternae around the nucleus. The hepatocytes had a moderate glycogen content. Distinct glycogen fields did exist, but the alpha rosettes were loosely packed. There was also some storage of fat, but only small inclusions. They were scattered through the cytoplasm instead of being restricted to the glycogen fields, as observed for larvae fed with decapsulated *Artemia* cysts. In addition, the lipid droplets of FD-fed larvae had a different morphology in that they possessed an electron-dense marginal ring.

The hepatocytes of larvae fed with MED + casein (Fig. 3) were smaller than the previously described cells (11-14 pm). The cell compartmentalization was satisfactory. There was a perinuclear layer of organelles (rER, mitochondria, peroxisomes, lysosomes and dictyosomes) and some peripheral stacks of rER. In the mitochondria, no swelling or disturbance of cristae arrangement was observed. They demonstrated a well-structured appearance just as in larvae fed with *Artemia* cysts. Dictyosomes were numerous. Very often a close topographical relationship between mitochondrion, dictyosome and lipid inclusion could be noted. Lysosomes and peroxisomes were few in number. A peculiar character of these hepatocytes was the presence of numerous membrane delimited inclusions of mitochondrial size and of moderate electron density, which showed matrices consisting of bubble-like vesicles. Occasionally, these structures were also found in larvae fed with the FD diet. The glycogen content of the hepatocytes of fishes fed with MED+ casein was low. The dominating storage product was lipid.

The hepatocytes of larvae fed with the pure MED (Fig. 4) were even smaller (9-12 μm) than those of larvae fed with MED+ casein. There was no clear development of a cellular compartmentalization. The mitochondrial morphology showed no degenerative alterations such as spherical swellings or changes in the arrangement of the cristae. The membranes of rER were few in number, and were located mainly near the nucleus. About 40% of the hepatocytes had numerous and prominent lysosomes, whereas the lysosome content of the other liver parenchymal cells was low. Glycogen was nearly absent in all cells. Lipid, however, was present in large amounts.

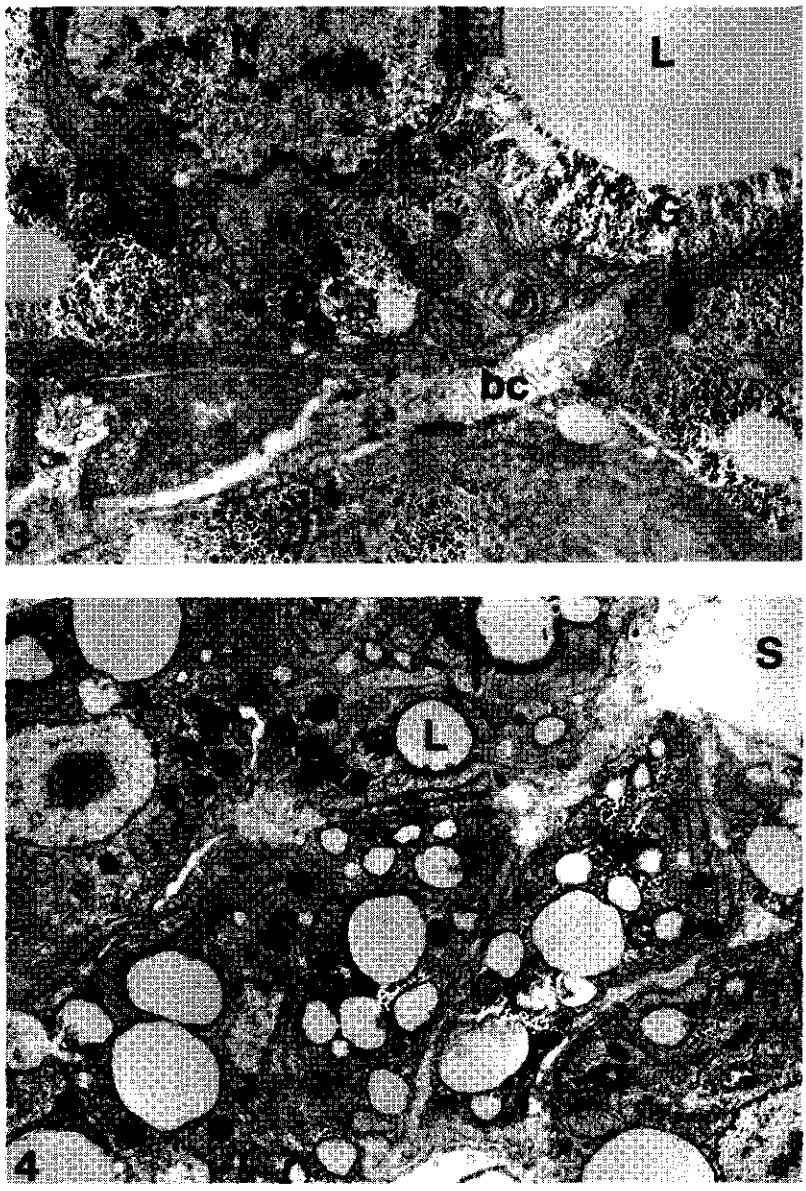

FIGURE 3 Electron micrograph of hepatocytes of *Clarias gariepinus* larvae fed with MED+ casein. Lipid is the main storage product. However, glycogen is deposited as well. Mitochondria show no pathological features and there is an internal cell organization. 10 900 X.

FIGURE 4 Electron micrograph of hepatocytes of *Clarias gariepinus* larvae fed with pure MED. The glycogen content is very low and lipid is almost the only energy reserve. No pathological alterations of cell organelles occur, but internal cell structure is relatively poor. 5200 X.

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Discussion and conclusions

The results indicate that the artificial dry feeds tested were not suitable for *Clarias gariepinus* larvae. However, decapsulated and dried artemia cysts seem to be an appropriate feed for larval rearing. The cysts have the appearance and the practical advantages of a dry feed. In contrast to *Artemia* nauplii (470-550 µm) their particle size (200-250 µm) is better suited for fish larvae. The elasticity of the cysts is high and their surface is very smooth. Dried and decapsulated cysts have a high floating capacity and sink only slowly to the bottom of the culture vessel. All the cysts have a balanced nutritional composition and they do not leach. The survival rate in our experiments was very high. Decapsulated cysts also gave the highest growth rate which was, however, much lower than the growth rate obtained by Verreth and Den Bieman (1987). Probably, in the present study, the husbandry system was sub-optimal. The fish density was four to five times higher than that used by Verreth and Den Bieman (1987), the aquaria were very small (2 l) and the recirculation system was less efficient, probably resulting in inferior water quality. Nevertheless, in the present study decapsulated *Artemia* cysts appeared to be the best diet and resulted in a considerable storage of glycogen. The additional deposition of lipid confirms the well-fed status of *Artemia*-fed larvae, demonstrating once more that *Artemia* is a good diet for larvae of *C. gariepinus*.

The Flüchter diet was clearly inadequate. In the first experiment, the growth rate was not significantly different from the best MED diets but the survival rate was much lower. The highest mortality occurred after 8-10 days of feeding. In experiment 2, the FD diet gave even poorer results than in experiment 1. Growth ceased and all fish died after one week. It is supposed that the low BHT concentration of the FD diet in the second experiment could have caused deterioration of the diet. With regard to the hepatocyte structure, two aspects are prominent. First, glycogen and lipid are deposited, indicating that the larvae are able to digest and metabolize the food. On the other hand, the observed structural aberrations of the mitochondria and the poorly developed cellular compartmentalization indicate a severe disturbance of the cellular metabolism. To evaluate the nutritional quality of a diet by means of the hepatocyte ultrastructure, the structural organization of the cell and the appearance of the organelles are much stronger criteria than the storage of energy reserves (Segner and Juario 1986). Thus, it must be concluded that the observed pathological changes are the expression of a deficiency of essential nutrients in the FD diet. Our results contradict those obtained by Flüchter (1982) with coregonid larvae. The poor results in this study may have been caused by a minor deviation in the diet preparation. In this respect, it should be stressed

that in the present study, shock-frozen *Artemia* (which is the basis for the FD preparation) gave a 50% reduction in growth compared to decapsulated *Artemia* cysts.

The micro-encapsulated egg diets resulted in a low growth rate but good survival of the fish larvae. Addition of casein gave consistently better growth and survival rate. Addition of lipid mix did not improve the MED diet performance. The hepatocytes were characterized by a dominance of lipid storage and only a small amount or nearly no glycogen. This is in contrast to the usual hepatocyte metabolism of *Clarias* larvae, which is based mainly on glycogen (Storch and Verreth, in prep.). Both egg diets evoke a shift to a lipid-oriented metabolism. A similar observation was made by Segner and Juario (1986) in *Chanos chanos* larvae. The high lipid content of the egg diets may explain why addition of casein yielded better growth. Henken *et al.* (1986) demonstrated that at 29°C the optimal ratio of crude protein to metabolizable energy for *C. gariepinus* juveniles of about 50 g amounted to 34.7 mg. kJ^{-1}. This coincides with a protein: fat ratio in the food of about 3:1, provided that all energy originates from these two sources. The best ratio attained in our MED diets was about 2:1. Paradoxically, the protein:fat ratio did not vary much between pure and casein-enriched MED, while the latter resulted in distinctly better growth. Possibly the egg proteins are less digestible than casein and/or have an unbalanced amino-acid composition. Using a dynamic growth model, Machiels and Henken (1987) demonstrated that the growth of *C. gariepinus* juveniles is directly related to the protein intake. According to this model, the maximal feed (or protein) intake is inversely related to the lipid content of the body mass, which in turn is determined by the fat content in the food. Consequently, in the long run, a high fat content in the food has a negative effect on feed ingestion. This hypothesis is further substantiated by the hepatocyte ultrastructure. The organelles do not exhibit pathological changes and the cell compartmentalization is, at least for the MED+ casein diet, very satisfactory, indicating that this diet provides all or nearly all essential nutrients. The low amount of storage products in the hepatocytes and the low growth rate of the larvae points to a low apparent energy content which can only be explained by a low digestibility and/or a low consumption of the diet.

The above findings stress the need for standardized experimental conditions in larval nutrition research. The final growth rate may be influenced seriously by the husbandry system. To quantify the effect of feed intake, nutritional experiments should be carried out at different feeding levels. To increase the comparability of the results, a standard reference diet, which allows a maximal growth rate, is needed. In our opinion, decapsulated and dried *Artemia* cysts of a known origin and processing batch can fulfil this role.

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CHAPTER 2

Quantitative feed requirements of African catfish *(Clarias gariepinus* Burchell) larvae fed with decapsulated cysts of *Artemia.* I. The effect of temperature and feeding level

Johan Verreth and Hans Den Bieman

Abstract

In a series of four experiments, the effects of temperature (range: 25.0-32.5 °C) and feeding level on the growth performance and survival rate of first-feeding larvae of the African catfish (*Clarias gariepinus* Burchell) was assessed. Feeding occurred with dried decapsulated cysts of *Artemia.* Growth performance was determined on the basis of growth rate, food conversion ratio, energy conversion efficiency, protein efficiency ratio, and apparent net protein utilization. A new approach to assess growth rate and feeding levels in larval nutrition studies is presented and discussed. The quantity of decapsulated *Artemia* cysts and the time needed to raise larvae of *Clarias gariepinus* up to a weight of 50 mg for different combinations of temperatures and feeding level were determined. Recommendations are made for optimal temperature and feeding level under practical farming conditions.

Key-words: Clarias gariepinus, Larval Nutrition, Growth, Feeding level, Temperature, Artemia

Introduction

The supply of fingerlings is the major bottleneck for the development of a commercial culture of the African catfish Clarias gariepinus (Burchell 1822) Hogendoorn 1979, 1980; Janssen 1984; Uys and Hecht 1985). In spite of many efforts to use artificial feeds (Hogendoorn 1980; Msiska 1981; Verreth *et al.* 1987) natural food organisms seem to be a prerequisite for the early larval rearing of the African catfish. Processed or inert food organisms may also be used. Uys and Hecht (1985) successfully used a dry feed based on *Torula* yeast and Verreth *et al.* (1987) obtained excellent results with dried decapsulated cysts of *Artemia.*

Artemia is often included as a reference diet in larval nutrition studies. The nutritional quality of *Artemia* nauplii may vary considerably according to the geographical strain,

processing batch and developmental stage (Léger *et al.* 1986). Further, by comparing living nauplii of *Artemia* with artificial test diets, the results may reflect a difference in feed ingestion more than in nutritional quality. Decapsulating and drying cysts of *Artemia* eliminates differences in physical properties and consequently in ingestion of the feed and increases the suitability of *Artemia* as a reference diet in larval nutrition studies (Verreth *et al.* 1987).

In order for such feed to be a valuable "reference" to other diets, the performance of fish larvae on decapsulated cysts should be investigated under different environmental and husbandry conditions. In this respect it is remarkable how rarely the effect of temperature and feeding level on larval fish growth has been documented. Flüchter (1980) found, at 18 °C, a food intake of *Artemia* by 37-mm-long Coregonus larvae of 2.8% of the body weight per meal. According to his results, these larvae require a minimal *Artemia* consumption of 800 nauplii prior to their metamorphosis. Baranova (1974, cited in Bryant and Matty 1980), found in carp larvae a maximal growth rate at 23-25 °C and at a feeding rate between 200 and 300% of the body weight. day^{-1}. In a detailed study, Bryant and Matty (1980) demonstrated that carp larvae attain maximal growth rate when fed *Artemia* nauplii at a feeding level of 200-250% of the body weight per day ($BW.d^{-1}$) during the first 5 days of exogenous feeding, followed by a rate of 100-125% $BW.d^{-1}$ for the following 5 days. Fuchs (1982) reports an increasing feeding rate of 10 to 600 *Artemia* $nauplii.day^{-1}$ per larval fish for Dover sole *(Solea solea)* during the first 15 days of exogenous feeding.

In this paper the effect of temperature and feeding level on the growth performance of *Clarias gariepinus* larvae, using decapsulated and dried *Artemia* cysts, is reported.

Material and methods

Facilities and fish

A total of four experiments was carried out in 45 L glass aquaria. The aquaria were part of two recirculation systems, each of which consisted of 12 aquaria, a 100 L sedimentation tank and a 100 L bio-filter of sand and gravel. Fresh water was supplied to the system at a rate of 0.6-0.8 $L.min^{-1}$. The water flow through each aquarium varied between 0.5 and 1.5 $L.min^{-1}$. Incubation of eggs and embryos (yolk sac larvae) was done at the experimental temperature. On the final day of yolk absorption, 1500 yolk sac larvae were stocked into each aquarium. The next morning feeding started.

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Feed and feeding levels

During all experiments, dried decapsulated cysts of *Artemia* (geographical origin: Burliu, (former) USSR) were used. Decapsulation and drying of the cysts was performed following standardized techniques (Vanhaecke and Sorgeloos 1982; Sorgeloos *et al.* 1983) at the *Artemia* Reference Centre, State University of Gent, Belgium. The particle size of the feed was 239.2 µm (W. Tackaert, ARC, pers. commun. 1986). The proximate composition of the cysts is given in Table 1.

Since in larval fishes the specific growth rate decreases continuously and since the daily feed ration, expressed as a percentage of the body weight, cannot be kept constant without changing its physiological value, it was decided to assess growth rates and feeding levels in another way. After a cube root transformation of the weight data, a linear relation (1) exists between the body weights and the length of the culture period (Hogendoorn 1980).

$$y_t^{1/3} = y_0^{1/3} + g.t \qquad (1)$$

where y = weight at time t
y_0 = weight at start
g = regression coefficient
t = time or length of culture period (days)

The slope of this regression line remains constant for the entire larval culture period and is a useful measure of the larval growth rate. The feeding levels in our experiments were chosen according to growth rates predicted by g = 0.05, 0.10, 0.20, 0.30 and 0.60 ($FL_{0.05}$ to $FL_{0.60}$). Preliminary experiments revealed that these levels encompass a range between (near) zero and maximal growth rate. The daily feed ratios were calculated on the basis of the expected growth for every particular day of culturing (2). Because of the rapidly changing dry matter content in fish larvae, it was decided to base all calculations on a dry weight basis.

$$R_t = \Delta y_t . \frac{DM_f}{DM_a} . FCR \qquad (2)$$

where R_t = the food requirements/fish on day t (mg wet weight)
Δy_t = the average individual growth on day t
= $y_{t+1} - y_t$ (mg wet weight)
DM_f = dry matter content of fish larvae
DM_a = dry matter content of *Artemia*
FCR = food conversion ratio

Since preliminary experiments revealed a FCR varying around 3 and because of the constant dry matter content of *Artemia*, the daily feed ration per aquarium was calculated by:

$$R_{it} = N_{it} \,.\, Rt = N_{it}\,(3.19\ \Delta y_t \,.\, DM_f) \quad (3)$$

where R_{it} = the quantity of food in aquarium 1 on day t (mg)
N_{it} = the number of fish larvae in aquarium i on day t
Δy_t and DM_f: see above

In order to adjust the food quantities for eventual deviations of the actual growth rate from the predicted one, the weights (y_t) on days 4, 7 and 10 were measured and used accordingly in equation (3). Feeding was done manually, four times a day (between 10.00 and 22.00 h).

Temperature

During each experiment all feeding levels were compared at one and the same temperature. To include a temperature effect, the experiments were repeated (in chronological order) at 27.5, 32.5, 30.0 and 25.0 °C. Heating occurred in the sedimentation tank. The temperature was controlled automatically and exhibited maximal fluctuations of 0.2°C.

TABLE 1. The proximate composition of the feed (*Artemia* cysts, strain Burliu)

Dry matter (%)	94.0
Protein content[1,2] (%)	51.6
Fat[1,3] (%)	10.9
Ash[1] (%)	5.2
Energy content[1,4] ($kJ.g^{-1}$)	22.3

[1] On dry weight basis.
[2] Measured as Kjeldahl-N x 6.25.
[3] Extracted in petroleum ether.
[4] Measured by bomb calorimetry.

Experimental procedure

Except for the experiment at 27.5 °C, all experiments were carried out with four replicates, divided into two pairs over two recirculation units. In every recirculation unit, the duplicates were assigned randomly to the aquaria. For technical reasons, the experiment at 27.5 °C was carried out in one recirculation unit with four feeding levels and three replicates. The lowest feeding level ($FL_{0.05}$) was dropped. The culture period varied between the experiments. The first experiment (27.5 °C) was terminated after 14 days. Because during the last 4 days of the experiment no extra information could be obtained, it was decided to stop the following experiments after 10 days. Due to a fungal infection, the experiment at 25.0 °C was stopped after seven days. Dead fishes were removed and counted

twice a day. Food remnants, faeces and debris were removed daily before feeding. Fish samples were taken on culture days 0, 3, 7, 10 and 14, each time before feeding. At the end of the experiment, the remaining fishes were counted.

Statistical analysis

On every sampling date, the following parameters were measured; individual fish weight, dry matter content, protein content and energy content of the fishes. The energy content of the fishes was measured through a COD procedure and the results corrected for incomplete oxidation according to Henken *et al.* (1986). The proximate composition of the feed (*Artemia*) was analyzed in the same way as the fishes (Table 1). For each aquarium, equation (1) was used to calculate the realised average growth rate (g). The daily quantity of food per fish was calculated from the daily amount of feed per aquarium divided by the surviving number of fishes (corrected daily for sampled and/or missing fishes). The daily food quantities were summed to obtain the total individual food ration per aquarium. The food conversion ratio (FCR) was defined as the total food ration (dry weight) per unit of dry fish weight gain. Further, the energy conversion efficiency (ECE = amount of energy retained per unit of energy intake), the protein efficiency ratio (PER = live weight gain per unit of protein intake) and the apparent net protein utilization (NPU = amount of protein retained per unit of protein intake) were calculated. As stated earlier, the feeding levels were expressed in relation to the fish growth rate they would induce, assuming a constant FCR. However, the FCR varied among aquaria and treatments. Further, the number of fishes (N_t) used in equation (3) was not corrected for sampled and/or missing fishes. After correction for these deviations, the actual feeding levels were obtained and used in the statistical analysis.

According to Bartlett's test (Sokal and Rohlf 1969) the variances of ECE, PER, NPU and survival rate were not homogeneous. The BMDP program 9D (Dixon *et al.* 1983) was used to apply the best transformation to the data (arctan$\sqrt{}$ECE, arctan$\sqrt{}$PER, arctan$\sqrt{}$NPU and arcsin$\sqrt{}$survival rate). At the lowest level (0.05) the data for ECE, PER and NPU were highly variable, most probably because the energy and protein content could not be measured accurately. Therefore, data for ECE, PER and NPU at this feeding level were excluded from further analysis. The data on growth rate, FCR, ECE, PER and NPU were analyzed with the BMDP program 1R (Dixon *et al.* 1983), using the following model:

$$y_i = b_o + b_1X_1 + b_2X_2 + b_3 (X_1 - \bar{X}_1)^2 + b_4 (X_2 - \bar{X}_2)^2 + e_i \quad (4)$$

where y_i = specific parameter
b_o = intercept
x_1 = temperature C)
x_2 = feeding level (FL)
$\overline{x_1}$ and $\overline{x_2}$ = respective weighted means
b_1 to b_4= regression coefficients
e_i = error term

Because the squared products were highly correlated with the linear terms ($r_{x1\ (x1)}^2 = 0.9997$ and $r_{x2\ (x2)}^2 = 0.9805$), the model was made additive by basing the squared terms on the deviations of the respective means.

To obtain a realistic estimation of the survival rate, the final number of fishes was adjusted for sampled and/or missing fishes. A mortality rate, derived from a time-frequency distribution of the daily mortality records, was attributed to the sampled number of fishes and the remaining number added to the count at the end of the experiment. The survival data were analyzed by a two way analysis of variance with temperature and feeding level as factors. To distinguish individual treatment combinations, Duncan's multiple range test was applied to the pooled replicates. Because the experiment at 25 °C had to be stopped early, it was not included in the analysis of the survival data.

Results

The results of the experiments are summarized in Tables 2 and 4. A summary of the results with respect to the regression analyses is presented in Table 3.

Growth rate

The linear relationship between the transformed mean weights of the samples and length of the culture period (equation 1) was highly significant ($P \leq 0.01$) in most aquaria ($0.94 \leq R^2 \leq 1.00$). The coefficient of determination R^2 was always higher than 0.79. The growth rate was highly significantly affected by temperature and feeding level (Table 3). According to the regression coefficients, differences in feeding level have a much stronger influence on growth rate than temperature. Growth rates are maximized at a feeding level between 0.45 and 0.50 and a temperature between 29.5 and 30 °C (Table 2, Fig. 1).

TABLE 2. The average growth rate, food conversion ratio (FCR), energy conversion efficiency (ECE), protein efficiency ratio (PER) and apparent net protein utilization (NPU) in *C. gariepinus* larvae fed with dried decapsulated cysts of *Artemia* at different combinations of temperature and feeding level

Temp. (°C)	Feeding level (FL)	Average growth rate (g)	Average FCR	Average ECE	Average PER	Average NPU
25.0	0.05	0.062	2.78	—	—	—
	0.10	0.104	2.65	—	—	—
	0.20	0.185	2.88	—	—	—
	0.30	0.205	4.13	—	—	—
27.5	0.10	0.119	2.93	0.372	4.79	0.470
	0.20	0.210	2.50	0.432	4.92	0.521
	0.30	0.267	3.50	0.306	3.32	0.379
	0.60	0.292	7.33	0.145	1.68	0.178
30.0	0.05	0.054	3.83	—	—	—
	0.10	0.116	2.90	0.388	5.40	0.505
	0.20	0.215	2.57	0.428	5.37	0.567
	0.30	0.281	3.05	0.351	4.13	0.453
	0.60	0.302	6.20	0.173	2.09	0.225
32.5	0.05	0.068	3.73	—	—	—
	0.10	0.123	3.00	0.361	5.12	0.480
	0.20	0.226	2.95	0.361	4.73	0.475
	0.30	0.226	4.65	0.222	2.93	0.303
	0.60	0.283	8.43	0.119	1.55	0.160

The dry matter content in the fish larvae increased continuously from ± 10% at the beginning to ± 14-16%, depending upon the final larval weight attained in the experiments (Fig.2).

Food conversion ratio

The food conversion ratio was significantly affected by temperature and by feeding level ($P < 0.0001$) (Table 3). With exception of the experiment at 25 °C, minimal food conversion ratios were consistently found at a feeding level of 0.20. At 25 °C the FCR was lower at a feeding level of 0.10, but did not differ much from the one at 0.20 (Table 2). The lowest food conversion ratio (on dry weight basis) was 2.50 (at 27.5 °C).

Body composition

At the onset of exogenous feeding, the average dry matter content of the fish larvae was

TABLE 3. The regression coefficients, t-values and multiple R^2 as determined by fitting the multiple regression model (equation 4, see text) to the experimental data

		Coefficient	*t*-values	R^2
Growth rate	b_0	-0.00255		0.94***
	b_1	$2.646^{*}10^{-3}$	2.81***	
	b_2	0.65103	27.88***	
	b_3	$-1.7980^{*}10^{-3}$	-4.26***	
	b_4	-1.44714	-14.54***	
FCR	b_0	-0.6969		0.85***
	b_1	0.0838	2.66*	
	b_2	4.8397	6.12***	
	b_3	0.0359	2.48*	
	b_4	20.4022	5.94***	
ECE	b_0	0.49799		0.87***
	b_1	0.00444	1.00	
	b_2	-0.29539	-5.12***	
	b_3	-0.00522	-3.15***	
	b_4	-0.55311	-2.55*	
PER	b_0	0.91035		0.91***
	b_1	0.01129	2.76**	
	b_2	-0.41312	-7.75***	
	b_3	-0.00582	-3.79***	
	b_4	-0.39627	-1.98	
NPU	b_0	0.41110		0.88***
	b_1	0.00967	2.13*	
	b_2	-0.32240	-5.46***	
	b_3	-0.00625	-3.68***	
	b_4	-0.58743	-2.64*	

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$.

10.4% (± 0.4 SD). During the course of the experiments, the dry matter content increased considerably. At the end of the feeding periods, it varied from 11.7% to 16.8% according to the feeding level and temperature combination. However, these differences reflect differences in growth (Fig. 2) rather than treatment effects. The body composition in the dry matter remains very stable during the feeding period. No treatment effect could be detected. The dry matter contained 69.1% ± 1.7 SD) protein, 12.5 % (± 0.6 SD) ash and 23.1 $kJ.g^{-1}$ ± 0.4 SD) energy.

Energy conversion efficiency

At all temperatures tested, the energy conversion efficiency (ECE) was higher at a

Verreth and den Bieman, 1987

feeding level of 0.20 and varied from 0.43 (27.5 °C) to 0.36 (32.5 °C). The highest feeding level (0.60) resulted consistently in the lowest ECE, varying from 0.17 to 0.12. The regression analysis revealed a significant temperature and feeding level effect (Table 3). The linear term of the temperature effect was not significant.

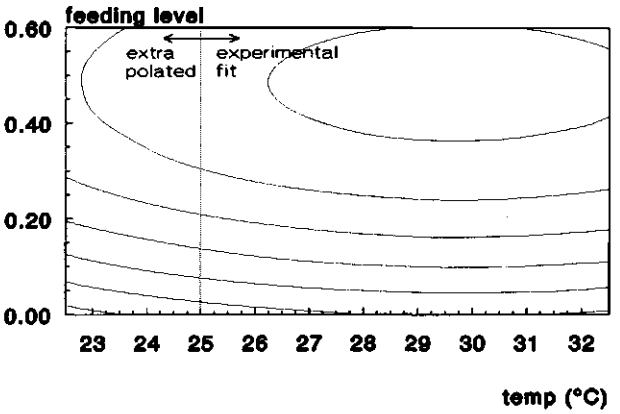

Figure 1. Isopleths of the growth rate (g) at different combinations of temperature and feeding level. The isopleths are computer drawn and were calculated using the multiple regression model which was determined by fitting equation 4 (see text) to the experimental data.

Protein efficiency ratio and apparent net protein utilization

At all temperatures the protein efficiency ratio (PER) decreased from ± 5 or more at a feeding level of 0.20, to 2 or less at a feeding level of 0.60 (Table 2). Differences between feeding levels 0.10 and 0.20 were small, indicating a rather constant PER at low feeding rates. The regression analysis revealed a highly significant temperature and feeding level effect. The quadratic term of the feeding level was not significant (Table 3), indicating that PER decreased linearly with increasing feeding level. The data on apparent net protein utilization (NPU) gave similar results. Both linear and quadratic regression coefficients of temperature and feeding level were significant. The highest apparent NPU's were obtained at lower feeding rates and varied between 0.48 and 0.57 according to the temperature. At the highest feeding level of 0.60, the apparent NPU varied between 0.16 and 0.23. The best protein conversion efficiencies were found at 30 °C (Table 2).

Survival

Because of its early termination, no data were available for the experiment at 25 °C. The statistical analysis was performed on the data from the three other temperatures. The analysis of variance revealed a highly significant feeding level and temperature effect on survival rate ($P < 0.01$). The lowest survival rates were obtained at the lowest feeding level of 0.05 (23.2% at 32.5 °C and 53.1% at 30 °C). In general the survival rates increased

considerably from the low feeding levels (0.05 and 0.10) to feeding levels above 0.20. For these higher feeding levels, Duncan's multiple range test revealed significantly better survival rates at 27.5 °C than at 30 and 32.5 °C. At 27.5 °C the survival rate varied between 93 and 96% for feeding levels between 0.20 and 0.60 (Table 4).

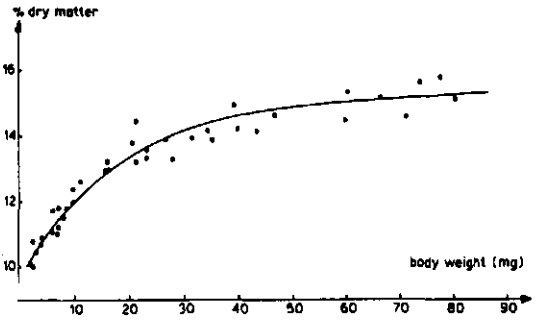

Figure 2. The average dry matter content (%) in growing larvae of *Clarias gariepinus* (Burchell).

Discussion and conclusions

Methodology

In fish nutrition studies, growth and feeding level are mostly expressed as a percentage of the live body weight per day (% BW.d^{-1}). For short culture periods the specific growth rate remains rather constant and as a consequence feeding level (expressed as % BW.d^{-1}) can be kept constant during those periods as well. The resulting growth performances may be compared by the specific growth rates. In larval fishes this procedure cannot be applied reasonably. Although the larval growth period is very short, in a few days the larval weight increases twenty- to fifty-fold, its dry matter content changes considerably and the specific growth rate decreases continuously. Bryant and Matty (1980) measured a reduction of ± 34% in the specific growth rate of carp larvae between the first 5 days and the following 5 days of the culture period. Hogendoorn (1980) reported a decreasing specific growth rate in larvae of *C. gariepinus* from 85% to less than 20% of the body weight.day^{-1} during the first 28 days of feeding. These observations imply that the daily feed ration should be adjusted accordingly and cannot be kept constant without changing its physiological value. In studying the effect of feeding level on growth and feed utilization in larval fishes, fixing the feeding levels as a % BW. d^{-1} would be meaningless. The procedure used in the present study is based on the assumption that growth of larval fishes can be linearized (hence a constant growth rate can be obtained) over the entire larval culture period by using equation (1). This procedure permits feeding levels to be fixed in terms of the predicted growth performance. From this study, it is clear that the larval growth of *C. gariepinus* can be

TABLE 4. The average final survival rate at different combinations of temperature and feeding level (Because of an early termination of the experiment, no data are available at 25 °C)

Temperature (°C)	Feeding level (FL)	Survival rate[1] (%)
27.5	0.10	69.7^{f}
27.5	0.20	95.7^{a}
27.5	0.30	93.3^{ab}
27.5	0.60	94.7^{ab}
30.0	0.05	53.1^{g}
30.0	0.10	79.0^{def}
30.0	0.20	88.1^{bcd}
30.0	0.30	84.3^{cde}
30.0	0.60	89.9^{abc}
32.5	0.05	32.2^{h}
32.5	0.10	53.5^{g}
32.5	0.20	68.2^{f}
32.5	0.30	76.1^{ef}
32.5	0.60	73.8^{ef}

[1] Results not followed by a letter in common differ significantly ($P \leq 0.05$) according to Duncan's multiple range test.

described excellently by equation (1). Except for a few aquaria at the lowest feeding levels, the regression analysis always yielded a R^2 above 0.94. Further, with the exception of the highest feeding level, the food conversion ratio fluctuated around 3. Both observations give a solid basis to the procedure used in the present study to determine feeding levels.

Growth

Equation (1) was used successfully to describe the growth of *C. gariepinus* larvae. As the cube root of weight refers to a length characteristic, Hogendoorn (1981) hypothesized that in *C. gariepinus,* length would increase linearly in time. This is substantiated by a length exponent of 2.9 in the length-weight relationship which was determined by Uys and Hecht (1985). Provided that similar observations could be done in other species as well, our approach may build a link between weight-based and length-based growth studies in fish larvae.

For the sake of comparability, the specific growth rate was calculated as well (Table 5). The average specific growth rates calculated over the first 10 days of feeding, increase asymptotically to a maximum of 40.7-41.9% of the body weight per day with increasing feeding level. This is higher than the average specific growth rate for carp larvae *(C. carpio)* as reported by Bryant and Matty (1980) over the same culture period (34%) or by

TABLE 5. The average specific growth rate of *C. gariepinus* larvae, after 10 days of rearing at different temperatures and fed at different feeding levels with dried decapsulated cysts of *Artemia* (Results are expressed as % BW.day^{-1}. Data between brackets refer to standard deviations. Data for starved (unfed) animals were obtained in separate experiments, not reported in the text. Specific growth rates of the experiment at 25 °C are lacking).

Feeding level	Temperature (°C)		
	27.5	30	32.5
starting	-4.7 (1.2)	-7.5 (1.0)	-4.7 (1.8)
0.10	21.7 (0.8)	20.0 (1.7)	20.6 (1.4)
0.20	33.1 (0.4)	32.4 (0.8)	34.0 (1.7)
0.30	39.0 (0.4)	39.1 (0.9)	38.1 (0.7)
0.60	41.9 (0.4)	41.7 (1.2)	40.7 (1.1)

Vanhaecke and Sorgeloos (1983) after 2 weeks (33.1-35%). However, both studies were carried out at lower temperatures (24 and 22.6 °C respectively). Further, it is remarkable how fast the specific growth rate in *C. gariepinus* declined compared to *Cyprinus carpio*. At 27.5 °C, the specific growth rate of *C. gariepinus* decreased from 56.1% between days 0-3 to 20.7% between days 7-10 for a feeding level of 0.30 and from 63.9% to 23.1% for a feeding level of 0.60. Bryant and Matty (1980) report a decrease for carp larvae from ± 45 % between days 0-2 to 32 % for the last two days (9-10). As the specific growth rate is physiologically dependent upon fish size, it is believed that this fast decrease reflects more the fast growth of the catfish larvae than a lower growth potential compared to carp larvae. This is further substantiated by the specific growth rates obtained by Huisman (1974, 1979) in carp and grass carp larvae, which declined from 60-65% during the first week of rearing to 20-25% for the second week.

The maximum growth rate, calculated according to equation (1), fluctuates around $g = 0.31$. This is slightly higher than the results of Hogendoorn (1980) who fed life *Artemia* nauplii to *C. gariepinus* and obtained a maximal growth rate of $g = 0.30$. At all temperatures, maximum growth rate was attained at a feeding level in between 0.30 and 0.60. According to the quadratic regression analysis, this maximum should be attained at a feeding level of 0.45-0.50 (Fig. 1).

Feed utilization

For feeding levels under 0.30 the food conversion ratios were remarkably low. If expressed on a wet weight basis, the lowest FCR found in our experiments (2.50) was only 0.42 (dry matter content was 15.8% in fish and 94.0% in food). Except at 32.5 °C, higher

feeding levels were utilized better at higher temperatures than at lower ones. As can be seen in Fig. 1 of Hogendoorn *et al.* (1983), the efficiency of feed utilization by *C. gariepinus* is not affected by the temperature. Temperature, however, has a tremendous effect on the maximal growth rate. Hence at lower temperatures a proportionally larger part of the food, administered in high feeding levels, is not consumed. Differences in FCR therefore reflect differences in feed intake and indicate that between 25 °C and 30 °C the scope for growth in larval *C. gariepinus* increases with increasing temperature, which corroborates the hypothesis of Hogendoorn *et al.*(1983).

This idea is further substantiated by the efficiency ratios: ECE, PER and apparent NPU which, for all feeding levels, were maximal at 30 °C, except for ECE at a feeding level of 0.20. However, it has to be remembered that the analysis did not include data at 25 °C nor at a feeding level of 0.05. Considering the low FCR at the two lowest feeding levels at 25 °C, ECE, PER and NPU would probably have been higher as well at this temperature for those low (0.05 and 0.10) feeding levels. Maximal PER and apparent NPU are very high (± 5.4 and > 0.5 respectively) confirming that *C. gariepinus* is a most efficient feed convertor. Quadratic regression analysis resulted in a significant quadratic term for feeding level. As is shown in Fig. 3, the deflection of the curves relating PER to feeding level occurred at the lowest feeding level (0.10) in the analysis. Inclusion of feeding levels lower than 0.10 in the analysis may result in a significant quadratic feed effect.

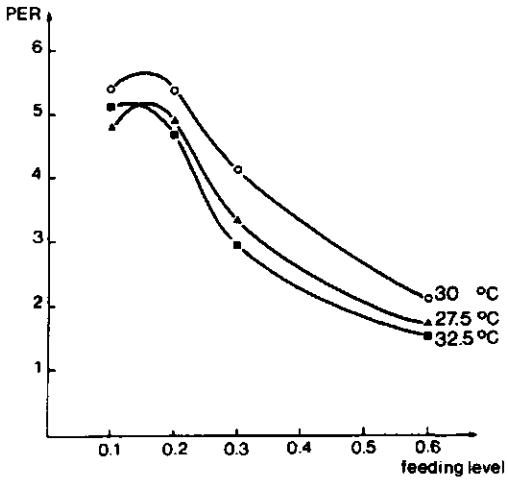

Figure 3. The average protein efficiency ratios (PER) attained in the present study at different feeding levels and temperatures. The curves are fitted by eye.

Application

The effect of temperature and feeding level on growth rate, FCR, PER, NPU and ECE was accurately described by the multiple regression model used in this study. As a consequence, the model may serve as a useful tool to compare growth performance of *C. gariepinus* larvae under different environmental conditions. As an example, the results of

TABLE 6 The amount of dried decapsulated cysts of *Artemia* and the time needed to raise *C. gariepinus* larvae up to a final weight of 50 mg at different combinations of temperature and feeding level. The upper number gives the total amount of *Artemia* cysts needed and the lower number the length of the culture period. All data were calculated on the basis of the multiple regression equation as determined in the present study

Temp. (° C)		Feeding level								
		0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50
22	mg	28.9	28.4	29.2	30.4	32.6	35.3	—	—	—
	days	-	47.2	26.2	18.2	14.8	12.4	—	—	—
23	mg	26.1	25.9	26.4	27.7	29.7	32.5	—	—	—
	days	78.7	29.5	18.2	14.8	12.4	10.7	—	—	—
24	mg	23.7	23.5	24.0	25.3	27.5	30.2	—	—	—
	days	47.2	23.6	16.9	13.1	11.2	10.3	—	—	—
25	mg	22.0	21.8	22.3	23.6	25.6	28.4	32.0	36.4	41.4
	days	39.3	19.7	14.8	11.8	10.3	9.4	8.7	8.4	8.4
26	mg	20.9	20.5	21.1	22.3	24.6	27.2	30.7	35.1	40.2
	days	29.5	18.2	13.1	11.2	9.8	8.7	8.4	8.1	8.1
27	mg	20.1	20.0	20.4	21.7	23.7	26.6	30.1	34.5	39.5
	days	26.2	16.9	12.4	10.4	9.1	8.4	8.1	7.9	7.9
28	mg	20.0	19.8	20.2	21.6	23.6	26.4	30.0	34.3	39.4
	days	23.6	15.7	11.8	10.3	9.1	8.1	7.6	7.6	7.4
29	mg	20.4	20.2	20.7	22.0	24.0	26.9	30.4	34.8	40.0
	days	23.6	15.7	11.8	9.8	8.7	8.1	7.6	7.6	7.4
30	mg	21.3	21.1	21.6	22.9	24.9	27.7	31.3	35.5	40.6
	days	23.6	14.8	11.8	9.8	8.7	8.1	7.6	7.4	7.4
31	mg	22.8	22.6	23.1	24.4	26.4	29.2	32.8	37.0	42.1
	days	23.6	15.7	11.8	10.3	8.7	8.1	7.9	7.6	7.6

Uys and Hecht (1985) may be taken. After 11 days of feeding with an artificial dry feed based on *Torula* yeast, a final weight of 15.87 mg in *C. gariepinus* larvae is reported. This coincides with a growth rate of $g = 0.10$. According to the regression equations found in this study, a maximal growth rate of $g = 0.26$ should be attainable at the temperature (24 °C) used in the experiments of Uys and Hecht (1985). The growth rate reported by these authors coincides with an *Artemia* feeding level of 0.15. These results can only be explained by either an inferior nutritional quality of the feed type used or by a low feeding level. Uys and Hecht (1985) report a constant feeding level in excess of 25% $BW.d^{-1}$ during the experiment. Obviously, at the beginning of the experiment, the larvae were severely underfed. In spite of the apparent constant feeding level, the physiological value of the feeding level was increased continuously throughout the rearing period.

