

## Stellingen

1. Differences in performance between decapsulated cysts and nauplii of *Artemia* as food for fish larvae are due to the presence of micro-nutrients and/or the quality of major components (e.g. protein) rather than to the macro-nutrient composition.  
*This thesis*
2. The proteolytic enzymes found in *Artemia* contribute only marginally to the total proteolytic activity measured in fish larvae fed decapsulated cysts.  
*This thesis*
3. To develop dry feeds for fish larvae, test diets should be based on *Artemia* rather than on ingredients commonly used in feeds for juvenile or adult fish.  
*This thesis*
4. When fish feeds are developed too much focus is given on finding good properties of diets rather than on searching explanations for a diet failure.
5. Give one fish to a man and you will feed him for the day, teach him how to grow fish in a sustainable way and you will feed him for the future.  
*After Chinese proverb*
6. Justifications from operators of computer networks in trouble confirm that we are really entering the era where man is dominated by the machine.
7. The Dutch saying *zet uw vis terug in het water en bewaar uw sport voor later* does not take the fish nor its flesh seriously.
8. The most relaxed pace of life is found in Mexico.  
*R. Levine (1997). A Geography of Time. Basic Books, New York, USA.*
9. Belgians talk when they have a problem, Dutchmen talk till they have a problem.
10. Mexicans believe lime cures almost everything and tequila almost everything else.
11. Mexicans abroad often end up talking about how nice the Mexican food is.

*Stellingen belonging to the thesis:*

"Nutritional value of decapsulated cysts of *Artemia* and their use as protein source in experimental microdiets for fish larvae"

Armando García Ortega,  
Wageningen, December 20, 1999.

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**Nutritional value of decapsulated cysts of *Artemia* and their use as protein source in experimental microdiets for fish larvae**

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### Abstract

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Live food dependence and the lack of suitable artificial diets are major constraints for the expansion of the larviculture in many fish species. The low digestibility and nutritional quality of artificial diets are factors that might explain their failure as starter food for fish. In this thesis, physiological aspects related to the capacity for digestion of food by fish larvae were studied. In addition, the protein quality in decapsulated cysts and nauplii of *Artemia* was evaluated, and the feasibility of using cysts as protein source in microdiets for fish larvae was studied. African catfish *Clarias gariepinus* was the experimental species. The results indicate that catfish larvae have the capacity to digest *Artemia*. The exogenous supply of digestive enzymes has a small contribution to the overall larval digestion. The protein in *Artemia* is mainly constituted by small size proteins, which might be more easily digested by the fish larvae than the proteins in artificial diets. It is suggested that the structure and size of the proteins in the fish food might have a very important role for its digestibility. *In vitro* studies on the protein digestibility of *Artemia* decapsulated cysts and nauplii, and of microbound diets made of decapsulated cysts revealed a higher digestibility of those diets compared to commercial diets. The use of decapsulated cyst of *Artemia* as protein source in microbound diets improved their performance as starter diet for fish larvae.

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## **General Introduction**

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## Aquaculture production and larviculture

Production of food by aquaculture has been constantly growing at an annual average rate of 10 % during most of the eighties and nineties, and it is expected to continue its present rate of growth during the first decade of the next century. This growth rate has made of aquaculture the fastest growing production system surpassing both terrestrial livestock (3 % per year) and fisheries (1.6 % per year) productions (Tacon, 1998). Improvements in culture techniques, in food technology and the introduction of new species are expected to expand further the industry.

Aquaculture production is based on the use of extensive or intensive fish culture systems. For several fish species under culture, the entire cycle of production has been completed, i.e. from the production of eggs and larvae to market size fish and broodstock. This allows an optimal and predictable production of fish throughout the year. For other fish species, especially marine fish, a complete production cycle is hampered by the lack of a predictable supply of high quality larvae and juveniles for on-growing farms. In the culture of some fish species, the larvae supply still relies on the capture from the wild. In the larviculture of those fish the critical period has been identified at the start of exogenous feeding (Watanabe and Kiron, 1994). Once the yolk sac reserves are depleted, the acquisition of nutrients in first feeding fish depends on the ingestion of zooplankton organisms found in their natural habitat. In larviculture, the most widely used live food organisms are the rotifer *Brachionus plicatilis* and the brine shrimp *Artemia*. They provide the best results in terms of fish larvae growth and survival in comparison to artificial diets (Jones et al., 1993; Watanabe and Kiron, 1994). Because the cultivation and maintenance of live food organisms is costly, labor intensive, unpredictable and in some cases could lead to the production of live food with sub-optimal nutritional quality, efforts are being done for their partial or total replacement. A suitable starter artificial diet is fundamental for the success in the larviculture of many fish species. However, so far the numerous attempts to replace live food by artificial diets had limited success. For the improvement of fish larvae rearing, a better knowledge with regard to the larval feeding and growth, as well as detailed information on the nutritional physiology of the early life stages of fish are necessary.

## Fish larvae nutritional physiology

In fish larvae, growth and survival are mainly influenced by nutritional aspects. During development, the fish larvae pass through important anatomical and physiological changes, which affect their nutritional requirements. After hatching, the fish larvae obtain all the required nutrients from their yolk sac. Once the yolk sac is absorbed, the fish initiates exogenous feeding. During the larval stage, the development of the digestive system differs among fish species (Govoni et al., 1986). For practical purposes, fish larvae can be divided in three groups according to the morphology of the digestive tract and the enzymes secreted into the gut (Dabrowski, 1986). In a first group are included fish larvae that have a functional stomach before switching from endogenous to exogenous feeding (e.g. salmonids). A second group consists of fish that lack a functional stomach or gastric glands secretions during the larval stage. In a third group, fish without stomach during the entire life cycle (e.g. cyprinids) are included. The second group of fish includes some freshwater and most marine fish under culture. To date most research has been focused on the physiological and nutritional aspects of the larval stages of those fish.

In the larviculture of fish species like salmonids, the first feeding phase can be realized with artificial diets. Other species require zooplankton organisms as initial food. In the fish without a functional stomach at the start of exogenous feeding the major changes in the digestive system during the larval period are related to the onset of stomach functions. Those changes involve switching from a trypsin-type enzymatic digestion of food as occurs when the stomach functions are absent, to a combination of acid secretion and pepsin-type of enzymatic digestion once the stomach becomes completely functional. Apparently, at the onset of exogenous feeding, the digestive capacities in those fish are sufficient to optimally digest and absorb nutrients from live food, but not from artificial diets. On the other hand, once the stomach has become functional, those fish species can be weaned successfully to artificial diets (Verreth et al., 1992; Pedersen and Falk-Petersen, 1992).

For the breakdown of proteins from artificial dry diets the action of the stomach is important for the process of denaturation and pepsin-type (acid) type of digestion (Segner et al., 1994). The lack of stomach functions is regarded as one important factor to explain the difficulties found when rearing fish larvae on artificial diets (Verreth et al., 1992; Segner et al., 1993). This is evidenced by the fact that fish that possess a functional stomach after the

yolk sac absorption can be reared with artificial dry diets from first feeding. Fish larvae that lack stomach functions rely on live food organisms at the start of exogenous feeding.

### **Live food versus artificial diets**

To explain the higher larval fish growth achieved with live food compared to artificial diets several hypotheses have been suggested. One hypothesis suggests that the digestive enzymes in live food (exogenous enzymes) play a key role in the utilization of nutrients by fish larvae (Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984; Munilla-Moran et al., 1990; Kolkovski et al., 1993). Those studies indicate that the enzymes derived from live food organisms play an important role in the digestion of nutrients due to a low enzyme production and a reduced digestive capacity in the fish larvae. On the other hand, Segner et al. (1993) and Cahu and Zambonino-Infante (1997) demonstrated that the reduced growth in fish larvae fed artificial diets cannot be attributed to a deficiency in the digestive capacity of the fish. Moreover, in a study on the digestive enzyme activity in sea bass larvae, it was demonstrated that fish larvae fed artificial diets or *Artemia* nauplii yielded the same enzymatic capacity (Zambonino-Infante and Cahu, 1994). This suggests that the fish larvae have the capacity for the digestion of food and that the enzymes in live food might not be essential for the protein digestion by fish larvae. However, more evidence is needed to verify how general this conclusion is for a wider group of fish species.

A second hypothesis proposed to explain the better utilization of nutrients from live food in comparison to those in artificial diets considers the form in which the protein is presented to the fish larvae. Eggs and yolk sac larvae of marine species are rich in free amino acids which are used for body protein synthesis and for energy metabolism (Rønnestad and Fyhn, 1993). Once the yolk reserves are depleted, the fish larvae rely on the supply of easy-to-digest proteins from live food. The free amino acid content of marine zooplankton is high (Fyhn et al., 1993). Marine fish larvae might be highly dependent of the supply of free amino acids at first feeding (Fyhn 1989; Rønnestad et al., 1993). Fyhn (1989) and Walford and Lam (1993) suggested also that dietary free amino acids or low molecular weight peptides in larval diets might increase their absorption by the fish larvae.