Practical fish farming is mainly interested in data on growth performance and

conversion ratios. In Table 6, the quantity of decapsulated *Artemia* cysts and the time needed to raise larvae of *C. gariepinus* up to a weight of 50 mg are listed for different combinations of temperature and feeding level. A final weight of 50 mg was chosen because it appeared to be the optimal weaning weight for *C. gariepinus*. At feeding levels from 0.30 onwards, the time needed to cover the mentioned weight range at 30 °C is only 0.5 day shorter than at 27 °C, but it requires 3-5% more food. Taking into account that at 27.5 °C the survival rates were generally 5-10% better than at 30 °C (except for a feeding level of 0.60), it is suggested that the optimal temperature for commercial rearing of larval *C. gariepinus* is 28°C. The feeding level to be used depends on local energy and investment costs, but should vary between 0.30 and 0.40. At 28 °C, feeding at 0.30 saves more than 20% in feeding costs but requires about 15-20% more time, i.e. extra energy and depreciation costs. Taking into account that the *Artemia* cysts used in this study (strain Burliu) lost ± 46% in weight by decapsulation (Sorgeloos, pers. commun. 1986), and based on the average European retail prices for *Artemia* cysts, our results reveal that, in our experiments, feeding *Artemia* cysts (28 °C, feeding level = 0.40) raised the production costs of *C. gariepinus* by 0.57 US$-cents per fish of 50 mg.

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CHAPTER 3

Weaning Time in *Clarias gariepinus* (Burchell) Larvae

Johan Verreth and Mathijs van Tongeren

Abstract

The present study attempts to assess for the African catfish, *Clarias gariepinus* (Burchell), the earliest possible weaning time from *Artemia* to crumbles of a commercial trout diet. At the onset of exogenous feeding, 24 aquaria were stocked with larvae, which were fed during varying periods with dried decapsulated cysts of *Artemia*. Depending upon the treatment group, the feeding of *Artemia* lasted 0, 2, 3, 5, 7 or 10 days. After these periods, the respective treatment groups were gradually weaned to the commercial diet. The experiment ended after 10 days of culture and the respective groups were compared on the basis of final weight, growth rate and survival rate. The final weights and growth rates of the groups weaned after 5 and 7 days did not differ significantly from the un-weaned group, attaining final weights between 70 and 76 mg and growth rates g between 0.31 and 0.32. The group receiving 100% *Artemia* feeding for only 2 days, attained a similar survival rate as the un-weaned control (96 %). The data were fitted to a quadratic plateau model, which allowed the statistical assessment of the earliest possible weaning time. Survival and growth rate reached the plateau level when the larvae were gradually weaned after 1.8 and 4.1 days of *Artemia* feeding, respectively.

Key-words: Clarias gariepinus, Larval Nutrition, Weaning time, Artemia, Dry food

Introduction

In studies on larval nutrition and growth, some confusion exists about the length of the larval period, and concurrently with it, the experimental duration varies considerably. In the case of the African catfish, *Clarias gariepinus,* Hogendoorn (1980) cultured the larvae during 4 weeks up to a size of 0.8-1.0 g, whereas Verreth and Den Bieman (1987) used periods of 7-14 days and a weaning size of 50 mg. Appelbaum and Van Damme (1988) and Hecht and Appelbaum (1987) reported experimental durations of 46 and 50 days, respectively, including a "post-larval" period. A clear definition regarding the length of the larval period would be beneficial for the comparability of different studies.

Until now, a detailed anatomical and physiological description of the larval ontogeny

of *C. gariepinus* is only partially available (Stroband and Kroon 1981; Hecht and Appelbaum 1987). Metamorphosis does not occur but during its ontogeny, the species gradually develops a functional stomach and the pH in the digestive tract changes from alkaline to acidic (Stroband and Kroon 1981). A fully differentiated median fin-fold, which marks the morphological end of the larval period (Balon 1975), was found at a total length of about 30 mm (Hecht and Appelbaum 1987). However, a stomach with a clear pylorus, functional glandular cells and a pH lower than 5 is present at a standard length of 11.5 mm and onwards (Stroband and Kroon 1981). As this point may mark the physiological end of the larval period, it is clear that the present status of knowledge of the anatomical and physiological ontogeny of this species does not give a clear indication of the length of the larval period.

For hatchery or research purposes, the minimal duration of feeding live food may be determined by assessing the time at which larvae can be weaned from *Artemia* or other live food to a commercial dry feed without significant effect on growth and/or survival. Because this weaning time depends partly on the quality of the commercial feed used, it may not coincide with the biological end of the larval period. Nevertheless, it is a practical method to determine the minimal duration of experiments on larval nutrition.

The present study was designed to find the earliest weaning time for larvae of the African catfish, *Clarias gariepinus.*

Materials and methods

Facilities and fish

The experiment was carried out in a recirculation system, consisting of 24 aquaria of 16.6 L water volume each, a sedimentation tank and a biofilter of lava-rock. Temperature was maintained at 27.5°C, which is considered to be optimal for larval rearing of the African catfish (Verreth and Den Bieman 1987). Water flow through the aquaria was maintained at 0.8 L/min. Eggs were incubated and yolk-sac fry were nursed at 30°C. At the final day of yolk absorption, each aquarium was stocked at a density of 50 larvae/L. Feeding started the same evening, when all yolk was absorbed.

Feeds and feeding

Larvae were fed either with dry decapsulated cysts of *Artemia,* or with a commercial

trout feed (Trouvit 000) which was first ground and then sieved to obtain a particle size fraction between 250 and 500 µm. Dry decapsulated *Artemia* cysts were used instead of live nauplii because they combine the nutritional quality of live *Artemia* nauplii and the physical appearance of dry diets (Verreth *et al.* 1987). Further, Verreth and Den Bieman (1987) demonstrated that the growth rate of *C. gariepinus* larvae, fed with decapsulated cysts, was slightly higher than the growth rate reported in the literature for live *Artemia* feeding. Feeding was done five times a day (between 08.00 and 22.00 h) with an automatic dispenser. Feeding level (FL) corresponded to a near-satiation level (FL = 0.35) and was calculated according to the procedure of Verreth and Den Bieman (1987).

Experimental procedure

Weaning from *Artemia* to a commercial pellet was initiated after 0, 2, 3, 5, 7 and 10 days of *Artemia* feeding. The first and the last of these treatments served as controls (0 days *Artemia* means only trout crumbles and 10 days *Artemia* means no weaning since the experiment stopped after 10 days). Weaning occurred gradually by decreasing the proportion of *Artemia* and increasing the proportion of the commercial diet (first day 75/25, second day 50/50, third day 25/75 and fourth day 0/100 ratio of *Artemia*/crumbles). With exception of the controls, a 50/50 ratio *Artemia*/crumbles was attained on days 4, 5, 7, and 9, respectively.

Each day, prior to feeding, aquaria were cleaned. Dead fishes were collected and counted twice a day. To calculate the daily feed ration, the average individual wet weight was measured daily by weighing two groups of five fishes and their dry matter content estimated from an earlier assessed relation between wet and dry weight of *C. gariepinus* larvae (Verreth and Den Bieman 1987). The calculation of the food conversion ratios was based on measurements of the dry matter content at the start and at the end of the experiment. The experiment was carried out with four replicates per treatment.

Parameters and analysis of data

At the start and at the end of the experiment, individual wet weights were measured on an analytical balance (accuracy 0.01 mg) after blotting the fishes individually on a tissue paper according to a standardized procedure. Dry matter content was measured in two groups of five fishes after drying overnight at 70°C followed by 4 h at 105°C. Food conversion ratio (FCR) was defined as the total food ration (dry weight) per unit of dry fish weight gain. Food conversion ratios and survival rates were calculated according to the procedures of Verreth and Den Bieman (1987).

Only average values per aquarium were used for the statistical analysis. Individual weight data were linearized by a third root transformation and the growth rate *g*, was assessed by regression of these data on time (Hogendoorn 1980; Verreth and Den Bieman 1987). Data were screened for deviations from normality and if necessary transformed through the SAS-procedure UNIVARIATE (SAS 1985a). Only survival rates needed to be transformed by an arcsine square root transformation. Statistical differences between treatments were tested by subjecting the data of final weight, growth rate, food conversion ratio and survival rate to Duncan's multiple range test (Steel and Torrie 1960). The effect of the time of weaning on these parameters was described by fitting the data to a quadratic plateau model. This model consists of two sections viz. a curvilinear section followed by a horizontal line which meet at a point, WT_{PL}. The first section of this curve was described by:

$$Y_{ij} = b_o + b_1 (WT) + b_2 (WT)^2 + e_{ij} \quad (1)$$

in which: Y_{ij} = dependant variable,
WT = weaning time (days),
b_0, b_2, b_2 = regression coefficients.

The second section (plateau) was described by:

$$Y_{PL} = b_o + b_1 (WT_{PL}) + b_2 (WT_{PL})_2 + e_{ij} \quad (2)$$

in which WT_{PL} is defined as $(-b_1)/(2b_2)$ (SAS 1985b, p. 595).
The nonlinear regression was done through the SAS procedure NLIN (SAS 1985b).

Results

The average final weights, growth rates, food conversion ratios and survival rates attained are presented in Table 1. After 10 days, the largest average weight (76.71 mg) was found in larvae which were weaned after 7 days of *Artemia* feeding; larvae fed only *Artemia* or weaned after 5 days all reached weights above 70 mg (Table 1). The maximal growth rate g (0.32) was attained when the larvae received decapsulated dry *Artemia* cysts for 7 days before weaning. Again, no significant differences were found with larvae weaned after day 5 and those receiving only *Artemia* (Table 1). The food conversion ratio improved as the

TABLE 1. The average final weight, growth rate, food conversion ratio and survival rate of *Clarias gariepinus* larvae in relation to the duration of *Artemia* feeding before the start of a gradual weaning to a dry commercial trout diet

Days of *Artemia* feeding	Final weight mg (± sd)	Growth rate *g* (± sd)	FCR (± sd)	Survival rate % (± sd)
0	13.63 (1.42)^{d}	0.111 (0.008)^{d}	28.82 (5.42)^{a}	17.9 (1.5)^{c}
2	55.12 (1.44)^{c}	0.267 (0.005)^{c}	5.61 (0.26)^{b}	95.6 (0.9)^{a}
3	63.32 (7.06)^{b}	0.294 (0.014)^{b}	5.46 (0.36)^{b}	90.7 (0.2)^{ab}
5	70.71 (4.42)^{a}	0.314 (0.008)^{a}	4.34 (0.62)^{b}	95.6 (1.2)^{a}
7	76.71 (3.42)^{a}	0.320 (0.006)^{a}	4.00 (0.30)^{b}	87.9 (0.2)^{b}
10	73.59 (3.60)^{a}	0.314 (0.008)^{a}	4.00 (0.18)^{b}	96.0 (0.1)^{a}

Results not followed by a common superscript are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

duration of *Artemia* feeding increased. It reached its plateau level at a FCR of about 4. With the exception of the group receiving commercial feed only, differences between treatments were not high and varied between 5.6 (weaning after 2 days) and 3.99 (weaning after day 7). With the exception of the groups which received just the commercial diet, average survival rates were consistently high and varied between 87.9% and 96.0%. In general, only the larvae receiving no *Artemia* at all showed very low growth and survival results.

TABLE 2. The regression coefficients, the plateau-level (Y_{PL}) and the time (WT_{PL}) at which this level is reached when a quadratic plateau model is fitted to the final weights, growth rate, food conversion ratio and survival rates of *C. gariepinus* larvae in relation to different weaning times

Coefficient and parameters	Final weight (mg)	Growth rate (*g*)	FCR	Survival rate
b_0	13.997	0.112	28.815	0.437
b_1	24.853	0.099	-18.965	0.958
b_2	-2.593	-0.012	3.680	-0.262
Y_{PL}	73.529	0.315	4.383	1.313[a]
WT_{PL} (days)	4.792	4.085	2.577	1.828

[a] If back-transformed, corresponds with 93.5% survival rate.

The results of the nonlinear regression analysis (regression coefficients, plateau-level for the various parameters and time when the plateau is reached) are shown in Table 2. The estimated plateau-levels coincide very well with the experimental results, which was corroborated by the high coefficients of determination (R^2) found in the different curve fittings (varying between 97 and 99%). Maximal final weight and growth rate was attained

if weaning did not start before 4.8 and 4.1 days of *Artemia* feeding, respectively. However, to maximize survival rate, only 1.8 days of *Artemia* feeding is required (Fig. 1).

Discussion

In this experiment, the growth and survival of the larvae of *Clarias gariepinus* compare very well with previous results obtained with this species (Hogendoorn 1980; Uys and Hecht 1985; Appelbaum and Van Damme 1988). The maximum average growth rates obtained in this study (g = 0.31-0.32) are similar to those found by Verreth and Den Bieman (1987) for larvae fed decapsulated cysts only. The quadratic plateau model, used in this study, adequately describes the effect of shorter weaning times on the growth and survival of *C. gariepinus*. Although other models (an asymptotic model and a model with one maximum) could also have been fitted to the data, they were considered not to be very appropriate. An asymptotic model was rejected because it never reaches a maximum, whereas, if food particle size is not adapted, the energy costs for feed intake may increase strongly with increasing fish size. A model with one maximum was not accepted because, in spite of the best growth results found in larvae which were fed *Artemia* cysts for 7 days, the prevalence of a peak could not be confirmed statistically.

The statistical procedure also enabled the precise estimation of the moment when the plateau level was reached (WT_{PL}), indicating the earliest possible weaning time for the parameter under consideration. The present study demonstrates clearly that *C. gariepinus* larvae can be weaned much earlier than hitherto assumed. To maximize the survival rate, only a 2-day pre-weaning period is necessary, whereas at 27.5°C, growth is maximized after a 4-5-day pre-weaning period. Apparently, after 2 days, the digestive system is not yet sufficiently developed to enable a good growth rate on commercial feed ingredients and the fish still require *Artemia*. The decreasing amount of *Artemia* in the ration of the treatment groups which were weaned after 2 days, resulted in a decreased growth rate although a good survival is maintained. Such a growth depression may not occur after a 4-day period of 100% *Artemia* feeding. This hypothesis is difficult to confirm with data presented in the literature. Stroband and Kroon (1981) gave a detailed description of the early ontogeny of the digestive tract in *C. gariepinus*. Unfortunately, weight related data were not given and, due to a different rearing temperature, the time scale of the events differs significantly. At the Zoological Institute, Heidelberg University, preliminary histological and enzymological

analyses of the digestive tract of *C. gariepinus* larvae, reared at 27.5°C, revealed a fully developed stomach, with differentiated pylorus, in 4-day-old larvae and only slight ontogenetic changes in enzyme activity (non specific esterase; amino-peptidase) could be detected between day 2 and day 4 (Segner, pers. commun. 1988). This information corroborates the results of the present study and asks for detailed anatomical and enzymological studies during the early development of *C. gariepinus*.

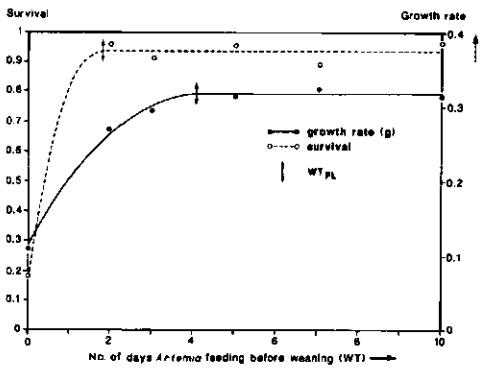

Figure 1. The survival rate and growth rate of *Clarias gariepinus* larvae, as assessed after a 10 day culture period in relation to a different number of days of *Artemia* feeding (WT) before the start of weaning. Weaning from *Artemia* to a commercial trout diet occurred gradually. The growth rate *g* is calculated by regression of the cube root of fish weight on rearing time. The lines represent the curves fitted through non-linear regression of a quadratic plateau model to the data. The vertical arrows indicate the earliest weaning time WT_{PL}, as assessed by the regression procedure.

It is obvious that the time of weaning is directly related to the ontogeny of the species. In the present study, the earliest weaning time to maximize the growth rate was 4.085 days (at 27.5°C). According to the estimated maximal growth rate (Fig. 1; Table 2), this weaning time corresponds to a body weight of 18.2 mg (taking into account an initial size of 2.4 mg). In the same way, it could be calculated that *C. gariepinus* can be weaned at a weight of 7.1 mg without any effect on the final survival. In spite of the rather advanced ontogenetic development of *C. gariepinus* at the onset of exogenous feeding, this weaning size is larger than the "adaptation weight" reported by Bryant and Matty (1981) for common carp *(Cyprinus carpio)* larvae (15 mg) and by Dabrowski (1984) for larvae of common carp, silver carp *(Hypophthalmichthys molitrix)*, bighead carp *(Aristichthys nobilis)* and grass carp (*Ctenopharyngodon idella*) (5-6 mg). However, these authors did not take into account the growth loss if compared with larvae fed solely on live food. In the present study, larvae weaned after 2 days still reach a final weight of approximately 55 mg at day 10. This is, however, considerably less than the 70-75 mg which is consistently attained if weaning starts after the earliest weaning time WT_{PL} for the parameters concerned (final weight and/or growth rate).

In conclusion, the results of the present study demonstrate that for the culture of the African catfish, the difficult and labour intensive period of *Artemia* feeding can be reduced

considerably without any loss in terms of growth and survival. The results confirm that, at 27.5°C, the African catfish, *Clarias gariepinus*, passes through a "larval" period during which the fish needs a specific larval diet. Depending upon whether the start of weaning or the 50/50 ratio of *Artemia*/dry food is taken as reference point, this period lasts 4 or 6 days. Until recently, only live food organisms were successfully applied in the larval rearing of *C. gariepinus* (Hogendoorn 1980; Msiska 1981). However, at this moment, suitable dry diets exist. These are either based on dried decapsulated cysts of *Artemia* (Verreth *et al.* 1987; Verreth and Den Bieman 1987) or on alkan yeast (Uys and Hecht 1985; Hecht and Appelbaum 1987). Recent modifications to the latter diet have improved its suitability considerably, yielding growth and survival results close to the ones obtained with dried decapsulated cysts of *Artemia* (Appelbaum and Van Damme 1988). The formulation of compound diets for larval fishes requires more information about the digestive and the metabolic system of the larvae. As has been demonstrated in this study, in *C. gariepinus* these investigations should focus on the first 4-6 days of exogenous feeding.

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Part 2

ONTOGENY OF DIGESTIVE AND METABOLIC FUNCTIONS IN *CLARIAS GARIEPINUS* LARVAE

CHAPTER 4

The development of a functional digestive system in the African catfish *Clarias gariepinus* (Burchell).

Johan Verreth, Els Torreele, Eljalill Spazier, Ad van der Sluiszen, Jan Rombout, Ronald Booms and Helmut Segner

Abstract

The morphological and physiological development of the intestine, stomach, liver and pancreas of the African catfish, *Clarias gariepinus* (Burchell 1822), was investigated from hatching until 9 days after the start of exogenous feeding by histological, histochemical and immuno-histochemical procedures. During the yolk sac period, lasting 48 hours at 30 °C, the digestive system develops from a rather undifferentiated system into a distinct liver and pancreas and a segmented intestine (parts I to III). Insulin and Glucagon producing cells were clearly found in the endocrine pancreatic islets from 14 hours after hatching onwards while at the start of exogenous feeding, the exocrine pancreas showed an intense zymogen coloration. Exogenous feeding started 3 days after fertilization, i.e. 48 hours after hatching. At that moment, the rearing temperature was lowered to 27.5 °C. The first day of feeding was referred to as day zero, changing into day 1 when the first 24 hours of feeding were completed. From the start of exogenous feeding onwards, amino-peptidase, non specific esterase and ATP-ase were detected in the digestive system. Morphologically, the stomach was completed at day 4 of exogenous feeding. Gastric acid secretion started on day 4 and lead to a Ph below 3.3 in the stomach on day 5. During the first four days of exogenous feeding, C-t-gastrin immuno-positive cells were found throughout the gut, including the gastric epithelium. After day 4, the C-t-gastrin cells were only detected in the anterior intestine. The completeness of the stomach development was also marked by the appearance of non specific esterase activity in the stomach epithelial cells on day 5. All data together clearly indicate that in *Clarias gariepinus*, the ontogeny of a functional digestive system has been completed on day 5 after the start of exogenous feeding, marking thereby the end of the larval period.

Key-words: Clarias gariepinus, Ontogeny, Digestive system, Stomach, Digestive Enzymes, Pancreas

Introduction

Fish larvae are characterized by an incomplete organogenesis, resulting in different organ functions compared to juvenile and adult fishes. As a consequence, they require specific larval diets, be it live food organisms or formulated feeds. Most fish larvae have difficulty in utilizing formulated diets. A good understanding of the fish ontogeny is

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essential for information on the developing abilities of the fish to cope with food ingredients and for comparison between species. For the African catfish, *Clarias gariepinus*, a detailed analysis of the anatomical and physiological ontogeny is not available. The length of the larval period is not clearly defined. Metamorphosis does not occur. Nevertheless, Hecht and Appelbaum (1987) found a fully differentiated median fin-fold at a total length of 30 mm. According to Balon (1975), this indicates the end of the larval period. In a detailed study, Stroband and Kroon (1981) demonstrated the presence of a stomach with a clear pylorus, functional glandular cells and a Ph lower than five at a standard length of 11.5 mm and onwards. Before that size, a stomach is lacking and this is at least one major difference with juvenile and adult *C. gariepinus*. In a study on the weaning time in *C. gariepinus*, the survival and growth rate were maximized after respectively two and four days of pure *Artemia* feeding (Verreth and van Tongeren 1989). Apparently, after two days, the digestive system of *Clarias gariepinus* was sufficiently developed to sustain an excellent survival rate with a commercial feed, but it took more than four days of *Artemia* feeding to enable maximal growth rates with a commercial diet afterwards.

This hypothesis is further tested during the present study. To elucidate the possible relation between weaning time and the development of digestive functions, a histological analysis was made of the anatomical development of the digestive tract, combined with an immuno-histochemical determination of some gastro-intestinal hormones and a histochemical analysis of some digestive enzymes during the early larval ontogeny of *C. gariepinus*.

Materials and Methods

Facilities and Fish

The experimental animals originated from the pooled offspring of four female breeders of the African catfish. Offspring were obtained through artificial breeding. Eggs and yolk-sac fry were incubated at 30 °C. Just prior to yolk absorption, the fish was stocked in 16.6 L aquaria at a density of 60/L. Rearing temperature during this period was 27.5 °C, which was considered to be optimal for larval growth of the African catfish (Verreth and Den Bieman 1987). Water flow through the aquaria was maintained at 0.8 L/min.

Feeding

The larvae were fed with instar I nauplii of *Artemia*. Feeding was done five times a day (between 08.00 and 22.00 h). Daily rations consisted of measured volumes of an *Artemia*

nauplii suspension of which the weight per unit volume was known. Each day, a fresh suspension of just hatched nauplii was stored and kept alive in the refrigerator according to the procedure of Léger et al. (1983). The weight per unit volume ratio was measured by weighing a known-volume sample out of this suspension. The feeding level (FL) corresponded to a near-satiation level (FL= 0.35) and was calculated according to the procedure of Verreth and Den Bieman (1987).

Sampling

The study period comprised different developmental stages. According to Balon (1975), the embryonal stage lasts from fertilization until the start of exogenous feeding. The embryonal period can further be subdivided into the egg stage (between fertilization and hatching) and the eleuthero-embryonal (yolk sac period) stage. The larval period starts with the onset of exogenous feeding. In *Clarias gariepinus*, at 30 °C, hatching occurs 24 hours after fertilization, and yolk sac absorption lasts 48 hours. Consequently, the larval period starts at an age of 3 days. Considering the objectives of the present study, the egg stage was considered as less important and sampling started from hatching onwards, e.g. during the eleuthero-embryonal period. Since ontogenetically, the embryonal (including the yolk sac period) and larval period are quite different, a separate sampling scheme for both periods was developed and all events were described in relation to the starting point of the respective developmental period: hatching for the yolk sac (or eleuthero-embryonal) period and the start of exogenous feeding for the larval period.

After hatching, 30 samples were taken for histological and immuno-histochemical analysis. During the yolk sac period, samples were taken every 12 hours, starting 2 hours after hatching. In total, four samples were taken before the onset of exogenous feeding. The larval period was covered by 11 samples, starting 2 hours after the first feed was given, followed by a sample at an age of 14 and 26 hours. Further, all samples were taken daily. The first day of exogenous feeding was considered as feeding day zero, changing into feeding day one, 24 hours after the start of exogenous feeding. Each day, a random sample of 10 fishes was used for length measurements and one of 15 fishes for weight measurements.

Histological analysis

The samples for histological and immuno-histochemical analysis were fixed in Bouin's fixative and subsequently embedded in paraffin. Serial sections of 5 µm were made sagitally, mounted and used either for histological or immuno-histochemical analysis. Each slide

contained parallel sections of five fishes. Per sample point, at least three slides were stained for histological and three slides for immuno-histochemical analysis. The slides for histological analysis were stained with Crossmon reagents (Bancroft and Stevens 1982) or PAS. The vacuoles present in the enterocytes of intestine I were assumed to contain lipid. In the staining methods used, lipid is dissolved and washed away. The mentioned vacuoles were not stained and had the appearance as holes in the slides. The intensity of lipid absorption was estimated on the basis of the presence and size of these vacuoles. Likewise, the presence of supranuclear vacuoles in intestine II was considered to indicate the occurrence of absorption in this part of the digestive system.

The slides for immuno-histochemical analysis were tested with rabbit antisera against human C-t Gastrin/CCK and Glucagon and with Guinea pig antiserum against porcine insulin in a "2 steps peroxidase (DAB-) method" according to the procedure of Rombout and Taverne-Thiele (1982). As second antibodies, goat-anti-Rabbit conjugated with Horse Radish Peroxidase (HRP) (IgG(H+L)) (dilution 1:200) (Bio-Rad Laboratories, Richmond, USA) and Rabbit-anti-Guinea pig - HRP conjugate (P141) (dilution 1:200) (DAKO-Immunoglobulins, Denmark) were used.

pH Measurements

From the start of exogenous feeding onwards, the pH of the gut was measured daily by micro-injection of methyl-red and/or congo red indicator fluid into the gut of living larvae.

Enzyme analysis

The occurrence of digestive enzymes (amino-peptidase, non specific esterase, ATP-ase) were tested histochemically. The enzymes were stained on cryostat sections from fresh material, according to the procedures of Segner et al (1989). For the amino-peptidase reaction however, only L-alanine-2-naphtylamide was used as a substrate. The samples for enzyme analysis originated from a previous, but similar experiment to the present one. Samples were only taken from the start of exogenous feeding onwards, on feeding days 0,1,2,4,5,7 and 10.

Results

Growth

Length and weight data are shown in Table 1. The final weight obtained (90 mg) coincides with a growth rate g = 0.35, which is more than the maximum growth rate

reported for larvae of *C. gariepinus* hitherto (Verreth and den Bieman 1987).

Morphological development

Just after hatching, the digestive tract consists of a straight undifferentiated tube, with a small lumen and closed oesophagus and anus. An undifferentiated pancreas and liver-anlage is present. In the pancreas, which consists of a clump of exocrine and endocrine cells, no distinct islets of Langerhans could be detected. During the first 24 hours after hatching, the gut, liver and pancreas develop strongly. The gut differentiates into a buccal cavity, an oesophagus anlage and an intestine. The anus remains closed. The intestine differentiates gradually into three sections that according to Stroband and Kroon (1981) coincide with intestine I, II and III (Fig. 1). In the pancreas, islets of Langerhans appear during the first 14 hours of the yolk sac stage.

At the onset of exogenous feeding, a stomach-anlage with isometric epithelial cells, but without a differentiated pyloric region, was present (Fig. 2). Two hours after first feeding, no food was detected in the stomach anlage. Twelve hours later, however, the larval gut was entirely filled with food particles. Intestine I shows many mucosal folds decreasing in height towards the end of intestine II. Intestine III consists of a straight part, with a thickened epithelial wall. At the start of exogenous feeding, a clear coloration of pancreas zymogen was apparent. During the first four days of exogenous feeding, the gastro-intestinal morphology shows very fast changes. Twenty four hours after first feeding, an intensive lipid absorption could be detected in the LM slides in the first part of the intestine, immediately behind the gastro-intestinal junction (Fig. 3). In the posterior half of intestine I, the size of these (lipid) vacuoles decreases strongly. The number and size of the vacuoles increases with increasing age. During the first day after the onset of exogenous feeding, supranuclear vacuoles are present in intestine II (Fig. 3). Only on day 2, a gall bladder became visible on the histological sections. At the third day after the start of exogenous feeding, a PAS-positive mucus layer is clearly visible on the luminal side of the stomach epithelium. The stomach attains its morphological completeness on day 4 when a pyloric sphincter is present.

Gastric pH

At the start of exogenous feeding, the pH of the gut was above 5.2 (Table 1). The first signs of pH decrease are found on feeding day 3 (72 hours after the start of exogenous feeding). During the next 48 hours, the gastric pH decreases further down to 3.3 (Table 1).

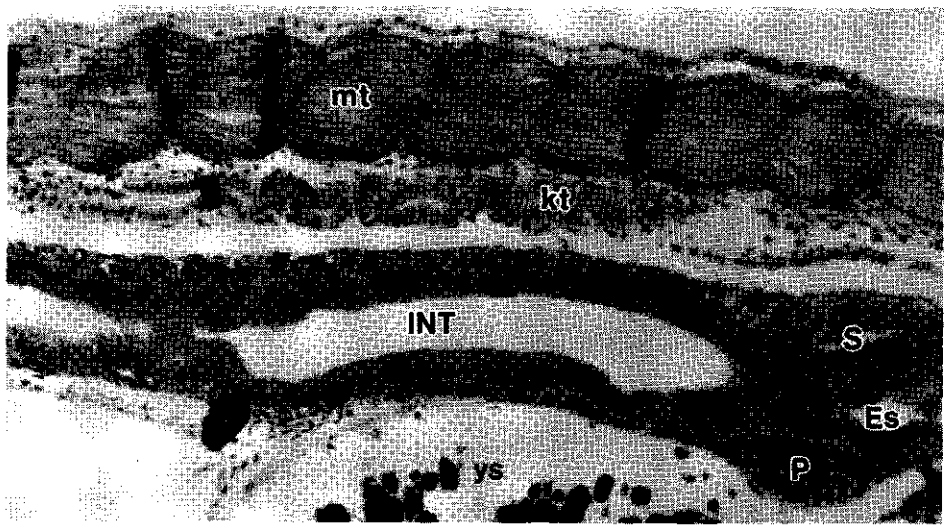

Figure 1. Sagittal section through the digestive tract of *Clarias gariepinus*, 14 hours after hatching (eleuthero-embryo). The lumen of the Oesophagus (ES) and intestine (INT) are open; pancreas (P) and future swimbladder (S) are present. kt = kidney tissue; mt = muscle tissue; ys = yolk sac. Staining: Crossmon. Magnification: 165 x.

Gastro-intestinal hormones

Immediately after hatching, a weak but clear staining of pancreatic endocrine cells which were randomly distributed throughout the organ, was obtained using the anti-insulin and anti-glucagon sera. The presence of insulin and glucagon producing cells could be demonstrated unambiguously as soon as the islets of Langerhans could be detected (14 hours after hatching) (Fig. 4). The first cells showing C-terminal gastrin/CCK-immunoreactivity, appeared in the gastro-intestinal epithelium during the second day of yolk absorption, prior to the start of exogenous feeding. Fourteen hours after the start of exogenous feeding, c-t-Gastrin immunoreactive cells were found in the epithelium of the stomach anlage and throughout the gut. However, during the first three days of exogenous feeding, this rather random distribution of the C-t-gastrin-immunoreactive cells changes towards a sharply defined location in the anterior part of intestine I. From feeding day 4 onwards, this location did not change any more.

Enzymes

Amino-peptidase. Histochemical evidence for the presence of amino-peptidase was found in intestine I and II. Both the stomach and intestine III reactions were negative. The

Table 1. A summary of the major events in the morpho-functional developments of the digestive system in larval African catfish, *Clarias gariepinus* (Burchell).

Age (days)	Length (mm)	Weight (mg)	Pancreas	Intestine	Stomach
EMBRYO					
0(2 h)	5.31	1.28	endo + exocrine cells; no islets; glucagon positive insulin positive	straight tube; no segmentation; anus closed	-
0.5	-	-	islets present	open lumen; parts I, II, III	-
1(26 h)	6.24	1.71		pharyngeal teeth anus open	-
1.5	-	-	zymogen granules	C-t-gastrin positive	gastric epithelium
LARVAE					
0	6.40	2.56		id.	stomach anlage; no pylorus
0.5	7.69	3.05		supranuclear vacuoles (int.II)	C-t-gastrin positive
1	9.20	4.18		fat vacuoles (int.I)	C-t-gastrin positive
2	9.63	7.54		id.	C-t-gastrin positive
3	11.38	11.62		id.	mucus layer; pH: between 5.2-4.4
4	12.07	17.88		C-t-gastrin positive in anterior gut	C-t-gastrin negative pH: between 4.4-3.3; pyloric sphincter present
5	17.20	23.95			pH: below 3.3
6	17.15	33.78			
7	19.19	50.26			
8	-	65.96			
9	-	90			

strongest staining was found in the caudal part of intestine I. The enzyme was located at the microvillus brush border of the enterocytes. Between feeding day 0 (start of exogenous feeding) and feeding day 10, only a weak increase of the staining activity could be found.

Non specific esterase. At the start of exogenous feeding, the activity of non specific esterase was very weak in the gut. However, in the pancreas and in the kidney tubuli, a distinct activity was present. In the stomach, a clear ontogenetic change of the esterase location occurred between feeding day 4 and 5. On day 4, esterase activity was found in the

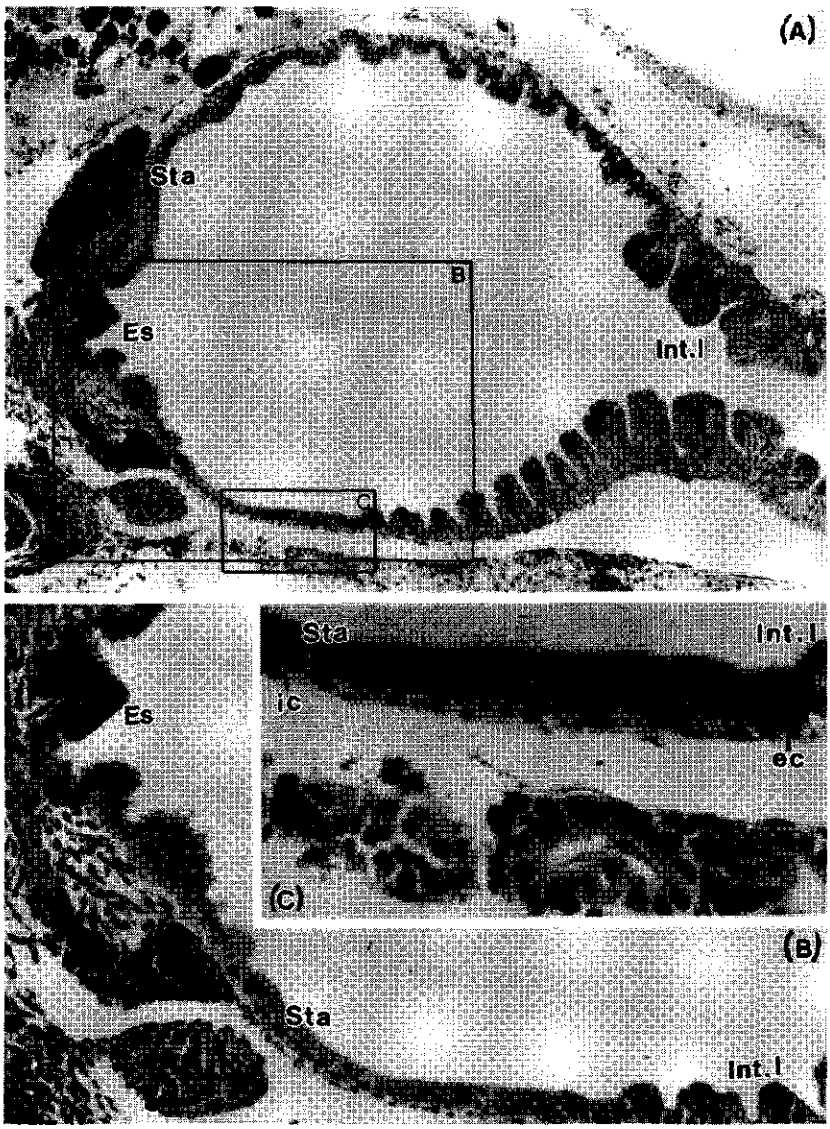

Figure 2. Sagittal section through the stomach and Intestine I region of *Clarias gariepinus* larvae, at the onset of exogenous feeding. Sampling was done two hours after the first food supply. 2A. Overview of the oesophagus (ES) stomach anlage (Sta) and intestine (Int. I). Stomach anlage and Intestine I are in open connection to each other. Intestine I is characterized by strong mucosal folds. At the moment of sampling, the gut did not contain food particles. Magnification: 165 x. Staining: Crossman. 2B. Focus on the section through oesophagus (ES), stomach anlage (Sta) and intestine I (Int. I). Magnification: 330 x. 2C. Detail of the border between stomach anlage (Sta) with isometric epithelial cells (ic) and intestine I (Int. I) with elongated cells (ec). Magnification: 660 x.

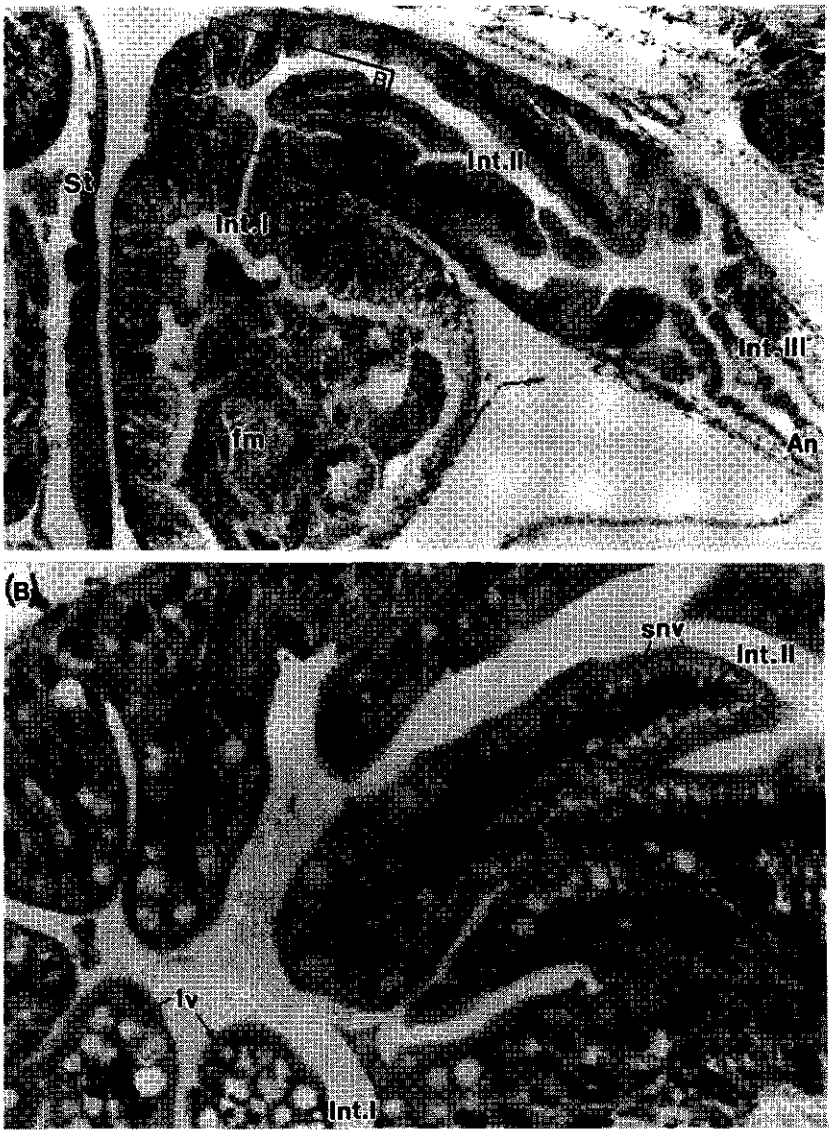

Figure 3. Sagittal section through the digestive tract of a two day old larva (2 days after the onset of exogenous feeding) of *Clarias gariepinus* (above). Overview of stomach (St), intestine (Int. I), intestine II (Int. II), intestine III (Int. III) and anus (An). fm = food material. Magnification: 125 x. 3B. Detail of Intestine I (Int. I) and II (Int. II) lv = lipid vacuoles; snv = supranuclear vacuoles. Magnification: 660 x.