## The brine shrimp *Artemia*

Nauplii of the small branchipod crustacean *Artemia* is the most widely used live food organism in larviculture of fish and shrimp (Léger et al., 1986). The size range of *Artemia* and its different physical forms (i.e. cyst or free-swimming nauplii and adults), make it very versatile for use in aquaculture. For the early life stages of fish, *Artemia* constitutes an excellent food item that meets the nutritional requirements of most fish. Decapsulated cysts of *Artemia* have been used successfully in the feeding of larvae of African catfish (Verreth et al., 1987; Pector et al., 1994), whitefish (Drouin et al., 1986), and carp (Vanhaecke et al., 1990). The use of decapsulated *Artemia* cysts as food for fish larvae has the advantages of combining some of the physical properties of a dry diet and the nutritional value of live nauplii. The cysts small particle size (100-250  $\mu\text{m}$ ) is adequate to be ingested by the fish larvae. The decapsulated cysts have a membrane that avoids the leaching of nutrients, in this way ensuring a balanced and constant nutritional composition in all food particles. Moreover, the energy content and individual dry weight of decapsulated cysts are higher than any of the stages of hatched nauplii (Vanhaecke et al., 1983; Léger et al., 1986). In the present study, both nauplii and decapsulated cysts of *Artemia* were used as model diets to avoid interactive effects with feed intake and digestibility, whereby decapsulated cysts served as model for artificial diets.

## Hypothesis and objectives of the research

From the available information, the following possible causes for a low growth and survival in fish larvae receiving artificial diets can be derived:

1. Live food has a better composition and it is better ingested than artificial diets. Nutrients in artificial diets might leach.
2. The digestive enzymes present in live food organisms assist in the digestion of food by the fish larvae.
3. The protein in live food has a higher affinity to the larval digestive enzymes and are more easily digested and absorbed than proteins used for the preparation of artificial diets.

Each of these hypotheses has been addressed in the literature and there is evidence that all three or a combination of them might be valid. In general, those studies emphasized the importance of food intake and food digestion. In this thesis research, possible bias derived

from the first aspect is avoided by using decapsulated cyst of *Artemia* in feeding experiments with catfish larvae. The problems related to food intake and feed particle quality can be solved with appropriate husbandry and feed processing techniques and by using a food item that is well accepted by fish larvae. Therefore, the research was focused on the physiological aspects (hypotheses 2 and 3). To assess the importance of the second hypothesis, the contribution of dietary proteolytic enzymes to the total digestion of food by the larvae has to be determined. The third hypothesis is based on the fact that there is an absence of acid denaturation and a proteolytic capacity in the larvae that differs from juvenile fish. The form in which the dietary proteins are supplied with the food might be of importance since live food organisms usually contain relative large amounts of free amino acids. Pre-treatment of food ingredients could improve the digestion and absorption of nutrients (e.g. protein) from artificial diets in the digestive system of fish larvae without a functional stomach. The experimental fish species used was the African catfish *Clarias gariepinus*. This fish was selected because it represents the group of fish that cannot be optimally reared with artificial diets during the larval stage. The larviculture of *C. gariepinus* is well established with good results in terms of growth and survival. Moreover, it is possible to perform standardized nutritional experiments during the catfish larval period.

Taking all this into consideration, the general objectives of the thesis research were to assess the importance of dietary enzymes on the digestion of food by fish larvae. Moreover, to test the hypothesis of whether pre-digestion of proteins in food improve their affinity to the larval digestive enzymes resulting in better digestion and absorption. In addition, the feasibility of using *Artemia* decapsulated cysts as protein source in dry experimental diets was tested as well.

## Outline of this thesis

In the present thesis different subjects were investigated to study some aspects which might induce the optimal digestion of nutrients (e.g. protein) in live food, compared to the artificial diets.

In **Chapter 1** the proximate composition, the amino acid and fatty acid contents, and the dry matter of cysts and nauplii of *Artemia* during development were determined. Additionally, a characterization of the proteolytic and specific enzyme activities was done to determine the amount of alkaline enzymes that are present in individual cysts and nauplii.

The effects of heat as food processing techniques on the protein quality of *Artemia* cysts were studied in **Chapter 2**. The results permitted us to obtain information of the effects of modifying the functional properties of the protein in the food for fish larvae.

In Chapters 3 and 4 the relative contribution of food enzymes to the total digestion of food by fish larvae was determined. In **Chapter 3** the food ingestion and food evacuation rate in fish larvae was determined by a new method using a natural marker (ascorbic acid sulfate) found in the cysts of *Artemia*. In **Chapter 4** the post-prandial variations in the proteolytic activity of fish larvae were determined and compared to starvation.

**Chapter 5** describes a new method for the determination of the protein digestibility in decapsulated cysts of *Artemia* and microdiets as measured *in vitro* simulating the conditions in which protein digestions occurs in stomachless fish larvae. In **Chapter 6** microdiets for fish larvae made of *Artemia* cysts or fishmeal were tested in a feeding experiment, the protein quality in the diet was evaluated and the differences in larval growth performance were related to diet protein structure. Moreover, the suitability of *Artemia* decapsulated cysts as protein source in artificial diets for fish larvae was discussed.

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## Chapter 1

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### **Biochemical and enzymatic characterization of decapsulated cysts and nauplii of the brine shrimp *Artemia* at different developmental stages**

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## Abstract

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Decapsulated *Artemia* cysts were used to study the factors which induce the superior performance of live organisms as food for fish larvae. The biochemical composition, the *in vitro* protein digestibility, and the total proteolytic and trypsin activities in cysts and nauplii of *Artemia* were determined at different developmental stages as a function of incubation time. Six different incubation times were studied: 1, 6, 11, 16, 21 and 25 h of development, which cover cyst and early nauplii stages. The individual dry weight of *Artemia* decreased through development. The individual protein and lipid content ( $\mu\text{g ind}^{-1}$ ) remained constant during development until the time of hatching after which they decreased slightly. However, no significant incubation effect was found. Small changes in amino acid and fatty acid composition were found during development, but is assumed that they are too small to be of nutritional importance to fish larvae. No major changes were observed in protease activities measured at acid and alkaline pH during the first 25 h of development. From the alkaline proteases, no significant change in trypsin activity was detected during cyst and early nauplii development. From the point of view of exogenous enzyme contribution to fish larvae, there seems to be no difference whether feeding decapsulated cysts or newly hatched nauplii, since no difference in qualitative protease composition was found during the first 25 h of *Artemia* development. The relative contribution of *Artemia* proteases to the digestion of food by fish larvae is discussed.

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**Keywords:** *Artemia* cysts; Proteolytic enzymes; Feeding and nutrition- fish, feed composition; Protein digestibility; Live food

## Introduction

Larvae of nearly all marine and of many freshwater fish species require live planktonic organisms as first food. In fish culture the use of live food organisms complicates the rearing techniques and increases the operational costs. For many fish species live food still gives better results in terms of growth and survival than artificial diets (Dabrowski, 1984). Several hypotheses have been proposed to explain this: e.g., food composition; structure and digestibility of dietary protein (Verreth, 1994); exogenous enzymes (Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984); chemical and visual stimuli from the live food organisms (Kolkovski et al., 1995). Studies to understand those factors are complicated by the fact that any comparison between live and artificial diets are confounded by behavioral aspects. Decapsulated *Artemia* cysts have been proposed to solve the latter (Verreth et al., 1987).

Decapsulated *Artemia* cysts have been tested successfully for larval rearing of African catfish (Verreth et al., 1987; Pector et al., 1994) and common carp (Vanhaecke et al., 1990). The cysts of *Artemia* present some advantages to artificial diets because they combine the physical properties of a dry artificial feed and the nutritional value of live *Artemia* nauplii. The question is whether this hypothesis is true. The nutritional value of decapsulated cysts might be sensitive to the quality of the decapsulation and subsequent drying procedures. During the decapsulation process the cysts are hydrated, as a consequence, the reversibly interrupted metabolism of the *Artemia* embryo resumes. This might result in a food with a biochemical and nutritional composition that diverge from both dry cysts and live nauplii. From previous studies on the biochemical composition and nutritional value of *Artemia*, only Hines et al. (1980) followed the biochemical composition during the development of both cysts and nauplii. For future studies on the role of dietary enzymes in larval digestion, the characterization and quantitative estimation of digestive enzymes from decapsulated cysts of *Artemia* are necessary to determine the enzyme input from live food to fish larvae. However, a combined analysis of the biochemical and enzymatic changes in *Artemia* during cyst development is not available. The present study was designed to provide such a characterization for the same batch of cysts during their development.