Figure 4. Sagittal section through the pancreas of 26 hours old yolk sac larvae (eleuthero-embryo) of *Clarias gariepinus*, Glucagon producing cells (see black arrows) can be distinguished in the islet of Langerhans. isLa = islet of Langerhans; eP = exocrine Pancreas. Magnification: 660 x.

glandular tissue, but not in the epithelium of the stomach. One day later (Fig. 5), also the stomach epithelium was clearly positive. A strong increase in the staining reaction of non specific esterase between day 0 (start of exogenous feeding) and feeding day 10 could be detected. Among the different gut segments, the strongest reaction occurred in the stomach and in the anterior half of intestine I. The staining activity gradually decreased towards the caudal part of intestine I. In intestine II, esterase activity was almost absent. In the intestine, the esterase was located intracellularly and remained restricted to the apical halves of the enterocytes, but it was absent from the brush border.

ATP-ase. This enzyme was present in the bile canaliculi and ducts, in the epithelial cells of the buccal cavity, oesophagus, in the glandular tissue of the stomach and in intestines I and II. The strongest reactions were obtained in the intestine, in which the staining activity decreased from the anterior part towards the caudal part. Also for this enzyme, a strong increase in the staining activity during the feeding period could be demonstrated.

Discussion and conclusions

Growth

From a methodological point of view, it is important that the morpho-functional development of the digestive system is described on the basis of fast growing animals. Poor growth may induce alterations in the intensity, speed and even the detectability of functional events. In the present study, the growth of *C. gariepinus* larvae was the highest reported hitherto (g = 0.35), which is probably related to the feeding of live *Artemia* nauplii compared to decapsulated cysts in earlier papers (Verreth and den Bieman 1987, Verreth and van Tongeren 1989). Further, also temperature has an obvious effect. For their study on the stomach development of *C. gariepinus*, Stroband and Kroon (1981) used larvae reared at 23°C. In practice, however, *C. gariepinus* larvae are reared at temperatures between 27.5 and 30 °C. As a result, the time path of their events does not compare with practical rearing conditions. In the present study, the larvae were reared at the optimal rearing temperature for *C. gariepinus* larvae (27.5 °C) (Verreth and den Bieman 1987). This difference in rearing temperature may partly explain the different sizes at which the stomach becomes functional in both studies (11.5 mm in the study of Stroband and Kroon (1981) versus 17.2 mm in the present study). If this hypothesis would hold, it would indicate that fish size is not the first determinant for the ontogenetic development. At the same time, some caution should be taken in comparing data of studies with such a different time path of ontogenetic events and using the methods as applied in both studies. In the present study, the morphological development of the stomach was completed on feeding day 4, at a fish size of 12.1 mm,

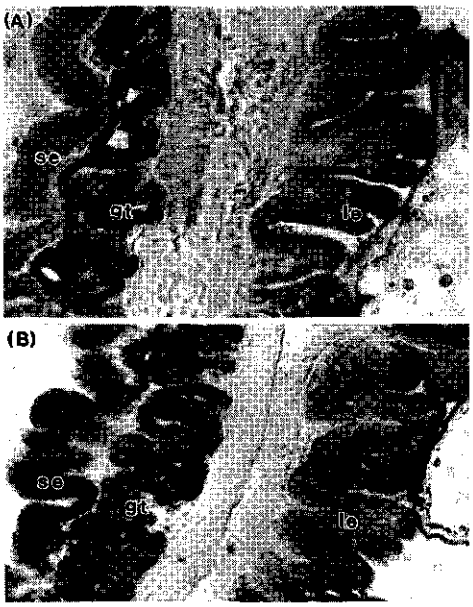

Figure 5. Histochemical assessment of non-specific esterase activity in the wall of stomach (STm) and intestine I (INT) of larvae of the African catfish *Clarias gariepinus*. The upper graph (A) depicts the situation at day 4, the lower graph (B) at day 5 after the start of exogenous feeding. The presence and amount of non-specific esterase is reflected by its black staining. In contrast to day 5, at day 4 there is almost no non-specific esterase activity in the stomach epithelium. se = stomach epithelium; gt = glandular tissue; Ie = epithelium of intestine I.

which is much closer to the size mentioned by Stroband and Kroon (1981) (Table 1). On day 4, only the coloration of the pH-indicator fluids in the stomach did not indicate a stable pH below 3.3. Besides the fact that this "indicator fluid method" leaves some space for subjective interpretation, the results may also have been influenced by the sampling frequency. At the higher temperature used in the present study, the ontogenetic events occur very fast. A fully functional stomach may have been completed on day 4, a few hours after sampling, but long before the next sampling point on day 5. Indirectly, the sampling frequency may have contributed to the mentioned differences in size between both studies.

Morphological and functional development

Just after hatching, the digestive functions of the African catfish *C. gariepinus* are poorly developed. Liver and pancreas are not yet differentiated into distinct organs, the digestive tube is undifferentiated and the anus is closed. Nevertheless, in the short period until the end of the yolk sac period (48 hours), a pronounced development of the digestive system took place. With exception of the stomach, the entire gastro-intestinal tract differentiated into its final segments.

The pancreatic and intestinal hormones, regulating the metabolism and digestive secretions (insulin, glucagon, c-t-gastrin/CCK) can be demonstrated within endocrine cells in 2 and 38-h-old yolk sac embryo. The exocrine pancreas produces and accumulates digestive enzymes as indicated by the presence of numerous, large zymogen granules. This hypothesis is corroborated by the results of Van Damme et al. (1989) who detected significant trypsin and chymotrypsin levels in larvae of *C. gariepinus* at the start of exogenous feeding. Significant trypsin and/or chymotrypsin levels were also found before or at the start of exogenous feeding in roach (Lauff and Hofer 1984), sturgeon (Buddington 1985) and striped bass (Baragi and Lovell 1986). The presence of active intestinal enzymes as indicated by the histochemical stains on amino-peptidase, non specific esterase and ATP-ase further substantiates the hypothesis of *C. gariepinus* having advanced digestive capacities at the start of exogenous feeding. Amino-peptidase enables the larvae to catabolize peptide's from the trypsin digestion further into smaller molecules, and as such its histochemical determination supports the hypothesis of an active trypsin and chymotrypsin production in the exocrine pancreas (Clark et al. 1987). In the intestine, non specific esterase catabolizes the breakdown of esters, which occur mainly in the lipid and carbohydrate metabolism. ATPase intervenes in the active transport of nutrients through the cell membranes (Toner 1968), and its presence in the intestine reflects the general absorptive capacity of the larvae. In this respect, the presence of many lipid vacuoles in the wall of intestine I, 14 hours after

first feeding, is illustrative, and points to an intense lipid absorption at this place. This coincides with the suggestions of Stroband and Dabrowski (1982) and Govoni et al. (1986), who emphasized the absorptive function of this intestine segment. However, it is striking, that at LM level, these lipid vacuoles are not visible from the start of exogenous feeding onwards. It might be very well possible that lipid absorption starts immediately after first feeding, but it may take some time before the formation of larger lipid droplets become visible at LM level.

In conclusion, at the start of exogenous feeding, larvae of the African catfish seem to be able to have an active protein and lipid digestion as suggested by the data on digestive enzymes, pancreatic hormones and the lipid absorption. Hence, the reduced growth and survival rate encountered in *C. gariepinus* larvae fed with commercial diets during the first two to four days of exogenous feeding, probably does not result from a lack of general digestive capacity. Also for larval walleye, Mitchell et al. (1986) concluded on the basis of enzyme studies that the problems encountered in feeding them formulated diets, would not be the result from a lack in digestive capacity. As more information on digestive enzyme activity in fish larvae becomes available (Govoni et al. 1986), the more this hypothesis seems to be confirmed and, as such, it may be common for freshwater species.

Nevertheless, at the onset of exogenous feeding, the morphological and functional development of the stomach is not yet completed. The pylorus is not developed yet and in spite of the presence of gastric epithelium, the pH is still too high to allow pepsin digestion. Before the pH drops below 4.4, on day 3 a PAS positive mucous layer appears on the luminal side of the stomach epithelium. Probably it protects the stomach wall against the gastric acid. The development of gastric acid secretion is presumably completed after five days of exogenous feeding, when the larvae have a mean size of 24 mg (17.2 mm) (Table 1). The morphological development of the digestive system reaches its completeness with the pyloric sphincter being fully developed on day 4. At the intracellular level, the functional development of the stomach is further demonstrated by the presence of non specific esterase in the stomach epithelium from feeding day 5 onwards. Non specific esterase often points to a functioning endoplasmatic reticulum (Böcking et al. 1974). Hence, its appearance on day 5 in the stomach epithelium clearly indicates that at that moment, the epithelial cells became functional. Similar results were obtained by Ferraris et al. (1987) in a study on milkfish larvae, where non-specific esterase in the stomach became active only after metamorphosis, when the stomach was functional. These data are further substantiated by the immuno-histochemical assessment of C-t-gastrin/CCK in the larval gut of *C. gariepinus*. The

distribution of the immunoreactive cells shows a remarkable change from a random pattern throughout gut and stomach anlage during the first two days of exogenous feeding towards a sharply defined location in the anterior part of the intestine. The disappearance of the C-t-gastrin/CCK immunoreactive cells from the stomach epithelium on day 4 may be explained by the production of a more stomach specific gastrin by the cells in the stomach epithelium, which may not be recognized by the antiserum used in the present study. This hypothesis is supported by the results of Boon et al. (1990). Using the same antiserum in fingerlings of *C. gariepinus*, they demonstrated c-t-gastrin immunoreactive cells only in the anterior intestine. Their suggestion to deal with a CCK-like rather than with a gastrin-like hormone, may hold also for the present study, at least for the immunoreactive cells found in the anterior intestine from feeding day 4 onwards. Whether the cells found in the stomach and gut epithelium during the first days of exogenous feeding also contained this CCK-like peptide or a less specific "precursor"-peptide, remains still to be clarified. Anyhow, the hypothesis that the change in location of these cells can be explained by a change towards the production of a more stomach specific gastrin, coincides well with the drop in gastric pH found in the period immediately after this change.

Physiologically, the formation of a functional stomach entails the increasing capacity of *C. gariepinus* larvae for pepsin digestion. Morpho-functional data corroborate with the changes in pepsin activity, measured in *C. gariepinus* by Van Damme et al. (1989) during the first four days of exogenous feeding. During this period, these authors found a strong increase in pepsin digestion from 5 to 42 mg tyrosine/g sample/10 minutes, with the strongest increase at the end of the four day period. The absence of a structurally and functionally fully developed stomach during the first days of exogenous feeding, will limit the amino acid availability in a lot of protein sources used in common fish feeds. Since the earliest weaning time as found by Verreth and van Tongeren (1989) coincides well with the onset of gastric acid secretion, the reduced growth rate resulting from earlier feeding with commercial diets, seems to be directly related to the absence of a functional stomach and the concurrent pepsin digestion. From the present study, no clear indications can be derived which morpho-functional event may explain the optimization of the survival rate with 2 days of *Artemia* feeding (Verreth and van Tongeren 1989). The most important changes during the very first two days are related with intestinal functions (lipid vacuoles, gall bladder formation). In this respect, it should be emphasized that the apparent lipid absorption in the first part of the intestine does not guarantee a well functioning transport and/or hepatic metabolism at this stage of development. An electron microscopic analysis of the liver ontogeny combined with enzyme analyses of the intermediary metabolism, may provide

more information to elucidate further the functional development of the digestive system.

In conclusion, the data presented in this study, indicate a fully functional digestive system in larvae of *C. gariepinus* after five days of exogenous feeding (at the end of feeding day 4). This coincides well with the weaning time found in a previous study (Verreth and van Tongeren 1989). Apparently, this point marks the physiological end of the larval period in the African catfish, *C. gariepinus*.

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Chapter 5

Metabolic enzyme activities in larvae of the African catfish, *Clarias gariepinus*: response to developmental and dietary influences

Helmut Segner and Johan Verreth

Abstract

The influence of ontogeny and nutrition on metabolic enzyme activities in larvae of the African catfish, *Clarias gariepinus*, was studied. After start of exogenous feeding, the larvae were reared for 10 days under three different nutritional conditions: *Artemia* nauplii, a dry starter diet, and starvation. The live feed gave the best growth (96 mg within 10 days) whereas the dry diet resulted in low growth (33 mg). The growth difference was reflected in larval RNA, DNA and glycogen concentrations, but not in the levels of soluble protein. Enzymes representing the following aspects of metabolism have been analyzed: NADPH generation (glucose-6-phosphate dehydrogenase G6PDH, malic enzyme ME), glycolysis (phospho-fructokinase PFK, pyruvate kinase PK), glyconeogenesis (fructose-1,6-diphosphatase FDPase), amino acid catabolism (glutamate-oxaloacetate transaminase GOT, glutamate pyruvate transaminase GPT) and oxidative catabolism (citrate synthase CS). All enzymes were present from the start of exogenous feeding onwards, but their maximum activities changed during ontogenesis. In *Artemia*-fed larvae, the activity of the NADPH-generating enzymes increased steadily with increasing age and weight of the larvae. ME activity became weight-independent beyond day 5. CS experienced an initial, fast enhancement and remained thereafter on a rather stable plateau. A similar early increase was found for the amino acid-degrading enzymes, but their levels strongly decreased between days 3- 5 or 10 - 20 mg body weight, respectively. The activities of the glycolytic and glyconeogenetic enzymes, on the other hand, started to increase only towards days 3 to 5. In *Artemia*-fed larvae, this period, therefore, seems to constitute a turning point in the ontogeny of larval metabolism. It coincides with other major developmental changes, e.g. muscle organization, gill morphology, respiration and stomach structure and function.

The different nutritional conditions applied in the present study affected enzyme maximum activities. Particularly, the levels of CS and of the amino acid-catabolizing enzymes were lower in the dry feed fed larvae than in live food fed larvae. Starvation resulted in an adaptive increase of CS and GOT. In addition, developmental patterns were modified by dietary conditions, with the dry diet resulting in the delayed development of glycolytic power and glyconeogenesis.

Key-words: Fish, *Clarias gariepinus*, Ontogenesis, Nutrition, Metabolism, NADPH generation, Aerobic metabolism Amino acid catabolism, Glycolysis, Glyconeogenesis

Fish Physiology and Biochemistry (submitted)

Introduction

During early life, fish have to pass through an extensive series of coordinated morphological, biochemical and behavioral alterations to develop from the larval into the juvenile stage. Due to the small body size, larvae encounter stronger viscosity forces than inertia (Osse and van den Boogaart 1994) what affects the larval mode of swimming and the coincident metabolic properties required for locomotion and food capturing (Forstner *et al.* 1983, Batty 1984, Hinterleitner *et al.* 1989). Further, organ systems involved in feed detection, digestion and nutrient metabolism undergo structural and functional changes during the larval period (Stroband and Dabrowski 1979, Govoni *et al.* 1986, Blaxter 1986, Avila and Juario 1987, Osse 1990, Segner *et al.* 1993, 1994a). Kotrschal *et al.* (1990) as well as Segner *et al.* (1994a) hypothesized that the specific characteristics of fish larvae could be an adaptation to the constraints set by the size of the animal. Fish species with large egg sizes, e.g. salmonids, can support a long-lasting yolk sac-phase and also large larval sizes, both factors favouring an advanced functional organogenesis at first feeding. In contrast, marine fish species which have very small egg and larval sizes (about 1 and 2-4 mm respectively), display a very incomplete organogenesis at the start of exogenous feeding (Cousin and Baudin-Laurencin 1985; Pittman *et al.* 1990, Kjørsvik *et al.* 1991, Pedersen and Falk-Petersen 1992).

Egg size in the African catfish, *Clarias gariepinus* is rather small (1.1 - 1.4 mm diameter) but at the onset of exogenous feeding the larvae measure 5.5 - 6.0 mm and weigh 2.2 - 2.4 mg which is rather common among freshwater fish. At a water temperature of 28 - 30 °C, fertilized eggs need 24 hours to hatch. The yolk sac period lasts 48 hours. The length of the larval period in this species is not clearly defined (Verreth *et al.* 1993). A basic knowledge about the structural and functional changes in digestive and metabolic properties of the early life stages is required for the design of optimal rearing and feeding procedures and to ensure a reliable supply of fingerlings for fish farms. An analysis of the functional development of the digestive tract of *C. gariepinus* showed that at the onset of exogenous feeding the intestine is subdivided into functionally different regions, possessing substantial activities of digestive enzymes, both from the pancreas and from the enterocytes, and being able to digest and to absorb lipids (Stroband and Kroon 1981; Verreth *et al.* 1992; Verreth *et al.* 1993). In addition, endocrine pancreatic hormones are present. At the start of exogenous feeding, a functional stomach is lacking. The histological (appearance of gastric glands) and functional (appearance of pepsin-like activity and acid pH) differentiation of the

stomach occurs only 3-5 days after the start of exogenous feeding (Stroband and Kroon 1981, Verreth *et al.* 1992). It is envisaged that these morpho-functional changes in the digestive system have a corollary in the metabolic properties of the larvae.

To test this hypothesis, in the present work, the ontogeny of various enzyme activities was investigated in larvae of the African catfish *Clarias gariepinus*. In the absence of precise biological information, the larval stage was thereby defined as the period between the onset of exogenous feeding (Balon 1975) and the moment that *C. gariepinus* is usually weaned to a dry diet in commercial fish hatcheries, e.g. after 10 days of exogenous feeding at a temperature of 27-28 °C (Verreth *et al.* 1993; Hecht and Oellerman 1994). The aims of the study were (1)to establish the ontogenetic patterns of several central metabolic pathways in young catfish; (2)to reveal if, on the basis of metabolism, a particular larval phase can be distinguished, and (3)of what nature the metabolic differences between larvae and juveniles are. Enzymes representing the following facets of metabolism have been studied: NADPH generation (glucose-6-phosphate dehydrogenase G6PDH, malic enzyme ME), glycolysis (phospho-fructokinase PFK, pyruvate kinase PK), glyconeogenesis (fructose-1,6-diphosphatase FDPase), amino acid catabolism (glutamate-oxaloacetate transaminase GOT, glutamate pyruvate transaminase GPT) and oxidative catabolism (citrate synthase CS). Since ontogenetic processes might be greatly modified by environmental factors, the analyses have been performed for three different nutritional conditions: live food (*Artemia* nauplii), dry food, and starvation. For juvenile and adult fishes it is well documented that enzyme activities are under strict regulation of nutritional conditions (Cowey *et al.* 1980, Moon 1983, Lupianez *et al.* 1989, Böhm *et al.* 1994). For larvae, information on dietary effects on enzyme activity is scarce. To our knowledge, only in larval Atlantic menhaden, *Brevoortia tyrannus*, the effect of dietary factors on citrate synthase and lactate dehydrogenase has been studied (Clarke *et al.* 1992).

Material and Methods

Facilities, Feeds and Fish

All experiments were carried out at the Experimental Hatchery "De Haar Vissen" of the Wageningen Agricultural University. Fish larvae were stocked in 17 L aquaria which were supplied with heated and well oxygenated water (temperature = 27.5°C) at a flow rate of about 0.8 - 1.0 L/min. For each experiment, eggs and larvae of the African catfish, *Clarias*

gariepinus, were obtained through artificial reproduction of 2-3 females and 1 - 2 males. Eggs and hatched larvae of all females were pooled during incubation. After hatching (24 hours post fertilization) the swimming larvae were flushed by the water current in a 180 L collector aquarium where they remained until yolk resorption. Upon yolk resorption (about 72 hours post fertilization), larvae were stocked at a density of 1000 animals/aquarium, i.e. about 60 animals/L and feeding started. In each experiment, three different dietary treatments were applied, e.g., live feed (*Artemia* nauplii), dry feed (Mainstream 00, BP Nutrition Aquaculture) and no feed (starvation). *Artemia* nauplii were obtained by incubation of cysts (strain Great Salt Lake, Utah, USA, Sanders Co.). To maintain optimal quality during feeding, each day a new batch of fresh nauplii was made and stored at temperatures below 5°C (Léger *et al.* 1986). Instar I nauplii of the mentioned strain of *Artemia* have a body length of 450-520 µm (Léger 1988, quoted in Dhert 1992). The proximate composition ranges from 42-47 % protein, 21-23 % lipid, 11-23 % carbohydrate and 7-10 % ash (Léger *et al.* 1986). The dry (Mainstream) diet had a particle size 0.4 mm, and according to the manufacturer's information, contained 54% protein, 15% fat, 1% fibre and 10% ash in the dry matter. Feeding of *Artemia* occurred at near satiation level so that the growth rate coefficient g (Verreth and den Bieman 1987) should reach a value between 0.30 and 0.35.

Experimental procedures

Four independent rearing trials were performed to measure ME and G6PDH, two experiments were carried out for the determination of GOT, GPT, FDPase, PK, protein, glycogen, RNA, DNA, and one experiment for the determination of PFK and CS. In addition, in one experiment with *Artemia*-fed larvae, the activities of NADP-dependent isocitrate dehydrogenase (IDH) and acetyl CoA carboxylase (ACC) were measured. Feeding was done four times a day between 9 a.m. and 12 p.m. The feeding level (FL) in the *Artemia*-fed groups corresponded to near-satiation level (FL = 0.3), and was calculated according to the procedure of Verreth and den Bieman (1987). Before feeding, feed remnants, faeces and dead larvae were removed from the aquaria and the feeding levels were adjusted accordingly (Verreth and van Tongeren 1989). Samples were taken at 0, 1, 3, 5, 8 and 10 days after start of exogenous feeding. Sampling time was between noon and 2 p.m. At sampling, 15 - 30 larvae were taken from each tank to determine individual wet and dry weight. For enzyme analysis, larvae were pooled to yield six samples of approximately 200 mg fresh weight per sampling date and per treatment. Upon sampling, the larvae were transferred to Eppendorf cups, frozen in liquid nitrogen and stored at -80°C until further processing (within 2 months after sampling).

Preparation of homogenates

Frozen samples were thawed and homogenized in ice-cold 0.15 M KCl using an Ultraturrax homogeniser. For analysis of CS activity, the homogenate was additionally subjected to sonification. An aliquot (100 µl) of the homogenate was transferred to perchloric acid (5% final concentration) for the determination of glycogen. A second aliquot (350 µl) was taken for RNA and DNA extraction. The remaining homogenate was centrifuged at 4°C for 15 minutes at 10 000 x g. The clear supernatant was analyzed for enzyme activities and concentration of soluble protein.

Enzyme analyses

Determination of enzyme activities were performed spectrophotometrically at a temperature of 25°C. Enzyme activities are given in units (1 unit = 1 µmol min^{-1}) per gram protein. The assay conditions were as follows (all concentrations are final concentrations):

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, measured according to Segner and Böhm 1994): 50 mM Tris, pH 7.5, 8.4 mM $MgCl_2$, 0.4 mM $NADP^+$, start by 1 mM glucose-6-phosphate; reaction was monitored at 340 nm.

Malic enzyme ME (E.C. 1.1.1.40, measured according to Segner and Böhm 1994): 50 mM Tris, pH 7.5, 8.4 mM $MgCl_2$, 0.4 mM $NADP^+$; start by 1 mM malate; reaction was monitored at 340 nm.

Phospho-fructokinase (PFK) (E.C. 2.7.1.11, modified from Mommsen *et al.* 1980): 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 U/ml glycerol-3-phosphate dehydrogenase, 1 U/ml aldolase, 2.5 U/ml triosephosphate isomerase, 0.12 mM NADH, 0.75 mM ATP; start by 6 mM fructose-6-phosphate; reaction was monitored at 340 nm.

Pyruvate kinase (PK) (E.C. 2.7.1.40, modified from Mommsen *et al.* 1980): 50 mM Tris, pH 7.5, 7.5 mM $MgCl_2$, 1.2 mM EDTA, 1 U/ml lactate dehydrogenase, o.12 mM NADH, 1 mM ADP; start by 1 mM phosphoenol pyruvate; reaction was monitored at 340 nm.

Fructose-1,6-diphosphatase (FDPase) (E.C. 3.1.3.11, measured according to Mommsen *et al.* 1980): 50 mM Tris, pH 7.5, 15 mM $MgCl_2$, 1 U/ml phospho-glucoisomerase, 1 U/ml glucose-6-phosphate dehydrogenase, 0.2 mM $NADP^+$; start by 0.1 mM fructose-1,6-bisphosphate; reaction was monitored at 340 nm.

Citrate synthase (CS) (E.C. 4.1.3.7, measured according to Mommsen *et al.* 1980): 50 mM Tris, pH 8.0, 0.1 mM acetyl-CoA, 0.1 mM DTNB (5,5'-dithiobis-(2-nitrobenzoate)); start by 0.5 mM oxaloacetate; reaction was monitored at 412 nm, ϵ_{DTNB} = 13.6.

Glutamate oxaloacetate transaminase (GOT) (E.C. 2.6.1.1, measured according to Mommsen *et al.* 1980): 50 mM Tris, pH 7.5, 40 mM aspartate, 25 µM pyridoxale phosphate, 8 U/ml

malate dehydrogenase, 0.12 mM NADH; start by 7 mM ∝-ketoglutarate; reaction was monitored at 340 nm.

Glutamate-pyruvate transaminase (GPT) (E.C. 2.6.1.2, measured according to Mommsen *et al.* 1980): 50 mM Tris, pH 7.5, 200 mM L-alanine, 25 µM pyridoxale phosphate, 1 U/ml malate dehydrogenase, 0.12 mM NADH; start by 7 mM ∝-ketoglutarate;

Controls were run by omitting the substrate. All co-enzymes as well as NADH and NADP were obtained from Boehringer Mannheim, FRG.

Other methods

Soluble protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Glycogen was determined by the amylase digestion method of Keppler and Decker (1984). The glycogen values measured were corrected for the body content of free glucose. The analysis of DNA and RNA followed the method of Rafael and Vsianski (1981). From these data, it was calculated the DNA/weight ratio as an estimate of cell number per unit weight, the protein/DNA ratio as an estimate of the cell size, and the RNA/DNA ratio as an estimate of the amount of RNA per cells (Foster *et al.* 1993, Böhm *et al.* 1994).

Statistics

All values are mean ± standard error. Comparison between dietary treatments or age groups were made using one way analysis of variance and the Newman-Keuls multiple range test (Sokal and Rohlf 1981), with $p < 0.05$ being used as the criterion of significance throughout.

Results

In Table 1, developmental and diet-related changes of body weight and body constituents of larval *Clarias gariepinus* are summarized. Larvae fed on *Artemia* grew in 10 days until an average weight of 96 mg, i.e. an average growth rate $g = 0.32$. For the experimental temperature used in this study, the coefficient $g = 0.32$ indicates an almost maximum growth of catfish larvae (Verreth and den Bieman 1987). The dry diet resulted in a significantly reduced growth (final body weight is about 33 mg) compared to *Artemia*-fed larvae. The body weight of starved catfish larvae decreased slightly in time as was expected. Five days after the start of exogenous feeding most starving larvae had died and therefore

this dietary regimen was stopped at that moment. Body levels of soluble protein remain fairly constant in fed larvae, regardless of the diet, but decline consistently in starved fishes. This difference in body protein levels of fed and starved animals is important when comparing protein-specific enzyme activities between these treatments.

Larval DNA concentrations can be regarded as an estimate for the number of cells per weight, whereas the ratio protein/DNA reflects more the size of a cell (Foster *et al.* 1993, Böhm *et al.* 1994). With values between 1.97 and 2.69 µg DNA mg^{-1} larval weight, the DNA concentrations are higher in fishes receiving dry food than in larvae fed on *Artemia* (1.73 - 2.11 µg DNA mg^{-1} larval weight; Table 1). This means that at a given body size larvae reared on the dry diet have more cells than Artemia-fed larvae. Concomitantly, the protein/DNA ratios in dry food fed fish are lower than in the live food fed groups (Table 1) which indicate that the cells are smaller in the dry food than in the live food groups. The elevated DNA concentrations (2.39 - 2.98 µg DNA mg^{-1} body weight) in starved larvae reflect the strong reduction of cell size under conditions of hunger. The RNA/DNA ratio as well as the RNA/protein ratio often are taken as biochemical growth rate indices (see Foster et al. 1993). *Artemia*-fed larvae possess the highest RNA/DNA values what correlates with the high growth rate of this group. Starved larvae, on the other hand, have the lowest RNA/DNA ratios. Larvae reared on Artemia show a scaling effect for the RNA/protein ratio (Houlihan et al. 1993), with slowly declining ratios at increasing body weight. For the dry food group, a scaling effect is not evident.

Whole body glycogen concentrations of catfish larvae fed with *Artemia* increased during the first 24 hours of feeding from 16 mg glycosyl units mg^{-1} larval weight to 40-45 mg glycosyl units mg^{-1} larval weight where it remained stable until day 5. It increased further to 72 and 79 mg glycosyl units mg^{-1} larval weight from day 5 to the end of the experimental period. Fishes receiving dry feed maintained a constantly low glycogen level throughout the experimental period.

Figures 1 to 5 present the changes in enzyme activities during the larval period of *C. gariepinus*. Since the timetable of developmental changes may be age-dependent as well as size-dependent, the data are presented both in relation to days after start of exogenous feeding and in relation to fresh body weight. The enzymes studied can be ascribed to the following aspects of metabolism:

Table 1. Ontogenetic changes of body constituents of *Clarias gariepinus* reared under different nutritional conditions. Soluble protein and DNA are expressed per unit larval weight, glycosyl per unit protein.

Day	Feed	Wet weight[1] (mg/ind.)	Protein[2] (mg/mgWW)	DNA[2] (µg/mgWW)	Protein/DNA[2] (µg/mg)	RNA/DNA[2] (µg/mg)	glycosyl[2] (mg/mg)
0		2.60 (0.30)	0.195 (0.016)	4.13 (0.66)	0.048 (0.08)	3.02 (0.39)	16.62 (3.32)
1	*Artemia*	4.52 (0.14)	0.079 (0.006)[a]	1.87 (0.27)[a]	0.042 (0.007)	2.79 (0.77)	44.52 (6.77)[a]
	Dry Food	4.00 (0.06)	0.098 (0.011)[ab]	2.21 (0.51)[a]	0.046 (0.007)	2.69 (0.77)	20.84 (4.80)[b]
	Starvation	2.92 ((0.13)	0.100 (0.009)[ab]	2.98 (0.45)[ab]	0.034 (0.003)[ab]	2.28 (0.51)[a]	11.55 (2.93)[b]
3	*Artemia*	10.99 (0.37)	0.121 (0.006)[a]	2.11 (0.41)	0.060 (0.012)[a]	3.92 (0.78)[a]	39.78 (5.52)
	Dry Food	8.24 (0.19)	0.114 (0.014)[a]	1.97 (0.43)	0.059 (0.009)[a]	3.14 (0.76)	25.25 (4.24)[b]
	Starvation	2.42 (0.07)	0.084 (0.005)[ab]	2.39 (0.65)	0.024 (0.006)[ab]	1.75 (0.70)[b]	11.03 (2.69)[b]
5	*Artemia*	23.22 (1.46)	0.123 (0.015)	2.07 (0.58)	0.065 (0.026)	3.76 (0.67)	44.07 (44.48)
	Dry Food	11.93 (0.59)	0.129 (0.040)	2.69 (0.64)[ab]	0.053 (0.031)[ab]	3.11 (0.68)[b]	20.85 (2.19)[b]
	Starvation	2.38 (0.09)	0.077 (0.005)[ab]	2.85 (0.52)[b]	0.028 (0.004)[b]	1.76 (0.45)[b]	14.06 (1.99)[b]
8	*Artemia*	55.25 (3.14)	0.143 (0.014)	2.03 (0.43)	0.073 (0.017)	4.26 (1.07)	71.80 (11.86)[a]
	Dry Food	17.60 (1.86)	0.130 (0.018)	2.59 (0.43)	0.051 (0.011)[b]	3.03 (0.93)[b]	13.36 (3.26)[ab]
10	*Artemia*	96.20 (5.15)	0.138 (0.025)	1.73 (0.36)	0.082 (0.014)	3.53 (0.65)	79.15 (7.32)
	Dry Food	32.79 (4.50)	0.125 (0.033)	2.40 (0.53)[b]	0.054 (0.018)[b]	3.11 (1.00)	22.76 (5.62)[ab]

[1]Mean values ± SD from 6 independent rearing trials
[2]Mean values ± SD from 2 independent rearing trials; within each trial 8 samples were measured per sampling date and treatment.
[a]Significant time effect (compared to previous sampling stage)
[b]Significant diet effect (compared to the *Artemia* group)
Significance level: $p<0.05$

1. NADPH-generating enzymes G6PDH (Figs. 1A,1,2) and ME (Figs. 1B,1,2).
   The activities of both enzymes steadily increase with the age of the fishes, with G6PDH, however, showing a transient decline on day 1. At a body mass between 10 and 20 mg, ME activity shows no further significant elevation but seems to reach a plateau level, i.e. becoming weight-independent. For G6PDH, activity continues to increase significantly beyond a body weight of 20 mg, although the rate of increase slows down. Starvation results in a significant decrease of both ME and G6PDH activities when compared to *Artemia* fed larvae. For G6PDH there exists no significant difference between larvae receiving dry or live feed, but ME levels are altered significantly. Also the height of the ME plateau as found for larvae of 20 mg body weight or more, is significantly different between the two dietary treatments.
2. Glycolytic enzymes PFK (Figs. 2A,1,2) and PK (Figs. 2B,1,2).
   In *Artemia*-fed catfish larvae, there occurs a strong elevation of PFK levels within the first 3 days after onset of exogenous feeding; thereafter, PFK activity levels off. This pattern is reflected also by the weight-related data; here, the turning point from the weight-dependent to the weight-independent phase is situated at approximately 10 mg

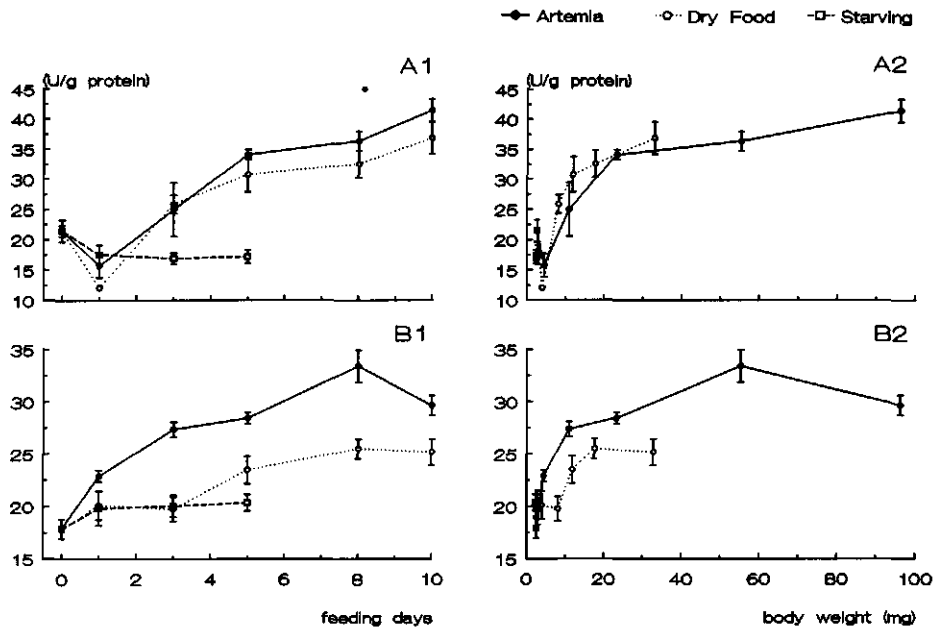

Figure 1 Development- and diet-related changes of the activities (U/g protein) of G6PDH (A1, A2) and ME (B1, B2) in larval *Clarias gariepinus*. The data are plotted versus age (in days; Figs. 1a) or versus body weight (in mg, Figs. 1b).

body weight. With the dry diet, PFK activity remains at a level as low as that of starving catfish, and starts to increase only beyond day 3 or a body mass of 10 mg, respectively. Within the period investigated, PFK activity of dry food-fed fishes did not reach a plateau level. The ontogenetic pattern of PK activities shows an increase starting at day 3 or at a body weight between 10 - 15 mg. PK does not attain a plateau level within the study period. Differences in PK activity between larvae fed on live or on dry feed become significant after 5 days of feeding. Although PFK and PK are situated along the same metabolic pathway (glycolysis), there exists a surprisingly high difference in their absolute activities (a factor of almost 100 !). To the best of our knowledge the only study with fish larvae presenting activity data on both PFK and PK, is the work of Forstner et al. (1983) on *Coregonus* sp.; interestingly, these authors observed a difference between PK and PFK activities within the same order of magnitude.

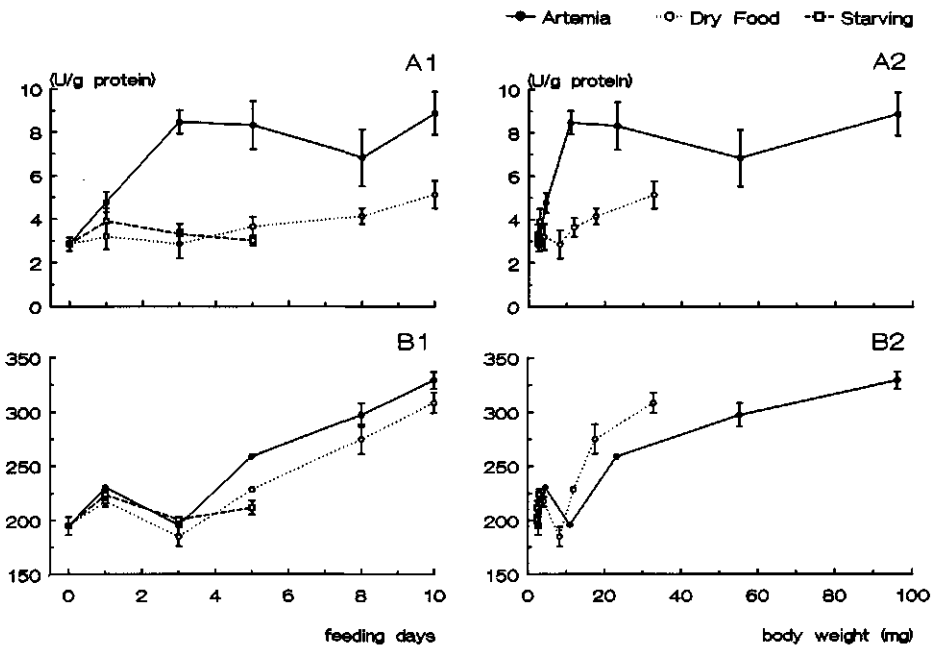

Figure 2 Development- and diet-related changes of the activities (U/g protein) of PFK (A1, A2) and PK (B1, B2) in larval *Clarias gariepinus*. The data are plotted versus age (in days; graphs at the left) or versus body weight (in mg, graphs at the right).

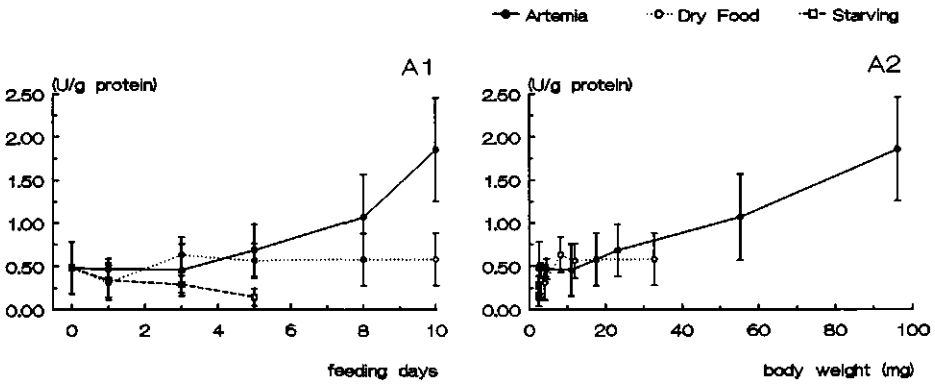

Figure 3 Development- and diet-related changes of the activity (U/g protein) of FDPase in larval *Clarias gariepinus*. The data are plotted versus age (in days: A1) or versus body weight (in mg: A2).