## Materials and methods

### *Hatching quality and cysts incubation*

The hatching rate and hatching percentage of the *Artemia* cysts were determined following the procedure described by Sorgeloos et al. (1986). The same batch of *Artemia* cysts (INVE Aquaculture N.V.; type EG batch number 5335) from Great Salt Lake (GSL), USA was used in all the analyses. The cysts were decapsulated with a solution of NaOCl, NaOH and water according to the method of Sorgeloos et al. (1986). In all analyses dry non-decapsulated cysts were used in parallel with decapsulated cysts to compare the changes in the embryo nutritional characteristics from the inactive dormant embryo to a cyst where the embryonic metabolism has been reactivated by incubation in seawater. After decapsulation, the cysts were incubated in hatching tanks with conical bottom to start the embryonic development process. The incubation conditions were: temperature 28°C, salinity 33‰, approximately 1200 lux of continuous illumination at the water surface, and medium aeration from the bottom of the tank to maintain the cysts in suspension. Six different incubation times were used: 0, 5, 10, 15, 20, and 24 h, which encompass cyst and early nauplii development. After incubation samples for enzyme analysis were shock-frozen with liquid nitrogen and stored at -80°C. Samples for the biochemical and digestibility analyses were stored at -20°C and later freeze-dried before further processing.

### *Biochemical composition*

Protein content was determined by Kjeldahl analysis according to ISO (International Organization for Standardization) 5983 (1979) procedures and calculated as nitrogen content multiplied by 6.25. Individual amino acids were measured with a Biotronic LC 5001 amino acid analyzer with a single column using an ion exchange resin. Amino acids were determined with ninhydrin as coloring reagent and quantified using an amino acid standard solution (Sigma AA-S-18), L-nor-leucine (Merck 24560) and L-tryptophan (Merck 8374). Cystine and methionine were analyzed after oxidation with performic acid and acid hydrolysis. Tryptophan was determined after alkaline hydrolysis. All other amino acids were determined after acid hydrolysis. The lipids in dry cysts samples were extracted following a modified procedure of Folch et al. (1957) using chloroform-methanol (2:1) and esterified with a mixture of 1% H<sub>2</sub>SO<sub>4</sub> in methanol. After fat extraction and esterification a fatty acid methyl ester (FAME) analysis was done according to the method described by Coutteau and Sorgeloos (1995) to obtain the HUFA profile of cysts samples. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC)

using a single-dimension double-development method (Tocher and Harvie, 1988). The lipid classes were quantified by charring followed by densitometry using a Sharp JX-325 scanner supported with ImageMaster<sup>TM</sup> Software (modified from Olsen and Henderson, 1989). Total carbohydrate was determined by the phenol-sulfuric acid reaction (Dubois et al., 1956) using glucose as a standard. Ash content was determined by burning oven-dried samples in a muffle furnace at 550°C according to ISO 5984 (1978). Individual dry weight was estimated by freeze-drying a known amount of cysts incubated in 2 L aerated containers at the different experimental times. The dry weight of the sample per volume was divided by the number of individuals counted in 250 µl samples taken during the incubation.

### ***In vitro protein digestibility***

A modified filtration method from Babinszky et al. (1990) was used to predict the protein digestibility *in vitro* of the cysts and nauplii samples. The samples were incubated in a two steps procedure with enzymes. In the first step the protein was digested with a solution of pepsin (Merck, 7190) and HCl (pepsin 2 g l<sup>-1</sup> in HCl 0.1 M, pH 1) during 1 h at 30°C. In the second step, the samples were further digested during 1 h with a solution of pancreatin (Merck, 7133), amylase (NOVO, Termamyl 120L), lipase (Sigma L3001) and bile salts (Sigma B8756) dissolved in a mixture of Na<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub>. After digestion in the multi-enzyme system, the digested fraction was vacuum-filtrated and the amount of undigested crude protein was determined by the Kjeldahl method. From the nitrogen content of the original cysts sample and the undigested protein from this analysis, the *in vitro* digestible protein content was calculated.

### ***Enzyme analyses***

The total protease was measured in cysts and nauplii homogenates. For homogenate preparation 75 mg of sample was ground in a potter blender and dissolved in 750 µl of distilled water. After homogenization the samples were sonified during 30 sec and centrifuged at 12000 × g during 10 min at 4°C. During preparation the homogenates were continuously kept on ice. Total protease activity was measured by a modified casein method from Walter (1984) using casein (10 mg ml<sup>-1</sup>) dissolved in 0.1 M Tris buffer at pH 8. The protease activity was determined at different pH values of: 3, 4, 6, 8 and 10. A citrate-phosphate buffer (0.1 M citric acid plus 0.2 M Na<sub>2</sub>HPO<sub>4</sub>) and a buffer composed of 0.2 M Tris and 0.1 N HCl were used to detect acidic and alkaline protease respectively with L-tyrosine as standard. The assay mixture, which consisted of 400 µl casein solution, 400 µl buffer and 200 µl supernatant of homogenate, was incubated at

30°C for 60 min, the reaction was stopped with trichloroacetic acid (0.3 M). After cooling on ice for 15 min, the assay mixtures were centrifuged at  $1700 \times g$  for 10 min at 4°C and the absorbance of the supernatant was measured at 280 nm with a spectrophotometer. The results are expressed as mg tyrosine liberated per g protein in sample per 60 min. Protein was determined by Kjeldahl analysis.

The trypsin activity was determined using a modified method from Hofer and Köck (1989) and Bergmeyer (1974) using the substrate BAPNA (N $\alpha$ -benzoyl-DL-arginine-4-nitroanilide, Merck 1670). The homogenate preparation for the trypsin measurements was similar to the casein assay with the difference that 100 mg of cyst or nauplii sample were ground and dissolved in 500  $\mu$ l of buffer. The assay mixture consisted of 3.9 mM BAPNA in a 0.1 M Tris buffer pH 8 with 0.2 M CaCl<sub>2</sub>. The enzymatic reaction was started by adding BAPNA to a mixture of buffer and homogenate. The change in absorbance during 5 min was measured at 405 nm. Trypsin activity is reported as Units g<sup>-1</sup> of sample (1 U = 1  $\mu$ mol BAPNA converted per min).

### ***Data analysis***

Four samples per incubation time were analyzed for total proteolytic activity and at least three for trypsin activity. Four samples per incubation time were analyzed for individual amino acid content except for cystine, methionine, tyrosine, histidine, phenylalanine and tryptophan which were determined with two samples. For protein, lipid, carbohydrate, fatty acid and ash content, protein digestibility and individual dry weight three samples per incubation time were analyzed. Lipid classes were determined with two samples per treatment. The change in individual weight was also considered when comparing changes in any nutritional component during the development of *Artemia* from cyst to nauplii. The data expressed as relative values were tested for normal distribution and submitted to square root arcsine transformation before a one-way ANOVA was performed. When significant differences were found, the means were compared with Duncan's multiple range test ( $P < 0.05$ ). The data expressed as absolute values were submitted to a non-parametric one-way ANOVA (Kruskal-Wallis test). The statistical analyses were done with SAS statistical software package (SAS Institute).

Prior to decapsulation the cysts were hydrated for 1 h and this hydration reactivates the metabolism of the dormant embryo in the cysts, therefore in the results 1 h is added to all incubation times and is indicated as hours of development.

## Results

### Hatching quality

The hatching efficiency corresponded to 144 377 nauplii g<sup>-1</sup> cysts and the hatching percentage was 70.1 ± 5.1%. From the hatching curve T<sub>10</sub> (time of 10% hatching) and T<sub>50</sub> (50% hatching) were graphically derived and corresponded to 14.5 and 17.3 h, respectively. The cysts showed 90% of their maximum hatchability (T<sub>90</sub>) at 21.8 h. The hatching synchrony (T<sub>s</sub> = T<sub>90</sub> - T<sub>10</sub>) was 7.3 h.

**Table 1.** Proximate composition, individual dry weight and *in vitro* protein digestibility of *Artemia* at different developmental stages as function of incubation time.

	Undeap. cysts	Time of development (h)					
		1	6	11	16	21	25
<b>Protein</b>							
% dw	55.8 ± 0.1	50.6 ± 0.1 <sup>a</sup>	53.8 ± 0.1 <sup>d</sup>	54.7 ± 0.1 <sup>c</sup>	55.7 ± 0.2 <sup>b</sup>	56.3 ± 0.1 <sup>a</sup>	56.2 ± 0.0 <sup>a</sup>
µg ind <sup>-1</sup> *	2.70 ± 0.1	1.73 ± 0.07	1.77 ± 0.08	1.67 ± 0.07	1.70 ± 0.08	1.48 ± 0.06	1.30 ± 0.06
<b>Lipid</b>							
% dw	11.2 ± 0.2	14.7 ± 0.1 <sup>d</sup>	16.2 ± 0.5 <sup>c</sup>	16.4 ± 0.3 <sup>bc</sup>	16.9 ± 0.3 <sup>b</sup>	18.0 ± 0.2 <sup>a</sup>	17.0 ± 0.3 <sup>b</sup>
µg ind <sup>-1</sup> *	0.54 ± 0.03	0.51 ± 0.02	0.53 ± 0.03	0.51 ± 0.02	0.52 ± 0.02	0.48 ± 0.02	0.40 ± 0.02
<b>Carbohydrate</b>							
% dw	6.9 ± 0.7	6.6 ± 0.5 <sup>a</sup>	6.6 ± 1.1 <sup>a</sup>	4.2 ± 0.3 <sup>b</sup>	4.3 ± 0.2 <sup>b</sup>	4.3 ± 0.7 <sup>b</sup>	3.6 ± 0.4 <sup>b</sup>
µg ind <sup>-1</sup> *	0.33 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.09 ± 0.01
<b>Ash</b>							
% dw	5.9 ± 0.2	10.6 ± 0.3 <sup>a</sup>	11.1 ± 0.2 <sup>a</sup>	9.0 ± 0.2 <sup>bc</sup>	9.8 ± 0.6 <sup>ab</sup>	7.2 ± 0.1 <sup>d</sup>	7.6 ± 1.2 <sup>cd</sup>
µg ind <sup>-1</sup> *	0.29 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.19±0.01	0.18 ± 0.01
<b>Individual dry weight</b>							
µg *	4.83 ± 0.2	3.42 ± 0.2	3.28 ± 0.2	3.06 ± 0.1	3.05 ± 0.1	2.62 ± 0.1	2.31 ± 0.1
<b>Digestible protein</b>							
% total protein	-	82.8 ± 1.3	79.7 ± 7.1	81.5 ± 4.0	79.5 ± 2.8	82.5 ± 1.0	77.8 ± 1.6

The time of development is the time of incubation plus one hour of hydration time during the decapsulation process.

Means in the same row with the same superscript are not significantly different (P>0.05) (% values)

\* No significant incubation effect was detected (P>0.05)

The composition of the samples taken from each of the incubation times were: 100% of cysts for 1, 6 and 11 h of development; 35% cysts and 65% nauplii (umbrella stages included) for 16 h; 33% cysts and 67% nauplii for 21 h; 30% cysts and 70% nauplii for 25 h. The latter 30% cysts can be regarded as unhatchable cysts which were present in all samples.

Table 2. Amino acid composition (g 100 g<sup>-1</sup> protein) of *Artemia* at different developmental stages as function of incubation time.

Amino acid	Uncap. cysts	Time of development (h)					
		1	6	11	16	21	25
Aspartic acid	8.6 ± 0.2	7.7 ± 0.2	7.5 ± 0.4	7.4 ± 0.0	7.5 ± 0.1	7.6 ± 0.3	7.6 ± 0.1
Threonine	4.1 ± 0.0	4.3 ± 0.2	4.4 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.1	4.3 ± 0.1
Serine	6.3 ± 0.1	5.7 ± 0.1 <sup>a</sup>	5.9 ± 0.1 <sup>a</sup>	5.5 ± 0.0 <sup>b</sup>	5.5 ± 0.0 <sup>b</sup>	5.5 ± 0.1 <sup>b</sup>	5.5 ± 0.0 <sup>b</sup>
Glutamic acid	10.3 ± 0.4	11.0 ± 0.4 <sup>ab</sup>	11.1 ± 0.2 <sup>ab</sup>	10.3 ± 0.1 <sup>c</sup>	10.9 ± 0.1 <sup>b</sup>	11.0 ± 0.1 <sup>ab</sup>	11.2 ± 0.1 <sup>a</sup>
Proline	5.0 ± 0.2	3.6 ± 0.1 <sup>c</sup>	3.6 ± 0.1 <sup>c</sup>	3.8 ± 0.2 <sup>b</sup>	4.0 ± 0.2 <sup>ab</sup>	4.0 ± 0.1 <sup>a</sup>	4.2 ± 0.1 <sup>a</sup>
Glycine	4.0 ± 0.1	3.9 ± 0.0 <sup>ab</sup>	3.9 ± 0.1 <sup>b</sup>	3.9 ± 0.0 <sup>b</sup>	4.0 ± 0.0 <sup>a</sup>	4.0 ± 0.1 <sup>a</sup>	4.0 ± 0.0 <sup>a</sup>
Alanine	5.1 ± 0.1	4.1 ± 0.0 <sup>c</sup>	4.3 ± 0.1 <sup>b</sup>	4.2 ± 0.0 <sup>c</sup>	4.2 ± 0.0 <sup>c</sup>	4.3 ± 0.1 <sup>b</sup>	4.4 ± 0.0 <sup>a</sup>
Cystine	1.3 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
Valine	4.9 ± 0.1	4.9 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.9 ± 0.1
Methionine	2.1 ± 0.0	2.5 ± 0.1	2.2 ± 0.4	2.4 ± 0.0	2.4 ± 0.0	2.4 ± 0.0	2.3 ± 0.0
Isoleucine	4.4 ± 0.1	4.8 ± 0.0 <sup>a</sup>	4.7 ± 0.1 <sup>ab</sup>	4.6 ± 0.0 <sup>b</sup>	4.6 ± 0.1 <sup>b</sup>	4.6 ± 0.1 <sup>b</sup>	4.7 ± 0.0 <sup>ab</sup>
Leucine	6.0 ± 0.2	6.6 ± 0.1 <sup>a</sup>	6.5 ± 0.1 <sup>ab</sup>	6.3 ± 0.0 <sup>c</sup>	6.4 ± 0.1 <sup>bc</sup>	6.4 ± 0.1 <sup>bc</sup>	6.5 ± 0.1 <sup>abc</sup>
Tyrosine	5.2 ± 0.1	3.3 ± 0.0 <sup>ab</sup>	3.1 ± 0.2 <sup>b</sup>	3.3 ± 0.0 <sup>ab</sup>	3.3 ± 0.0 <sup>ab</sup>	3.3 ± 0.0 <sup>ab</sup>	3.4 ± 0.0 <sup>a</sup>
Phenylalanine	3.7 ± 0.0	3.9 ± 0.0 <sup>a</sup>	3.8 ± 0.0 <sup>bc</sup>	3.8 ± 0.0 <sup>c</sup>	3.8 ± 0.0 <sup>c</sup>	3.8 ± 0.0 <sup>bc</sup>	3.9 ± 0.0 <sup>ab</sup>
Histidine	2.7 ± 0.1	2.7 ± 0.0 <sup>a</sup>	2.7 ± 0.0 <sup>a</sup>	2.6 ± 0.0 <sup>b</sup>	2.4 ± 0.0 <sup>c</sup>	2.5 ± 0.0 <sup>c</sup>	2.5 ± 0.0 <sup>bc</sup>
Lysine	7.1 ± 0.2	7.4 ± 0.1 <sup>a</sup>	7.3 ± 0.1 <sup>ab</sup>	6.9 ± 0.1 <sup>d</sup>	7.2 ± 0.0 <sup>c</sup>	7.2 ± 0.1 <sup>bc</sup>	7.3 ± 0.1 <sup>ab</sup>
Arginine	6.4 ± 0.2	7.0 ± 0.0 <sup>a</sup>	7.0 ± 0.1 <sup>b</sup>	6.7 ± 0.0 <sup>d</sup>	6.7 ± 0.0 <sup>cd</sup>	6.7 ± 0.1 <sup>cd</sup>	6.8 ± 0.0 <sup>c</sup>
Tryptophan	0.9 ± 0.1	1.3 ± 0.1 <sup>a</sup>	1.2 ± 0.0 <sup>ab</sup>	1.2 ± 0.0 <sup>ab</sup>	1.1 ± 0.1 <sup>b</sup>	1.2 ± 0.1 <sup>ab</sup>	1.2 ± 0.1 <sup>ab</sup>
Total amino acids	88.1 ± 1.5	85.8 ± 0.6 <sup>a</sup>	85.0 ± 0.5 <sup>ab</sup>	82.9 ± 0.6 <sup>d</sup>	84.0 ± 0.3 <sup>bc</sup>	84.7 ± 1.0 <sup>cd</sup>	85.8 ± 0.5 <sup>a</sup>

The time indicated as hours of development is the time of incubation plus one hour of hydration time during the decapsulation process

Means in the same row with the same superscript are not significantly different ( $P > 0.05$ )

Standard deviation values were rounded to decimals

### Proximate analysis

The protein, lipid, carbohydrate and ash composition of cysts and nauplii of *Artemia* are given in Table 1 in relative (% dw) and absolute values ( $\mu\text{g ind}^{-1}$ ). The relative concentration in the dry matter (% dw) of all four nutritional components changed significantly ( $P < 0.05$ ) during cyst development. However, for the absolute values no significant incubation effect was found ( $P > 0.05$ ). In uncapsulated cysts the individual protein content was higher than in all subsequent developing stages. However, during incubation (e.g. embryonal development) individual protein and lipid contents remained constant, exceptions were at 21 and 25 h which corresponded with the times of the highest presence of nauplii in the samples. Individual carbohydrate and ash contents also decreased towards the naupliar stage, attaining low values at 25 h of development.