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3. Glyconeogenetic enzyme FDPase (Figs. 3, A1, A2).
   Before day 3, an FDPase activity is almost not detectable. Thereafter, in *Artemia*-fed animals the activity of FDPase increases steadily. On a weight basis, the turning point in the pattern of FDPase activity occurs around 10 mg body weight. With the dry diet, no elevation of FDPase activity can be observed during the investigated period. In starved fishes, the enzyme activity declines continuously.
4. Amino-acid catabolizing enzymes GOT (Figs. 4, A1,A2) and GPT (Figs. 4, B1,B2).
   The activity levels of the two enzymes differ by a factor of approximately 10. However, their developmental pattern shows a remarkable similarity. Both for the live feed- and the dry feed-groups, maximum enzyme activities are found in larvae of less than 20 mg body weight. However, these maximum levels differ greatly in altitude between the two dietary treatments.GPT activities is clearly weight- and not age-dependent. Beyond a body mass of approximately 20 mg, the activities of the two amino acid-catabolizing enzymes reach a fairly constant plateau, i.e. they become weight-independent, and the diet-induced differences in activity seem to disappear.
5. Aerobic enzyme CS (Figs. 5, A1,A2).
   Similar to the activities of GOT and GPT, also CS activity increases fast to a maximum once exogenous feeding starts. When compared on the basis of weight-related data, the enzyme activity pattern in dry food-fed larvae is slightly delayed compared to the *Artemia*-group. As observed also for GOT and GPT, the changes of CS activity in starved larvae are very similar to those in larvae fed with the dry diet.

Discussion

The data used for the present study originated from 6 independent rearing trials which were carried out over a period of almost two years. To ensure compatibility of the experiments, all trials were performed according to the standard protocols for rearing and feeding of larval *Clarias gariepinus* as developed by Verreth & den Bieman (1987) and Verreth *et al.* (1987). In the case of the groups reared with Artemia, which serve as a reference group, the feeding levels applied were designed to yield a predicted growth rate g of 0.3 (Verreth and den Bieman 1987). The value of the growth coefficient *g* actually obtained in the 6 rearing trials varied between 0.30 to 0.35, thus demonstrating that growth of the larvae from the different experiments in fact is comparable. Moreover, the results also demonstrate that in the present study growth of the *Artemia* fed groups is comparable or

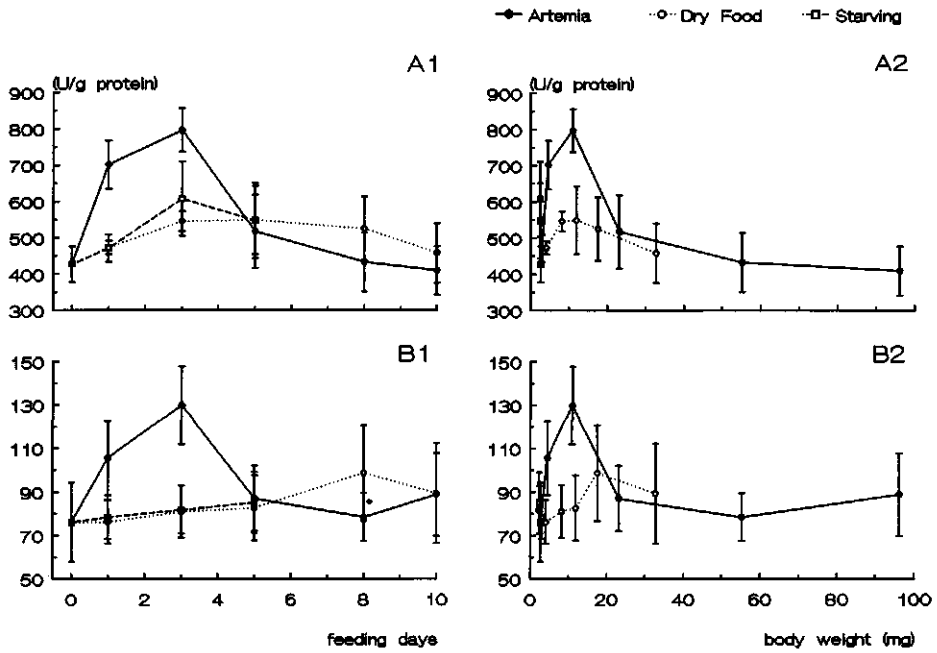

Figure 4 Development- and diet-related changes of the activities (U/g protein) of GOT (A) and GPT (B) in larval *Clarias gariepinus*. The data are plotted versus age (in days: Figs A1, B1) or versus body weight (in mg, Figs. A2, B2)

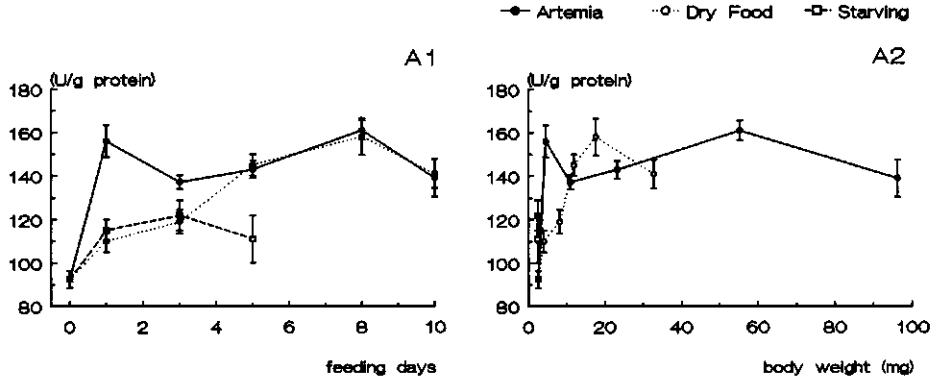

Figure 5 Development- and diet-related changes of the activity (U/g protein) of CS in larval *Clarias gariepinus*. The data are plotted versus age (in days; Fig. A1) or versus body weight (in mg, Fig. A2).

even higher than in similar experiments with *C. gariepinus* reported earlier (Verreth and den Bieman 1987; Verreth and van Tongeren 1989; Verreth *et al.* 1994a), providing a sound basis to the experimental data used in this study.

The response of larval metabolic capacities to ontogenetic and dietary factors were estimated by measuring the maximum activities of selected enzymes under optimized *in vitro* assay conditions. The selected enzymes are rate limiting for the metabolic pathways of interest. Their activities provide a quantitative indication of the maximum flux through a pathway *in vivo* (Newsholme and Paul 1983, Moon and Mommsen 1987). Changing activities reveal variations in the demand for specific metabolic functions at different developmental stages or under different environmental situations (Mommsen *et al.* 1980, Newsholme & Paul 1983, Kiessling *et al.* 1991). The main objectives of the present study were (a) to describe the ontogenesis of central metabolic pathways in *Clarias gariepinus* early life stages, (b) to learn in what aspects larval metabolism differs from that of older stages, (c) to reveal whether ontogenetic changes of metabolism coincide with other developmental events, (d) to analyze the influence of different dietary conditions on larval metabolism and its ontogeny. The major question in this context is whether larvae fed with different diets and, thus, showing different growth rates reach the same stage of enzyme development at a given body weight.

NADPH generation

G6PDH is the key enzyme regulating the carbon flux through the pentose shunt. ME catalyses the oxidative decarboxylation of malate to pyruvate. The activities of the two enzymes provide an estimate of the intensity of NADPH production for biosynthesis processes in the developing larvae. Metabolic processes which require NADPH include xenobiotic metabolism by mixed function oxidases, fatty acid desaturation and elongation and *de novo* synthesis of fatty acids (lipogenesis). These metabolic processes are already present in larvae. An inducible mixed function oxidase activity has been demonstrated for cod (*Gadus morhua*) larvae (Goksøyr and Solberg 1987, Norrgren *et al.* 1993) and a capacity for fatty acid desaturation and elongation has been shown for larvae of the African catfish, (Verreth *et al.* 1994a,b). This species is also able to perform *de novo* synthesis of fatty acids. The latter is evidenced by the low but unequivocally detectable levels (0.4 - 0.8 U/g protein, Segner and Verreth, unpublished) of the key enzyme for lipogenesis, acetyl CoA carboxylase (ACC), throughout the larval stage. Obviously, in catfish larvae a substantial capacity for NADPH generation exists from the onset of exogenous feeding onwards.

Therefore, the aforementioned metabolic processes should not be limited by an insufficient supply of reducing equivalents. In contrast to the present results with *C. gariepinus*, Munilla-Moran *et al.* (1989) reported the absence of detectable levels of G6PDH in turbot (*Scophthalmus maximus*) larvae. The authors concluded that the pentose shunt is inoperative during early life of this species. Up to 80% of the NADPH-producing capacity in larval turbot was found to based on the NADP-dependent isocitrate dehydrogenase (IDH) (Munilla-Moran *et al.* 1989), whereas only minor contributions came from ME and G6PDH. In catfish larvae, however, IDH is of less importance since the activity level observed for this enzyme (10 -30 U/g protein, Segner and Verreth, unpublished) is lower than the activity range observed for G6PDH and ME (20 - 40 U/g protein).

Apart from a reduction of NADPH-producing capacity, an inoperative pentose shunt affects the synthesis of RNA and DNA. The pentose phosphate pathway produces ribose units which are an essential component for nucleotide synthesis, a process of particular importance for fast growing larvae (for instance, the minimum daily increase of DNA between day 0 and day 10 in *Artemia*-fed catfish larvae is 6.1 µg DNA per fish, see also Table 1). To date, it is not clarified whether fish larvae can perform endogenous nucleotide synthesis or whether they have to rely on exogenous sources (Dabrowski and Kaushik 1982). The presence of a functional pentose shunt in catfish larvae proves that there exists at least the possibility for endogenous production of ribose units.

In adult fishes, levels of G6PDH and ME are strongly affected by diet quantity and quality. Starvation or restricted rations induce a pronounced decline of the activities of NADPH-generating enzymes (e.g. Walzem et al. 1991, Bastrop et al. 1992, Böhm et al. 1994). Also in catfish larvae, food deprivation evoked a reduction of G6PDH and ME activities. ME activity was significantly depressed also by the dry diet, but no consistent difference between dry and live feed was obvious for G6PDH. On the other hand, an ontogenetic effect on G6PDH activity was found. The substrate for G6PDH is glucose-6-phosphate. As a consequence, the developmental increase of G6PDH levels may be correlated with the ontogenetic enhancement of glycogen stores. However, the increase in G6PDH activity occurs earlier than the increase of glycogen depots. Further, G6PDH activities increase both with dry and live feed, whereas an elevation of glycogen stores is only realized in the *Artemia*-group. Thus, the ontogenetic effect on G6PDH activity in larval catfish can hardly be explained by differences in the substrate supply.

Energy metabolism

The capacity for energy metabolism in *C. gariepinus* larvae was assessed by measuring the activities of CS as an estimator of aerobic power (Forstner *et al.* 1983, Hinterleitner *et al.* 1987, 1989, Power and Walsh 1992) and by measuring the activities of PFK and PK as estimators of anaerobic power (Forstner *et al.* 1983, Hinterleitner *et al.* 1987, 1989, Kiessling *et al.* 1991). CS catalyses the initial reaction in the citrate cycle, PFK is the rate-limiting step which controls entrance of hexose substrates into glycolysis, and PK is the rate-limiting enzyme controlling the pyruvate output from glycolysis. Activity changes of the two glycolytic enzymes have not necessarily to be in parallel, as substrates such as glycerol can leave or enter the glycolytic pathway at the triose level in between the two control points (Dworkin and Dworkin-Rastl 1991, Segner *et al.* 1994b).

CS activities increased immediately after the start of exogenous feeding and commencement of active swimming. In *Artemia*-fed larvae, the enzyme reaches a more or less constant plateau level at a body weight of approximately 5 mg. In contrast to the pattern in CS, the glycolytic enzymes PFK and PK showed a delayed ontogenetic increase with PFK activity in *Artemia*-fed larvae reaching a plateau at approximately 10 mg body weight, and with PK reaching no plateau within the experimental period. The developmental changes in aerobic and anaerobic power of *Clarias gariepinus* larvae as found in the present study agree with observations on other teleost species such as *Coregonus* sp. (Forstner *et al.* 1983), *Scophthalmus maximus* (Segner *et al.* 1994a), and particularly *Rutilus rutilus* (Hinterleitner *et al.* 1987, 1989). The different time pattern of the activities of CS and of both glycolytic enzymes reflect a change from a pre-dominantly aerobic metabolism during the very early stage to a mixed aerobic/anaerobic metabolism during later stages of development. As shown by Forstner *et al.* (1983) for *Coregonus* sp. and by El-Fiky *et al.* (1987) for cyprinids, the shift in energy metabolism indicates an ontogenetic reorganization of the muscle tissues. In larvae, both outer (superficial red layer) and inner muscle (embryonic white) layers are considered to be aerobic, older life stage possess outer aerobic (red) and inner glycolytic (white) muscles. The differentiation process starts at the transition form the larva to the juvenile (Forstner *et al.* 1983, El-Fiky *et al.* 1987) and can continue up to 1 year of age (Kiessling *et al.* 1991). The parallel ontogenetic reorganization of muscle fibres and energy metabolism occurs also in *Clarias gariepinus*. Adult-type muscles start to develop in *Clarias gariepinus* only at a body length of about 11 mm (Akster *et al.* 1994). According to Verreth *et al.* (1992) this corresponds to 10 - 15 mg body weight or to a 3-day-feeding on *Artemia*. At the same size, PFK activity reaches its plateau level and PK activity starts to increase.

The development of PK activity in catfish is mainly size-dependent, but shows little responsiveness to dietary influence. On the other hand, PFK activity is strongly influenced by nutritional conditions. There are reports that PFK activity of fish varies with changes in food consumption or growth rate (Hinterleitner *et al.* 1987, Kiessling *et al.* 1991). Since dry diets result in diminished food consumption and reduced growth of larvae, this may well explain the lowered PFK levels observed in catfish larvae reared on dry food.

Glyconeogenesis

The glyconeogenetic capacity of *C. gariepinus* larvae was estimated by measuring FDPase, the enzyme catalyzing the conversion of fructose-1,6-bisphosphate in fructose-6-phosphate. This enzyme is the rate-limiting control point at the end of the glyconeogenetic pathway. In early feeding larvae, FDPase activity was very low but it started to increase at a body weight of approximately 20 mg. The development of the glyconeogenetic pathway took place in relation to the ontogeny of glycolytic power in muscle tissue of catfish early life stages. This coordinate development enables the larval organism to re-convert the product of anaerobic glycolysis - lactate - into glucose *via* glyconeogenesis. The dietary effect on the ontogenetic pattern of FDPase activity can be ascribed to diet-induced differences in the status of carbohydrate metabolism. Catfish larvae receiving the dry diet experienced continuously low glycogen concentrations, together with a reduced or delayed increase of glucose-catabolizing PFK activity. As a consequence, in these larvae there was no need to develop the ability for glucose regeneration by glyconeogenesis that early.

Amino acid catabolism

In fish, from a quantitative point of view, GOT and GPT are the most important transamination enzymes (Cowey and Walton 1989, Kim et al. 1992). Both enzymes transfer the amino group of respectively aspartate (although not exclusively specific for this amino acid) (GOT) and alanine (GPT) to an alpha-keto acid resulting in the formation of another keto-acid and an amino acid. The resulting keto-acid is used as an energy source and is oxidized to CO_2 and H_2O *via* the citrate cycle, or it is used for synthesis processes such as glyconeogenesis. The resulting amino acid is deaminated either by the transdeamination pathway or by the purine nucleotide cycle to form ammonia (Cowey and Walton 1989). In the liver of teleosts, GOT and GPT levels are usually within the same order of magnitude, whereas in the muscle tissues of fish their activities differ greatly (Jürss and Nicolai 1976, Mommsen *et al.* 1980). In the present study where whole body homogenates of *C. gariepinus* larvae were used, a marked difference between the activities of GOT and GPT was observed, confirming that in larval whole body homogenates we mainly measure muscle enzymes.

Amino acids are considered as an important fuel for teleost larvae (Dabrowski 1986, 1989, Fyhn 1989, Rønnestadt *et al.* 1992). This may explain the high activities of GOT and GPT in catfish larvae right from the onset of exogenous feeding onwards. The findings of Dabrowski (1986, 1989) on the ever decreasing role of amino acid catabolism for energy generation during fish ontogeny agrees with the reduction of GOT and GPT activities in larvae of 20 mg body weight an more.

The diet exerted a pronounced influence on the transaminase activities of the catfish larvae, indicating differences in the intensity of amino acid catabolism. The reason why this effect is specifically found during the very early feeding period may be a lower amino acid availability from the dry feed. Verreth et al. (1992, 1993) hypothesized that the absence of a functional stomach during the early developmental stages of *Clarias gariepinus* may affect the digestibility of dry diets. As a matter of fact, the diet-induced difference in GOT and GPT activities found in larvae fed on live or on dry feed disappears at a body weight of approximately 20 mg, i.e. the stage when the stomach becomes functional (Verreth et al. 1992). On the other hand, onset of stomach functions does not corroborate with increased activities of GOT and GPT in the dry food group but in contrast to this, in reduced activities in the live food group. This suggests that also other factors than amino acid availability are involved in the dietary effect on GOT and GPT activities. An alternative explanation may be found in the locomotor activity of the larvae. Because amino acids are a major energy substrate in fish larvae, an increased swimming activity results in increased ammonia excretion (Dabrowski 1986). Catfish larvae receiving live feed show a high swimming activity in search and capture of food and, therefore, have to enhance the rates of amino acid oxidation, i.e. expressing elevated activities of GOT, GPT as well as CS. As soon as the larval swimming metabolism shifts from a mainly aerobic type to a mixed aerobic/anaerobic one, other energy sources than amino acids (e.g., glycogen and glucose) gain importance and the diet-related effects on GOT, GPT and CS disappear.

In the present study on larval *C. gariepinus*, diet effects were not only found in the protein catabolism but also in the protein anabolism. The general decline in RNA/DNA- as well as RNA/protein ratios with dry feed may be an indication for lower protein synthesis rates (Foster *et al.* 1993, Houlihan *et al.* 1993). Contrary to the results related to protein catabolism, the diet-related difference in protein anabolism continues over the complete experimental period and does not disappear beyond 20 mg of body mass ! The metabolism of juvenile fishes is able to adapt to food deprivation (Mendez and Wieser 1993). In juvenile roach, *Rutilus rutilus*, the adaptive phase is characterized by stability of most of the enzymes

of aerobic catabolism and an elevated amino acid catabolism as indicated by increased activity of GPT (Mendez and Wieser 1993). In catfish larvae, starvation induced an increase in GPT, GOT and CS levels what points to a surprisingly well developed adaptive capacity of the larval life stage.

Effect of body size

Verreth et al. (1992, 1993) reported that in larval *C. gariepinus*, the stomach differentiates at a body weight of approximately 20 mg, corresponding to an *Artemia* feeding period of 3 to 5 days at a temperature of 27.5 °C. Also in the present study, several changes in the larval metabolism were found around a body weight of 20 mg. PFK reached a plateau level, the activity of PK and FDPase started to increase, glycogen concentrations in the body raised and early peak levels of GOT and GPT activity decreased again. Also other ontogenetic changes take place at a body weight of 20 mg, e.g., differentiation of the muscles into adult-like fibre types (Akster *et al.* 1994) or development of secondary lamellae on the gills (Segner and Verreth, unpublished observations), indicating the shift from cutaneous to branchial respiration. Apparently a size of 20 mg body weight constitutes a "turning point" in the ontogeny of *Clarias gariepinus*. Although certain functions such as air-breathing develop only later, from a viewpoint of nutritional physiology, the larval period ends with the described morphological and physiological changes. This conclusion is confirmed by the results of weaning studies which show that from that developmental stage onward catfish larvae can be reared equally well with dry as with live feeds, i.e. they do not need any longer a specific larval diet (Verreth and van Tongeren 1989; Verreth *et al.* 1993).

Conclusions

Our questions for the present study were : (a) to describe the ontogenesis of central metabolic pathways in *Clarias gariepinus* early life stages, (b) to learn in what aspects larval metabolism differs from that of older stages, (c) to reveal whether ontogenetic changes of metabolism coincide with other developmental events, (d) to analyze the influence of different dietary conditions on larval metabolism and its ontogeny.

The investigated enzymes of the central intermediary metabolism were all present from the start of exogenous feeding onwards. The difference between various developmental stages is not qualitative, e.g., the absence or presence of a pathway, but is of quantitative

nature. Important ontogenetic changes were found in glycolysis, glyconeogenesis, glycogen storage, and amino acid catabolism. The turning point for metabolic changes occurs at a body weight of approximately 20 mg, or after 3-5 days of *Artemia* feeding. The changes in the metabolism coincide with changes of muscle organization, gill morphology, respiration and stomach structure and function. The combined change of various morphological and physiological parameters indicates that the larval period of *Clarias gariepinus* ends at a body weight of approximately 20 mg. Dietary conditions are able to alter the developmental patterns. Catfish larvae reared on dry instead of live feed delayed the development of glycolytic power and glyconeogenesis beyond the "turning of 20 mg body weight.

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ABBREVIATIONS:

CS: citrate synthase
FDPase: Fructose-1,6-diphosphatase
GOT: glutamate oxaloacetate transaminase
GPT: glutamate pyruvate transaminase
G6PDH: glucose-6-phosphate dehydrogenase
ME: malic enzyme
PFK: phosphofructokinase
PK: pyruvate kinase

Part 3

LIPID METABOLISM IN EARLY LIFE STAGES OF *CLARIAS GARIEPINUS*

CHAPTER 6

The metabolism of neutral and polar lipids in eleuthero-embryos and starving larvae of the African catfish *Clarias gariepinus* (Burchell).

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Abstract

Lipid class analysis was carried out on developing eggs, eleuthero-embryos (yolk sac larvae) and starving larvae of the freshwater species *Clarias gariepinus* (Burchell), using thin layer chromatography. Samples were taken at fixed intervals from a large pool of fertilized eggs obtained through induced reproduction of several parent fish. The total lipid content of fertilized eggs fluctuated around 22% of the dry weight and decreased from 21% at hatching to about 12.5% at yolk absorption. In starving larvae, the amount of total lipid per individual remained relatively constant. Polar lipids (phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) together accounted for 73.6 to 80% of total lipid. PC was by far the most abundant lipid class during the entire experimental period (70-75% of total lipid). PC was catabolized proportionally to total lipid, demonstrating its role as the main energy supplier. All yolk PE was converted to body tissue. The neutral lipids consisted of triglycerides (TAG), cholesterol and cholesteryl esters (respectively 12.5, 10 and 3% of total lipid in newly fertilized eggs). All TAG were depleted before complete yolk absorption.

Introduction

In most fish species, yolk material provides the substrates for energy and growth during the egg and eleuthero-embryonic (yolk sac) stage. The general model assumes that yolk lipid is the main energy substrate whereas yolk protein provides amino acids for tissue synthesis (Heming and Buddington 1988). In marine fish eggs energy is partly derived from the free amino acid pool (Fyhn 1993, Rønnestad *et al.* 1992) while phospholipids fulfil other essential functions for growth and survival (Kanazawa *et al.* 1985; Olsen *et al.* 1991; Sargent *et al.* 1993). In freshwater fish species, the free amino acid pool in the eggs is considerably lower and during the early life stages energy is mainly fuelled by combustion of lipid (Kamler 1976; Polat *et al.* 1994).

The lipid composition of eggs and the lipid metabolism in early life stages of fish differ strongly among species (Kaitaranta and Ackman 1981). These differences include the amount and composition of lipid in the yolk, the time and level of lipid combustion, the classes of lipid used for either combustion or tissue synthesis and the role of different fatty acids. In freshwater fish, the lipid content of fish eggs vary between 2.5 and 10% of the wet weight (Henderson and Tocher 1987). Higher lipid contents in eggs are associated with high percentages of neutral lipid, whereas high concentrations of polar lipid (60-86% of the total lipid) occur mainly in eggs with lower total lipid content (up to 5% of the wet weight) (Henderson and Tocher 1987; Sargent *et al.* 1989). In species with a relative high phospholipid concentration, phosphatidylcholine (PC) was the major lipid class, ranging from 63-83% of the total phospholipid (Tocher and Sargent 1984). The importance of this lipid class as a dietary constituent for larval survival and growth has been confirmed in marine fish species (Kanazawa *et al.* 1985; Teshima *et al.* 1987; Olsen *et al.* 1991; Kanazawa 1993). Neutral lipids, especially triacylglycerols (TAG) and wax esters are assumed to be the main energy sources in eggs of many fish species (Anderson *et al.* 1990).

Most of the information cited above is derived from studies with marine fish species. Data on the lipid dynamics in early life stages of freshwater species are scarce. In the present study the lipid class composition during embryonic and early larval development of the freshwater catfish *Clarias gariepinus* (Burchell) has been investigated.

Material and methods

Facilities and fish

Selected male and female breeders (individual wet weights varying between 0.9 and 2 kg) were put individually in 70 L aquaria (25 °C) for induced reproduction. Eggs were obtained by hypophysation of 5 females plus 2 males according to standard procedures (Hogendoorn and Vismans 1980). Eggs from the five females were pooled, fertilized and incubated at 27.6 °C (± 0.3 °C) in sixteen aquaria (V=16 L). After complete yolk absorption, larvae were kept without feeding until 50% mortality, when the experiment stopped.

Sampling

Samples were taken at fixed intervals. To enable comparison with other studies done at slightly different temperatures, time was expressed as physiological day-degrees (PD°),

according to the equation:

$$PD° = \frac{Temp*(hours/24)}{q} \quad (1)$$

where : PD° = physiological day degrees
Temp = temperature (°C)
q = Winberg coefficient to rate physiological processes at 20 °C (Winberg 1956; Huisman 1974). For 27.6 °C, q=0.554.

The first sample was taken immediately after fertilization (PD° = 0). Subsequently 3 more samples were taken during the egg stage at 12.8, 24.4 and 42.7 PD° after fertilization. Hatching occurred around 50 PD°. Yolk sac larvae were sampled at 55.9, 75.0, 100.0, 124.7 and 151.0 PD°. In the design of the present study, the first sampling point where the yolk sac could not be separated from the embryonic body (176.3 PD°) was considered to belong to the starvation period. The starving larvae were sampled at 176.3, 204.7, 259.8, 315.0 and 358.9 PD°.

During the yolk sac phase, samples were taken both from entire animals and embryonic bodies without yolk. Yolk was eliminated by puncturing the yolk sac and vortexing the punctured animals in a soap solution ($3x10^{-3}$ ml/ml) twice during 20 seconds, followed by three times rinsing in distilled water. During sampling activities, samples were kept on ice. For lipid analysis, bulk samples of about 350-450 mg estimated dry weight were taken at each sampling point and stored in the freezer at -18 °C for further processing.

Analyses

Wet and dry weight were determined on three separate samples of about 100 animals each. Wet weight of embryonic bodies was not measured because of practical problems. Wet weight was measured by sieving, blotting and weighing a group of fish and counting the number of fish afterwards. Dry weight was determined on the same samples by drying during 4 hours at 70 °C, followed by 4 hours at 105 °C.

At the end of the experiment, the bulk samples for lipid analysis were freeze dried, pulverized in a mortar and stored in screw cap flasks at -18 °C. When needed for extraction of lipid, the samples were exposed to air to obtain air stable dry weights. From this material, three subsamples of 50 mg each were weighed and dried in the oven to obtain the dry matter content in the freeze dried material. Lipid extraction was performed according to the procedure of Bligh and Dyer (1959) in five subsamples of 40 mg (air stable dry weight) per

sampling point. Total lipid was determined gravimetrically after evaporating the chloroform in a rotary evaporator (Heidolph VV2000) at 30 °C and raising the vacuum by nitrogen flushing. The lipid was subsequently redissolved in three times 0.5 ml (total volume = 1.5 ml) chloroform, transferred into 4 ml screw cap vials and stored at -18 °C.

To separate lipid classes, a unidirectional TLC with two solvent mixtures was used on 20x20 cm glass plates coated with 0.25 mm silica gel 60. These plates with a concentration zone and without a fluorescent indicator were obtained from Merck. All plates were prewashed in chloroform/methanol (1:1, v/v) and activated at 100 °C for 30 min. To separate polar lipids, the plates were developed first in a chloroform/ methanol/water (65:27:4, v/v/v) mixture, followed by a second development in an n-hexane/diethyl ether/acetic acid (80:20:1, v/v/v) mixture to separate neutral lipids. Samples were applied with a 5 µl Brand transferpettor at 1 cm of the lower edge of the plate. On each plate, three times 5 µl of three samples (n=9) were applied. Depending upon the total lipid content in each sample, the lipid mass applied in this way varied roughly from 15 to 30 µg per stroke. After separation, the plates were air dried during 5 minutes, dipped in 10% (w/v) $CuSO_4$ in 8% (v/v) H_3PO_4, and charred at 160 °C during 10 minutes.

The lipid classes were quantified by scanning in a Laser densitometer (LKB 222-010 Ultroscan XL) (5 scans per subsample of 5 µl) and averaged. Peak areas were integrated with the Gel Scan XL™ 2.0 Programme. Peaks were identified and area% converted into relative weights by comparing with parallel TLC separation of known amounts of standard lipids. L-α-phosphatidyl-L-serine, L-α-phosphatidylinositol, L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, sphingomyelin, cholesteryl stearate and triolein were obtained from Sigma Co. Cholesterol was obtained from Boehringer Mannheim, GmbH.

Data analysis

All data were analyzed using the LOTUS 123 (rel. 3.1) spreadsheet programme. To detect preferential usage of individual lipid classes, changes in µg of each lipid class were compared with changes in total lipid amounts. Relative rates of use were determined by expressing the amounts of each lipid class as a fraction of its initial amount at PD° = 0. Conversion efficiencies were calculated according to the formula:

$$CE = \frac{(B_{151pD} - B_{55.9pD})}{(Y_{55.9pD} - Y_{151pD})} * 100\% \qquad (2)$$

Where : CE = conversion efficiency (%)
B = weight of lipid class in body (mg/ind)
Y = weight of lipid class in yolk (mg/ind)

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Results

Wet and dry weight

The wet weight of whole organisms increased slightly during the first 13 PD° from 1.47 to 1.54 mg/ind. (Table 1). It decreased again between 42.7 and 55.9 PD°, when hatching occurred. The maximum alevin weight was attained at 204.7 PD° (2.7 mg), i.e. shortly after complete yolk absorption. During the egg stage, dry weight remained stable and varied around 0.35 mg/ind. The dry weight decreased continuously from 0.35 mg/ind at hatching (PD°= 55.9) to 0.17 mg/ind at the end of the experimental period. Linear regression of the dry weight values against time yielded an average rate of dry weight decline of 0.55 μg/ind/PD°. From the data of Table 1, changes in yolk amount and composition were calculated. The yolk dry weight is declining from 0.3 mg at 55.9 PD° to 0.1 mg at 151 PD° (Figure 1), i.e. a yolk absorption rate of 2.1 μg/ind/PD° (R^2 = 0.99). According to this average yolk absorption rate, complete yolk absorption would have occurred at 199.8 PD°. The conversion efficiency of yolk dry weight to body tissue, calculated according to equation 2, was 60.5%.

Total Lipid

Total lipid in eggs, whole organisms and embryonic bodies are presented in Table 1 and Figure 2. During the egg stage the amount of lipid fluctuated around 21% or 75 μg/ind. During the yolk sac period, a strong decline of the total lipid was observed, especially between 100 and 204.7 PD°. In starving larvae, the amount of total lipid remained constant. Apparently, due to selective utilization of non-lipid compounds, the concentration of total lipid in the dry weight increased at the same time (Table 1). The conversion efficiency of total lipid from yolk to body tissue was 48.5%.

Lipid classes

Table 2 shows the lipid class composition at the different sampling points. Polar lipids, consisting of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), accounted for 75 to 80% of total lipid. The concentration of PC in total lipids remained rather constant during the whole experimental period and fluctuated between 72 and 76% of total lipid. PC was mainly consumed during the embryonic (egg plus yolk sac) period and decreased from 47 μg/ind at 42.7 PD° to 28 μg/ind at 176.3 PD°. The concentration of PE in the total lipid increased slightly during the starvation period, whereas there was a relative decrease of other lipids. In absolute amounts, PE was mainly used during the egg stage (from 2.3 μg/ind to

Table 1. **Wet weight, dry weight and total lipid content in eggs, yolk sac larvae and starving larvae of the African catfish, *Clarias gariepinus*, raised at 27.6 °C. PD° = physiological day degrees; Data between brackets are standard deviations. Standard deviations smaller than 0.005 are set as zero (-).**

Stage	Time (PD°)	Wet weight (mg/ind)	Dry weight (mg/ind)	Total Lipid (%DW)	
				mean (s.d)	n
Egg	0.0	1.47 (0.02)	0.35 (-)	21.87 (1.98)	4
	12.8	1.54 (0.03)	0.36 (0.01)	22.48 (2.55)	5
	24.5	1.54 (0.01)	0.35 (-)	21.92 (1.28)	5
	42.7	1.55 (0.02)	0.35 (-)	19.30 (1.71)	5
Yolk sac	55.9	1.25 (0.08)	0.37 (0.03)	20.92 (1.31)	5
	75.1	1.33 (0.02)	0.34 (0.01)	21.06 (1.70)	5
	100.0	1.55 (0.02)	0.32 (-)	21.90 (1.67)	5
	124.7	1.87 (0.03)	0.31 (-)	20.11 (1.07)	5
	151.0	2.22 (0.03)	0.29 (0.01)	18.69 (2.58)	5
Starvation	176.3	2.56 (0.04)	0.27 (-)	14.58 (1.67)	5
	204.7	2.71 (0.03)	0.26 (-)	12.35 (0.69)	5
	259.3	2.49 (0.01)	0.23 (0.01)	11.78 (1.16)	5
	315.0	2.44 (0.04)	0.20 (-)	15.28 (1.75)	5
	358.9	2.33 (0.04)	0.17 (-)	15.58 (2.60)	5
			Embryonic bodies		
Yolk sac	55.9		0.063 (0.006)	18.74 (1.06)	5
	75.1		0.065 (0.006)	19.00 (1.09)	5
	100.0		0.096 (0.015)	18.34 (1.46)	5
	124.7		0.158 (0.010)	16.80 (0.44)	4
	151.0		0.183 (0.013)	18.19 (1.83)	5

1.9 µg/ind between 0 and 42.7 PD°), and thereafter, the amount of PE remained rather constant. The amount of PE in embryonic bodies increased strongly form 0.38 µg/ind at 55.9 PD° to 1.62 µg/ind at 151 PD°. The neutral lipids consisted of triacylglycerols (TAG), cholesterol (FC) and cholesteryl-esters (CE) (Table 2). At fertilization, egg total lipid consisted of about 10% cholesterol, 13% TAG and 3% CE. All triacylglycerols (TAG) were used during the yolk sac absorption. During the yolk sac period, CE levels increased from 1.96 µg/ind at 42.7 PD° to 4.18 µg/ind at 151 PD°, whereafter they decrease again to 1.25 µg/ind at 204.7 PD°. The amount of cholesterol fluctuated between fertilization and the last yolk sac sample (176.3 PD°) from about 7.5 to about 6 µg/ind. In the period around complete yolk absorption (between 176.3 and 204.7 PD°), FC decreased from 6.9 to 4.7 µg/ind. During the larval starvation period, it remained rather constant.

Total lipid had a lower conversion efficiency from yolk to body tissue than dry weight (Figure 4), indicating that it was preferentially used for combustion compared to other compounds in the dry matter. The conversion efficiency of TAG was -9%, indicating a

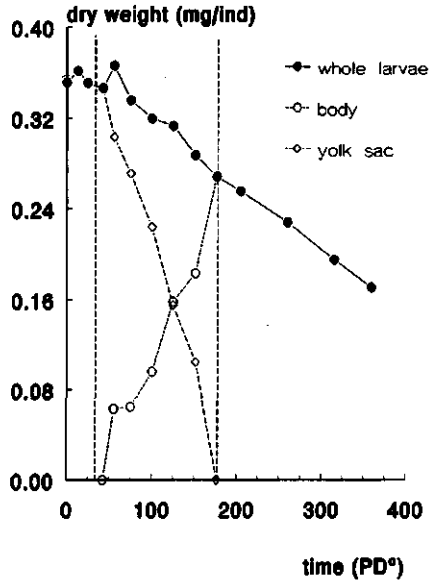

Figure 1 The changes in individual dry weight of the whole organism, of the yolk sac and of the embryonic body during the early life stages of the African catfish *Clarias gariepinus*.

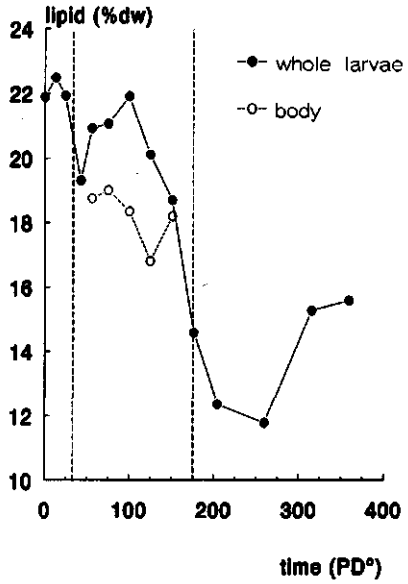

Figure 2 The total lipid content (as a % of the dry weight) in whole organisms and in embryonic bodies during the early life stages of the African catfish *Clarias gariepinus*.

complete catabolization of yolk TAG. All yolk PE was converted to body tissue (conversion efficiency was 104.3%). The conversion efficiency of CE was 152%, indicating CE synthesis during the yolk sac period.

Discussion

Although in the present study, eggs and larvae of several parent fish and many individuals for each sample were used, the standard deviations found reflect rather the analytical quality than the biological variation in the species *Clarias gariepinus*. At each sampling point, one bulk sample was taken for lipid analysis which was divided into different subsamples after freeze drying. Further, all material was obtained through one reproduction trial of several breeders and as such, the data reflect the nutritional and gonadal status of those breeders at that time. In *Clarias gariepinus*, the size of the eggs may differ from batch to batch. Under Dutch hatchery conditions egg size varied from 0.3 to 0.4 mg/ind

Table 2. **The lipid class composition expressed as a % of total lipids, in developing eggs, yolk sac larvae and starving larvae of the African catfish, *Clarias gariepinus*. PD° = physiological day degrees, s.d. = standard deviation; n.d. = not detectable.**

Stage	Time (PD°)	PC (%)	PE(%)	FC (%_)	TAG (%)	CE (%)
Egg	0.0	71.63 (1.95)	3.00 (0.24)	9.77 (0.78)	12.70 (1.51)	2.91 (0.46)
	12.8	74.32 (0.52)	2.59 (0.06)	7.88 (0.14)	12.57 (0.34)	2.63 (0.20)
	24.5	71.54 (0.20)	2.71 (0.13)	9.43 (0.24)	13.39 (0.32)	2.93 (0.18)
	42.7	71.71 (0.49)	2.86 (0.13)	6.83 (0.06)	15.57 (0.30)	3.01 (0.13)
Yolk sac	55.9	70.99 (1.07)	2.49 (0.03)	9.77 (0.21)	12.23 (0.14)	4.52 (0.95)
	75.1	72.32 (0.14)	2.50 (0.16)	9.67 (0.09)	11.36 (0.18)	4.16 (0.35)
	100.0	72.70 (0.34)	3.24 (0.07)	8.84 (0.03)	10.06 (0.20)	5.16 (0.11)
	124.7	72.68 (0.23)	2.93 (0.02)	8.98 (0.09)	9.70 (0.21)	5.70 (0.17)
	151.0	72.40 (0.34)	3.62 (-)	12.81 (0.24)	3.39 (0.45)	7.77 (0.21)
Starvation	176.3	72.32 (0.66)	4.77 (0.26)	17.66 (0.18)	n.d	5.24 (0.22)
	204.7	75.78 (0.20)	5.29 (0.06)	14.97 (0.09)		3.95 (0.26)
	259.8	73.65 (1.03)	5.50 (0.06)	17.63 (1.26)		3.22 (0.19)
	315.0	73.24 (1.13)	5.97 (0.24)	18.18 (0.67)		2.61 (0.26)
	358.9	74.22 (0.58)	5.91 (0.08)	17.85 (0.50)		2.02 (0.06)
				Embryonic bodies		
Yolk sac	55.9	76.01 (0.67)	3.19 (0.10)	10.33 (0.24)	6.71 (0.43)	3.76 (0.08)
	75.1	78.22 (0.38)	3.90 (0.07)	9.54 (0.12)	3.12 (0.03)	5.22 (0.37)
	100.0	75.41 (0.34)	4.44 (0.04)	12.65 (0.31)	1.18 (0.06)	6.33 (0.03)
	124.7	76.29 (1.36)	4.56 (0.21)	10.55 (0.60)	1.19 (0.08)	7.41 (0.50)
	151.0	74.52 (0.35)	4.85 (0.08)	12.52 (0.17)	0.48 (0.08)	7.62 (0.30)

dry weight (Polat *et al.* 1994, Verreth, unpublished data). However, when compared with natural populations, the variability in egg quality and quantity due to differences in reproductive cycle, temperature and feeding conditions are strongly reduced in the hatchery conditions applied in Wageningen (Richter *et al.* 1987). Therefore, the present data represent the lipid class mobilization which occurs typically during the early life stages of *C. gariepinus*. To obtain information on the effect of broodstock nutrition, environmental factors, reproductive cycle etc., specific studies should be planned and designed.