The individual dry weight was highest for unencapsulated cysts, and decreased during development (Table 1).

### *Amino acid composition*

In general, small differences were found among individual amino acids (Table 2). The differences were larger for lysine and arginine for which smaller values were measured at 11 h of development, just before hatching. No significant differences were detected for aspartic acid, threonine, cystine, valine and methionine. Tryptophan was detected at all times of development and its values ranged from 0.9 g 100 g<sup>-1</sup> protein in unencapsulated cysts up to 1.3 g 100 g<sup>-1</sup> protein at 1 h. The total amino acid content remained constant during development with exceptions at 11 and 16 h when it decreased and corresponded to the time before and after hatching, respectively.

### *Fatty acid composition and lipid classes*

As indicated in Table 3, the fatty acid composition during cysts and nauplii development presented small differences. A slight decreasing trend was detected in samples at 25 h of incubation; for most of the fatty acids, the values of nauplii were smaller than in the previous developmental stages. Most of the lipid classes remained constant during development (Table 4). Free fatty acids increased strongly towards 25 h of development. Total polar lipid increased towards 21 h of development, while the total neutral lipid did not vary.

### *In vitro protein digestibility*

High protein digestibility (expressed as percentage of total protein content) was found for all the developmental stages (Table 1). It ranged from 77.8% at 25 h to 82.8% at 1 h. No difference in digestibility was found between cysts and nauplii samples.



**Table 3.** Fatty acid composition (mg g<sup>-1</sup> DW) of *Artemia* at different developmental stages as function of incubation time. The time of development is the time of incubation plus one hour of hydration time during the decapsulation process.

Fatty acid	Uncap. cysts	Time of development (h)					
		1	6	11	16	21	25
14:0	1.3 ± 0.1	1.6 ± 0.1 <sup>a</sup>	1.8 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>a</sup>	1.8 ± 0.1 <sup>a</sup>	1.7 ± 0.4 <sup>a</sup>	1.2 ± 0.1 <sup>b</sup>
14:1n-5	1.1 ± 0.1	1.3 ± 0.1 <sup>ab</sup>	1.5 ± 0.1 <sup>a</sup>	1.5 ± 0.1 <sup>a</sup>	1.6 ± 0.1 <sup>a</sup>	1.4 ± 0.4 <sup>a</sup>	1.1 ± 0.0 <sup>b</sup>
15:0	0.3 ± 0.0	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.1 <sup>b</sup>	0.3 ± 0.0 <sup>c</sup>
15:1n-5	0.6 ± 0.1	0.7 ± 0.0 <sup>ab</sup>	0.8 ± 0.0 <sup>a</sup>	0.8 ± 0.1 <sup>a</sup>	0.8 ± 0.1 <sup>a</sup>	0.8 ± 0.2 <sup>a</sup>	0.6 ± 0.0 <sup>b</sup>
16:0	12.7 ± 1.3	16.1 ± 0.4 <sup>a</sup>	17.8 ± 0.8 <sup>a</sup>	17.7 ± 0.8 <sup>a</sup>	18.6 ± 1.4 <sup>a</sup>	16.5 ± 4.0 <sup>a</sup>	13.2 ± 0.6 <sup>b</sup>
16:1n-7	3.9 ± 0.4	5.0 ± 0.1 <sup>a</sup>	5.6 ± 0.2 <sup>a</sup>	5.6 ± 0.2 <sup>a</sup>	5.9 ± 0.5 <sup>a</sup>	5.2 ± 1.3 <sup>a</sup>	4.1 ± 0.2 <sup>b</sup>
17:0	0.9 ± 0.1	1.1 ± 0.1 <sup>ab</sup>	1.2 ± 0.1 <sup>a</sup>	1.1 ± 0.1 <sup>a</sup>	1.3 ± 0.1 <sup>a</sup>	1.1 ± 0.3 <sup>ab</sup>	0.9 ± 0.0 <sup>b</sup>
17:1n-7	1.1 ± 0.1	1.4 ± 0.0 <sup>ab</sup>	1.6 ± 0.1 <sup>a</sup>	1.6 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>a</sup>	1.5 ± 0.4 <sup>a</sup>	1.2 ± 0.1 <sup>b</sup>
18:0	4.4 ± 0.4	5.3 ± 0.1 <sup>ab</sup>	5.9 ± 0.3 <sup>a</sup>	5.8 ± 0.2 <sup>a</sup>	6.3 ± 0.5 <sup>a</sup>	5.7 ± 1.3 <sup>ab</sup>	4.8 ± 0.2 <sup>b</sup>
18:1n-9	19.1 ± 1.9	24.2 ± 0.5 <sup>ab</sup>	26.9 ± 1.2 <sup>a</sup>	26.7 ± 1.2 <sup>a</sup>	28.3 ± 2.0 <sup>a</sup>	25.1 ± 6.2 <sup>a</sup>	20.3 ± 0.8 <sup>b</sup>
18:1n-7	6.7 ± 0.7	8.4 ± 0.2 <sup>bc</sup>	9.4 ± 0.3 <sup>ab</sup>	9.6 ± 0.4 <sup>ab</sup>	10.3 ± 0.7 <sup>a</sup>	9.2 ± 2.3 <sup>ab</sup>	7.4 ± 0.3 <sup>c</sup>
18:2n-6-1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
18:2n-6-c	5.5 ± 0.5	6.9 ± 0.2 <sup>a</sup>	7.6 ± 0.3 <sup>a</sup>	7.6 ± 0.3 <sup>a</sup>	8.1 ± 0.6 <sup>a</sup>	7.1 ± 1.8 <sup>a</sup>	5.7 ± 0.3 <sup>b</sup>
18:3n-6	0.5 ± 0.1	0.6 ± 0.0 <sup>ab</sup>	0.7 ± 0.1 <sup>a</sup>	0.6 ± 0.1 <sup>ab</sup>	0.6 ± 0.1 <sup>a</sup>	0.6 ± 0.1 <sup>ab</sup>	0.5 ± 0.1 <sup>b</sup>
18:3n-3	27.3 ± 2.8	34.2 ± 0.8 <sup>ab</sup>	37.9 ± 1.1 <sup>a</sup>	37.8 ± 1.7 <sup>a</sup>	39.6 ± 2.5 <sup>a</sup>	35.6 ± 9.0 <sup>a</sup>	28.6 ± 1.2 <sup>b</sup>
18:4n-3	4.6 ± 0.5	5.6 ± 0.1 <sup>a</sup>	6.2 ± 0.2 <sup>a</sup>	6.0 ± 0.3 <sup>a</sup>	6.2 ± 0.4 <sup>a</sup>	5.5 ± 1.4 <sup>a</sup>	4.5 ± 0.2 <sup>b</sup>
20:0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n-9	0.4 ± 0.1	0.4 ± 0.0 <sup>b</sup>	0.5 ± 0.1 <sup>ab</sup>	0.5 ± 0.1 <sup>a</sup>	0.5 ± 0.1 <sup>a</sup>	0.5 ± 0.1 <sup>ab</sup>	0.4 ± 0.0 <sup>b</sup>
20:4n-6	0.6 ± 0.0	0.7 ± 0.1 <sup>ab</sup>	0.8 ± 0.1 <sup>a</sup>	0.8 ± 0.1 <sup>a</sup>	0.8 ± 0.1 <sup>a</sup>	0.8 ± 0.2 <sup>ab</sup>	0.6 ± 0.1 <sup>b</sup>
20:3n-3	0.6 ± 0.0	0.7 ± 0.0 <sup>ab</sup>	0.8 ± 0.1 <sup>ab</sup>	0.8 ± 0.0 <sup>a</sup>	0.8 ± 0.1 <sup>a</sup>	0.8 ± 0.2 <sup>ab</sup>	0.6 ± 0.1 <sup>b</sup>
20:4n-3	0.7 ± 0.1	0.8 ± 0.0 <sup>ab</sup>	0.9 ± 0.0 <sup>a</sup>	0.8 ± 0.1 <sup>ab</sup>	0.9 ± 0.1 <sup>a</sup>	0.8 ± 0.2 <sup>b</sup>	0.7 ± 0.0 <sup>b</sup>
22:0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
20:5n-3	3.2 ± 0.3	4.2 ± 0.2 <sup>a</sup>	4.7 ± 0.2 <sup>a</sup>	4.7 ± 0.2 <sup>a</sup>	4.9 ± 0.3 <sup>a</sup>	4.4 ± 1.1 <sup>a</sup>	3.5 ± 0.2 <sup>b</sup>
22:6n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	tr	tr	tr	tr
Σ n-3 >or= 20:3n-3	4.6	5.8	6.5	6.3	6.6	6.0	4.8
Σ n-6 >or= 18:2n-6-1	6.8	8.4	9.3	9.2	9.7	8.8	7.0
Total FAME	100.0 ± 7.1	125.9 ± 2.9 <sup>ab</sup>	139.4 ± 5.0 <sup>a</sup>	139.3 ± 6.0 <sup>a</sup>	146.8 ± 10.1 <sup>a</sup>	130.8 ± 32.2 <sup>a</sup>	105.6 ± 4.3 <sup>b</sup>

Means in the same row with the same superscript are not significantly different ( $P > 0.05$ )

Standard deviation values were rounded to decimals

tr = trace

Table 4. Lipid classes (mg g<sup>-1</sup> DW) in *Artemia* at different developmental stages as function of incubation time.

Lipid class	Undevelop. cysts	Time of development (h)							
		1	6	11	16	21	25		
<b>Polar</b>									
Sphingomyelin	0.5 ± 0.0	nd	2.7 ± 0.0 <sup>a</sup>	3.0 ± 1.2 <sup>a</sup>	2.9 ± 0.1 <sup>a</sup>	3.0 ± 0.3 <sup>a</sup>	2.6 ± 0.1 <sup>a</sup>		
Lysophosphatidylcholine	nd	nd	nd	0.8 ± 0.1 <sup>a</sup>	0.4 ± 0.1 <sup>b</sup>	0.8 ± 0.1 <sup>a</sup>	0.7 ± 0.0 <sup>b</sup>		
Phosphatidylcholine	16.7 ± 1.2	24.7 ± 1.8 <sup>a</sup>	24.3 ± 1.2 <sup>a</sup>	23.4 ± 0.2 <sup>ab</sup>	23.5 ± 0.5 <sup>ab</sup>	24.4 ± 0.1 <sup>a</sup>	21.5 ± 0.3 <sup>b</sup>		
Phosphatidylserine	0.8 ± 0.2	0.5 ± 0.2 <sup>c</sup>	2.9 ± 0.0 <sup>b</sup>	2.8 ± 0.0 <sup>b</sup>	2.8 ± 0.4 <sup>b</sup>	4.8 ± 0.3 <sup>a</sup>	3.0 ± 0.1 <sup>b</sup>		
Phosphatidylinositol	2.6 ± 0.4	3.9 ± 0.4 <sup>c</sup>	4.5 ± 2.0 <sup>bc</sup>	6.0 ± 0.0 <sup>ab</sup>	6.0 ± 0.1 <sup>ab</sup>	7.8 ± 0.0 <sup>a</sup>	6.6 ± 0.2 <sup>ab</sup>		
Phosphatidic acid + cardiolipine	2.5 ± 0.3	2.8 ± 0.2 <sup>c</sup>	3.9 ± 1.6 <sup>bc</sup>	4.9 ± 0.2 <sup>ab</sup>	5.1 ± 0.1 <sup>ab</sup>	6.2 ± 0.4 <sup>a</sup>	4.8 ± 0.1 <sup>ab</sup>		
Phosphatidylethanolamine	7.6 ± 0.5	11.4 ± 0.4 <sup>d</sup>	13.1 ± 1.0 <sup>bc</sup>	13.7 ± 0.2 <sup>bc</sup>	14.3 ± 0.2 <sup>ab</sup>	15.1 ± 0.4 <sup>a</sup>	12.4 ± 0.3 <sup>cd</sup>		
<b>Neutral</b>									
Pigments	5.0 ± 0.2	4.6 ± 0.1 <sup>b</sup>	7.6 ± 3.8 <sup>ab</sup>	10.1 ± 0.6 <sup>a</sup>	9.9 ± 0.4 <sup>a</sup>	9.9 ± 0.9 <sup>a</sup>	11.5 ± 0.3 <sup>a</sup>		
Cholesterol	8.4 ± 0.2	11.5 ± 0.5 <sup>b</sup>	13.0 ± 0.6 <sup>a</sup>	11.2 ± 0.3 <sup>b</sup>	12.0 ± 0.9 <sup>ab</sup>	12.1 ± 0.2 <sup>ab</sup>	11.0 ± 0.6 <sup>b</sup>		
Free fatty acids	4.6 ± 0.1	3.5 ± 0.3 <sup>d</sup>	6.1 ± 4.5 <sup>cd</sup>	8.9 ± 0.3 <sup>bc</sup>	9.7 ± 0.4 <sup>bc</sup>	13.1 ± 1.2 <sup>b</sup>	23.1 ± 1.4 <sup>a</sup>		
Triglycerides	49.9 ± 1.1	67.9 ± 2.0 <sup>a</sup>	72.0 ± 9.1 <sup>a</sup>	65.1 ± 0.3 <sup>a</sup>	64.9 ± 1.4 <sup>a</sup>	65.0 ± 0.1 <sup>a</sup>	52.5 ± 0.2 <sup>b</sup>		
Cholesterol esters	14.3 ± 0.9	16.3 ± 1.4	14.1 ± 7.8	8.9 ± 0.4	10.8 ± 0.5	11.9 ± 1.2	12.9 ± 0.0		
Total polar lipid	29.7 ± 2.3	43.3 ± 3.0 <sup>c</sup>	49.2 ± 8.0 <sup>bc</sup>	54.6 ± 0.4 <sup>ab</sup>	55.1 ± 0.1 <sup>ab</sup>	62.1 ± 0.6 <sup>a</sup>	51.6 ± 0.4 <sup>bc</sup>		
Total neutral lipid	82.2 ± 2.3	103.8 ± 3.1 <sup>a</sup>	112.8 ± 7.9 <sup>a</sup>	104.2 ± 0.5 <sup>c</sup>	107.3 ± 0.0 <sup>a</sup>	112.0 ± 0.9 <sup>a</sup>	111.0 ± 0.9 <sup>a</sup>		

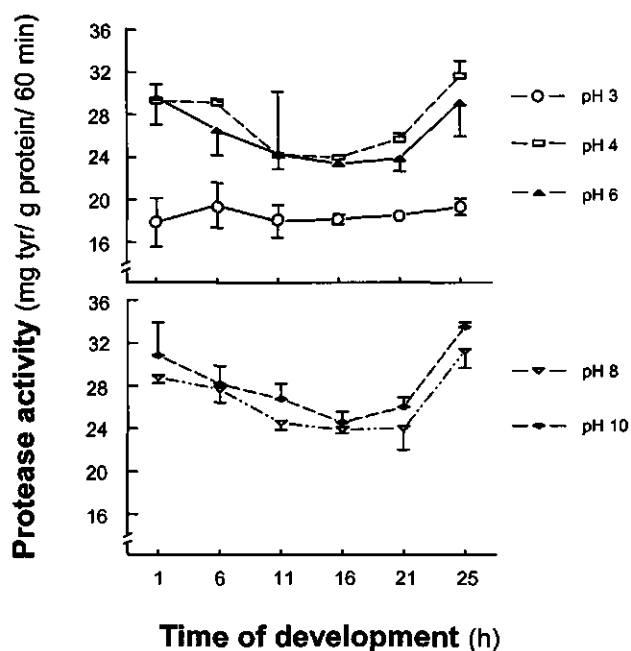
The time indicated as hours of development is the time of incubation plus one hour of hydration time during the decapsulation process.

Means in the same row with the same superscript are not significantly different (P>0.05)

nd = not detected

### Enzyme analyses

The total proteolytic activity in cysts and nauplii measured at acid and alkaline pH is presented in Fig. 1. The protease activity in undecapsulated cysts at pH 8 was  $11.9 \text{ mg tyr g}^{-1} \text{ protein } 60 \text{ min}^{-1}$  compared to 28.7 for 1 h incubated cysts. This indicates a sharp increase in enzymatic activity after the reactivation of the embryo metabolism by hydration of cysts. The protease activity during cyst and early nauplii development differed significantly when measured at pH 4, 6, 8 and 10. The lowest protease activity was observed around 16 h of development. The effect of pH on the measurement of protease activity was similar in all the treatments, i.e. only the values at acid pH were lower. For trypsin activity (Fig. 2) no significant differences were found between the studied developmental stages.



**Figure 1.** Total proteolytic activity during early development of cysts and nauplii of *Artemia* measured at acid and alkaline pH. The time of development is the time of incubation plus 1 h of hydration during the decapsulation process.