Hatching occurred at approximately 50 PD° and complete yolk absorption was estimated at approximately 200 PD°. This is in accordance with earlier data (Polat *et al.* 1994, Conceição *et al.* 1993). However, yolk conversion efficiencies were calculated on the basis of measured data of both yolk and embryonic tissue and therefore, in the present study data were treated as if yolk was absorbed between 151 and 176 PD°. Maximum wet weight was

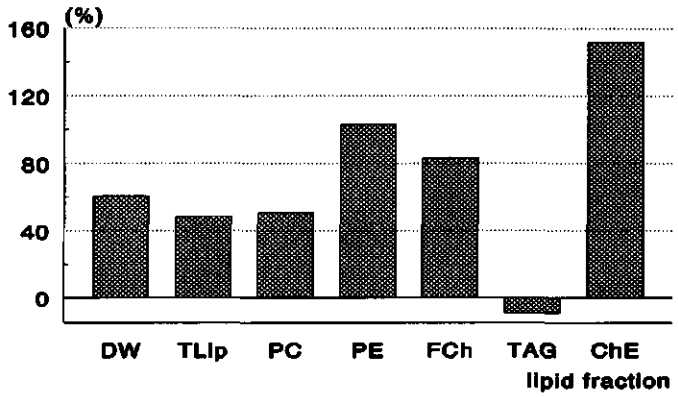

Figure 3 Conversion efficiencies of dry weight, total lipid and different lipid classes from yolk to body tissue during the eleuthero-embryonic period of the African catfish *Clarias gariepinus*. DW=dry matter; TL=total lipid; PC=phosphatidylcholine; PE=phosphatidylethanolamine; FC=cholesterol; TAG=triacylglycerol; CE=cholesteryl-ester.

obtained at the start of the starvation period (204.7 PD°). According to the simulation model of early larval growth in *C. gariepinus* (Conceição *et al.* 1993), and according to data from many other species (Heming and Buddington 1988) maximum wet weight should be attained before complete yolk absorption. This may be explained by differences in the experimental design of the present study (see above) and the theoretical assumptions underlying the mentioned model. In the present study, a linear yolk absorption curve was fitted to the experimental data, whereas both Heming and Buddington (1988) and Conceição *et al.* (1993) assumed a sigmoid curve. As a result, in the present study the estimated time of complete yolk absorption may have been advanced slightly.

The polar lipid content of the eggs is consistent with Henderson and Tocher (1987) who stated that high polar lipid concentrations (up to 60-86% of the total lipid) in eggs may be associated with lower lipid contents (2.5-5% of the wet weight). In the present study, the total lipid of the eggs was 5.2% of the wet weight. The PC concentration in the total lipid remained rather constant during the whole experimental period, indicating that PC is used proportionally to total lipid. Apparently, PC is not preferentially used, yet the absolute amounts of PC decline with about 60%. Being the most abundant lipid class, it suggests that in *C. gariepinus* PC is an important energy source. This coincides with similar conclusions for Atlantic salmon (Cowey *et al.* 1985), Atlantic halibut (Falk-Petersen *et al.* 1986, 1989)

and Atlantic herring (Tocher *et al.* 1985). In cod, highly unsaturated fatty acids (HUFA) such as 22:6n-3 (DHA) which originated from depleted PC were incorporated into the body TAG (Fraser *et al.* 1988). Taking into account that in the present study TAG was completely catabolized, a similar function of PC as a HUFA source is questionable. In a similar study on Atlantic herring, a concurrent strong decrease of polar and total lipids was found between fertilization and yolk absorption (Tocher *et al.* 1985). The authors suggested a metabolic role for PC to explain the high concentrations of this lipid class in herring eggs, e.g. as a supplier of inorganic phosphate for the intermediary metabolism and/or of choline for neurotransmission. The functions for which PC is used in the early life stages of *C. gariepinus* are not known.

In the yolk sac stage, all yolk PE is converted to body tissue. PE constitutes the most abundant lipid class in neural tissues of rainbow trout and cod (Tocher and Harvie 1988), and contained high concentrations of DHA which is known to be essential for the development of brain and vision in larval fish. The same functions may explain why in *C. gariepinus* eleuthero-embryos, the relative low amounts of PE are strongly conserved during development. Because of the concurrent strong decline in PC amounts, the ratio PC:PE declined from 23.9 at PD°=0 to 12.6 at 358.9 PD°. Such a decrease in the PC:PE ratio was also found in Atlantic salmon (Cowey *et al.* 1985), Atlantic halibut (Falk-Petersen *et al.* 1989) and cod (Fraser *et al.* 1989) and reflect the high initial requirements for PC.

TAG is used as an energy source and was totally depleted during the yolk sac period. This differs from data from marine species where TAG combustion occurs often after yolk absorption (Fraser *et al.* 1988; 1989). During the yolk sac stage, CE was initially synthesized, but decreased again when TAG was almost depleted. This suggests a role of CE as energy reserve. However, catabolization of CE may result in the liberation of free fatty acids and cholesterol which are used for other functions than energy fuel. Cholesterol may function as a precursor for bile salts. In *C. gariepinus*, lipid absorption occurs immediately after the first food is given (Verreth *et al.* 1992), hence it is reasonable to assume that emulsifying agents such as bile salts are produced shortly before first feeding starts, e.g. at the end of the yolk sac period. The sudden decrease of FC between 176.3 and 204.7 PD° and the concomitant decrease of CE support this hypothesis.

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CHAPTER 7

The Dynamics of Fatty Acids in developing Eggs, Yolk sac larvae and Starving larvae of the African catfish *Clarias gariepinus* (Burchell).

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Abstract

Eggs, yolk sac larvae and starving larvae of the African catfish *Clarias gariepinus* (Burchell) were sampled at different moments during their development and analyzed for their wet weight, dry weight, total lipid content and fatty acid content. The total lipid content in the eggs was about 6% of the wet weight. During the yolk sac period total lipid content decreased from about 18% of the dry weight at hatching to about 13% of the dry weight at complete yolk absorption. The most abundant fatty acids in the eggs were 16:0, 22:6n-3, 18:1n-9, 18:0, 20:5n-3 and 18:2n-6 (respectively 28.5, 19.4, 16.6, 10.5, 6.1 and 5.4% of the total fatty acid composition). Most yolk fatty acids were absorbed at 0.5% of the initial amount present in the egg per physiological day degree (PD°). The amounts of fatty acids (µg) per individual decreased over the experimental period. A significant positive correlation existed between initial amount of a fatty acid in the egg and the rate of depletion. Statistical analysis did not reveal significant differences between the relative depletion rate of the different fatty acids and of the dry weight of the organism, suggesting that there was no strong preference for individual fatty acids to be combusted or to be "saved" in the body tissues. Depletion rates differed however strongly between developmental stages. During the embryonal period (egg plus yolk sac), some fatty acids (especially 20:4n-6, 20:5n-3 and 22:6n-3) were synthesized. The transfer efficiency of most other fatty acids from yolk to body tissue was lower than 60%.

Key-words: Clarias gariepinus, fatty acids, eggs, larvae, catfish, metabolism

Introduction

Most commercial fish species require live food organisms during the initial days of exogenous feeding, limiting thereby a reliable production of fingerlings for the fattening farms. Development of alternative, e.g. artificial diets requires a detailed knowledge of the nutritional requirements in the early life stages of the fish. Live food organisms are relative inflexible to changes in their biochemical composition and therefore not well suited to study these nutritional requirements. On the other hand, the dry larval diets available to date result mostly in relative low growth rates, and are therefore not preferred. The nutrient dynamics

in egg and yolk sac stages can provide a model to overcome these problems. As these stages constitute a closed system in which all nutrients needed by the growing larva must be derived from the egg yolk, the nutrient dynamics during this period can give information on the nutritional requirements at the start of exogenous feeding.

During the embryonic stages of fish, both protein and lipid are major energy fuels (Heming and Buddington 1988, Fyhn 1993, Polat *et al.* 1994, Sargent 1994, Verreth *et al.* 1994c). However, most information on the protein and lipid metabolism in fish is derived from studies on juvenile and adult specimens. Based on these studies, it is generally assumed that lipid is the preferred source of energy for most fish species, and dietary requirements for essential fatty acids are concomitantly rather low (0.5-1% of the diet) (Castell *et al.* 1972, Sargent *et al.* 1989, Koven *et al* 1992, Mourente and Tocher 1993). Fish need (n-3) polyunsaturated fatty acids (PUFA) and probably also some (n-6)PUFA, e.g. arachidonic acid (20:4n-6) (Sargent *et al.* 1989, Sargent 1994). In marine fish the C20 and C22 homologues of (n-3)PUFA, e.g. eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3) are considered to be essential, whereas freshwater fish are capable to synthesize these important highly unsaturated fatty acids (HUFA) from their C18 precursors, e.g. linoleic acid (18:2n-6) and linolenic acid (18:3n-3) (Henderson and Tocher 1987, Sargent *et al.* 1989). (n-3)PUFA are very important for brain and retina development in fish (Tocher and Harvie 1988, Sargent *et al.* 1993a, Sargent *et al.* 1993b, Sargent 1994). In growing embryos and larvae of fish, neural tissues and organs, especially the eyes, constitute a relative large proportion of the body mass (10-20%) (Sargent *et al.* 1993a). Therefore, in early life stages of fish the requirements for (n-3)PUFA may be relatively high.

Sargent (1994) hypothesized that the different requirements for (n-3)HUFA between marine and freshwater fish would be caused by differences in feeding habits rather than by differences in water salinity. Most studies on marine species concerned predatory and slow growing fish and demonstrated the need for elevated dietary concentrations of 22:6(n-3). Also their natural food contains high levels of this fatty acid. In slow growing fish however, the nutrient requirements per unit body mass are relatively low. Sargent (1994) suggested therefore that in this situation the abundance of DHA in the natural diet makes the development of biosynthesis capacities unnecessary. The African catfish, *Clarias gariepinus* is a typical example of fast growing tropical freshwater fish with an omnivorous predatory feeding habit (Bruton 1979). According to the hypothesis of Sargent (1994), it can be expected that this species is capable of elongation and desaturation of C18 precursor fatty

acids. Verreth *et al.* (1994a) indeed found evidence for biosynthesis of (n-6) and (n-3)HUFA in larvae of this species, but the results varied according to age and growth rate of the larvae. They hypothesized that the enzymes needed for conversion of C18 precursor fatty acids into their C20 and C22 homologues are especially active in a later stage of the larval period. This means that in African catfish larvae, the fatty acid requirements would change throughout the larval period. Under such circumstances it is very difficult to assess the fatty acid requirements by conventional feeding experiments. Therefore, in the present study the fatty acid dynamics were investigated in eggs and yolk sac larvae. The conversion of fatty acids from yolk to body tissue was investigated in an attempt to elucidate fatty acid requirements during the first period of exogenous feeding of *Clarias gariepinus* larvae.

Material and methods

Facilities and Fish

From a broodstock population of 100 one year old males and females which were raised and maintained under standard conditions (Richter *et al.* 1994), four females and two males were selected for induced reproduction. The female breeders had a size of respectively 1.6, 1.2, 1.1 and 0.8 kg. Maturation and ovulation of eggs was induced by injection of a carp pituitary suspension (4 mg/kg body weight), eggs were stripped and fertilized with the sperm of the two males according to standard procedures described by Hogendoorn and Vismans (1980). Eggs were incubated in sixteen aquaria (V=16 L; bottom surface = 30x40 cm) at a density of 10 g/aquarium (i.e. about 6000 eggs) and a water flow of 1 L/min/aquarium. Upon hatching, free moving larvae were automatically flushed by the water current into a collector aquarium (V = 180 L), where the larvae of all aquaria were mixed and maintained throughout the yolk sac and starvation period. The experiment was stopped at 50% mortality (8 days after hatching). Throughout the experiment, the water temperature was maintained at 28 ± 0.3 °C.

Sampling

Samples were taken at intervals of about 6, 12 and 24 hours during respectively the egg, the yolk sac and the subsequent larval stage. In this way, 4 to 5 samples were taken in each developmental stage. To enable comparison with other studies done at different temperatures, time was expressed as physiological day-degrees (PD°) according to the equation:

$$PD° = \frac{Temp * (hours/24)}{q} \quad (1)$$

where :

PD°	=	physiological day degrees
Temp	=	temperature (°C)
q	=	Winberg coefficient to rate physiological processes at 20 °C (Winberg 1956). For 28 °C, q = 0.520.

The first sample (PD° = 0) was taken in unfertilized eggs. During the egg stage (from fertilization to hatching), three more samples were taken at 13.5, 27.5 and 43.5 PD°. Yolk sac larvae were sampled at 61.1, 79.1, 106.0, 132.4 and 159.9 PD°. The first point where the yolk sac could not be separated from the embryonal body was considered to belong to the subsequent starvation period. The starving larvae were sampled at 184.5, 238.4, 292.2, 346.1 and 399.4 PD°. In the subsequent paragraphs, these sampling times will be rounded to the nearest whole number. During the yolk sac phase, samples were taken both from entire animals and embryonal bodies without yolk. Yolk was removed by puncturing the yolk sac and vortexing the punctured animals twice in a soap solution ($3x10^{-3}$ ml/ml) for 20 seconds, followed by rinsing three times in distilled water. During sampling activities, animals were kept in an ice-water solution to reduce metabolic activities. The primary objective of the present study was to estimate fatty acid dynamics in African catfish in relation to development. Therefore, it is essential to obtain maximal accuracy at each sampling moment in time. Preliminary analyses of the total lipid content in four independent and large batches of eggs from different groups of broodstock animals revealed that the variation between samples is low (mean total lipid: 19.36% of the dry weight, standard deviation: 1.72) (Verreth, unpublished). This variation included sampling error, analytical error and biological variation. On the other hand, the variation in the total lipid content of egg and larval samples is affected by the sample size with sample weights below 40 mg dry weight giving high levels of inaccuracy. Variation in these results were related to the difficulties in measuring the weights of the samples (Ticheler and Verreth, unpublished). Therefore, in the present study bulk samples of about 500 mg estimated dry weight were taken to obtain a large homogenous batch from which subsamples were taken for lipid analysis. All samples were transferred to petri dishes and stored at -18 °C until further processing.

Analyses

Weight measurements included both wet and dry weight. Wet weight of eggs and

larvae was measured in three separate samples of 0.10 to 0.25 g each per sampling point. The number of fish in the different samples varied from 39 to 128. Wet weight of embryonal bodies was not measured because the adhering water made measurements inaccurate. Measurements were done by sieving, blotting and weighing the sampled fish as a group and counting them afterwards. Dry weight was determined on the same samples by drying overnight at 70 °C, followed by 4 hours at 105 °C.

Chemical analyses included measurements of total lipid and fatty acids. The bulk samples taken for this purpose were homogenized with ultraturrax, freeze dried and subsequently pulverized in a mortar. To obtain an air stable dry weight, the powder was exposed to air for 2 hours. This procedure was followed because air stabilization of dried samples resulted in a high precision of sample weight determination. In between all subsequent analyses, the samples were stored under nitrogen at - 18 °C. Preliminary tests to evaluate the different steps in the methodology revealed no statistically significant reduction in total lipid or fatty acid content due to freeze drying (comparison with storage in liquid nitrogen) or because of the short exposure to air (Ticheler and Verreth, unpublished). From each freeze dried and air stable bulk sample, three subsamples of about 40 mg each were weighed and dried in the oven (105 °C, 4 h) to obtain the dry matter content in the air stable material. Lipids were extracted in another batch of 4 to 6 subsamples of 40 mg each (air stable material) according to a modified Bligh and Dyer (1959) procedure. Total lipid was measured gravimetrically ($\pm 10^{-4}$ g) after evaporating the chloroform in a rotary evaporator (Heidolph VV2000) at 30 °C and raising the vacuum with nitrogen. The dried lipid was redissolved in 2.5 ml of a methanol-toluene solution (3:2 v/v), transferred into a 12 ml screw cap vial and flushed with nitrogen before storage.

Fatty acids were transesterified according to the acid-catalyzed transmethylation procedure of Lepage and Roy (1984) modified by Coutteau and Sorgeloos(1994) with 2.5 ml of a freshly prepared acetyl chloride-methanol mixture (5:100 v/v) at 100 °C. Esterification was stopped after 1 hour with 2.5 ml of distilled water. Subsequently, methylesters of heptadecanoic acid (C17:0) and behenic acid (C22:0) were added as internal standards. For this purpose, two stock solutions of these standards were made by dissolving C17:0 (4.788 mg/ml) and C22:0 (4.998 mg/ml) in hexane and pipetting fixed volumes of these solutions into the fatty acid methyl ester (FAME) solutions. The added volumes of the standard solutions were gravimetrically determined (0.186 ml) and corresponded with 0.891 mg of C17:0 and 0.930 mg of C22:0 respectively. The FAMEs were extracted three times in hexane and possible water contamination was eliminated using a waterfree Na_2SO_4

filter. Hexane was evaporated at 30 °C in a rotary evaporator, the vacuum raised by a stream of nitrogen and the remaining solvents eliminated with additional nitrogen flushing. Finally the FAMEs were redissolved in 1 ml hexane before gas chromatographic (GC) analysis.

Analysis of the FAMEs was carried out on GC (Varian 3600) equipped with a DB-23 fused capillary column (30m x 0.258 mm i.d.) (J&W Scientific Inc.) using hydrogen as carrier gas and a temperature gradient programmed from 150 °C to 205 °C (4 °C/min). The temperature of the injector and of the detector was respectively 225 and 240 °C. Methylesters were identified by comparison with a known standard mixture of fatty acids (SUPELCO) and with previously analyzed *Artemia* samples (provided by courtesy of Prof.dr.P. Sorgeloos, Artemia Reference Centre, Gent, Belgium). The quality of the GC analysis was evaluated by plotting the logarithms of the retention times of a homologous series of fatty acids against chain length (Christie 1989). Area percentages were converted to weights by comparison with the internal standards. Since the area% of both standards were not in a constant proportion to each other, it was decided to quantify the FAMEs with retention times smaller than C17:0 by comparison with C17:0 only and those having retention times larger than C22:0 by comparison with C22:0 only. For each sample, the Response Factor (Rf) of C17:0 was set at 1.0 and the corresponding Rf of C22:0 was calculated according to the formula:

$$Rf_{22:0} = \frac{A_{22:0} * W_{17:0}}{A_{17:0} * W_{22:0}} \qquad (2)$$

where :

$Rf_{22:0}$ = Response factor of C22:0 standard
$A_{17:0,22:0}$ = Area% of each standard
$W_{17:0,22:0}$ = Weight of each standard.

For fatty acids in between C17:0 and C22:0 a weighed average by linear intrapolation of the Rf's of both standards was used to convert area% into weights.

The analytical procedure was evaluated by analyzing the FAME content in the same *Artemia* samples both at our laboratory and elsewhere (the Artemia Reference Centre, Gent, Belgium). Comparison of the results did not reveal significant differences in the fatty acid composition (%). The present procedure gave consistently 80% of the fatty acid amounts found at the Artemia Reference Centre. According to a recent ICES intercalibration study, such a deviation can be considered as normal when analytical procedures between two

laboratories are compared (Léger *et al.* 1989, Coutteau and Sorgeloos 1994). Therefore, the data presented in this study are regarded as reliable.

Data handling and statistical analysis

Unless otherwise specified, all data were expressed per individual organism. For data of wet weight, dry weight and total lipid, suspected outliers were selected with Dixon's test (Sokal and Rohlf, 1969) (α=0.1) and omitted for analysis when significant. The amount of fatty acid in the yolk was determined by subtracting the amount of fatty acid in the body from that present in whole larvae. Fatty acid absorption was estimated from the decreasing amounts in the yolk. Rates of absorption and depletion of fatty acids during the different developmental stages (i.e. egg, yolk sac and starvation period) were calculated by:

$$\frac{dW}{dt} = \frac{W_{t_1} - W_{t_2}}{t_1 - t_2} \qquad (3)$$

where : dW/dt = rate of change of fatty acid amount (µg/PD°)
$W_{t1,2}$ = weight of fatty acid at t1 or t2 (µg)

Absorption rates and deposition rates (i.e. changes in amounts in the embryonal bodies) were calculated for the total embryonal period only (i.e. between fertilization and PD°=160 (see above)). Depletion was calculated for the entire experimental period (PD° from 0 to 399 (see above)). Relative rates of absorption were calculated by expressing the depletion of each fatty acid from the yolk as a fraction of the amount present at PD° = 0, divided by "time" in physiological day degrees (ng/µg/PD°). Relative rates of depletion were calculated in a similar way. To avoid that only two points (final and initial amounts) would determine the results, relative depletion rates were calculated by parameter estimation in an analysis of covariance using the fractional amounts of the fatty acids at each sampling moment ($Weight\text{-}FA_{i,t}/Weight\text{-}FA_{i,t0}$) as input data. To detect selective combustion or deposition of individual fatty acids, the relative depletion rate of each fatty acid was compared with the relative depletion in dry weight of the animal calculating linear contrasts between the two parameters. Conversion efficiencies of a fatty acid from yolk to body were calculated by:

$$CE = \frac{(W_{t_2} - W_{t_1})_{body}}{(W_{t_1} - W_{t_2})_{yolk}} * 100\% \qquad (4)$$

where: CE = Conversion efficiency (%)
$W_{t1,2}$ = Amount of fatty acid at times 1 or 2 (µg) (for the yolk sac period, t_1 = 61 and t_2 = 160 PD°).

Statistical analysis of the data was done using the GLM procedure of SAS (SAS Inc. 1985).

Results

Wet and dry weight

Egg wet weight increased during the first 14 PD° from 1.37 to 1.71 mg (Table 1) and attained a maximum of about 1.8 mg at 28 PD°. Between 43 PD° and 61 PD° (when hatching occurred) individual wet weight decreased by about 20% . Maximum wet weight of the whole organism (the so-called maximum alevin weight) was attained at 185 PD° (2.8 mg), i.e. close to complete yolk absorption. Dry weight of the egg fluctuated around 0.42 mg. Between 43 PD° and 61 PD°, individual dry weight decreased from 0.42 to 0.36 mg. During the first part of the yolk sac period (from 61 until 132 PD°), the individual dry weight remained rather stable and fluctuated around 0.38 mg whereafter it decreased continuously to 0.19 mg at the end of the experimental period. Linear regression of dry weight values over the whole experimental period yielded an average dry weight depletion rate of 60 μg/PD° (R^2 = 0.97). During the yolk sac period, the yolk absorption rate was calculated in the same way and attained 2.28 μg/PD°. According to this regression, complete yolk absorption would have occurred at 206 PD°. During the yolk sac period, the conversion efficiency of yolk dry weight to body tissue was 88.7%. When calculated for the whole embryonal period (PD° = 0 - 160), the conversion efficiency was 73.3%.

Total Lipid

Total lipid in eggs, whole yolk sac larvae, embryonal bodies and in starving larvae are presented in Table 1. From fertilization until 106 PD° (47 h post fertilization, e.g., about 1 day after hatching), the total lipid content fluctuated around 18.5% of the dry weight, whereafter it declined during the second part of the yolk sac period to about 13%. In starving larvae, the lipid concentration remained rather stable and fluctuated between 12.4% and 13.7% of the dry weight. The conversion efficiency of total lipid from yolk to body tissue during the yolk sac period was 65.1%. For the period between fertilization and 160 PD° this was 53%.

Fatty acids

The fatty acid contents (μg) in eggs, whole yolk sac larvae, embryonal bodies and starving larvae at the different sampling points are given in Table 2. From these data relative absorption rates (ng/μg/PD°) were estimated for each individual fatty acid (Table 3). As is shown in Table 3, the absorption rates of all fatty acids clustered around - 4-5 ng/μg/PD° for the period between fertilization (PD°= 0) and PD°=160. Exceptions were the

Table 1. **Wet weight (WW), Dry weight (DW) and total lipid (TL) content in developing embryos and starving larvae of the African catfish *Clarias gariepinus*. Figures between brackets refer to standard deviations between samples (weight measurements) or subsamples (lipid measurements). Data are based on three, respectively four to six replicates for weight and total lipid measurements. Age was expressed both in hours post fertilization and in physiological day degrees. Fertilization was considered as time zero. For further explanation see text.**

			whole organism		
Stage	PD°	hours	WW (µg)	DW (µg)	TL (µg)
Egg	0.0	0	1372 (29.2)	430 (14.3)	82 (5.3)
	13.5	6	1707 (25.7)	412 (9.1)	74 (1.9)
	27.5	12	1815 (7.6)	425 (3.3)	80 (5.8)
	43.2	19	1787 (77.1)	421 (20.3)	67 (7.3)
Yolk sac	61.1	27	1408 (36.2)	362 (123.9)	67 (1.9)
	79.1	35	1488 (18.6)	381 (9.4)	71 (2.6)
	106.0	47	1754 (16.0)	365 (5.7)	69 (3.1)
	132.4	59	2118 (49.1)	378 (22.7)	62 (3.7)
	159.9	71	2535 (106.0)	336 (18.9)	52 (2.8)
Starvation	184.5	82	2826 (48.0)	316 (9.6)	43 (9.9)
	238.4	106	2811 (24.7)	293 (4.1)	36 (1.7)
	292.2	130	2754 (51.7)	254 (3.2)	35 (1.7)
	346.1	154	2666 (19.0)	217 (2.1)	27 (2.6)
	399.4	178	2315 (29.5)	191 (4.5)	25 (2.6)
			embryonal bodies		
	PD°	hours	WW (µg)	DW (µg)	TL (µg)
Yolk sac	61.1	27	n.d.	53 (1.0)	6 (0.1)
	79.1	35	n.d.	79 (10.9)	10 (0.7)
	106.0	47	n.d.	134 (3.7)	17 (0.8)
	132.4	59	n.d.	181 (0.7)	25 (0.9)
	159.9	71	n.d.	258 (2.8)	34 (1.3)

fatty acids 18:4 (-1.9 ng/µg/PD°), 24:0 (-1.7 ng/µg/PD°) and 22:5n-3 (-6.6 ng/µg/PD°). Depletion rates (ng/PD°) are an estimation of the amount of each fatty acid that is combusted and are shown in Figures 1A (whole experimental period) and 1B (from fertilization to end of yolk sac period). The largest depletion rates are found in the most abundant fatty acids,

Table 2. The fatty acid content (μg) in eggs, yolk sac larvae, embryonal bodies and starving larvae of the African catfish, *Clarias gariepinus*, at different moments of development. Age is expressed in physiological day degrees. For comparison with time in hours, see Table 1.

PD°:	0	14	28	43	61	79	106	132	160	185	238	292	346	399
Fatty Acid														
14:0	0.716	0.713	0.719	0.781	0.585	0.594	0.548	0.500	0.307	0.180	0.135	0.063	0.062	0.045
14:1	0.066	0.072	0.074	0.113	0.061	0.067	0.039	0.036	0.029	0.009	0.018	0.010	0.015	0.005
15:0	0.329	0.311	0.315	0.333	0.252	0.302	0.248	0.255	0.188	0.164	0.140	0.104	0.086	0.072
15:1	0.259	0.192	0.237	0.202	0.171	0.192	0.194	0.201	0.205	0.225	0.229	0.203	0.199	0.162
16:0	11.450	10.247	10.501	10.512	8.852	9.975	9.321	8.879	6.772	5.178	4.108	2.889	2.354	2.038
16:1n-7	1.119	0.998	1.072	1.049	0.981	0.964	0.829	0.717	0.415	0.218	0.123	0.065	0.051	0.044
18:0	4.207	3.450	3.701	3.636	3.134	3.342	3.335	3.153	3.095	2.418	2.171	1.845	1.560	1.434
18:1n-9	6.683	5.860	6.237	6.177	5.176	5.513	5.289	4.850	3.753	2.517	1.959	1.492	1.246	1.156
18:1n-7	1.250	1.036	1.178	1.159	0.964	1.049	1.011	0.936	0.769	0.504	0.368	0.257	0.201	0.171
18:2n-6	2.171	1.995	2.173	2.052	1.615	1.779	1.676	1.382	1.049	0.616	0.399	0.236	0.164	0.144
18:3n-6	0.095	0.085	0.112	0.098	0.088	0.097	0.101	0.069	0.069	0.049	0.042	0.042	0.041	0.037
18:3n-3	0.283	0.255	0.308	0.272	0.204	0.220	0.226	0.157	0.127	0.052	0.033	0.015	0.013	0.012
18:4	0.075	0.066	0.090	0.098	0.058	0.079	0.065	0.046	0.068	0.017	0.021	-	-	0.007
20:3n-6	0.338	0.329	0.320	0.381	0.265	-	0.304	0.264	0.255	0.303	0.207	0.134	0.092	0.074
20:4n-6	0.404	0.379	0.353	0.479	0.304	0.380	0.425	0.428	0.541	0.561	0.554	0.428	0.367	0.331
20:5n-3	2.469	2.383	2.906	2.671	1.721	2.403	2.399	2.088	2.126	2.021	1.799	1.041	0.710	0.597
22:4n-6	0.066	0.087	0.201	0.269	0.123	0.128	0.120	0.070	0.107	0.022	0.013	0.033	0.018	0.022
24:0	0.086	0.120	0.168	0.152	0.063	0.085	0.074	0.086	0.119	0.135	0.056	0.045	0.044	0.031
22:5n-3	0.727	0.710	1.016	0.977	0.588	0.628	0.637	0.683	0.557	0.478	0.320	0.207	0.180	0.134
22:6n-3	7.799	7.079	9.854	8.424	5.499	6.309	7.122	5.863	6.870	6.522	5.348	4.188	3.363	3.083
saturates	16.79	14.84	15.40	15.41	12.89	14.30	13.53	12.87	10.48	8.07	6.61	4.95	4.10	3.62
monoenes	9.38	8.16	8.80	8.70	7.35	7.78	7.36	6.74	5.17	3.47	2.70	2.03	1.71	1.54
(n-3)	11.28	10.43	14.08	12.34	8.01	9.56	10.38	8.79	9.68	9.07	7.50	5.45	4.26	3.83
(n-6)	3.07	2.88	3.16	3.28	2.40	2.38	2.63	2.21	2.02	1.55	1.21	0.87	0.68	0.61
n-3/n-6	3.67	3.63	4.46	3.76	3.35	4.01	3.95	3.97	4.79	5.86	6.17	6.25	6.26	6.30

	Embryonal bodies				
Fatty Acid					
14:0	0.537	0.528	0.427	0.342	0.151
14:1	0.047	0.062	0.025	0.013	0.024
15:0	0.222	0.257	0.173	0.147	0.073
15:1	0.145	0.137	0.091	0.098	0.054
16:0	7.98	8.553	6.898	5.63	2.502
16:1n-7	0.917	0.874	0.678	0.528	0.193
18:0	2.868	2.812	2.351	1.836	1.01
18:1n-9	4.678	4.728	3.931	3.181	1.494
18:1n-7	0.875	0.89	0.732	0.592	0.3
18:2n-6	1.524	1.539	1.332	0.948	0.451
18:3n-6	0.077	0.082	0.079	0.029	0.025
18:3n-3	0.194	0.196	0.186	0.111	0.075
18:4	0.048	0.068	0.044	0.025	0.052
20:3n-6	0.251	-0.05	0.233	0.154	0.04
20:4n-6	0.282	0.301	0.323	0.244	0.105
20:5n-3	1.652	2.071	2.046	1.376	0.706
22:4n-6	0.117	0.116	0.112	0.055	0.012
24:0	0.06	0.073	0.063	0.064	0.063
22:5n-3	0.559	0.508	0.536	0.294	-0.04
22:6n-3	5.345	5.384	6.051	3.469	1.807
Total saturates:	11.67	12.22	9.912	8.02	3.799
Total monoenes:	6.662	6.692	5.457	4.413	2.064
Total (n-3):	7.751	8.159	8.82	5.249	2.55
Total (n-6):	2.25	1.989	2.079	1.43	0.633
(n-3)/(n-6):	3.445	4.102	4.243	3.67	4.031

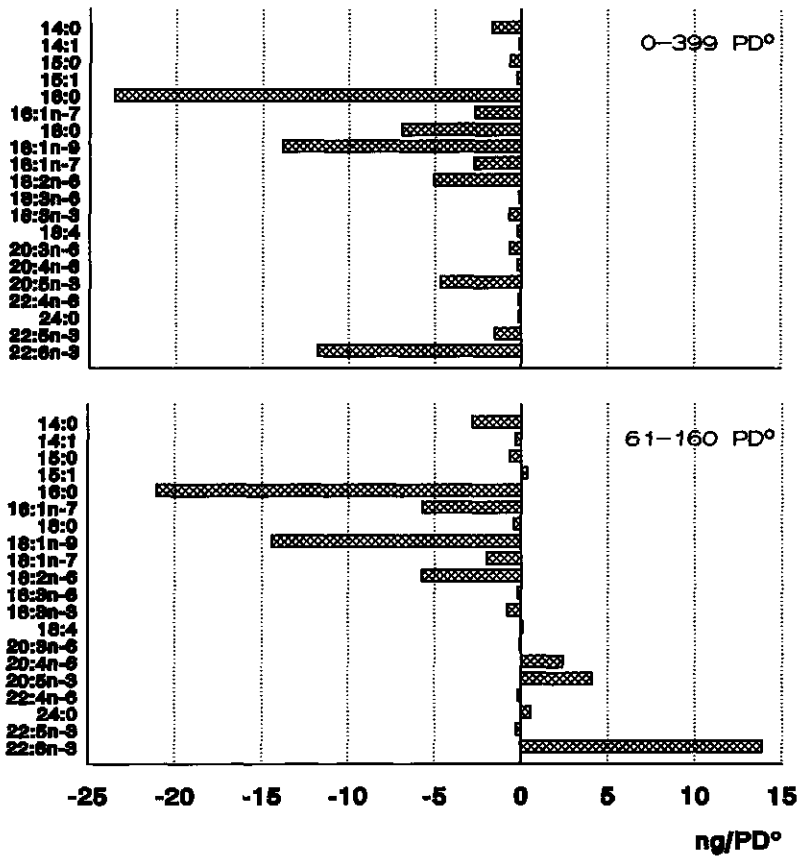

Figure 1 The average depletion rates (ng/PD°) of individual fatty acids during the early life stages of the African catfish *C. gariepinus*. Top: overall depletion rates as measured over the whole experimental period. Bottom: the same data for the yolk sac period only.

e.g. 16:0 (23.6 ng/PD°), 18:1n-9 (13.8 ng/PD°) and 22:6n-3 (11.8 ng/PD°) (Figure 1A). There is a strong positive correlation between the initial amounts in unfertilized eggs and the overall depletion rates during the experimental period (Figure 2). The residuals (the deviations of each fatty acid from the regression line) give a first indication of the selective combustion: the points above the regression line in Figure 2 represent fatty acids which are combusted at a higher rate than the average, i.e. 16:0, 18:1n-9 and 18:2n-6. The relative depletion rates in whole organisms as mentioned in Table 3 suggest that most fatty acids and fatty acid groups were depleted at a higher rate than dry weight, with exception of the fatty acids 15:1 and 20:4n-6. In general, the amount of (n-3)PUFA's in whole organisms

decreased at a lower rate than saturates, monoenes or (n-6)PUFA. When depletion rates were calculated for the yolk sac period (61 - 160 PD°) only (Figure 1B), a quite different picture appeared. Most 20C PUFA's showed a net increase during this period (respectively 2.4, 4.1 and 13.9 ng/PD° for 20:4n-6, 20:5n-3 and 22:6n-3) while on the other hand 16:0 and 18:1n-9 were declining fast (21.1 and 14.4 ng/PD° respectively). Statistical analysis ($p<0.05$) of the relative depletion rates revealed a selective combustion of 14:0, 14:1, 16:1n-7, 18:3n-3 and 22:4n-6 while 20:4n-6 was selectively spared when compared with the decline in dry weight (Table 3).

The conversion efficiencies from yolk to body tissue during the yolk sac period for the different fatty acids is presented in Figure 3. The HUFA's arachidonic acid, EPA and DHA were converted at efficiencies above 100%, indicating that they were synthesized. Most other fatty acids were converted at efficiencies below 60%.

Discussion

In the present study, eggs and larvae of several parent fish were used and each sample contained many individuals. Since the objective of the present study was to investigate the changes of fatty acids in time rather than the individual or the intraspecific variation, it is important to have very homogenous material to draw samples from. Therefore, for the lipid analyses one bulk sample per sampling time was taken and individual (sub)samples were weighed after freeze drying and homogenisation. This procedure reduced weighing errors and enabled maximal accuracy in the quantification of the fatty acids. However, it reduced also the biological variation among the samples. The intraspecific variation in lipid content and composition is correlated with differences in reproductive cycle, temperature and feeding conditions. All these factors are strongly reduced under the standard hatchery conditions of the experimental facility were the samples of the present study were taken. Preliminary tests in independent egg batches taken at different times in the year yielded a coefficient of variation (CV) of 8.9% for total lipid content. This is only slightly higher than the CV found in the present study (6.45%) which reflects mainly the analytical variation. Therefore, the present data can be regarded as exemplary for the fatty acid dynamics which occurs during the early life stages of *C. gariepinus*. When the intraspecific variation rather than changes in time are subject of study, specific experiments should be designed, taking into account parental, feeding and environmental effects.

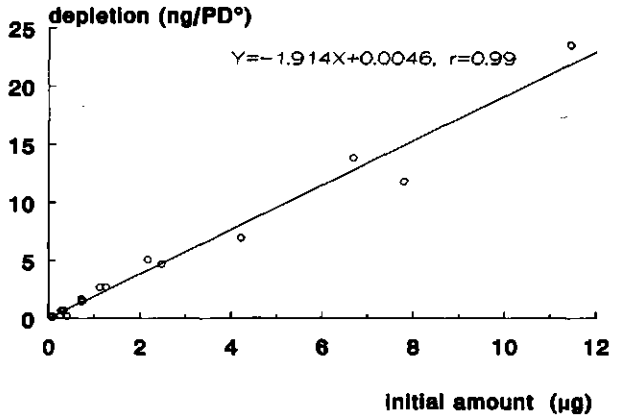

Figure 2 The fatty acid depletion rates over the entire experimental period in relation to the initial amounts of these fatty acids present in the eggs.

Hatching occurred in between the sampling points 43 and 61 PD°. The moment of 50% hatching was estimated at 51.6 PD°. Yolk absorption was estimated to be completed at 205.8 PD° and maximum alevin weight was found at 184.5 PD°. These staging results agree well with earlier information on the same species (Verreth *et al.* 1994b, Polat *et al.* 1994) and support the assumption of Conceição *et al.* (1993) that in *C. gariepinus* hatching and complete yolk absorption can be approximately fixed at respectively 50 and 200 PD°. Slight differences may occur due to differences in temperature and size of the eggs. In the present study, the dry weight of the eggs (0.43 mg) was slightly higher than those in the previous study of Verreth *et al.* (1994b) which may explain the slightly longer duration of yolk absorption in the present study (205.8 vs 199.8 PD° respectively).

The lipid content in teleost eggs varies considerably between species (Heming and Buddington 1988, Henderson and Tocher 1987, Sargent *et al.* 1989). In the present study, the lipid content of unfertilized eggs of *C. gariepinus* amounted to 19.1% of the dry weight, i.e. about 6% of the wet weight. This is slightly higher than found in earlier studies on the same species, e.g. 5.2% (Verreth *et al.* 1994b), 5.75% (Polat *et al.* 1994) and 4.5% (unpublished data) of the wet weight. More than 70% of the egg lipid consist of phosphatidylcholine (Verreth *et al.* 1994b). These data characterize *C. gariepinus* as a species with eggs containing moderate amounts of lipid of a very polar nature (Kaitaranta and Ackman 1981, Henderson and Tocher 1987, Verreth *et al* 1994b). In line with previous

Table 3. Yolk absorption rates and depletion rates of fatty acids in the African catfish, *Clarias gariepinus*. The t-values test the hypothesis that the relative rate of depletion of the fatty acid under concern is not different from the relative rate of depletion of the dry weight.

	Absorption rate (0-160 PD°) (ng/µg/PD°)	Depletion rate (0-399 PD°) (ng/µg/PD°)	t-value
dry matter	n.d	1.40	-
14:0	- 4.96	2.90	4.25*
14:1	- 4.02	3.23	6.33*
15:0	- 4.88	2.17	1.14
15:1	- 4.97	0.28	2.35
16:0	- 4.91	2.23	1.32
16:1n-7	- 5.20	2.88	4.16*
18:0	- 4.78	1.54	0.04
18:1n-9	- 4.88	2.29	1.53
18:1n-7	- 4.78	2.35	1.71
18:2n-6	- 4.98	2.72	3.29
18:3n-6	- 4.67	1.92	0.52
18:3n-3	- 4.63	2.87	4.11*
18:4	- 1.88	2.88	3.38
20:3n-6	- 5.54	2.04	0.78
20:4n-6	- 4.65	0.11	4.27*
20:5n-3	- 4.49	2.04	0.79
22:4n-6	- 5.11	5.93	38.77***
24:0	- 1.67	2.67	3.08
22:5n-3	- 6.61	2.68	3.10
22:6n-3	- 4.83	1.62	0.09
saturates	n.d	2.09	0.90
monoenes	n.d	2.32	1.62
(n-3)	n.d	1.81	0.33
(n-6)	n.d	2.28	1.48

n.d = not determined;
* = significant t-value at $p<0.05$; *** = significant t-value at $p<0.002$

results (Polat *et al.* 1994, Verreth *et al.* 1994b,c, Conceição *et al.* 1993), lipid is preferentially combusted during the second half of the yolk sac period (106 to 185 PD°) (Table 1) which may be related to an increased swimming activity during this period (Heming and Buddington 1988).

The fatty acid composition shows a remarkable high percentage of 18:0 (10.5%) in the lipid of unfertilized *C. gariepinus* eggs. In eggs of many other marine and freshwater fish, 18:0 percentages range from 2.3-3.4% (Kaitaranta and Linko 1984, Tocher *et al.* 1985, Ulvund and Grahl-Nielsen, 1988). Also the levels of 16:0 are high (28.5%) whereas the 18:3n-3 levels (0.70%) are lower than in most investigated fish species. Total PUFA

accounted only for 35% of the total fatty acids, which is extremely low, compared to most other marine and freshwater fish (Henderson and Tocher 1987, Sargent *et al.* 1989) and taking into account that the egg lipid of *C. gariepinus* is highly polar (Verreth *et al.* 1994b). The fatty acid composition of *C. gariepinus* larvae at 185 PD°, (i.e. close to complete yolk absorption) agrees very well with the fatty acid profile in larvae of the same species at start of exogenous feeding (Verreth *et al.* 1994a). The most abundant fatty acids at that moment are 22:6n-3 (29.4% in this study, versus 28.2% in the study of Verreth *et al.* 1994a), followed by 16:0 (23.5%), 18:1n-9 (13.7%), 18:0 (11%) and 20:5n-3 (9%).

In line with data reported on other fish species (Sargent *et al.* 1989, Sargent 1994), the major (n-3)PUFA in eggs of *C. gariepinus* were 20:5n-3 and 22:6n-3 (Table 2). However, in the present study the ratio of EPA/DHA in eggs was 1:3 rather than the 1:2 ratio which is usually found in eggs of other species (Sargent 1994). 18:2n-6 is by far the most important (n-6)PUFA in eggs of *C. gariepinus* and its concentration was almost 8 times that of 18:3n-3. The ratio of (n-3)/(n-6)PUFA in the egg samples was 3.7 which is very low compared to similar ratios in eggs of many other species, both marine and freshwater (Sargent 1994, Henderson and Tocher 1987). Considering the relative high proportion of 22:6n-3 in the (n-3)PUFA, this low (n-3)/(n-6) ratios should be attributed to the elevated levels of 18:2(n-6). Although freshwater fish generally contain higher (n-6)PUFA levels than marine fish, the results found in this study may also reflect the fatty acid composition of the broodstock diets, which may have contained elevated levels of linoleic acid from vegetable oils and of DHA from fish oils.

As is shown by the data assembled in Table 3, most fatty acids are absorbed from the yolk at very similar rates, e.g. about 0.5% of the initial amount per PD°, indicating that the lipases present in yolk and/or syncytium are generally unspecific. At 185 PD° no separation of yolk and embryonal body was possible and the absorption rates were calculated for the period until 160 PD°. However, the total lipid analysis showed that lipid depletion (and possibly also absorption) increased during the second half of the yolk sac period (Table 1), and this period was not fully covered in the calculations of the fatty acid absorption rates. Therefore, the presented absorption rates may be underestimated. The depletion rates differed strongly among the fatty acids (Figure 1A,B). There is a significant relation between total depletion rates over the whole experimental duration and initial amounts of the fatty acids in the egg lipid, indicating that the most abundant fatty acids, e.g 16:0, 18:0,

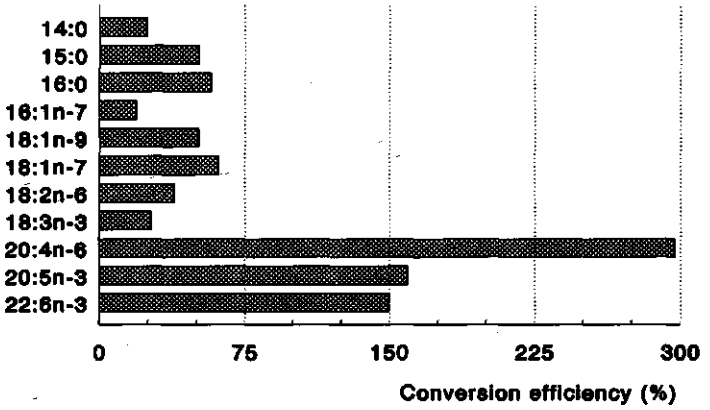

Figure 3 The conversion efficiencies for different fatty acids from yolk to body tissue during the embryonal (from fertilization until yolk absorption) period.

18:1n-9 and 22:6n-3 contribute most to energy fuelling. This is further substantiated by the results of the statistical comparison between the relative depletion rate of each fatty acid with the one of dry weight (Table 3). For most fatty acids, the relative depletion rate was not statistically different from the overall dry weight decline. In addition, the applied statistical procedure (calculating linear contrasts, and thus repeated t-tests) increases the risk for type I error (false rejection of the null hypothesis) (Sokal and Rohlf, 1969). A conservative approach would be to apply a significance level of α= (0.05/25 comparisons)= 0.002. In that case, only 22:4n-6 was depleted at a significantly higher rate than dry weight. Therefore it seems safe to conclude that in early life stages of *C. gariepinus* all fatty acids are used in a similar way for combustion and/or deposition, although there are trends which indicate a slight "saving" of arachidonic acid (20:4n-6) and a slight preference for combustion of some lower chain saturates and monoenes.

At the same time, these relative depletion rates were affected by changes in the opposite direction during the different developmental periods. As is shown in Figure 1B, during the yolk sac period, the net amount of several fatty acids, especially the 20C HUFA's, was increasing in stead of decreasing. This means that the estimated depletion rates must have been strongly affected by a high depletion during starvation, when the yolk was absorbed. Apparently the role of the different fatty acids change with the metabolic status of the fish. In periods of starvation, the main role of lipids is energy fuelling, and according to the data

collected in Table 3, all types of fatty acids, e.g. saturates, monoenes, and PUFA are used for this purpose. In periods of growth, such as during the yolk sac period, fatty acids are needed for tissue formation, besides energy fuelling. The data presented in Figure 1B and Figure 3 (conversion efficiencies) show that especially 20C homologues of (n-3) and (n-6) fatty acids are spared or even synthesized during this period.

As mentioned earlier, Verreth *et al.* (1994b) demonstrated that more than 70% of the lipid in eggs and yolk sac larvae of *C. gariepinus* consisted of phosphatidylcholine (PC) and that it was depleted proportionally to the dry weight. All phosphatidylethanolamine (PE) present in the yolk was converted to body tissue and all triacylglycerides (TAG) were combusted during the yolk sac period. In the present study no distinction was made between the fatty acid composition of the different lipid classes. However, combining the present data with the results of Verreth *et al.* (1994b), one may speculate that the most abundant fatty acids, e.g. 16:0, 18:0 and 22:6n-3 constitute the major components of PC. Arachidonic acid is found often at the s-2 position of PI, a lipid class which was possibly present but not detected in eggs or yolk sac larvae of *C. gariepinus* (Verreth *et al.* 1994b).

The calculated conversion efficiencies (Figure 3) demonstrate the synthesis of the most important HUFA's, e.g. 20:4(n-6), 20:5(n-3) and 22:6(n-3). This is in agreement with the general assumption that freshwater fish can biosynthesize these fatty acids from their 18C precursors. However, it remains remarkable that synthesis occurs when high levels are initially present in the eggs, e.g. 22:6(n-3). These results suggest that the enzymes needed for elongation and desaturation of 18:2(n-6) and 18:3(n-3) were active in the growing yolk sac larvae. In a study on the fatty acid dynamics in feeding larvae, Verreth *et al.* (1994a) speculated that in *C. gariepinus* these enzymes become active at a later stage of the larval period. According to the present results, this hypothesis can not be hold on. Detailed studies using radiolabeled precursor fatty acids could provide more insight in this regard.

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CHAPTER 8

The effect of low hufa and high hufa enriched *Artemia*, fed at different feeding levels, on growth, survival, tissue fatty acids and liver histology of *Clarias gariepinus* larvae.

Johan Verreth, Johan Coppoolse and Helmut Segner

Abstract

The present study investigated the effect of two diets containing different levels of HUFA, given at three different feeding levels on the growth performance, fatty acid composition and liver structure of African catfish, *Clarias gariepinus* (Burchell), larvae. The larvae were raised at 27.5°C until a final weight of 50 mg. The fish were fed *Artemia* which was enriched with an emulsion low in *n*-3 HUFA (coconut-oil) or high in *n*-3 HUFA (SELCO). Both feed types had similar protein and ash levels, but differed mainly in their fatty acid composition. The level of the *n*-3 HUFA in SELCO-enriched *Artemia* was three times higher (14.5 mg/g dry weight) than in the coconut-oil enriched *Artemia* (5 mg/g dry weight). Feed type did not affect growth or survival. Dietary HUFA levels affected the fatty acid composition of the fish but this feed type effect was statistically not significant. The specific growth rate ranged, depending upon the feeding level, from 17 to 52% body weight.day^{-1} and survival rate varied between 79 and 89%. The total amount of food given to the fish did affect significantly the dry matter content, tissue fatty acid composition and the liver lipid content in the fish. At the start of exogenous feeding, docosahexaenoic acid (22:6 *n*-3) was the most abundant fatty acid in the fish (17.1 mg/g dry weight or 28.2% of total fatty acids), but its level decreased to 2-4 mg/g dry weight at the end of the experiment. Fatty acid retention percentages revealed the capacity to synthesize HUFA. Feeding the larvae at optimal feeding level or higher resulted in accumulation of lipid in the liver (> 40% of the hepatocyte volume consisted of lipid). Overall, feeding level had a more pronounced effect than feed type.

Introduction

For research on feeding and growth of larval *Clarias gariepinus*, dried and decapsulated *Artemia* cysts can be used as a reference diet (Verreth et al., 1987). Using this diet, optimal feeding level, rearing temperature and weaning time for catfish larvae were established (Verreth and den Bieman, 1987; Verreth and van Tongeren, 1989). The nutritional value of

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Artemia as a food source for fish larvae is strongly related to its fatty acid composition, and especially to the *n*-3 HUFA content (Watanabe et al., 1983, Léger et al., 1986, 1987). In marine fish, *n*-3 HUFA which are essential, e.g., DHA (22:6*n*-3) and EPA (20:5*n*-3) are an important constituent of cell membranes, especially in brain and retina (Sargent et al., 1989; Watanabe, 1993), and are therefore required during early life stages to assure normal visual and neural development (Sargent et al., 1993). In contrast to marine fish, freshwater species may possess the desaturases and elongases necessary to synthesize these fatty acids from 18:3 precursors. As a consequence, larvae of freshwater species tend to have lower requirements for *n*-3 HUFA than marine fish larvae. The *n*-3 fatty acid profile in *Artemia* is highly variable (Watanabe et al., 1978; Lèger et al., 1986; Bengtson et al., 1991), depending on strains and the diets which were eaten by the harvested *Artemia* population (Lavens et al., 1989). The levels of these fatty acids in *Artemia* can be insufficient to maintain good growth and survival in marine larvae. Léger et al. (1986) developed a technique to "bio-enrich" *Artemia* nauplii with fatty acids in order to optimize their fatty acid profile. Although freshwater fish larvae may be less affected by the variable fatty acid compositions in *Artemia* (Bengtson et al., 1991), also in these species, different dietary fatty acid profiles may alter the growth response of the larvae, and thereby the value of *Artemia* as a "reference diet" (Verreth and den Bieman, 1987) for nutritional studies. In the present study, the "enrichment" technique was used to test the effect of different dietary *n*-3 HUFA levels on the growth performance, fatty acid composition, liver structure and energy stores in larvae of the African catfish, *Clarias gariepinus*, when fed at different feeding levels.

Materials and methods

Larvae of the African catfish were obtained through artificial reproduction of 3 female and 1 male breeders. Upon yolk resorption, the larvae were stocked in the aquaria. At that time, the larvae weighed 2.3 mg with a dry matter content of 12.8%. The experiment was carried out in two separate recirculation units, each containing 9 aquaria (volume = 17 l), a sedimentation tank and a gravel filter. Temperature was kept at 27.5 °C, the optimal rearing temperature for larval *C. gariepinus* (Verreth and der Bieman, 1987). The larvae were fed *Artemia* (strain Great Salt Lake, Utah, USA), bio-enriched with an emulsion of either coconut oil or SELCO (*Artemia Systems NV*, Baasrode, Belgium) according to the procedure of Léger et al. (1986). Feeding was done five times a day, at 3-h intervals between 12 a.m. and 12 p.m. Each day, a newly-enriched and cold-stored batch of *Artemia* was used.

Feeding occurred at three feeding levels, which were determined according to the procedure of Verreth and den Bieman (1987): daily weight increase was predicted by using the linear regression equation between the cube root of wet weight as dependant variable and days of feeding as independent variable, using regression coefficients (FL) of 0.1, 0.2 and 0.3 respectively. The daily ration of *Artemia* (in mg) was calculated assuming a food conversion ratio of 3 on a dry matter basis. Basically, these feeding levels corresponded with a "slightly above maintenance" level (FL = 0.1), an optimal (in terms of conversion efficiency) level (FL = 0.2) and a near satiation level (FL = 0.3) (Verreth and den Bieman, 1987). The total feed ration (mg dry matter of *Artemia* per fish) given during the entire rearing period, was obtained by adding all daily individual rations, and used to test the effect of feed intake. Each combination of feed type (n_i=1,2) (coconut-oil and SELCO-enriched *Artemia*) and feeding level (n_j=1,..3) (FL= 0.1, 0.2 and 0.3) was tested with three repetitions. The six treatment combinations were assigned to both recirculation units according to an unbalanced scheme, e.g. alternating 2 repetitions in one unit plus one repetition in the other unit. The experiment was carried out at pair growth, i.e. fish were reared until an average final weight of 50 mg, irrespective of the feeding level. This final weight was chosen because it is the commonly used weight for weaning in catfish hatcheries (Verreth and den Bieman, 1987). Different feeding levels induce different growth rates. Hence, the rearing time required to reach the final weight varied according to the feeding level: 9 days for the highest feeding level (FL = 0.3), 12 days for the intermediate level (FL = 0.2) and 21 days for the lowest level (FL = 0.1). These different rearing periods forced us to apply different stocking densities. Due to the longer rearing period at lower feeding levels, a larger number of intermediate samples were needed to check the calculations of the daily feed rations. To compensate for this different "sampling mortality", larvae were stocked at densities of 800, 700 and 600 larvae for the feeding levels FL = 0.1, 0.2 and 0.3, respectively.

The two types of *Artemia* were analyzed for dry matter, protein, energy and ash content in addition to their fatty acid composition. Earlier experiments (Verreth and den Bieman, 1987 and unpublished results) revealed that the dry matter composition in the body of *Artemia* fed *C. gariepinus* larvae was not significantly affected by differences in feeding level, temperature or husbandry conditions. Therefore, in the present study, fish was only analyzed for dry matter and fatty acid composition. To obtain information about the average composition of the feed (*Artemia*) during the course of the experiment, every other day, a sample of freshly bio-enriched *Artemia* was taken and pooled with the previous *Artemia* samples. For the fish, pooled samples per treatment group were taken at the end of the experiment. Dry matter, protein, ash and energy contents were measured according to the

procedures of Henken et al. (1986). All analyses were done on groups of at least 5 - 10 larvae or, as for protein, on 20 mg freeze dried material. Protein data are based on Kjehldahl nitrogen (N x 6.25) and energy on COD measurements. The fatty acid composition was measured by weighing 50 mg freeze-dried material into a 5 ml tube, to which 1 ml heptane was added. The fatty acids were esterified with 100 µl of 2 M sodium methyl acetate, according to the procedure of Badings and DeJong (1983) and analyzed on GC (Hewlett Packard 5710A) with a 2 m x 4 mm^2 column packed with 2.4 g 15% ChromPack-sil 84 coated on chromosorb WHP, 100/120 mesh. One µl of the FAME mixture was injected using an autosampler (HP 7671A) at 225 °C and subsequently analyzed isothermically at 200 °C. Each sample was injected twice to have a check on the quality of the GC analysis. The standard deviations among these duplicate analyses varied between 0.5 and 1% of the mean. The average of both measurements was used as sample (i.e. one per aquarium) value. Fatty acid levels were quantified by comparison with behenic acid (C22:0) as internal standard. Fatty acid retention was calculated by dividing the change in µg fatty acid per fish between the start of exogenous feeding and the end of the experiment by the total amount of fatty acid given as food (µg fatty acid growth/µg fatty acid intake x 100%). At the end of the experimental period, four fish per treatment group (n = 4x6 = 24 fish) were sampled for electron microscopical analysis of the hepatocyte structure. Semi-thin and ultra-thin sections were prepared and examined according to the procedures of Segner and Braunbeck (1990). Quantitative estimations of the relative lipid and glycogen volumes within the liver cells (volume density) were made by means of stereology (Segner and Braunbeck, 1988, 1990). The point counting method of Weibel (1979) was employed. Hepatocyte volume was chosen as reference volume during these volume density estimations.

Survival rates were determined according to the procedure of Verreth and den Bieman (1987) and include a correction for the sampling mortality. Growth rate was assessed both by the specific growth rate ($100\%(\ln W_t - \ln W_0)/t$) and the growth coefficient *g* (Verreth and den Bieman, 1987). The effect on the growth and tissue fatty acids was tested by a covariance analysis according to the model :

$$Y_{ijk} = \mu + RU_i + (FT)_j + (RU \times FT)_{ij} + b \times X_k + (FT \times X)_{jk} + e_{ijk} \qquad (1)$$

with:
- Y_{ijk} = dependant variable (growth or fatty acid content)
- μ = mean
- RU_i = recirculation unit (i = 1..2)
- FT_j = feed type (j = 1..2)
- X = total feed ration (recalculated in mg dry matter.$fish^{-1}$)
- (RU x FT) and (FT x X) = interaction terms
- e_{ijk} = error term

The effect on glycogen and lipid content in the liver parenchymal cells was tested by :

$$Y_{ijk} = \mu + (\text{feed type})_i + (\text{feeding level})_{j:i} + e_{ij} \quad (2)$$

with: Y_{ij} = glycogen or lipid volume density
μ = mean
feed type (i=1,2) = low- or high HUFA enriched *Artemia*
feeding level (j=1..3) = FL 0.1,...0.3., nested within feed types
e_{ij} = error term

Heterogeneity of variances was investigated by plotting residuals against predicted values. Durban-Watson's D coefficient served to test the presence of autocorrelation among independent variables. All analyses were done using SAS procedure GLM (SAS Inc., 1985).

Results

The proximate and total fatty acid composition of the two types of enriched *Artemia* is given in Table 1. As expected, both feed types differed mainly in their HUFA content (Table 1), and more particular in their *n*-3 HUFA content (14.5 vs 5 mg/g dry weight for the SELCO-enriched and coconut-oil enriched *Artemia* respectively) (see also Table 3). The results of the larval growth in the different treatment groups are provided in Table 2.

TABLE 1. Proximate and total fatty acid composition of *Artemia*, enriched with either a coconut-oil or a SELCO emulsion to obtain respectively low HUFA and high HUFA levels. All data are expressed on dry matter basis.

Enrichment Emulsion	Energy (kJ/g)	Protein (%)	ash (%)	total/FAME (mg/g)	total/PUFA (mg/g)	total/Hufa (mg/g)
SELCO	28.7	54.8	11.7	178.7	80.6	15.1
Coconut-oil	23.7	53.4	11.5	172.4	67.0	5.6

Because the unit in which the larvae were reared had no significant effect on the results (Table 4), only feeding level and feed type data are given. The specific growth rate was significantly (P=0.0004) influenced by total feed ration, not by feed type (Table 4). The growth coefficient "g" indicates that larvae grew according to the expected growth rate, as preset in the calculation of feed rations. Survival was not affected by any of the treatments. The average survival rate varied between 83 and 89% with exception of the survival in the

TABLE 2. Feed ration and growth rate of larvae of *Clarias gariepinus* fed with two differently enriched types of *Artemia* at three feeding levels. Growth rate was calculated both on the basis of the exponential growth model (specific growth rate SGR) and of the cube root model (growth coefficient *g*) (Verreth and den Bieman, 1987). Standard deviations are given between brackets.

	Low HUFA diet			High HUFA diet		
Feeding level:	0.1	0.2	0.3	0.1	0.2	0.3
Number of samples	3	3	3	3	3	3
Feed ration (mg dm.fish-1)	15.5 (4.2)	17.8 (0.7)	23.3 (3.5)	16.4 (4.2)	20.7 (4.0)	37.0 (15.8)
SGR (% BW/d)	16.8 (1.8)	33.5 (0.9)	49.1 (1.4)	16.9 (1.9)	33.7 (2.7)	52.2 (3.4)
Growth coefficient *g*	0.11 (0.02)	0.21 (0.01)	0.29 (0.01)	0.11 (0.02)	0.21 (0.02)	0.32 (0.03)
Dry matter larvae (%)	16.0 (0.9)	17.3 (0.2)	17.5 (0.4)	16.6 (0.9)	17.6 (0.5)	17.8 (0.6)

larvae receiving high HUFA enriched *Artemia* at the highest feeding level (FL = 0.3). Due to a disease problem the average survival in this treatment group was 79%.

The fatty acid composition of the bio-enriched *Artemia*, used as food, and the fatty acid composition of the larvae at the start and at the end of the rearing period is given in Table 3. At the start of exogenous feeding, docosahexaenoic acid (DHA) content was 17.1 mg/g dry weight but it decreased to 1.1 - 4.3 mg/g dry weight during rearing. The opposite was found for linoleic and linolenic acid, which increased from 1.9 and 1.5 mg/g dry weight at the start to about 6 and 15 mg/g dry weight at the end, respectively. Total feed ration affected the total HUFA, *n*-3 HUFA and DHA levels in the fish significantly. The statistical analysis revealed no significant treatment effects on total FAME and PUFA levels. Feed type had no significant effect on any of the tested parameters mentioned in Table 4. However, the statistical analysis of the growth, dry matter and *n*-3 HUFA data yielded F-values which were close to significant values ($0.1>P>0.05$) for a feed type effect (SGR) and for the interaction of feed type with ration. The presence of an interaction effect signifies that the effect of ration differed according to the feed type, hence there was at least a tendency for an indirect feed type effect on the tested parameters (Table 4). The type of enriched *Artemia* did have a significant ($P=0.0338$) effect on the retention of dietary EPA (20:5*n*-3) in the fish body. These retention values or conversion efficiencies of the different fatty acids are presented in Table 5. For most of the fatty acids, the retention varied from 10 to 40%. Only for 20:4 and 22:5, retention values close to or above 100% were found. Taking into account a digestibility efficiency for fat of 90-95%, retention values above 90% reflect the occurrence of biosynthesis.

TABLE 3: The fatty acid composition (mg/g dry weight) in feed (*Artemia*) and in fish larvae. Data are means of different samples. Standard deviations were omitted for clarity, but varied mostly between 5 and 10% of the mean.

	Feed		Fish						
				low HUFA			high HUFA		
Fatty Acid	Cocos	SELCO	Initial	0.1	0.2	0.3	0.1	0.2	0.3
N of samples	5	6	6	3	3	2	3	3	3
12:	13.0	0.2	-	0.3	3.1	3.0	0.1	0.1	0.1
14:0 + 14:1	13.6	4.4	0.3	2.7	6.8	6.8	2.2	3.7	2.6
15:0 + 15:1	-	-	0.1	-	-	-	-	-	-
16:0	23.6	21.9	13.9	11.5	18.1	16.9	14.8	19.2	15.0
16:1	5.4	7.5	0.2	1.6	3.5	3.1	3.3	5.3	3.5
18:0	9.6	9.6	9.2	7.1	9.7	8.6	8.3	9.8	8.5
18:1	53.3	54.1	9.6	19.0	34.3	30.6	27.3	38.9	28.4
18:2	12.9	12.3	1.9	4.5	7.6	6.1	5.6	7.7	5.4.
18:3 + 20:1	44.6	44.7	1.5	10.2	20.2	14.3	15.9	22.0	14.5
18:4 + 20:2	3.5	3.4	-	0.7	2.0	0.9	1.1	1.6	0.8
22:1	1.3	2.1	1.6	1.0	1.1	0.7	1.2	1.3	0.8
20:4	0.6	0.7	-	0.6	1.3	0.9	0.5	0.8	0.6
20:5	5.0	10.7	3.7	3.3	3.3	1.8	4.2	4.7	3.0
22:4	-	-	0.2	-	-	-	-	-	-
22:5	-	0.4	0.6	1.1	1.1	0.5	1.1	1.1	0.7
22:6	-	3.4	17.1	3.2	2.9	1.1	4.3	3.4	1.9
n.i.p.	-	0.1	0.7	-	0.3	-	0.1	-	
total PUFA	67.0	80.6	25.6	24.7	39.4	26.3	33.7	42.5	27.7
n-3 HUFA	5.0	14.5	21.4	7.6	7.3	3.4	9.6	9.2	5.6
Total FAME	172.4	178.7	60.6	67.6	115.1	95.2	89.6	119.6	85.8

Hepatic lipid and glycogen volume densities are given in Table 6. Both feed type and feeding level had a significant effect on these volume densities. Feeding level 0.1 resulted in low lipid content and a relative high glycogen content (Fig. 1). Increasing feeding levels led to a decrease of glycogen volume density and an increase of lipid (Fig. 2). Low HUFA-fed larvae showed higher values for glycogen than high HUFA-fed larvae. Lipid values were not different between the two feed types with exception for FL = 0.1 when low HUFA-fed larvae showed lower values for lipid.

Discussion

To assure a sound interpretation of nutrition results, survival and growth rates should approximate "normal" to maximal values. In the present study, survival rates varied roughly between 80 and 90% which was also found in other studies with these catfish larvae (Verreth

TABLE 4. Results of the statistical analysis (mean squares) on growth, dry matter content and fatty acid composition in larvae of *C. gariepinus*, when raised until a final weight of 50 mg. Mean squares are based on type III Sum of Squares. RU = recirculation unit, FT = feed type.

Parameter	Growth rate	Dry matter	Total Fame	Total PUFA	Total HUFA	*n*-3 HUFA	22:6*n*-3
RU	63.490	0.052	150.966	3.273	1.961	2.521	0.315
FT	377.135^{1}	1.506	1304.826	59.830	1.992	1.992	0.417
(RU x FT)	274.761^{1}	0.846	886.249	71.064	0.227	0.001	0.053
Ration	1954.864***	4.863***	113.616	8.254	21.324*	23.914**	6.590***
(FT x Ration)	542.348*	1.471^{1}	1203.565	29.948	6.886	7.936^{1}	1.638
Remainder (MSE)	83.573	0.412	461.513	58.539	2.497	2.171	0.515
df Remainder	12	12	11	11	11	11	11

*,** and *** significant at respectively $P < 0.05$, $P < 0.01$ and $P < 0.05$
^{1} $P < 0.10$

TABLE 5. The fatty acid retention (%) in larvae of the African catfish *Clarias gariepinus*, when fed low HUFA and high HUFA enriched *Artemia* at three different feeding levels.

	low HUFA			high HUFA		
Fatty acid	0.1	0.2	0.3	0.1	0.2	0.3
12:	4.1	12.7	10.1	9.9	30.8	19.5
14:0 + 14:1	10.3	26.8	22.3	18.1	39.9	21.6
16:0	23.8	39.9	31.3	23.0	40.7	24.8
16:1	15.0	34.0	25.7	15.9	33.3	17.3
18:0	35.7	51.9	38.9	28.9	47.3	31.8
18:1	17.9	34.0	25.4	17.9	34.0	19.3
18:2	17.5	31.0	21.0	16.3	29.4	16.1
18:3 + 20:1	11.7	24.1	14.5	12.9	23.4	11.9
18:4 + 20:2	10.8	29.7	11.9	11.7	21.9	8.9
22:1	36.0	44.7	21.0	18.5	27.1	13.7
20:4	54.6	113.4	65.0	23.8	50.9	32.0
20:5	32.3	34.4	14.6	13.2	20.5	10.1
22:5	-	-	-	90.9	129.2	62.9
22:6	-	-	-	34.5	40.7	16.1

and den Bieman, 1987; Haylor, 1992). Growth rates were of course affected by feeding levels but the obtained results approximated the values predetermined in the experimental design and therefore can be regarded as markers for the reliability of the experimental results.

The dry matter, protein and energy content of the two types of *Artemia* nauplii were only slightly affected by the enrichment with either a coconut-oil or with SELCO emulsion (Table 1). The main difference between both feed types was their *n*-3 HUFA content. As a

consequence, the treatment, e.g., feed type effects can indeed be attributed to the intended differences in dietary fatty acid composition. In cocos-enriched *Artemia*, the level of *n*-3 HUFA was approximately three times lower than in SELCO-enriched *Artemia*. Nevertheless, EFA-deficiency signs such as impaired growth, scoliosis or histopathological alterations of the liver were not observed in the course of the present experiment in larvae receiving cocos-enriched *Artemia*. Light microscopic and enzyme histochemical investigations on stomach or stomach anlage and on the intestine (data not presented here) gave no evidence for nutrition-related lesions. In addition, the present study did not reveal any treatment-related effects on larval survival. Apparently, fatty acids, essential for larvae of *Clarias gariepinus* were sufficiently available in both feed types.

The present study was designed to test whether changes in dietary fatty acid composition could alter the growth response of *Clarias gariepinus* larvae to *Artemia* as a proposed reference diet, and this effect was tested at three different feeding levels. The effect of feed type (dietary fatty acid profile) on production parameters such as growth and survival was not significant. As a matter of fact, only the fatty acid profile (area%) in the fish body (data not presented here) and 20:5*n*-3 retention was affected by the differences in feed type. At the onset of exogenous feeding, high levels of *n*-3 HUFA were found in larval *Clarias gariepinus* (Table 3). This is in agreement with published data on endogenous fatty acid profiles of just hatched teleost larvae (both marine and freshwater), which all show high concentrations of *n*-3 HUFA (Witt et al., 1984; Henderson and Tocher, 1987; Fraser et al., 1988; Watanabe, 1993). The strong decrease in HUFA levels during the experimental period may reflect the low dietary requirements of these freshwater larvae for *n*-3 and *n*-6 HUFA. This is further corroborated by the low retention percentages for most fatty acids (Table 5), suggesting that the majority of the ingested fatty acids are catabolized, and/or converted. At the same time these data may also suggest that in larvae of this catfish the bioconversion of C18 precursor fatty acids to HUFA occurs only at very low rates. This hypothesis would also explain the high concentrations of endogenous HUFA (especially 22:6*n*-3) found at the onset of exogenous feeding, e.g. they may have a "reserve" function, providing the larvae with these essential nutritional compounds during early life until the bioconversion pathways become functional. More evidence for this hypothesis can be found by investigating the different HUFA levels in body tissues of fish raised at different feeding levels (Table 3). At lower feeding levels, the period to reach the final weight was considerably longer than at high feeding levels. When the development of a functional system of desaturases and elongases to convert PUFA into HUFA is ontogenetically determined, the start of this bioconversion may be age rather than size related. In the aquaria with lower feeding levels

and extended rearing periods, later biosynthesis of HUFA may have affected the net decrease measured over the total rearing period. In this context, it is interesting to find evidence for biosynthesis of HUFA (20:4 and 22:5) in fish raised at lower feeding levels and not in fish raised at higher feeding levels (FL=0.3). At the same time, the concentration of these fatty acids, e.g., 22:5 in the body tissue was lower in fish raised at the higher feeding level. Although retention values suggest that at the highest feeding level, dietary inputs of 22:5 were given in excess, they were not sufficient to increase its tissue concentration to the levels found when biosynthesis occurred. In the same way, also the data of 22:6*n*-3 provide some evidence for the mentioned hypothesis. In younger animals (raised at higher feeding levels), DHA levels are consistently lower than in older animals. Further, in older animals (e.g. those raised at lower feeding levels), the presence or absence of dietary 22:6*n*-3 in the diet did not affect the levels of this fatty acid in the body fat (Table 3). Apparently, when the enzymes needed for biosynthesis of 22:6*n*-3 become functional, the larvae of *C. gariepinus* maintain a certain level of this fatty acid in their body fat, even when fed a diet lacking it. The relative unimportance of the dietary DHA input is further evidenced by the small but striking difference in body concentration of DHA (1.1 vs 1.9 mg/g) in fish receiving both diets at the highest feeding level (Table 3). Assuming that in these young fish no synthesis occurred, the enrichment of *Artemia* with substantial levels of DHA was not very effective in increasing body levels of this fatty acid.

The values for the fatty acid retention (Table 5) provide further indications for the fatty acid metabolism in *C. gariepinus* larvae. Fatty acids which show a low retention may be preferentially oxidized by the fish, while a high retention indicates a selective deposition. When comparing fatty acids in this way, also the overall dry matter conversion ratio should be taken into account. In the present study a dry:dry food conversion ratio of 3 was assumed in the experimental design. Previous studies (e.g., Verreth and den Bieman, 1987) demonstrated that feeding levels have a strong impact on the final food conversion ratios, with the highest ratios (2.5) at intermediate feeding levels (FL=0.2). Translated to the present study, it explains why the highest retentions were consistently found at the optimal feeding level (FL=0.2). At the same time, comparison with the overall dry matter conversion ratio provide a cue for assessing the relative importance of the different fatty acids in larval *C. gariepinus*. Fatty acids which are deposited in the larval body at lower rates than the overall dry matter conversion ratio, e.g., 30-40%, are presumably preferentially catabolized or converted into other compounds. In the present study this was the case for most saturates, monoenes and the C18-fatty acids. The low retention values of the C18-fatty acids are probably a reflection of their bioconversion to C20 and C22 HUFA. In this respect, it is

interesting to note that fatty acids with a retention value above the assumed overall dry matter conversion (indicating active deposition) belonged all to the C20 and C22 family (Table 5). The relative low retention values for *n*-3 HUFA such as EPA (20:5) and DHA (22:6) are an indication that dietary levels of these fatty acids were above requirement levels for this freshwater fish or that the dietary fatty acids were not presented in a form suitable for deposition in body tissues. Presumably, the enrichment procedure results in increased levels of triglycerides or of the free fatty acid pool in *Artemia*, while the fish larvae may have a stronger preference for fatty acids esterified to phospholipids (Sargent et al., 1993). Also in *C. gariepinus* this may be the case. Verreth et al. (1994) revealed that phospholipids accounted for 75 to 80% of total lipid in eggs and yolk sac larvae of this species and that all yolk triglycerides were preferentially catabolized before yolk absorption. Providing elevated dietary HUFA levels as free fatty acids or as triglycerides may result in a relative large proportion of catabolization of these fatty acids and decrease the effectiveness of the enrichment procedure.

The results of 22:5 are quite remarkable. The data reveal unambiguously that synthesis of 22:5 occurred. Unfortunately, the GC analysis did not differentiate between the *n*-3 and *n*-6 homologues. Synthesis of 22:5*n*-6 would fit well with the evidence for synthesis of 20:4*n*-6, and would corroborate with the higher requirements for *n*-6 PUFA found in other tropical freshwater fish (f.ex. tilapia species) (Kanazawa et al., 1980, in: Sargent et al., 1989). On the other hand, there are two arguments to deny this hypothesis. First, in the present study the tissue concentrations of 22:5 seem to increase and decrease in proportion to 20:5 (the ratios between 20:5 and 22:5 were 3-3.6 and 3.8-4.3 for low HUFA and high HUFA fed larvae respectively) and not to 20:4, suggesting that it is 22:5*n*-3. Second, *C. gariepinus* is an omnivorous predatory fish, which feeds mainly on fish, crustaceans and minor amounts of insect larvae (Bruton, 1979). A prominent role of *n*-6 PUFA in the diet would suggest an herbivorous feeding mode and this is not the case for this species. Obviously, more detailed research is necessary to elucidate the specific requirements and the role of the different *n*-6 and *n*-3 PUFA in *C. gariepinus*.

Feed type also influenced the deposition of lipid in the liver of larval *Clarias gariepinus*. For the feeding level FL = 0.1, lipid volume densities in the liver of larvae fed with high HUFA enriched *Artemia* were markedly higher than in livers of larvae fed with low HUFA enriched *Artemia*. They were slightly higher for feeding levels FL = 0.2 and 0.3. The higher hepatic lipid content of larvae fed with high HUFA enriched *Artemia* may be explained by energetic and digestibility considerations. Austreng et al. (1979) and Takeuchi

TABLE 6. Volume densities (cm^3/cm^3) of hepatocellular lipid droplets and glycogen fields (mean ± SD), as determined through stereometric assessment. As reference volume, the total hepatocyte volume was taken.

Feed type	Feeding level	Glycogen	Lipid
Cocos	0.1	0.35 (± 0.10)	0.02 (± 0.006)
	0.2	0.34 (± 0.12)	0.41 (± 0.03)
	0.3	0.25 (± 0.05)	0.41 (± 0.04)
SELCO	0.1	0.28 (± 0.09)	0.18 (± 0.08)
	0.2	0.13 (± 0.02)	0.44 (± 0.07)
	0.3	0.13 (± 0.03)	0.42 (± 0.05)

et al. (1979) demonstrated that in fish, unsaturated lipids are better digested than saturated ones because of a preferential hydrolysis by the pancreatic lipases of glycerolipids which contain unsaturated fatty acids (Lie et al., 1986). Consequently, absorption of lipids from high HUFA enriched *Artemia* may be higher than those from the low HUFA diet. Additionally, the energy content of high HUFA enriched *Artemia* (29 kJ/g) was higher than that of low HUFA enriched *Artemia* (24 kJ/g) (Table 1). Both factors together may have resulted in a higher energy and lipid input when the high HUFA diet was given, leading to a higher hepatic lipid storage (Schwarz et al., 1988). Obviously, this feed type effect is more prominent at lower intake levels of dietary lipid, e.g. FL=0.1, where the high HUFA diet resulted in a lipid volume density of 0.18 cm^3/cm^3 versus only 0.02 cm^3/cm^3 for the group receiving the low HUFA diet. At higher feeding levels, the importance of this feed type effect on liver lipid density decreased suggesting another factor to be more determinant for liver lipid storage. In the present study, feeding level proved to be the most decisive parameter for larval growth and metabolic performance of the liver. In both types of *Artemia*, higher feeding levels resulted in increased volume densities of hepatic lipid while glycogen densities were decreasing. Increased lipid deposition with increased feeding level may be a consequence of the enhanced lipid intake of the fish. At low feeding levels, the comparatively small amount of ingested lipid may be almost completely oxidized for energy expenditure. With increasing lipid intake, an increasing "surplus" of lipid is ingested and deposited in the liver. However, at the higher feeding levels, the volume densities of lipid in the liver cells of larval *C. gariepinus* are remarkably high : about 40 % of the cell volume consists of lipid, while in adult specimens of *C. gariepinus*, the lipid volume density in the liver was only 5 % (Segner, unpublished data). Therefore, besides the effect of "surplus" energy intake, other factors such as ontogenetic effects may have influenced the results of the present study. The present study does not allow to distinguish between dietary and ontogenetic effects since at the end of the experiment, the age of the fish differed according

Figure 1. EM view (6800 x) of the hepatocyte ultrastructure in larvae of the African catfish *C. gariepinus*, which received low amounts (FL=0.1) of SELCO-enriched *Artemia*, at the end of the experimental period, i.e. 21 days after start of exogenous feeding and a larval weight of 50 mg/ind. The dominant energy reserve is glycogen. N = nucleus; m = mitochondrion; gl = glycogen.

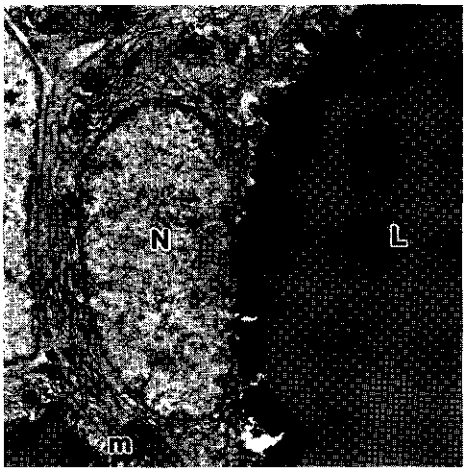

Figure 2. EM view (7500 x) of the hepatocyte ultrastructure in larvae of the African catfish *C. gariepinus*, which received large amounts (FL=0.3) of SELCO-enriched *Artemia*, at the end of the experiment, i.e. 9 days after start of exogenous feeding and a larval weight of 50 mg. The dominant energy reserve is lipid. N = nucleus; m = mitochondrion; L=lipid vacuole.

to the feeding level. The decreasing values for glycogen must not be related to concomitant changes in the biochemical glycogen concentration. The stereological procedure applied in the present study results in a relative measurement of glycogen, especially at the strong changes in lipid volume densities occurring in the present study. Higher feeding levels affected significantly the dry matter content of the fish larvae, thereby suggesting that the body lipid content increased with increased lipid intake. However, assuming that total FAME values would give an indication for body lipid content, the results (Table 3) were not conclusive.