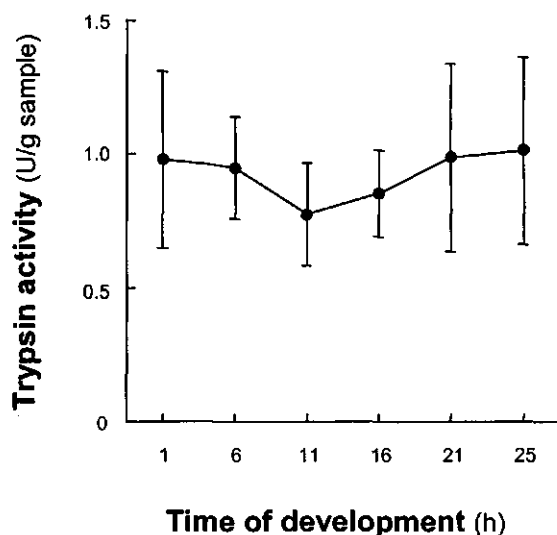
## Discussion

The hatching rate and hatching synchrony obtained in the present study indicate that good quality cysts were used.

The nutritional quality in *Artemia* varies considerably. This variation might be related to the geographical origin of *Artemia* (Léger et al., 1986), to differences among different batches of cysts from the same origin, and to the methods of analysis. Because of this variation, in nutritional studies with fish larvae it is important to determine the biochemical composition of the live food being used. The high protein content in undecapsulated cysts is due to the presence of the chorion or external layer composed of lipoprotein impregnated with chitin and hematin (Sorgeloos et al., 1986). The chorion is removed during decapsulation, resulting in a lower protein content and individual weight measured in decapsulated cysts and in newly hatched nauplii. The proximate composition in cysts and nauplii varied according to changes in individual dry weight. To determine the nutritional quality of *Artemia* it is important to study changes in proximate composition on individual weight basis and not exclusively in relative terms (% dw). The individual protein and fat content, and most notoriously the individual carbohydrate content decreased towards the naupliar stage. However no significant differences in individual proximate composition were found during development. All constituents were used during development, most probably for energy purposes. In general, cysts constitute a food item with a consistent biochemical composition. During development from cyst to nauplius, no drastic changes in the biochemical properties occur. Stronger changes in biochemical composition might be expected between different strains of *Artemia* (Léger et al., 1986).

*Artemia* nauplii of different origin have different amino acid profiles (Watanabe et al., 1983; Dendrinos and Thorpe, 1987). For GSL cysts, Landau and Riehm (1985) found a higher relative amount of tryptophan (8.3% total amino acids) compared to 1.1% in this study. Since no other authors have analyzed or found tryptophan, no definitive conclusion can be made with regard to its proportional presence in *Artemia*. Methionine was not detected in starved adults of *Artemia* (Claus et al., 1979). However, in the present study methionine was detected in cysts and in early nauplii. According to Watanabe et al. (1983) *Artemia* nauplii are deficient in histidine, methionine, phenylalanine and threonine, whereas adults are rich in all essential amino acids. In the present study, that deficiency was not evident, at least in quantitative terms. The small decrease in individual fat content from cysts to early nauplii is probably due to the utilization of lipid reserves. This, together with the decrease in individual protein content, reflects the need of

feeding the nauplii with micro algae or yeast when *Artemia* are being used as food for fish larvae. No previous reports exist on the HUFA composition of *Artemia* during development of cysts and only the 1 and 21 h samples, which corresponded to decapsulated cysts and early nauplii, respectively, can be compared with previous data on GSL *Artemia*. It is concluded that the small changes in amino acid and fatty acid composition during the development of *Artemia* cyst and early nauplii might have no relevance regarding its nutritional value as food for fish larvae. The total amino acid content of samples at 1h (cysts) and 25 h (naupliar stage) was not significantly different, and the total fatty acid content of nauplii was lower than in cysts. However, *Artemia* nauplii yielded higher growth than cysts when fed to catfish larvae (García-Ortega et al., 1995). The superior performance of live food is probably more related to food intake and digestibility than to the biochemical composition of the food.



**Figure 2.** Trypsin activity during early development of cysts and nauplii of *Artemia*. The time of development is the time of incubation plus 1 h of hydration during the decapsulation process.

No major changes occurred in protease activities during cyst development. There was a small tendency for a transient decrease around 16 h, coinciding with the hatching time. However, this might not represent a relevant aspect with respect to the nutritional importance for digestive processes in fish larvae. The protease composition did not differ between decapsulated cysts and nauplii of *Artemia*. As a consequence, as far as it concerns the hypothetical contribution of exogenous enzymes to fish larvae, there is no difference in feeding decapsulated cysts or early nauplii. From the protease activity in *Artemia* embryos and nauplii, over 90% is related to a cysteine protease that is used as hatching enzyme, in yolk utilization and as a digestive enzyme when the nauplii begins to eat (Warner et al., 1995). The importance and potential activity of this protease for fish larvae depends on the pH of the larval gut. Cysteine proteases are not active at alkaline pH (Warner and Shridhar, 1985). Thus the supply of these proteases might be meaningless to the fish larvae if the pH in the larval intestine is around 8.

Recently, Cahu et al. (1995) and Kurokawa et al. (1997) demonstrated that proteases derived from live food had only a small contribution to the enzymatic activity measured in sea bass and sardine larvae respectively. A post-hatching activation of trypsin-like proteases in *Artemia* (Pan et al., 1991) might not be of much importance to the nutritional quality of nauplii, and thus can not be used as an argument to use *Artemia* nauplii instead of decapsulated cysts as food for fish larvae. More important differences might be expected from other factors such as the structure and digestibility of proteins and the food intake stimulation derived from live nauplii. Additional information in this regard is required to define the factors that influence the better utilization of live food by the fish larvae when compared to artificial diets.

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## Chapter 2

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### **Heat treatment affects protein quality and protease activity in decapsulated cysts of *Artemia* when used as starter food for larvae of African catfish *Clarias gariepinus* (Burchell)**

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**Abstract** 

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Decapsulated cysts of *Artemia* subjected to different heat treatments (40, 60, 80 and 96°C) were fed to African catfish *Clarias gariepinus* larvae. Heated cysts, untreated cysts and live *Artemia* nauplii as control constituted the experimental diets. Protein denaturation and solubility, total alkaline protease and specific trypsin activities in the cyst diets were evaluated. The growth of catfish larvae and the proteolytic activity of larval samples during development were also determined. Heat treatment of cysts increased protein denaturation and decreased protein solubility. The protease activity in the cyst diets decreased with higher heating temperatures. The growth of catfish larvae differed according to the diet; higher fish growth was achieved with nauplii and cysts heated at 40°C. The digestive enzyme activity in larval samples remained similar in all dietary treatments during larval development. The quality of food protein and the way this protein is processed might be more important for successful larval growth than an exogenous enzyme supply.

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**Keywords:** *Artemia* cysts, protein quality, protein denaturation, proteolytic enzymes, fish larvae, *Clarias gariepinus*.

## Introduction

The main interest for the development of artificial diets for fish larvae is to reduce dependence on live planktonic organisms as starter food. Together with technological studies on diet manufacturing, physiological studies are needed to assess the digestive capacity of the developing larvae in different species. Larvae of the African catfish (*Clarias gariepinus*) can be raised using artificial dry diets as starter food. However, the growth and survival rates are lower than when live food is used (Verreth et al., 1987). According to several authors, the digestive enzymes from live food organisms play an important role in the digestion of nutrients by fish larvae (Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984; Walford and Lam, 1993). However, other studies demonstrated that the poor growth of fish larvae fed artificial diets can not be attributed to a deficiency of the digestive capacity in the fish (Segner et al., 1993; Cahu and Zambonino Infante, 1997). Thus, the relative importance of dietary enzymes for larval digestion requires further investigation. In the African catfish most digestive and metabolic enzymes are detected at the start of exogenous feeding (Verreth et al., 1992; Segner et al., 1993; Segner and Verreth, 1995). Taking into account that live food organisms contain mainly protein in their dry matter, the proteolytic enzyme capacity can be regarded as the most important one during early life of the fish. In the African catfish, the gastro-intestinal tract (GIT) is fully differentiated at the start of exogenous feeding, with exception of the stomach (Verreth et al., 1992). Hence, before onset of stomach functions, protein digestion in the larvae depends entirely on trypsin. Interestingly, in this species the earliest weaning from live food to dry food coincides with the onset of stomach functions (Verreth and van Tongeren, 1989). The major change in the larval digestive system occurring at this moment is the change from trypsin to a combined trypsin/pepsin digestion for protein breakdown (Segner et al., 1993; Segner and Verreth, 1995). In addition, the total proteolytic activity in the GIT of catfish larvae does not change quantitatively by the onset of stomach functions suggesting that the mentioned change in proteolytic capacity may affect the type of proteins which can be digested rather than the amount (Verreth, 1994). The latter author hypothesized that before onset of stomach functions the complexity of the protein molecules in the food may be of crucial importance for the digestion by the fish larvae.