In conclusion, rearing larval *Clarias gariepinus* with a diet low in HUFA (coconut-oil enriched *Artemia*) did not reduce growth nor survival when compared to larvae receiving a diet rich in HUFA (SELCO-enriched *Artemia*). The data suggest the presence of HUFA biosynthesis in larvae which were raised to the final size over a longer period, implying that the enzymes needed for desaturation and elongation of dietary precursor fatty acids become functional in a later stage of the larval period. The effect of increased dietary *n*-3 HUFA

levels on liver energy stores was restricted to an enhanced lipid deposition at a low feed intake in the groups receiving high dietary HUFA levels. The feeding level affected both growth and metabolic parameters. Feeding the larvae at optimal (FL = 0.2) or satiation (FL = 0.3) levels resulted in an extremely high liver lipid storage. A parallel increase of body lipid concentration could be assumed from increased body dry matter content, although in this respect, total FAME values were not conclusive. At low feeding level (FL = 0.1), glycogen was the dominant liver energy store. Therefore, we conclude that the fatty acid composition of *Artemia* will not alter its performance as a reference diet in nutritional studies with larval African catfish.

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GENERAL DISCUSSION

Development and nutrition in fish larvae, with special reference to *Clarias gariepinus*

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Introduction

The objective of the present study was to assess which aspects in the development of larval African catfish *Clarias gariepinus* are critical for the formulation of a (dry) experimental diet. Our hypothesis was that changes in larval performance and their ability to use juvenile diets are associated with changes in development and metabolism. Therefore, this relation was the subject of the present research project. First we analyzed the conditions needed for optimal growth and rearing (Chapters 1 and 2). Thereafter, the morpho-functional and physiological development of the digestive system (Chapters 3 to 5) was investigated. Finally, the dynamics of lipids and fatty acids in the larvae (Chapters 6 to 8) were studied in relation to development. In accordance with these objectives and working hypothesis, in the present chapter the following questions will be addressed: (a)what is the relation between larval development and digestive and metabolic capacities of the growing larva; (b)what is the impact of these ontogenetic aspects on the design of larval nutrition studies and on the strategies for dry diet formulation; (c)what is the impact of development on larval nutrient requirements, with special reference to lipids; (d)are the results of the present study extendable to other fish species and if so, what precautions have to be taken.

Developmental aspects in *Clarias gariepinus* larvae.

In the past, the necessity to feed fish larvae with living organisms and the high sensitivity of the larvae to stress and mortality were attributed to the larva's "poor" functional development. This concept is not correct. Fish larvae are specialized organisms which evolved numerous and specific adaptations to the constraints set by their tiny size and the environment (Osse and van den Boogaart 1994; Segner et al. 1994; Dabrowski and Culver 1991). They are not "poorly" but well adapted to these constraints. A detailed knowledge of these adaptations is desirable to design adequate rearing and feeding strategies. The present thesis contains some contributions to this endeavour (Chapters 3, 4 and 5) for the specific case of the African catfish, *Clarias gariepinus*. Additional information on this species is accessible in the literature. Vandewalle et al. (1985), Surlemont and Vandewalle (1991) and Vandewalle et al. (1993) reported on the development of the skull and the associated mechanics for buccal and opercular respiration. General information on the embryonal development can be obtained from Bruton (1979), and Légendre and Teugels (1991). The stomach development was described by Stroband and Kroon (1981), and the muscle development and swimming capacities by Akster et al. (1994). Finally, several MSc

thesis reports (e.g., Torreele 1990; Spazier 1990; Unger 1990; Flach 1991; Schwager 1992, Bergwerff 1992 a.o., unpublished) were carried out as part of the present research and contain information on the functional development of *C. gariepinus* larvae which has still to be published. The picture that emerges from all these studies is of a fish species which hatches at a rather early stage of development. It undergoes a very fast (about 2 weeks) development into a young juvenile with functional sensory, digestive, locomotive and (air-) breathing organs. As such, it remains in line with the majority of freshwater fish species which have a distinct larval period (Balon 1975, Dabrowski 1984).

To assess the relation between development and nutritional physiology of the fish larvae, it is necessary to characterize the larval development and to assess where larvae differ from the embryos and juveniles. In line with the concept of saltatory ontogeny (Balon 1975, 1984) two thresholds delineate the start and the end of the larval period, e.g., the transition of endotrophy to exotrophy and the metamorphosis from the larval into the juvenile period. In the subsequent paragraphs, these two thresholds will be treated separately.

TRANSITION FROM ENDOGENOUS TO EXOGENOUS FEEDING

Early development

At hatching, *Clarias gariepinus* (± 4 mm) has no mouth or anus. The alimentary canal consists of a straight and histologically undifferentiated tube lined by a monolayer of epithelial cells. Goblet cells are absent. Liver and pancreas are present as a clump of cells instead of as distinct organs. The heart is beating already and blood vessels are visible on light microscopical sections (Torreele 1990, Spierts 1993, unpublished). Ten hours later (35 h post fertilization), the larvae possess a mouth, a nose sac, eyes, incomplete gill arches and an alimentary canal with open lumen which is differentiated in foregut, midgut and hindgut. According to the nomenclature of Stroband and Dabrowski (1982) the midgut and hindgut are also differentiated in intestine I (absorption zone, generally comparable with midgut), intestine II (a zone with many supranuclear vacuoles and prevailing pinocytosis) and intestine III (with no distinct absorptive structures). Also kidney tubuli start to develop.

Changes related to feed intake

One day after hatching (48 h post fertilization) the yolk sac larvae have an open mouth and some mandibular teeth. Also the operculum appears at this stage. Head and jaw movements needed for buccal aspiration and for capturing prey are not yet feasible (Surlemont and Vandewalle 1991). A swimbladder with ductus pneumaticus is present and

this may facilitate buoyancy and swimming. According to Spierts (1993), swimming is still anguilliform and normal speed levels attain about 2 Body Lengths/sec. Because of the low Reynolds numbers, viscous forces dominate in the animal's environment and because of the drag of the surrounding water locomotion costs much energy. On the second day of the yolk sac period (from 48 to 72 hours post fertilization), the cartilaginous skull develops further into a distinct neuro- and splanchnocranium, allowing the buccal pump to function and thus the animal to capture prey by suction feeding (Drost and van den Boogaart 1986). However, the movements related with prey suction imply a good coordination of the cranial muscles. According to Surlemont and Vandewalle (1991), at this stage the innervation of the cranial muscles is not yet completed. These authors hypothesize that at this stage, prey ingestion through suction forces is still inefficient. The liver and pancreas are developed into distinct organs. The oesophagus and the whole gut lumen is open, indicating the physical ability of the embryo to ingest food. Pharyngeal teeth needed to crush the ingested food, start to develop as well. Mucus producing cells and mucus needed in the oesophagus to transport food from the buccal cavity into the stomach anlage appear somewhat later (at the end of the yolk sac period (66-75 h post fertilization).

Changes related to feed digestion and absorption

At the end of the yolk sac stage, the digestive tract consists of a rather straight tube, which is differentiated into a pharynx, an oesophagus, a stomach anlage and three intestinal regions (Stroband and Kroon 1981; Verreth et al. 1992). The length of the intestine is about 36% of total fish length which is rather short but very similar to the relative intestinal lengths in turbot (*Scophthalmus maximus*), whitefish (*Coregonus lavaretus*) (Segner et al. 1993) and roach (*Rutilus rutilus*) (Hofer and Nasir Uddin 1985). In spite of the short passage times of chyme through the gut, the digestive and absorptive surfaces are increased by small foldings of the intestinal mucosa and microvilli at the luminal border of the enterocytes. Also the enterocytes in intestine I are fully ready for nutrient uptake (Table 1). Also in other teleost species the morpho-functional development of the intestine is ready for nutrient uptake at the transition of endotrophy to exotrophy (Stroband and Dabrowski 1982; Govoni et al. 1986; Deplano et al. 1991; Segner et al. 1993, 1994). Further, in *C. gariepinus* Schwager (1992, unpublished data) revealed also intense lipid absorption a few hours after the start of exogenous feeding.

Also from a physiological point of view, the animals have developed the capacities to digest and absorb at the onset of exogenous feeding (Chapters 4, 5, Verreth et al. 1993; Segner et al. 1993). At the end of the yolk sac stage, small amounts of zymogen granules are found in the exocrine pancreas and their presence becomes very distinct when exogenous

Table 1. Characteristics of the enterocytes in larval African catfish at the start of exogenous feeding. X=present; XX=well developed or abundant; 0=absent.

Characteristics and cell components	
microvilli	XX
folding of intestinal mucosa	X
terminal web	X
rough endoplasmatic reticulum	XX
smooth endoplasmatic reticulum	XX
Mitochondria	XX
Golgi apparatus	X
Cytoplasmic Lipid droplets	0

feeding starts. Segner et al. (1993) and Verreth et al. (1993) presented data on the development of pancreatic proteolytic activity in larval *C. gariepinus*. At the start of exogenous feeding, the activity of alkaline proteases (trypsin, chymotrypsin) is well developed (18.7 mg tyrosine/h/g fresh sample) (Segner et al. 1993). Also several brush border enzymes, e.g., aminopeptidase, alkaline phosphatase and maltase, were histochemically detected (Chapter 4; Segner et al. 1993).

Identification of several gastro-entero-pancreatic (GEP) hormones which regulate gut motility and the secretion of pancreatic juices (Holmgren 1985), indicate that at the start of exogenous feeding, the larvae possess the ability for hormonal control of the digestive process (unpublished data). In the gut, the hormone producing cells for metenkephalin and serotonin were found from the start of exogenous feeding onwards (Spazier 1990), indicating that the digestion process was associated with gut motility. The presence of gastrin/CCK like producing cells in the intestine shows a coordinated digestive process, e.g., the hormone regulated secretion of pancreatic enzymes and bile. The three pancreatic hormone producing cells, e.g., insulin, glucagon and somatostatin, were all detected in the endocrine pancreas before the completion of the yolk sac period (Verreth et al. 1992; Spazier, 1990; Unger 1992), showing the possibility to control the metabolic process from the start of exogenous feeding onwards. The liver, the most important organ for nutrient metabolism, is fully differentiated at the start of exogenous feeding (Segner et al. 1993; Flach 1990, unpublished). Finally, key enzymes of most pathways of the intermediary metabolism are functioning from the start of feeding onwards (Chapter 5).

To conclude, the yolk sac period is used for organ formation and preparing the animal for exotrophous life. At the start of exogenous feeding, the catfish larvae are capable to

detect, capture, crush and ingest prey and to digest, absorb and utilize the major nutrients. From the point of view of natural history, this is quite logic. The only way to overcome a period of dependence on exogenous food sources is by having the necessary functioning organs and enzymes for digestion, absorption and metabolism. This happens at this stage. Similar results were found in several other larval species, e.g. whitefish, *Coregonus lavaretus* (Segner et al. 1989, 1993); turbot, *Scophthalmus maximus* (Cousin and Baudin-Laurencin 1985; Cousin et al. 1987; Segner et al. 1994); cod, *Gadus morhua* (Kjørsvik et al. 1991); halibut *Hippoglossus hippoglossus* (Pittman et al. 1990); seabass, *Dicentrarchus labrax* (Diaz et al. 1989, Deplano et al. 1991) etc. The question remains why most fish species need live food at the start of exogenous feeding. To answer that question, also the differences between larvae and juveniles need to be investigated.

TRANSITION FROM LARVAL TO JUVENILE CHARACTERISTICS

Changes related to feed intake

To our knowledge, to date there are no reports on the development of the neural and sensory system in the African catfish *C. gariepinus*. In contrast to the situation in marine fish, in this catfish species most developmental changes in the sensory system may occur in the embryonal period. In any case, at the onset of exogenous feeding, functional eyes are present, but there is no information on the stage of development of the retina. A similar lack of knowledge exists related to the tactile and olfactory sensory systems. More information is available about changes in the systems for locomotion and respiration. Akster et al (1993) and Spierts (1993, unpublished data) analyzed the swimming behaviour and associated hydrodynamics in *C. gariepinus* larvae. At sizes above 10-11 mm (i.e. about 3-5 days after the start of exogenous feeding) the fish larvae swim in an environment where inertial forces dominate and where the drag forces of the water become less important. Concomitant with this, also the mode of swimming changes from the earlier anguilliform and continuous swimming to a carangiform, e.g., a more juvenile type of swimming.

In the growing *C. gariepinus* larva, the change in swimming mode is accompanied by a relative increase in the white muscle mass in the body (Akster et al. 1993; Spierts 1993, unpublished data). Between a size of 5 to 15 mm body length, the ratio red/white muscles decreased steeply from about 0.16 to about 0.05 whereafter the decline levelled off. The muscle fibre differentiation showed a parallel development. Juvenile red muscle fibres appear during the first five days of exogenous feeding, and in the same period the embryonic inner white muscle mass differentiates into juvenile white muscle fibres. In contrast to smaller sizes, at a body length of ± 10 mm, almost no enzymes representative for aerobic

metabolism, could be detected in the white muscle fibres (Berbner 1993, unpublished data). From this size onwards, white muscle tissue starts to develop strongly.

This ontogenetic development of muscle differentiation and swimming performance coincides with the transition from cutaneous to gill respiration which occurs at a size of about 20 mg (Segner, unpublished data; see also chapter 5). Such a functional relationship between muscle and gill development has been demonstrated in many different fish species (Hinterleitner et al. 1987, El-Fiky and Wieser 1988, Segner et al. 1994) and is regarded as a basic aspect of fish metamorphosis (Forstner et al. 1983).

Changes related to feed digestion

At the onset of exogenous feeding the morpho-functional development of the digestive system has not been completed yet in *C. gariepinus* (see also chapter 4). At start feeding, the stomach is not differentiated and not functional (Stroband and Kroon 1981; chapter 4). However, at temperatures of 28-30 °C, it takes only 4-5 days from start feeding to complete the morphological and physiological development of the stomach. Pepsin-like activity was detected from the third day after start feeding onwards (Verreth et al. 1993), which coincides with the first appearance of active glandular tissue in the stomach wall (Segner et al. 1993) and with the decrease of the intra-luminal stomach pH. The experimental assays for pepsin-like activity reached a maximum of 35 mg tyrosine/h/g, a few days (on day 8 after the onset of exogenous feeding) after the completion of stomach functions (Segner et al. 1993). At that age (day 8 after start of feeding) the activities of acid and alkaline proteases were about the same, giving a total proteolytic activity of approximately 70 mg tyrosine/h/g. Interestingly, this is about the same level of total proteolytic activity as found on day 3 after the start of exogenous feeding. As in most fish larvae, during the first days of exogenous feeding, only tryptic activity was detected. It increased to a peak value of about 70 mg tyrosine/h/g at day 3 after the start of exogenous feeding where-after it decreased continuously during the rest of the larval period (Verreth et al. 1993). This suggests that the increasing activities of pepsin were rather replacing than adding to the existing trypsin activity. In other words, the onset of stomach functions changed the total proteolytic capacity of the growing larva only qualitatively and not quantitatively. This is a most interesting conclusion, because it suggests that at this stage of development, not the quantity but the "type" of the ingested proteins which can be digested is affected by the stomach functions. The significance of this development in terms of life history traits may be a sudden widening of the food spectrum for the larvae (Forstner et al. 1983; Segner et al. 1993, 1994). The onset of stomach functions may enlarge the feeding niche of the larvae significantly, thereby increasing their "ecological flexibility".

THE CONCEPT OF METAMORPHOSIS IN *CLARIAS GARIEPINUS*

At the onset of exogenous feeding, *Clarias* larvae can detect, capture, ingest and digest food items. They have functional sensory (eyes, olfactory tissue, barbels) and digestive (intestine, pancreas, liver) organs. They differ from juveniles by the absence of branchial respiration, juvenile muscle types and the associated anaerobic energy fuelling, and by the absence of a functional stomach. All the latter functions develop at a size of 10-15 mm, e.g. a weight of 15-20 mg. Such a simultaneous development of functions is typical for a "threshold" in the concept of saltatory ontogeny (Balon 1975, 1984). Whether it can be regarded as metamorphosis in *C. gariepinus*, is still unclear. Life history specialists define usually the transition from a larva into a juvenile by morphological characteristics, e.g., differentiation of the median finfold, the presence of paired fins, calcification of the axial skeleton. In *C. gariepinus*, the median finfold differentiation and the fin ray formation is completed at a size of about 30 mm, i.e. about 15 days after the onset of exogenous feeding (18 days post fertilization) (Hecht and Appelbaum 1987, Legendre and Teugels 1991). Haylor (1992) stated that the onset of air breathing in African catfish coincides with the differentiation of the fins. He considered the onset of air breathing as a useful marker for the end of the larval period. Haylor and Oyegunwa (1993) provided quantitative relations between temperature, fish size and the onset of air breathing. Using these relations, at 27.5 °C the larval period in *C. gariepinus* would end at a total length of 21 mm or a weight of 90 mg. This coincides with the usual duration of the experiments in the present study (10 days of exogenous feeding) and also with the rearing and feeding protocols applied in practice (see General Introduction). From a physiological point of view, it can be argued whether the mentioned changes can be considered as distinguishing factors between the larval and juvenile period. Which switches in functions are reflected by these ontogenetic events ? When discussing this question, the ecological significance of the different functional changes should receive special attention. The change from a reactive to a resistive form of swimming which represents a genuine change in swimming mode (Akster et al. 1993) opens new possibilities to capture prey, to escape predation and thus to enter new habitats. The coinciding differentiation of the muscle fibres into adult-like types, coupled with the change to glycolysis and with the switch from cutaneous to branchial respiration may constitute another major bench mark in the interaction between environment and organism. Further, the presence of a stomach widens the feeding niche of the animal and increases thereby its fitness to survive. The fact that the onset of stomach functions, gill respiration, the glycolytic pathway, differentiation into adult-like muscle types and the changes in metabolic enzyme activities, all occur simultaneously, provides a strong argument to use these functional changes as the delineation of the larval period, e.g. metamorphosis.

The impact of development on larval nutrition studies

In larval nutrition, a correct definition of developmental stages is obvious for the design of experiments and for the interpretation of results (this thesis, Balon 1984). The problem however is how to apply this knowledge in experimental conditions. The easiest and thus most widely applied approach is by using age as a marker for development. Also in the present research project, this approach was followed, and all experiments had a duration of 10 days after the start of feeding. For practical rearing conditions, scaling developmental stages and the concurrent changes in feeding protocols by time is attractive because of its simplicity and the possibility to relate working schedules to it. Research on larval nutrition, with the aim to elucidate patterns and mechanisms needs better parameters. The question is which parameter for staging should then be selected; how generally applicable is this parameter (e.g., what is the effect of different temperatures) and how to apply such a parameter in the design and execution of nutritional experiments with larvae.

Size and Development

From studies of enzyme activities and development (see Chapter 5) and from studies of muscle fibre differentiation (Akster et al. 1993) it is clear that size (length and/or weight) is a better parameter for development than age. The size at which developmental events in *C. gariepinus* occurred remained rather constant in contrast to age which varied according to differences in growth rate under different feeding regimes (chapter 3; Akster et al. 1994).

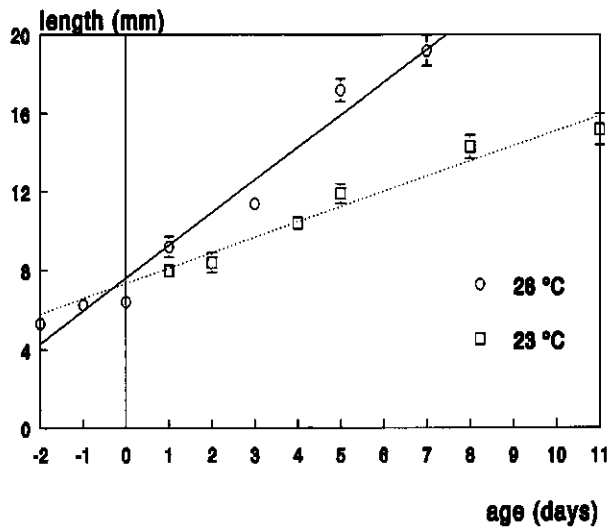

Figure 1 Growth rate of *C. gariepinus* larvae reared at 23 °C (Stroband and Kroon 1981) and at 28 °C (Verreth et al. 1992). Lines are regression curves for the respective studies. Day 0 refers to the start of exogenous feeding.

However, for a given species also size at development is not constant. In a study on developmental responses of carp, *Cyprinus carpio* to different dietary

Table 2. Relation between egg size and female size in different studies on *C. gariepinus*. With exception of one study (Kamler et al. 1994), all data are derived from reproduction trials at the experimental facility in Wageningen, The Netherlands (the same genetic background).

Data base	Eggs	Females		
	Diameter (mm)	Dry Weight (mg)[1]	n	Wet Weight (g)[2]
		0.354	2	
		0.480	2	
Wageningen (1993)	>1	0.297	2	(1013-1380)
Wageningen (1985)	1.27	0.284		
	1.22	0.287		
	1.2	0.297	3	
	1.25	0.290		
	1.33	0.308		(900-1100)
	1.3	0.319	3	
Wageningen (1986)		0.356	3	(521-705)
Wageningen (1993)		0.38	4	(1075-1468)
Wageningen (1992)	>1.1	0.350	5	(862-1998)
Wageningen (1991)	>1.05	0.43	4	(772-1556)
Wageningen (1994)		0.398	5	(822-1080)
Wageningen (1991)		0.385	6	(600-900)
Poland		0.413	1	2800

[1] Means of minimal 3 samples
[2] minimum and maximum sizes used

regimes, Kamler et al. (1990) showed that diet, hence growth rate, affected the size (both in terms of weight and length) at which the different developmental steps were attained. Other evidence for this conclusion can be derived by comparing the studies of Stroband and Kroon (1981) and Verreth et al. (1992, chapter 4). Both studies describe the development of a functional stomach in *C. gariepinus*, but were carried out at different temperatures (23 vs 27.5 °C). In the former study, the development of a functional stomach was completed at 11.5 mm. In the study of Verreth et al. (1992), this size was attained after 3 days of exogenous feeding (Figure 2). In the latter study, gastric glands were found but the intraluminal pH in the stomach was still higher than 4. Apparently, environmental factors, e.g., food and temperature, have different effects on growth rates and on developmental rates. Also biotic factors affect the relationship between size and development. At hatching, *C. gariepinus* larvae measure 3.6-5.3 mm depending upon the source and experiment. Similar high variations are reported for sizes at later stages in development. Surlemont and Vandewalle (1991) reported a size of 4.7 mm at 38 hours post fertilization while in our own studies, larval length at this age varied from about 5 to 5.8 mm. Similarly, our experience reveals that at the onset of exogenous feeding larval wet weight varies between 2.2 and 3.2 mg. Osse and van den Boogaart (1994) compared egg size and fish lengths at different

ontogenetic events in 11 salmonoid species. They showed that size at hatching, at the start of exogenous feeding, at the transition to the juvenile stage all increase with increasing egg weight. This relation between egg and larval size may also be present for intraspecific variation in egg size. In our experience, egg sizes in *C. gariepinus* vary strongly from batch to batch (Table 2). This may be related to the size of the broodstock, but as Table 2 demonstrates, also other factors interfere, e.g., age of the postvitellogenic oocytes, genetic background, etc. (Richter et al. 1994).

In conclusion, relating developmental events with size and/or age must be done with caution. For the design of larval nutrition experiments, the best approach would be to base the time of sampling and feeding schemes on ontogenetic stages. This implies morpho-functional parameters. Such parameters are not easily incorporated within the experiments. Staging by size remains then the next best solution.

Temperature and Development

In poikilothermic animals, temperature has an overruling effect on all metabolic rates, including development. According to Fry (1947, cited by Blaxter 1992), five categories of temperature effects on early life stages of fish can be defined, e.g. lethal, controlling, directing, limiting and masking effects. In the context of the present discussion, only the controlling category, i.e. those effects which pace the biochemical and physiological reactions (Blaxter 1992), are of importance.

To adjust for temperature differences between different species, locations and experiments, the rate of development is usually expressed as *Day-degrees* (D°), i.e. the product of average temperature and development time (in days). However, day-degrees do not correct sufficiently for changes in temperature. As an alternative, in the present study the concept of *Physiological Day-degrees* (PD°) was used (see chapters 6 and 7) as a temperature independent parameter for development time. The concept was introduced by Huisman (1974) to estimate the time needed to complete vitellogenesis in carp breeders which were held at ambient temperatures. Basically, it adjusts the number of day-degrees to a standard temperature of 20 °C by using the correction factor (q) derived by Winberg (1956) from the "normal curve" of Ege and Krogh (1914). This curve relates temperature to oxygen consumption at routine metabolism. Winberg (1956) recognized that values of this curve can be approximated by using adequate Q_{10}-factor values for the different temperature intervals. This opened the way for adjusting experimental data to a theoretical standard temperature. Following these relations, he calculated the conversion factors q to

rate experimental data at a standard temperature of 20 °C. Several studies which were carried out in the course of the present research project revealed that development time (f.ex. the time needed to reach complete yolk absorption, i.e. to pass the embryonal period) is reasonably stabilized by PD° (Conceição et al. 1993, Verreth et al. 1994, see also chapters 6 and 7). The embryonal period (from fertilization to yolk absorption) lasts 200 PD°, but among individual experiments it ranges from 190 to 210 PD°, and the last yolk remnants disappear even later, around 240-250 PD°. In the same way, also other developmental intervals, e.g. from fertilization to hatching or to metamorphosis, can be estimated and used in experimental designs. In a recent study on temperature induced changes of early development in *C. gariepinus*, Kamler et al. (1994) compared different parameters to rate developmental advancement. They concluded that none of the mentioned parameters, D°, PD° or Q_{10} are suitable to make development time completely temperature independent. They propose a new index, e.g., *Effective day-degrees*, $D°_{eff} = t(T-T_0)$, where T is the actual temperature, T_0 is the threshold temperature at which development is arrested and t the time (in days) to reach a given developmental stage. In *C. gariepinus*, the threshold temperature would be 17.36°C (Kamler et al. 1994). When recalculating some of our data, the suggestion of Kamler et al. (1994) that $D°_{eff}$ is a more temperature independent index of developmental advancement, seems to be confirmed. It is therefore suggested to apply this parameter in the design of experiments and for determining sampling times in larval nutrition studies.

GROWTH AND DEVELOPMENT

An interesting example of the interaction between development and larval growth is presented by Haylor (1992). In an attempt to prove that the cube root growth model, as applied in the present study (Chapter 2), is not a valid alternative for the exponential growth model, he demonstrated that in larval *C. gariepinus* growth rate fluctuates strongly during the larval period, irrespective of the growth model applied. Interestingly, two dips in growth rate can be observed, one at the very start of exogenous feeding and one at the onset of air breathing. These two points coincide with the two ontogenetic thresholds marking the start and (possibly) the end of the larval period (see above). Our own experiments are not long enough to confirm the latter dip in growth rate. Usually increased growth rates are found during the last days of our feeding experiments, e.g., once the larvae have developed a functional stomach (between days 6 and 10)(Verreth, unpublished data). On the other hand, a temporary decrease in growth rates of the catfish larvae can always be observed when they are weaned from one diet type to another. As was shown earlier, under the prevailing conditions in our hatchery, the time of weaning is close to the onset of air breathing.

The first dip in growth rate may be a general feature in the development of larval fish, as it has been found in other larval species as well (Wieser et al. 1988b). Also in our experiments it was found regularly. Especially during the very first day of exogenous feeding, the larvae seem to have problems in starting to eat. This observation corroborates with the suggestion of Surlemont and Vandewalle (1991) that at the start of exogenous feeding, the movements of mouth, jaw and buccal aspiration are still not well coordinated and therefore may decrease the efficiency of prey capture. As in most fishes, in *C. gariepinus* exogenous feeding starts when still some remnants of the yolk sac are present. In *Clarias*, this mixed endo-exotrophic period lasts only a few hours. However, in many species it lasts considerably longer, e.g., 14-16 days in pike-perch (*Stizostedion lucioperca*) (Mani-Ponset et al. 1994). Studies on digestive enzymes have shown that in many species, the enzymatic activities increase only gradually after the start of exogenous feeding, and it takes some time before peak values are obtained (Cousin et al. 1987; Dabrowski and Culver 1991). Also in *C. gariepinus* the total proteolytic activity increases from about 19 to 70 mg tyrosine/h/g during the first three days after the start of exogenous feeding (Segner et al. 1993; Verreth et al. 1993). During the extended endo-exotrophic phase in pike-perch, low initial synthesis levels of pancreatic lipase or of bile salts were also related to a temporary reduction in lipid absorption, which occurred in spite of a sufficient lipid intake (Mani-Ponset et al. 1994). Such detailed information is not available for the African catfish. However, unpublished information on ultrastructural aspects of larval lipid absorption (Schwager 1992) showed also in *C. gariepinus* the first signs of lipid absorption only 6-8 hours after the first food supply. In conclusion, although at the onset of exogenous feeding the morpho-functional development is ready to enable food ingestion and digestion, in practice the animal seems to have difficulties in "switching on" these functions, both in terms of ingestion as digestion. The dip in growth rate found at the transition of endotrophy to exotrophy may be related to these factors.

Design of experiments in larval nutrition

Since growth can be considered as the net resultant of all positive and negative factors, the mentioned interactions between development, temperature and size will affect the way growth can and should be measured in nutritional experiments with larvae. Most studies on larviculture present growth results as increments of body length (mm) or of body weight (mg) during the experimental period. Such an approach is suitable to determine : "which of the tested treatments gives the highest growth". It does not provide any background information of the underlying dynamics. Rates of growth are more suitable for this purpose. In fish nutrition studies, mostly the exponential growth model is applied, and over short

weight intervals, the relative (specific) growth rate remains constant (Brett 1979). This is not so for larger weight ranges because, as in most animals, growth rate in fish changes allometrically with body size ($W^{0.8}$). When compared to body size, the weight increase in larvae is of the same order of magnitude as in juveniles. As a consequence, also in larvae the specific growth rate decreases with size (Verreth 1987, see also chapter 2). This has a very practical implication on the design of larval feeding experiments because feeding levels should be adjusted daily or food has to be supplied in excess (*ad libitum*). The latter method is particularly suited when maximal growth rates are desirable and when differences in feed intake are of less importance, e.g., for studies on biological features. For studies on doses-response relations between dietary inputs and biochemical, physiological and growth outputs, graded levels of restricted feeding levels are to be preferred. The method developed by Verreth and den Bieman (1987, chapter 2) is of particular value in this respect, because it entails a daily adjustment of the feeding level to the changes in relative growth rate. This method has more a practical (e.g., for research purposes) than a scientific value. An interesting alternative could be the concept of *Metabolic Weight*, i.e. applying the allometric growth relation in the design of feeding experiments (Heinsbroek 1987). The concept has not been applied yet in studies on larval fish nutrition, probably because it is not sure whether the allometric relations derived from juvenile fish (Winberg 1956; Huisman 1974; Brett and Groves 1979; Hogendoorn et al. 1983; Heinsbroek 1987) are also valid in larvae. In a review study on the relations between body weight and energy metabolism in fish, Heinsbroek (1987) suggested that in larval fish, the weight exponent would be higher than in juvenile fish. The few literature data available on this aspect are conflicting and questionable. Unpublished data from our own experiments (van der Ploeg 1985) indicate a weight exponent for yolk sac larvae varying between 0.7 and 0.9. Outputs of the dynamic simulation model for larval growth of *C. gariepinus* (Conceição et al. 1993) suggest a weight exponent of 0.96. Until today there are no undisputable empirical data on this exponent.

The foregoing discussion is based on the classical approach for designing feeding experiments, e.g., measuring (growth) responses on variation in feed inputs, whereby the amount of feed is used as independent variable. However, because of the impact of developmental stages in larval nutrition studies, feeding experiments may be preferably designed according to a *pair growth* approach (cfr. chapter 8). In that way, side effects induced by differences in body size and the concomitant differences in development may be avoided.

The impact of development on dry diet formulation

The results of chapter 3 demonstrate that the mentioned developmental changes coincide with the feasibility of using a juvenile diet instead of live food in the feeding strategies for *C. gariepinus*. Obviously, the question of early weaning or replacement of live food by a dry larval diet is related to the changes occurring at the transition from the larval to the juvenile period. Although many functions change, the one which affects most the digestive properties of the animal is the appearance of a functional stomach.

The stomach is most important for dietary protein breakdown. It exerts its function in different ways. First, the gut passage time is strongly increased by the so-called storage function of the stomach. In *C. gariepinus*, this storage function was noticed at the 2nd day of exogenous feeding already, long before the onset of the gastric acid secretion (Verreth et al. 1993; Torreele 1990, unpublished data). The stomach contractions induce a strong mixing of feed particles with gastric juices and helps in the reduction of the food particle size which is passed on to the intestine. In *C. gariepinus*, the detection of metenkephalin and serotonin producing cells before the onset of exogenous feeding (Spazier 1990 and Unger 1990, unpublished data) indicate that gut motility is not a limiting factor in the larval food digestion. When the gastric glands are functional, stomach pH decreases to levels below 3 and food proteins are acidified and denaturated. Additional pepsin digestion splits the proteins into smaller peptides before they are released into the intestine for further intestinal digestion and absorption. This facilitates their hydrolysation by subsequent proteolytic breakdown in the intestine (Jany 1976 in Segner et al. 1994). Therefore, it can be concluded that the presence or absence of stomach functions seems to affect mainly the accessability of dietary protein for intestinal lysis. This conclusion does not yet explain why in the absence of a functional stomach, larvae grow very well on live food organisms and not or less on commercial (fish meal containing) diets. Several possible explanations have been suggested: (a)the enzymes present in the ingested prey organisms provide additional digestive capacity to the fish larvae (Dabrowski and Glogowski 1977); (b)endogenous secretion of pancreatic enzymes is insufficiently stimulated by dry diets (Segner et al. 1992); (c)in the absence of stomach proteolysis, free amino acids must be provided through the diet. Dabrowski and Glogowski (1977) and Lauff and Hofer (1984) have shown that enzymes from prey organisms contribute significantly to the total proteolytic activity in cyprinid fish larvae. Adding exogenous enzymes to formulated diets has been tested with variable results. Recently Kolkovski et al. (1993) obtained reasonable growth results in *Sparus aurata* after addition of pig pancreatin to the a formulated larval diet. Evidence for the second explanation has also been found. Hjelmeland et al. (1988) and Pedersen and Andersen

(1992) demonstrated that in herring larvae secretion of trypsinogen can be stimulated by feeding polystyrene spheres, indicating a physical induction of enzyme release, based on intestinal receptors. The water-rich and voluminous live food organisms may induce stronger endogenous enzyme secretions than a dry diet. The question of free amino acids is most intriguing but seems to influence mainly the physiology of marine fish larvae (Fyhn 1993, Rønnestad et al. 1992). Nevertheless, both freshwater and marine planktonic organisms contain relative large proportions of free amino acids (Dabrowski and Rusiecki 1983; Fyhn et al. 1993; Helland et al. 1994). The relative successful application of SCP diets in *C. gariepinus* (Uys and Hecht 1985; Van Damme et al. 1990) may also be related to this aspect. However, for this particular example, the availability of yeast amino acids to the intestinal proteolysis in the catfish larvae may be a better explanation. In this respect the remark of Hecht (1994) that during manufacturing, the single cell yeast proteins are denaturated and partially hydrolysed, is most important. A fourth possible explanation which has not been documented yet in larval fish, is the effect of differences in buffering capacity of different feed ingredients. In a study on piglets, Makkink (1993) showed clear differences in the gastric acid requirements to reduce the pH in products of animal and vegetable origin. A similar effect may also in fish larvae affect the accessability of organic matter to enzymatic action.

Nutrient requirements in larval fish

Protein and Energy

As all other animal organisms, fish larvae need protein, lipid, energy and a mixture of vitamins and minerals to maintain their body functions and to grow and survive. After water, protein is the most abundant component of a fish body and in most species, it exceeds other body components, e.g., lipids, by a factor 2 or 3. Fish derive a large part of their energy needs from protein oxidation. During the embryonal period of *C. gariepinus*, about 50% of the total energy expenditure consisted of protein energy (Polat et al. 1994; Verreth et al. 1994). Obviously, energy and protein requirements are linked to each other. A major contributor to the energy metabolism in fish is the cost of protein synthesis and thus of growth (Houlihan et al. 1993)

This is of particular importance to larval fish, which exhibit a very high growth rate. Because of the small size and the concomitant relatively low maintenance costs, energetic expenditures in fish larvae are dominated by the expenses for tissue synthesis and, especially

in larvae swimming at low Reynold numbers, also for locomotion. Not much is known about these aspects in larval fish, although an increasing amount of experimental data are becoming available (Fauconneau 1985; Wieser 1985; Wieser et al. 1988a,b; Houlihan 1991; Houlihan et al. 1994). These data gave rise to an interesting discussion and conflicting views on the larval strategy for growth and survival. Wieser et al. (1988b) showed that, contrary to the situation found in large fish, in larval cyprinids growth rate and the mass specific oxygen consumption were not proportional to each other. These authors concluded that in larvae, the metabolic scope is too limited to accommodate all energy consuming functions at the same time, forcing them to optimize their energy needs for activity (foraging) and growth. The question now is how efficient fish larvae use these limited resources. According to Kiørboe (1989), fish larvae grow at the maximal rate of efficiency enabled by the chemical reactions of anabolism. In other words, they reduce maintenance costs, e.g. protein turnover, as much as possible and channel all available energy into growth. This implies also that protein accretion (growth) almost equals protein synthesis. Opposite views are defended by Fauconneau (1985) and Fauconneau et al. (1986), who found high turnover rates in larval carp ($300\%.day^{-1}$) and in larval coregonids. This would imply that high growth rates are realized by increasing the difference between protein synthesis and protein degradation at high turnover rates. This is however not a very efficient way of growing. Houlihan et al. (1992, 1993, 1994) provided empirical evidence for several fish species that larval growth efficiency is not much different from the situation in juveniles and adults, at least when differences in body weight are taken into account. In larvae, the retention efficiency of protein synthesis ranged around 50-60% (Houlihan et al. 1994). In yolk sac larvae of *C. gariepinus*, protein synthesis rates ranged in the order of magnitude of $150\text{-}180\%.day^{-1}$ which is about 1.5 times the daily (protein) growth rate at that stage of development (Conceição et al. 1994, Conceição, Houlihan and Verreth, unpublished data). Using a different approach (by constructing an energy balance), Verreth et al. (1994) showed that the growth efficiency, as measured by Kg (RE/MEp), varied around 75% in yolk sac larvae of *C. gariepinus*, which is far below the levels suggested by Kiørboe (1989) (90%).

What is the significance of these aspects in larval nutrition ? High synthesis rates, especially when turnover rates are low, imply a sufficiently large influx of absorbed amino acids to the body free pool. For this, however, the digestion and absorption of protein might be limiting in early feeding larvae. In this context, the ratios of the flux of absorbed amino acids to the total amount of amino acids in the body free pool is important to understand the buffering capacity of the animal to changes in amino acid influx. Further, to optimize the retention efficiency of synthesized protein, both the composition and the concentrations of the amino acid influx are important. Any limitation in this regard may increase degradation

of body protein and thus decrease the growth efficiency (Houlihan 1991; Houlihan et al. 1994) and larval survival. This may be of particular importance for larvae of *C. gariepinus* in which daily influx of dietary amino acids may exceed the relative small body free pool (3-4% of the total amount of amino acids) (Polat, Conceição and Verreth, unpublished data). Also in chicks, the growth efficiency was affected by the "fit" of the dietary composition to the animal's digestive capacity (Schreurs et al. 1994). It may be a general phenomenon for most fast growing animals.

The relation between growth and protein and energy metabolism in fish larvae is not well investigated. Because of the presumed importance of the amino acid digestion and metabolism (see earlier) in *C. gariepinus* larvae, also in the framework of the present research project, investigations in this field were started. Studies have been carried out and/or are underway on respiration (van Herwaarden 1993, unpublished), energy and nitrogen balances (Verreth et al. 1994), amino acid dynamics (Polat et al. 1994, Polat, Conceição and Verreth, unpublished data) and protein synthesis and turnover rates (Conceição, Houlihan and Verreth, unpublished data).

LIPIDS

In contrast to proteins and amino acids, lipids vary strongly in fish and their metabolism is highly complex. Possibly because of the high importance of the dietary (*n*-3)HUFA's for marine fish larvae, a vast body of literature on lipid requirements in fish larvae exists. Nevertheless, from these studies it is not clear which functions the different fatty acids have in early life stages of fish and neither why the fatty acid requirements differ among larval species.

Some generalities can be derived from the considerable body of data that exists on lipids. A first point is that (marine) fish larvae tend to be more sensible for essential fatty acid (EFA) deficiency than juveniles and adults. This could be interpreted as a tendency for higher relative EFA requirements in larval fish (Sargent et al. 1989). It is generally accepted that the dietary requirements for (*n*-3)HUFA (20:5*n*-3, EPA and 22:6*n*-3, DHA), which are essential in most marine fish species, vary between 0.5 and 1.0% (Castell et al. 1972; Watanabe 1993; Sargent 1994), both in larval and juvenile fish. Only a few species would have higher dietary requirements for these fatty acids (Watanabe 1993). However, most of these data are derived from studies on small juvenile fish and it is reasonable to assume that the quantitative requirements are relatively much higher in the fast growing larval stages (Sargent et al. 1989). Chatain (1994) report higher requirements for larvae of the European

seabass, *Dicentrarchus labrax* (minimally 3% EFA and 12% total lipid in the diet). Based on the composition of marine plankton, i.e. the natural diet for most marine fish larvae, Sargent et al. (1989) inferred about dietary (*n*-3) PUFA requirements of about 10% on dry weight basis. Determining quantitative fatty acid requirements without the expedient of a semi-purified diet, as it happens in larval fish, is most difficult and may lead to biased conclusions. The requirements for individual fatty acids are very dependent upon the concentrations of other fatty acids in the lipid, of the nutritional background of the animal and of environmental conditions under which the requirements are tested (Sargent 1994). For example, the conversion of 22:6(*n*-3) from 20:5(*n*-3) and ultimately from 18:3(*n*-3) is affected by the concentrations and ratios of 18:2(*n*-6), 20:4(*n*-6) and 22:5(*n*-6) and of 18:1(*n*-9) and 20:3(*n*-9) through the competitive use of the Δ-5 and Δ-6 fatty acid desaturases needed for these conversions (Sargent 1994). A high (*n*-6)/(*n*-3) ratio, and/or high concentrations of f.ex. linoleic acid may lead to a relative overproduction of arachidonic acid at the expense of the conversion to the biologically active (*n*-3) fatty acids. The common live food organisms used in larviculture and in larval nutrition studies, e.g., *Artemia* and *Brachionus*, are typically rich in 18:3(*n*-3) and 20:5(*n*-3), have variable concentrations of 18:2(*n*-6), and 20:4(*n*-6), and are generally deficient in 22:6(*n*-3). In the same way, one may speculate about the possible effects of broodstock nutrition on the fatty acid composition in the egg and thus on the fatty acid dynamics in feeding larvae later on. Although it is assumed that during vitellogenesis, irrespective of their nutritional background, female fish tenaciously deposit (*n*-3)PUFA in the oocytes, most of this information is based on experiments of short duration. It is very well possible that elevated (*n*-6) fatty acid levels in the adipose tissue of the mother fish are transferred to the oocyte during vitellogenesis. In the case of a completely domesticated broodstock, e.g., as with the breeders of *C. gariepinus* used in the present project, generations of fish being raised on commercial diets containing substantial levels of vegetable oils (soya-oil, soya-lecithin, etc.) may result in eggs which contain higher levels of (*n*-6) fatty acids than their wild conspecifics. This raises the question how much of the data presented in part 3 are under genetic control (and thus related to developmental and/or physiological aspects) or how much are environmentally induced (and thus related to the experimental design). At present, there is no clear answer to this. In this respect, it would be most interesting to compare the results of the studies reported in Part 3 of this thesis with material derived from wild *C. gariepinus*.

Another general point which can be derived from the literature is that eggs usually contain substantial amounts of (*n*-3)PUFA's, preferably phospholipid, and in particular of DHA (22:6(*n*-3)). This was also found in *C. gariepinus* (chapters 6 and 7). This is generally related to the importance of these fatty acids as essential constituents of the

phospholipid in cell membranes. Because during embryogenesis and larval development cells proliferate and differentiate, it is vital that a sufficient supply of these constituents is available in the egg. Especially DHA is of particular importance for a normal development of neural (retina) and brain tissues (Sargent et al. 1993). In fish eggs, DHA is mainly found in phosphatidylcholine and the fish larva incorporates it in neural tissue phosphatidylethanolamine. As has been stated earlier, at hatching most fish species have not yet completed the morpho-functional development of brains and eyes, and in the case of marine fish, it may even extend into the exotrophic phase. The practical importance of these findings can be exemplified by the case of turbot, *Scophthalmus maximus*. A major constraint for the commercial development of turbot culture is the high proportion of malpigmented or albinistic fish in the stock, leading to a reduced marketability. This malpigmentation is the result of an insufficient functioning of rhodopsin in the rods of the retina, which stimulates the secretion of the melanophore-stimulating hormone. Since DHA is a main precursor of fish rhodopsin, deficient dietary supplies of this fatty acid during the development of the retina leads ultimately to malpigmented and low priced market fish (Kanazawa 1993).

A second important function of essential fatty acids, in particular of arachidonic acid 20:4(*n*-6), is their role as precursors of eicosanoids (Sargent 1994). In fish, 20:4(*n*-6) is preferentially incorporated into phosphatidylinositol (PI), which is usually found in small amounts (1-4% of total lipid). PI is thought to have an important role in signal transduction in the cell membrane. The importance of PI in larval fish diets has long been overlooked but recently a strong correlation was found between dietary PI concentration and larval survival and growth in the freshwater fish *C. carpio* (Geurden et al. 1994). This is most intriguing because generally freshwater fish larvae possess the capacity to synthesize arachidonic acid from the linoleic precursor. It emphasizes the need for more research in this field.

To summarize, the important role of lipid classes and fatty acids in early life stages of fish seems mainly related to the biochemical needs for organogenesis in the animal. In this regard it is a question whether and how the results of Part 3 of this thesis can also be interpreted from that viewpoint. As most other fish species, *C. gariepinus* deposits high concentrations of (*n*-3)HUFA, especially DHA, in the eggs. As in most other fish species, the major polar lipid class is PC. However, the proportion of both components is at the higher level of the range usually found in fish, and certainly in the eggs of freshwater fish species (Henderson and Tocher 1987). As in many marine fish species, the majority of PC is catabolized but in contrast to marine species, e.g., herring (Tocher et al. 1985), it does not

coincide with a concomitant conversion of (*n*-3)PUFA from PC to TAG, because the latter is entirely used during the yolk sac period. The question is then why during vitellogenesis such high concentrations of PC and (*n*-3)PUFA are deposited in the egg yolk. Tocher et al. (1985) suggested that PC catabolization could also be a mean to produce choline for the developing embryos and larvae. Choline interferes in many metabolic functions. It is hard to say how important the catabolized choline from PC is and which role it has in larval catfish. Similarly, the high proportions of DHA found in larvae at start feeding (Chapters 7 and 8) are also difficult to explain from the point of view of a life history strategy. The species has evolved the capacity to synthesize this fatty acid from the 18C and 20C precursors (see also chapter 7 and 8). Therefore, from that point of view this "storage" of DHA in the egg yolk and in the embryonal body tissues seems superfluous, unless the hypothesis (as suggested in Chapter 8) that the biosynthesis capacities are activated only at a later stage in the larval period, holds true.

CONCLUSIONS

Obviously, there is still much lacking in our understanding of the biochemical metabolism of this fish (and of fish larvae in general), and thus in our ability to formulate adequate diets and to design adequate feeding strategies. The previous paragraphs suggest a clear difference between the nutrient requirements for proteins and lipids. The problem of protein and amino acid requirements is basically of a quantitative nature. In contrast to this, the dietary requirements of lipids is highly qualitative. There is no direct quantitative relation between growth and the dietary content of individual fatty acids (see also Chapter 8) as long as a certain threshold level is maintained. For the optimization of larval growth and survival, the overall dietary lipid content is secondary to the lipid composition. As long as the biochemical and physiological functions of the individual lipid classes and fatty acids in fish larvae are not well understood, real "balanced" larval diets will be difficult to formulate.

However, as the discussion on choline may have indicated, other (especially minor) nutrients should not be overlooked in a study on nutrient requirements in fish larvae. The next two examples may further illustrate this. The first example deals also with the complexities of the lipid metabolism, but even more with the general metabolic status of the fish through its interference with intracellular acetylCoA-levels, e.g. L-Carnitine. L-Carnitine is responsible for fatty acid transport through the mitochondrial membrane and influences thereby the rate of ß-oxidation of fatty acids. Torreele et al. (1993) showed a clear growth stimulating effect of L-Carnitine additions to the diet of juvenile *C. gariepinus*.

In mammals and in human, problems with L-carnitine synthesis occur mainly in foetal and/or newborn animals. When dietary supplements of L-carnitine can exert such a positive effect on growth of juvenile catfish, it is most likely that the biosynthesis of this compound is limiting for larval growth. The second example deals with another small substance but with broad interference in the intermediary metabolism, e.g., vitamin C. In a joint study with the Laboratory for Aquaculture, University of Gent, Belgium, different levels of dietary ascorbic acid were fed to larval African catfish via enriched *Artemia*. Dietary ascorbic acid levels varied from the natural level (about 0.5 mg/g) to a fourfold increased level (2 mg/g dry weight). The results showed a clear positive correlation between the elevated ascorbic acid levels in *Artemia* and growth, concentration of AA in the larval tissues and larval stress resistance (Merchie et al. 1994). It is our conviction that answering these and other questions related to the mechanisms of early metabolism in fish will be of paramount value for the development of better feeding strategies and better larval diets.

Final Remarks and Conclusions

In the present study, emphasis was put on the interaction between ontogenetic and nutritional aspects in the African catfish *Clarias gariepinus*. The basic hypothesis of the study was that nutritional problems in larvae are mainly, but not only, a matter of dealing with small sized fish (i.e. a quantitative scaling aspect). Additional ontogenetic factors induce also qualitative differences with juveniles and adults. The study provided sufficient evidence to support this hypothesis.

A good example is the protein metabolism. In quantitative terms, on a weight basis, protein dynamics in larvae seem to be very similar to the situation found in juveniles and adults. As shown in chapter 2, protein retention and protein efficiency ratios are in the order of magnitude which could be expected when size differences were taken into account. This view is corroborated by the general pattern of protein synthesis efficiency found in larval fish (Houlihan et al. 1993; 1994) : it fluctuates around 50% and changes allometrically with weight in the same way as most other physiological rates (Jobling 1993; Houlihan et al. 1994). Also *C. gariepinus* larvae seem to fit into this pattern (Conceição et al. 1994; Conceição, Houlihan and Verreth, unpublished). However, in qualitative terms, the absence of a functional stomach during the first days of exogenous feeding affects the processes of digestion and absorption considerably. It may lead to an imbalanced amino acid influx if the diet preparation is not geared to these differences in the basic physiological processes.

The design of adequate larval rearing and feeding strategies and the formulation of dry larval diets need a comprehensive analysis of the ontogenetic changes occurring during the early life stages of the species under concern. Such an approach was used in the present project. This analysis provided cues for a better definition of metamorphosis in (freshwater) fish. The concept which prevails in life history studies, e.g., that metamorphosis can be measured by the differentiation of the (truly larval) median finfold into distinct (adult-like) fins, is not fully supported by the physiological development of *C. gariepinus*. The results of the present research suggest that larvae of the African catfish pass through a series of substantial and simultaneously occurring developmental changes at a size of roughly 11-15 mm, i.e. 10-20 mg. According to literature data, the classical markers for metamorphosis occur at a size of 25-30 mm. The changes described in this thesis invoke new and/or more versatile functions, especially with regard to the animal's capacities to interact with its outside world. Therefore, they may constitute better markers for metamorphosis. Which of both approaches is the most appropriate one, has still to be clarified. The basic point is that the concept of metamorphosis deserves a reconceptualization.

The onset of stomach functions was presented as a key factor in this process of "metamorphosis". Together with the change in swimming mode, the most pronounced effect of a functional stomach is the widened food spectrum for the animals compared to the earlier stages. Interestingly, in *C. gariepinus*, it coincided also with the moment of weaning to a common grow-out diet. Similar findings have been done for other fish. Also in marine fish species, weaning to a dry diet seems to be possible only when the stomach is functional. It shows a direction for future research on formulated dry diets for larvae. It may be preferable to incorporate denaturated or predigested protein, free amino acids, or any protein substrate which is more accessible to the larval digestive system in the diet. However, care has to be taken with pure technical solutions. Some very intriguing question marks remain. The ontogenetic pattern of *C. gariepinus*, as described in this thesis, is strikingly similar to the one described for a very different marine species, e.g., turbot *Scophthalmus maximus* (Segner et al. 1994). Nevertheless, until today there are no indications that diets with denaturated protein such as the SCP diets, can be utilized by the turbot larvae before their stomach development has completed. Apparently, what counts for *Clarias* may not be valid for another species. Further, it calls for detailed research to explore the reasons why stomach digestion makes such differences as found in many larval fish species.

This brings us to the question what the validity of this work is for larval nutrition in general. We have then to return back to the original hypothesis. As was stated in the introduction, problems in larval nutrition can be understood, and thus possibly solved, when

the factors causing the differences with juvenile nutrition are known. The hypothesis was that these factors are mainly related to size differences (scaling factors), but they are superimposed by ontogenetic factors. The latter ones are the most difficult to understand, and are thus paramount for further progress in larval nutrition. In this regard, it is our conviction that differences in nutritional aspects between different larval species may be less puzzling and less rigid when regarded from an ontogenetic perspective. Indeed, the same mechanisms may cause the problems in the nutrition of most larval fish species, and differences between species may be explained by differences in the level of development at hatching and/or at the start of exogenous feeding. In the next paragraph, this idea will be further elaborated.

On the developmental continuum from a fertilized oocyte to a juvenile fish, most marine fish species seem to hatch and to start exogenous feeding at an earlier stage in development than freshwater fish species. This may explain why marine fish species are so sensitive to (*n*-3)HUFA deficiencies and why they seem to have higher relative (*n*-3)HUFA requirements then most freshwater fish species. When hatching at an earlier stage of development, the development of neural tissues is extended beyond the start of exogenous feeding, and relatively more lipid is required to ensure this organogenesis then in those species where this development took place during the embryonal period. In other words, positioning the species under concern on the continuum of life history periods, ranging from the generalist (altricial) to the specialist (precocial) species (Balon 1985), the necessary research strategies for elucidating larval nutritional requirements may be strongly narrowed. Hecht (1994) illustrated this predictive role of early life history and ontogeny for the larval nutrition of different Silurid (catfish) species, with *Clarias gariepinus* at the altricial side and the channel catfish, *Ictalurus punctatus*, at the precocial side. This concept may also be valid for a wider range of taxonomic groups.

The research strategy to be followed when testing this hypothesis would be to select a few target species, preferably all belonging to the group of those species which have no functional stomach at the start of exogenous feeding but which develop it later-on (Dabrowski 1984). At the altricial side, a few marine species, e.g. turbot and european seabass could be selected because of their very distinct life styles and the concomitant different epigenetic factors. At the precocial side, the African catfish and coregonids would be most suitable. Herring and pike-perch would be species of an intermediate position, probably with the freshwater pike-perch as more altricial than the marine herring. Research should focus on an integrative analysis of the morphological, physiological and biochemical development of functions related to feed intake and to feed digestion and utilization, in relation to different dietary factors. Such a comparative research would not only provide the

necessary cues to develop adequate diets for larval fish, it would also generate a much better understanding of the essence of larval life. For *Clarias gariepinus*, a first attempt in this direction has been made. Since growth is the ultimate resultant of all in- and outputs in the organism, a mechanistic simulation model for growth of larval African catfish was developed (Conceição et al. 1993). Since it is based on the biochemical pathways of anabolism and catabolism, it integrates most of the physiological and biochemical information which has been collected during the course of the present research project. Calibrating and validating this model to other, e.g., marine species, would turn it into a versatile and powerful tool in developing optimal rearing and feeding strategies for larval fish.

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SUMMARY

SAMENVATTING

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Critical aspects in the larval nutrition of the african catfish, *Clarias gariepinus* (Burchell), in relation to development: a summary.

INTRODUCTION

For many fish species, further growth of the farming industry is hampered by the absence of a reliable production of young fish needed for stocking grow-out tanks. This constraint is strongly related to an insufficient knowledge of the nutritional aspects in rearing the larval stages of those species. Most fish species require live food organisms as first food, hampering thereby the development of more dependable larval production techniques. To overcome this problem, more appropriate (formulated) feeds and feeding procedures for larval fish are required. Because in fish larvae, the development of organs and functions is not yet completed, larval rearing and feeding procedures can only be developed when the biological properties of the developing young animal are taken into account. In the present thesis, this philosophy was applied for the case of the African catfish, *Clarias gariepinus* (Burchell). The study aimed (a) at the development of appropriate rearing and feeding procedures and of a suitable dry starter diet, which could also be used in nutritional research with fish larvae and (b) to elucidate the relation between problems in larval nutrition and the associated ontogenetic aspects. As many other fish species, larvae of the African catfish have no stomach at the onset of exogenous feeding but develop it later on. The basic assumption behind the study was that differences in nutritional requirements between larval and juvenile fish would provide the cues for the development of such a formulated diet. As a working hypothesis the mentioned differences between larvae and juveniles were supposed to be induced both by the small size of larvae (a quantitative scaling effect) and by qualitative differences in digestive and metabolic properties because of ontogenetic effects. The research project was subdivided in three phases, which dealt respectively with the development of a standardized husbandry system, the ontogeny of the digestive and metabolic properties of the growing larvae and with the lipid metabolism in the early life stages.

PHASE ONE

During the first phase of the study, standardized rearing and feeding conditions for nutritional experiments with larval catfish were developed. At the start of the project, the nutritional value of several dry test diets for larvae of *C. gariepinus* was determined. Dried and decapsulated cysts of *Artemia* gave consistently the best growth and survival rate. Micro-encapsulated (chicken)-egg diets, especially when enriched with casein and a vitamin/mineral mixture, resulted in high survival rates but low growth rates. Analysis of the hepatocyte ultrastructure in the different test groups suggested that the egg diets contained all essential nutrients. Apparently not only the quality but also the intake of the larval diets is important. In comparative studies with dry formulated diets, behavioral influences on feed intake can be avoided by using dried and decapsulated cysts of *Artemia*, instead of just hatched nauplii. This first study did not include a dry diet composed of Single Cell Protein (yeast cells) which has been reported to produce good growth and survival in larval *C. gariepinus* (Appelbaum and van Damme 1988; Van Damme et al. 1990). However, later and unreported tests with the latter diet in our laboratory yielded consistently lower growth rates than in groups fed with *Artemia*. Hence, the conclusion that *C. gariepinus* larvae need live food organisms as first food to maximize growth rates, is still valid. For nutritional experiments, *Artemia* was suggested as a reference diet.

In a second study, the growth performance of larval *C. gariepinus* under different conditions of feeding rates and temperature was investigated. A method for determining feeding levels in larval nutrition studies was developed. It entails a daily adjustment of the feeding level to changes in relative growth rate and in dry matter content of the larvae. Using this method, a classic dosage-response relation between feed ration and growth was obtained. Larval growth rate was maximized (growth coefficient $g = 0.30\text{-}0.31$) when food rations were calculated according to predicted growth coefficients of 0.3-0.5. The optimal temperature was set at 27.5 °C.

In a third study, the length of the experimental duration was investigated. The study showed that larvae of *C. gariepinus* can be weaned from *Artemia* to crumbles of a commercial trout diet without loss of growth and of survival, provided that they had first received live food for four days. Taking into account that weaning occurred gradually, these results make it clear that eight days after the start of exogenous feeding, the larvae are not restricted by a larval diet, and therefore may be regarded as small juvenile fish. This finding gives a good support to the duration of the experiments which was used throughout the present study, i.e. 10 days.

PHASE TWO

During the second phase of the research project the hypothesis that in *C. gariepinus*, the larval period is ending at an age of approximately 5-8 days was further elaborated. In two separate studies, the morpho-functional development of the digestive system and the metabolic development of *C. gariepinus* was elucidated. At the onset of exogenous feeding, the intestine is subdivided into three functionally different regions, possessing substantial activities of digestive enzymes, both from the pancreas and from the enterocytes. The intestine is capable to digest and absorb lipids. Immunohistochemical evidence for the presence of pancreatic hormones, e.g., insulin and glucagon, and for the presence of a gastrin/CCK-like hormone demonstrated the capacity for a coordinated digestive and metabolic process from the start of exogenous feeding onwards. Apparently, at the start of exogenous feeding, the animals are capable to ingest, digest, and absorb nutrients. A major difference with the juvenile period is the absence of a functional stomach at the start of exogenous feeding. The morphological and functional development of the stomach is completed about 5 days after the start of exogenous feeding. Other studies which are not included in this thesis (Segner et al. 1993; Verreth et al. 1993) revealed that the onset of stomach functions coincided with a switch from an alkaline proteolytic digestion to a combined acid and alkaline proteolytic digestion. Pepsin-like activity reached a maximum, eight days after the start of exogenous feeding. The stomach differentiation seems to coincide with the earliest possible weaning time as determined in the previous study, e.g., after an *Artemia* feeding period of about 5 days at 27.5 °C or at a size of about 20 mg. Also the next study, e.g., on the ontogeny of metabolic functions as exemplified by enzyme activities, showed that this size of 20 mg is a kind of "turning point" in the development of *C. gariepinus*. All investigated enzymes of the central intermediary metabolism were present from the start of exogenous feeding onwards. Important ontogenetic changes were found in the activities of those enzymes which are characteristic for glycolysis, glyconeogenesis, glycogen storage and amino acid catabolism. All these changes were quantitative of nature and occurred at a size of about 20 mg, i.e. after 3-5 days of *Artemia* feeding. These changes in metabolism coincide with changes of muscle organization, gill morphology, respiration, and as shown earlier, with changes in stomach functions. The combined change of the various morphological and physiological parameters indicates that the larval period in *C. gariepinus* ends at a body size of approximately 20 mg.

PHASE THREE

The third phase of the research project focused on the assessment of nutrient requirements in larval *C. gariepinus*. The present thesis includes three studies on the lipid metabolism in the early life stages of this species. The aim was to analyze the relative importance of endogenous and of exogenous sources of lipids and fatty acids for larvae of the African catfish. The first of these three papers deals with the conversion of the different neutral and polar lipids in the yolk to the body tissues of the growing yolk sac larvae and their fate in subsequently starving larvae. The eggs of *C. gariepinus* can be characterized as having moderate amounts of lipid (approximately 5%) of a highly polar nature (80% of the lipid), with phosphatidylcholine as the most dominant lipid class (about 70-75% of the total lipid). Phosphatidylcholine was catabolized proportionally to total lipid, demonstrating its role as main energy supplier. All yolk phosphatidylethanolamine (PE) was converted into body PE. The neutral lipids consisted of triglycerides (TAG), cholesterol and cholesteryl esters. All TAG were depleted before complete yolk absorption. The most abundant fatty acids in the eggs were 16:0, 22:6*n*-3, 18:1*n*-9, 18:0, 20:5*n*-3 and 18:2*n*-6 (respectively about 29, 19, 17, 11, 6 and 5% of the total fatty acid composition). Most yolk fatty acids were absorbed proportional to the total fatty acids, e.g., at approximately 0.5% of their initial amount per time unit (physiological day degree). Apparently during the yolk sac period there is no strong preference for individual fatty acids to be catabolized or to be converted into body tissue. The overall conversion efficiency of most fatty acids from yolk to body tissue was lower than 60%. Nevertheless, during the yolk sac phase, some fatty acids were clearly synthesized, showing conversion efficiencies above 100%: 20:4*n*-6; 20:5*n*-3 and 22:6*n*-3. These three fatty acids are essential for marine fish and crucial for a good organogenesis and functional development of any fish (Sargent 1994). At the end of the yolk sac period, 22:6*n*-3 is the most abundant fatty acid in the lipid (about 28% of the total fatty acids), suggesting a crucial role of this fatty acid for *C. gariepinus* as well. Nevertheless in a feeding experiment with *Artemia*, enriched with either low or high levels of highly unsaturated fatty acids (HUFA), dietary *n*-3 HUFA levels did not affect growth or survival rates in *C. gariepinus*. Irrespective of the dietary levels, the tissue levels of 22:6*n*-3 in the fish decreased from about 17 mg/g dry weight at the start of exogenous feeding to 2-4 mg/g dry weight at the end of the experiment (at a body size of about 50 mg wet weight). Fatty acid retention percentages revealed a capacity to synthesize HUFA. The dietary fatty acid levels seem of less importance to the growing larvae because of abundant levels of essential fatty acids resulting from the yolk reserves.

CONCLUSIONS

In conclusion, during the present research project suitable experimental conditions for nutritional research on larvae of the African catfish *C. gariepinus* were developed. Important reference data on growth, growth efficiency and survival in larval catfish were collected. However, a direct solution for the problem of a reliable fish seed supply through the development of a formulated dry diet is not yet available. However the study narrowed the research questions considerably and provided several suggestions for a future orientation of research in larval nutrition. For species like this freshwater catfish, which are characterized by the absence of a functional stomach at the onset of exogenous feeding, the availability of dietary amino acids seems to be crucial for the development of formulated feeds. In the absence of stomach functions, predigested, denaturated proteins and/or free amino acids may be required for the formulation of a successful dry larval diet.

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Critische aspecten in de larvale voeding van de Afrikaanse meerval, *Clarias gariepinus* (Burchell), in relatie tot zijn ontwikkeling: een samenvatting.

INLEIDING

De teelt van vele vissoorten wordt beperkt door het ontbreken van een bedrijfs-zekere pootvisproductie. Dit probleem is gerelateerd aan een gebrekkige kennis van de larvale voeding bij deze soorten. De meeste vissoorten kunnen als larve uitsluitend met levende prooi-organismen in leven worden gehouden. De ontwikkeling van aangepaste droogvoeders en voederschema's zou een oplossing kunnen bieden. Dit kan slechts gerealiseerd worden als meer kennis van de functionele ontwikkeling en van de voedingsfysiologie van vislarven beschikbaar is. Vislarven worden immers gekenmerkt door een onvoltooide ontwikkeling van de orgaanstelsels en dit heeft gevolgen voor de voedingsfysiologie. In de u voorliggende studie wordt deze gedachte toegepast op de Afrikaanse meerval, *Clarias gariepinus* (Burchell). Doel van deze studie was tweeërlei: (a) het ontwikkelen van een geschikte voederstrategie en van een droog startvoeder dat ook voor nutritioneel onderzoek kan gebruikt worden en (b) vaststellen in hoeverre de voedingsproblemen bij larvale vissen gerelateerd zijn aan ontwikkelings-biologische factoren. Zoals vele andere vissoorten beschikt de Afrikaanse meerval niet over een maag wanneer het dier overschakelt van dooierresorptie naar exogene voedselopname. De maag wordt slechts in een later stadium van de ontwikkeling voltooid. Bij deze studie werd aangenomen dat de formulering van larvenvoeders kan afgeleid worden uit (voedings-)fysiologische verschillen tussen de larvale en juveniele levensstadia. Verder werd ervan uitgegaan dat deze verschillen zowel van kwantitatieve als van kwalitatieve aard zijn: kwantitatieve verschillen worden veroorzaakt door de kleine afmetingen en gewicht van de larven, terwijl de kwalitatieve verschillen veroorzaakt worden door ontwikkelingsbiologische factoren. Het onderzoeksproject werd in drie delen opgesplitst. Tijdens het eerste deel werd gewerkt aan een gestandaardiseerde houderij van de larven, tijdens het tweede deel aan ontwikkelingsbiologische aspecten van de spijsvertering en tijdens het derde deel werd het vetmetabolisme en de vetbehoeften tijdens de vroege ontwikkeling van de meerval onderzocht.

DEEL ÉÉN

Tijdens het eerste deel van de studie werd gezocht naar een gestandaardiseerde houderij voor larven van de Afrikaanse meerval. In eerste instantie werd de kwaliteit van verschillende droge startvoeders vergeleken in een poging om voor het verdere onderzoek een referentie voeder vast te stellen. Gedecapsuleerde en gedroogde cysten van *Artemia* gaven de beste groei en overleving. Voeders gebaseerd op kippe-eieren (de zgn. "micro-encapsulated egg diets") resulteerden in een hoge overleving maar slechte groei, ook wanneer ze waren aangerijkt met caseïne en een vitamine-mineralen mix. Niettemin wees electronenmicroscopische analyse van de levercellen uit dat deze ei-voeders over alle essentiele voedingsstoffen beschikten. Het succes van een droog startvoeder wordt vermoedelijk ook in grote mate door de opname beïnvloed. Teneinde verschillen in opname van levend en droog voeder uit te schakelen kan met gedecapsuleerde en gedroogde cysten van *Artemia* gewerkt worden. Overigens werden in deze eerste studie geen voeders op basis van gist (de zgn. Single Cell Protein voeders) getest. Volgens latere literatuurbronnen (Appelbaum en Van Damme 1988; Van Damme et al. 1990) leveren deze SCP-voeders juist wèl een goede groei en overleving op bij larven van *C. gariepinus*. Nader onderzoek in de eigen accomodatie (deze gegevens zijn niet in deze dissertatie opgenomen) kon dit niet bevestigen. Bijgevolg wordt vastgehouden aan de conclusie dat larven van *C. gariepinus* afhankelijk zijn van levende voedselorganismen om een maximale groei te realiseren. Op basis van dit eerste onderzoek werd *Artemia* als referentie dieet voor voedingsonderzoek gesuggereerd.

Tijdens een tweede onderzoek werd de groei van *C. gariepinus* larven gemeten onder verschillende omstandigheden van voederniveau en temperatuur. Daarbij werd een methode ontwikkeld om voederniveau's voor larvaal voedingsonderzoek vast te stellen. De kleine afmetingen en hoge groeisnelheden van vislarven maakt het gebruik van voederniveau's op basis van een vaste fractie van het lichaamsgewicht onmogelijk. De hier ontwikkelde methode omzeilt dit probleem en maakte het mogelijk om een klassieke dosis-respons relatie tussen voedergift en groei voor larvale *C. gariepinus* vast te stellen. Maximale groei (groeicoëfficiënt g = 0.30-0.31) werd gerealiseerd wanneer de voedergift werd berekend op basis van een voorspelde groeicoëfficiënt van 0.3 - 0.5. De optimale temperatuur was 27.5 °C.

Het derde onderzoek evalueerde de duur van de voederexperimenten in deze studie. Larven van *C. gariepinus* kunnen zonder groeiverlies omgezet worden van levend voedsel naar een gemalen commercieel forellenvoeder op voorwaarde dat ze eerst vier dagen uitsluitend met *Artemia* werden gevoederd. Het omzetten van levend naar droog voeder werd over drie dagen gespreid. Derhalve kan geconcludeerd worden dat na acht dagen (bij

27.5 °C) jonge meerval volledig onafhankelijk is geworden van levende voedselorganismen. Op basis van deze resultaten werd vastgesteld dat de gebruikelijke duur van de voederproeven in dit onderzoeksproject (10 dagen) betrouwbaar was.

DEEL TWEE

De hypothese dat de larvale periode bij Afrikaanse meerval slechts 5-8 dagen duurt werd verder onderzocht in de tweede fase van het project. Daarbij werd vooral ingegaan op de morfo-functionele ontwikkeling van het spijsverteringsstelsel en op de ontwikkeling van een aantal stofwisselingprocessen. Bij het begin van exogene voedering beschikt de larve van *C. gariepinus* over een darm die in drie functionele delen kan opgesplitst worden. Verder beschikt de larve over een set van actieve spijsverteringsenzymen, zowel afkomstig van de alvleesklier als van de darmwandcellen. Het dier is verder in staat vetten te verteren en te absorberen. Met behulp van immunohistochemische technieken werd de aanwezigheid van insuline, glucagon en van een gastrine/CCK-achtig hormoon vastgesteld wat wijst op een hormonale controle van het spijsverterings- en stofwis-selingsproces. Op basis van al het voorgaande werd geconcludeerd dat bij de aanvang van exogene voedering, larven van *C. gariepinus* voedsel kunnen opnemen, verteren en absorberen. Het grote verschil met de latere juveniele fase is het ontbreken van een functionele maag. De morfologische en functionele ontwikkeling van de maag duurt ongeveer 5 dagen vanaf het begin van de exogene voedering. Andere studies die in dit proefschrift niet zijn opgenomen (Segner et al. 1993; Verreth et al. 1993) toonden aan dat deze maagontwikkeling gepaard gaat met een omschakeling van een eiwitvertering op basis van trypsine naar een vertering op basis van zowel pepsine als trypsine. De zuurproteolytische activiteit bereikte een maximum na ongeveer 8 dagen (na begin van exogene voedering). Opvallend is dat de differentiatie van de maag samenloopt met de eerder vastgestelde periode van spenen. De voltooiing van de maagontwikkeling en de mogelijkheid tot omzetten van levend naar droog voeder gebeurt bij 27.5 °C ongeveer 5 dagen na het begin van exogene voedering of bij een visgewicht van ongeveer 20 mg.

Ook tijdens het vervolgonderzoek naar de ontwikkeling van enzymactiviteiten bleek dit lichaamsgewicht van 20 mg een soort "keerpunt" te zijn in de ontwikkeling van *C. gariepinus*. Bij dat gewicht werd de activiteit van de meeste geteste enzymen, die karakteristiek zijn voor allerlei functies in de stofwisseling, gewichts-onafhankelijk. Alle geteste enzymen waren actief vanaf het begin van exogene voedselopname, maar hun specifieke activiteit nam toe met toenemend visgewicht tot vermeld keerpunt. Deze ontwikkelingsbiologische veranderingen werden vastgesteld voor enzymen die snelheids-

bepalend zijn voor glucolyse, gluconeogenese, aminozuur catabolisme en voor NAPDH productie. Ze vallen samen met veranderingen in de spierontwikkeling, de morfologie van de kieuwen, respiratie en met het eerder vastgestelde verloop van de maagontwikkeling. Vooral deze koppeling in veranderingen van verschillende morfologische en fysiologische parameters is een indicatie dat de larvale periode in de Afrikaanse meerval eindigt bij een lichaamsgewicht van ongeveer 20 mg.

DEEL DRIE

Tijdens de derde fase van het onderzoeksproject werd aandacht besteed aan de vetstofwisseling gedurende de vroege ontwikkelingsstadia van *C. gariepinus*. Het doel van deze studies was na te gaan in welke mate larven van *C. gariepinus* afhankelijk zijn van endogene (uit de dooier) dan wel exogene (uit het voeder) bronnen van vetten en vetzuren. In een eerste onderzoek werd het metabolisme van diverse vetsoorten bestudeerd tijdens de dooierzakfase en in hongerende larven. De tweede studie gaat in op het vetzuur-metabolisme tijdens deze periode. In een derde en laatste studie werd onderzocht of larvale meerval, conform de situatie bij mariene vislarven, verhoogde gehaltes aan onverzadigde vetzuren in hun dieet nodig hebben.

De eieren van *C. gariepinus* bevatten matige hoeveelheden vet (5% op versgewicht basis), dat evenwel zéér polair is (80% van het totale vetgehalte bestaat uit polaire vetten). Eén vettype (phosphatidylcholine, PC) domineert het vet in de eieren van de Afrikaanse meerval (70-75% van het totale vet bestaat uit PC). Gedurende de embryonale fase (van bevruchting tot dooierzakresorptie) en tijdens de latere hongerende larvale fase wordt PC proportioneel aan het totale vet gecatabolizeerd. Blijkbaar is PC vooral een energieleverancier. Alle phosphatidylethanolamine (PE) uit de dooier wordt omgezet in lichaams-PE. Alle triglyceriden (TAG) verdwenen uit het organisme vóór het einde van de dooierzakfase en speelden blijkbaar uitsluitend een rol als energiebron. Qua hoeveelheid zijn de belangrijkste vetzuren in eieren van *C. gariepinus*: 16:0, 22:6*n*-3, 18:1*n*-9, 18:0, 20:5*n*-3 and 18:2*n*-6 (respectievelijk 29, 19, 17, 11, 6 and 5% van de totale vetzuursamenstelling). In deze fase van de ontwikkeling worden individuele vetzuren niet bij voorkeur geselecteerd voor verbranding of voor omzetting in lichaamsweefsel. Alle vetzuren uit de dooier werden aan een gelijke snelheid geabsorbeerd, i.e. ongeveer 0.5% van hun hoeveelheid in het ei per tijdseenheid (fysiologische dag-graden). Voor de meeste vetzuren geldt dat minder dan 60% van de oorspronkelijke hoeveelheid in eidooier wordt omgezet in lichaamsweefsel. Niettemin worden sommige vetzuren tijdens de dooierzakfase gesynthetiseerd, zoals bleek uit de omzettingsefficienties voor 20:4*n*-6; 20:5*n*-3 and 22:6*n*-3. Deze drie HUFA (resp.

arachidonzuur, *eicosapentaenoic acid*, EPA en *docosahexaenoic acid*, DHA) zijn cruciaal voor een goede ontwikkeling van organen en functies in vis (Sargent 1994). Aangezien zeevissen ze niet kunnen synthetiseren, zijn ze essentieel voor en spelen ze een uiterst belangrijke rol in de kweek van larvale zeevis. Bij de Afrikaanse meerval werd vastgesteld dat aan het eind van de dooierzakperiode, DHA (22:6*n*-3) het meest overvloedige vetzuur is (28% van de totale vetzuursamenstelling bestond uit DHA). Dit suggereert dat *n*-3 onverzadigde vetzuren ook bij *C. gariepinus* een belangrijke rol kunnen spelen in de voeding. Niettemin hadden de concentraties aan *n*-3 HUFA in het voeder geen invloed op groei of overleving tijdens de larvale periode. Ongeacht de gehaltes in het voeder daalde de DHA concentratie in het lichaamsweefsel van *C. gariepinus* van 17 mg/g droge stof bij het begin van exogene voedering tot ongeveer 2-4 mg/g droge stof bij een lichaamsgewicht van 50 mg. Verder bleken larven van *C. gariepinus* in staat deze meervoudig onverzadigde vetzuren te synthetiseren. Daarom kan besloten worden dat de gehaltes aan hoogwaardig onverzadigde vetzuren in larvale voeders van *C. gariepinus* minder belangrijk zijn, vermoedelijk door de hoge reserves die tijdens de dooierzakfase uit de dooier naar het lichaam worden omgezet.

CONCLUSIES

Samenvattend kan vastgesteld worden dat tijdens dit onderzoek een houderij- en voederstrategie ontwikkeld is die geschikt is voor voedingsonderzoek bij larvale *C. gariepinus*. Verder werden belangrijke referentiegegevens mbt groei, groei-efficientie en overleving verzameld. Een direkte oplossing van het pootvisprobleem in de vorm van een geschikt droogvoeder voor deze larven is echter nog niet voorhanden. Het in dit proefschrift beschreven onderzoek heeft echter de betreffende vraagstelling sterk vernauwd. Verder leverde het verscheidene suggesties op in welke richting het onderzoek in de voeding van vislarven zich kan ontwikkelen. Voor soorten die, net zoals de Afrikaanse meerval, over geen functionele maag beschikken bij het begin van de exogene voedering, is de verteerbaarheid van het eiwit in het voeder van eminent belang. Teneinde aminozuren in het voeder bereikbaar en beschikbaar te maken voor een vislarve zonder maag is het aan te bevelen voorverteerde, gedenatureerde en/of vrije aminozuren in de voedersamenstelling op te nemen.

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Some foreigners are less foreign than the others. Back in 1981, during a visit of a group of german students, a young finely dressed Professor jumped into my office and uttered that we should collaborate. He showed some micrographs and tried to convince me about some important black dots. It was no use; I was simply puzzled. Nevertheless, it constituted the very start of a long, enjoyful and most fruitful collaboration with the Zoological Institute of the University of Heidelberg. The professor was dr. V. Storch, director of that Institute. Volker, your contribution is not so apparent on the first glance, but we know better. Your support was crucial to establish the Erasmus project which became the lubricating oil to my research. Your professoral authority has largely influenced my attitude towards doing science.

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Johan

Curriculum vitae

Johan (Alfons Jozef) Verreth (1952, Mechelen, Belgium) followed his secondary studies (Latin-Greek humaniora) at the St.Gabriël Institute, St. Kathelijne Waver (B), and studied subsequently Biology at the State University of Gent (B). He graduated in 1974 as Zoologist, study direction Morphology, Systematics and Ecology with specialisations in aquatic ecology and aquaculture. After his graduation, he further specialized in fish culture, fisheries and water quality assessment via specific training courses in Germany and Hungary and via several short term assignments at the University of Gent and for FAO (associate expertship) in Venezuela and Colombia. Since 1980, Johan Verreth is employed by the Wageningen Agricultural University, as University Lecturer at the Department of Fish Culture and Fisheries. He is responsible for the field of pond fish culture. Since 1992 he coordinates the field of nutrition and nutrient management in fish production systems. His function as university lecturer encompassed teaching, course development, curriculum reformulation, MSc and PhD research, consultancies for FA0, DGIS (Dutch Ministry of Technical Cooperation), Nuffic and IFS, and administrative/managerial tasks such as the chairmanship of the educational committee of the study direction of Animal Production, the coordination of an Erasmus project in the field of fish (larvae) nutrition, etc. In his research, Johan Verreth focused on larviculture in ponds (1981-1986) and in indoor hatcheries (1986 to present). The present thesis covers a significant part of the latter research effort. Over the last 10 years, he (co-)authored more than 40 publications, out of which 27 full papers in peer reviewed journals and 5 papers in conference proceedings and/or international professional journals. He was co-founder and first president (1984-1987) of the Dutch Aquaculture Society. In 1993 the University of Gent (B) appointed him as guest professor at the Faculty of Agricultural and Applied Biological Sciences. Johan Verreth is married and has two daughters.