The aim of this work is to evaluate if a reduction in the proteolytic activity in the live food has an effect on larval fish growth. This reduction in proteolytic enzyme activity was intentionally induced by heat treatment of the food. Heating constitutes one of the most important operations in food processing. However, processing food protein with heat changes also the protein structure (i.e. in the arrangement of the polypeptide chains within the protein

molecule) and consequently its nutritive quality (Boye et al., 1997). These changes in the protein structure can be either beneficial or detrimental depending on the conditions of heating (de Wet, 1983) and on the preferences of the larval digestive system. Therefore, the effects of heat processing on the protein quality of decapsulated cysts were assessed as well.

In the present study, decapsulated *Artemia* cysts were used as model diet. The use of decapsulated, dried or brine-dehydrated *Artemia* cysts as experimental diets have been tested successfully in the larval rearing of *C. gariepinus* (Verreth et al., 1987; Pector et al., 1994) and other fish species (Drouin et al., 1986; Vanhaecke et al., 1990). Using decapsulated *Artemia* cysts as direct food for fish larvae presents the practical advantages of combining some of the physical properties of a dry feed and the nutritional value of live nauplii. The cysts of *Artemia* have a particle size (200-250  $\mu\text{m}$ ) small enough to be ingested by catfish larvae. Dried cysts float on the water surface and sink only slowly to the bottom upon hydration. Cysts do not leach nutrients, consequently, a balanced nutritional composition can be ensured in all food particles (Verreth et al., 1987). On an individual basis, there is no difference in the proximate composition between decapsulated cysts and freshly hatched nauplii (García-Ortega, et al., 1998). Additionally the energy content and individual dry weight of decapsulated cysts are higher than any of the stages of hatched nauplii (Vanhaecke et al., 1983; Léger et al., 1987).

## Materials and Methods

### *Fish larvae and heat treatment of cysts*

African catfish eggs and larvae were obtained by artificial reproduction. The larvae were reared in a recirculation system with 17 L aquaria at a density of 1000 larvae per aquarium. Water temperature was kept constant at 28°C. Cysts of *Artemia* (type EG; INVE Aquaculture N.V., Baasrode, Belgium) from Great Salt Lake, USA were decapsulated with a chlorine solution according to the method of Sorgeloos et al. (1986) and stored for 30 minutes at 4°C before being submitted to heat treatment. The heat treatment consisted of heating equal portions (approx. 400 g each) of hydrated decapsulated cysts in water at 40, 60, 80 and 96°C, respectively for five min. During heating, the cysts were kept in suspension by moderate aeration. As a control one of the portions of decapsulated cysts was not heat-treated. After the heat treatment the cysts were dehydrated with saturated brine ( $>330 \text{ g NaCl L}^{-1}$ ) for 24 h and stored at 4°C in fresh brine.

### ***Experimental procedure and sampling***

Five cyst diets and a control diet (live *Artemia* nauplii) were fed to the catfish larvae. The cyst diets consisted of decapsulated cysts subjected to one of the heat treatments: untreated, C-40, C-60, C-80, C-96. Before being offered to the larvae, the cysts were suspended in freshwater for a few minutes to allow for cyst hydration and to rinse the brine. Fish were fed two days after hatching, every four hours (five times every 24 h) for ten days. Each diet treatment was tested with three replicate groups. The amount of food was calculated according to the predicted maximum growth (Verreth and Den Bieman, 1987). The initial wet weight of the larvae at the start of exogenous feeding was 2.5 mg. The larval weight was determined by sampling 20 larvae from each replicate treatment at the start of exogenous feeding and every three days. Excess water in fish samples was removed with tissue paper, and wet weight was determined. To obtain the dry weight the same fish samples were dried overnight in an oven at 70°C, with a subsequent drying at 103°C for 4 h. Total protease and trypsin activities were measured in the five cyst diets and in samples of whole catfish larvae taken before the first daily feeding. Larval samples for enzyme analysis were taken at days 1, 4, 7 and 9 after the start of exogenous feeding. The samples were shock-frozen with liquid nitrogen and stored at -80°C until analysis.

### ***Protein denaturation and solubility***

Differential scanning calorimetry (DSC) and the protein solubility index (PSI) are useful to evaluate the effects of processing on food ingredients. Protein denaturation in the diets was estimated by DSC (Wright, 1982 and 1984). A calorimeter (DSC 12-E, Mettler-Toledo, Tiel, The Netherlands) was used to determine the enthalpy in cyst samples. Fifteen to 18 mg of each diet was weighed into a medium pressure crucible (Mettler-Toledo), and approximately 30 mg of water was added before sealing the crucible. The cyst diets were hydrated for one hour at room temperature. A second crucible filled with aluminum foil was used as a reference. Subsequently the sample crucible and the reference were subjected to a controlled temperature program in the calorimeter. An initial period of five min. at constant temperature of 20°C was applied, and then the temperature was increased from 20 to 160°C at a rate of 10°C min<sup>-1</sup>. The energy input into the sample was measured and compared with that of the reference. The difference in energy absorption of both sample and reference was considered as an indication of the protein denaturation in the cysts sample. The higher the energy absorption or enthalpy measured the lower the amount of denatured protein was present in the

sample. The protein denaturation is expressed as relative percentage to the level of denaturation in untreated cysts; the latter was set at 0%.

Protein solubility was estimated with the protein solubility index (PSI) (Morr et al., 1985). One ml distilled water and 0.5 g of cysts sample were homogenized in a potter blender. The homogenate was placed in a covered centrifuge tube and water was added up to a total volume of 20 ml. The suspension was stirred for one hour at 30°C and transferred to a volumetric flask. Once the particles were settled, 40 ml of the liquid phase was centrifuged for 10 min at 1800 g. The resulting supernatant was filtered. The crude protein of the original sample and of the filtrate were determined by the Kjeldahl method according to ISO 5983 (1979). The solubility of the protein was calculated as: PSI (%) equal to protein content in the supernatant divided by the protein content of the original sample multiplied by 100.

### *Proteolytic enzymes analysis*

Proteolytic enzymes are important for the digestion of protein. The analysis of enzyme activity in fish provides a good indication of the nutritional condition of the larvae (Pedersen et al., 1987). Thus, the total proteolytic activity and the specific trypsin activity in fish samples were determined. Fifty to 100 mg of larval fish sample was ground in a potter blender with 500 to 1000  $\mu$ l respectively of 0.1 M Tris buffer at pH 8. To prepare cysts homogenates, 75 mg of material was used. After homogenization the samples were sonified for five seconds and centrifuged at 12000 g for 10 min at 4°C. During preparation the homogenates were always kept on ice. Total protease activity in cysts and in fish homogenates was measured by a modified casein method from Walter (1984) using casein (10 mg ml<sup>-1</sup>) dissolved in 0.1 M Tris buffer at pH 8. A buffer composed of 0.2 M Tris and 0.1 N HCl was used to detect alkaline protease activity at pH 8 using L-tyrosine as the standard. The assay mixtures were incubated at 30°C for 30 min, the reaction was halted with trichloroacetic acid. After cooling on ice for 15 min, the assay mixtures were centrifuged at 1700 g for 10 min at 4°C and the absorbance of the supernatant was measured at 280 nm. The results are expressed as mg tyrosine liberated g<sup>-1</sup> protein in sample 30 min<sup>-1</sup>.

For the determination of trypsin activity, the cysts and fish homogenates were prepared similarly as for the total protease measurements. The trypsin activity was determined using a modified method from Hofer and Köck (1989) and Bergmeyer and Graßl (1983), using BAPNA (N $\alpha$ -benzoyl-DL-arginine-4-nitroanilide, E-Merck, Darmstadt, Germany). The assay cocktail consisted of 0.42 mM BAPNA in a 0.1 M Tris buffer pH 8 with 0.2 M CaCl<sub>2</sub>. Adding

BAPNA solution to a mixture of homogenate and buffer started the reaction. The change in absorbance during 5 min was measured at 405 nm. Trypsin activity is reported as mU mg<sup>-1</sup> of cysts (1 U = 1 µmol BAPNA converted per min).

### ***Data analysis***

All the data were tested for normal distribution before being subjected to analysis of variance. A one-way analysis of variance was used to determine if significant differences existed between treatments in each of the sampling days. When differences were found, the means were compared with Duncan's multiple range test ( $P < 0.05$ ). The statistical analysis was performed with the data on DSC, PSI and total proteolytic activity with two replicates from each cyst diet. The trypsin activity of the diets was analyzed with four replicates. The fish growth, total protease activity and trypsin activity in larval samples were analyzed with three replicates per diet treatment.

## **Results**

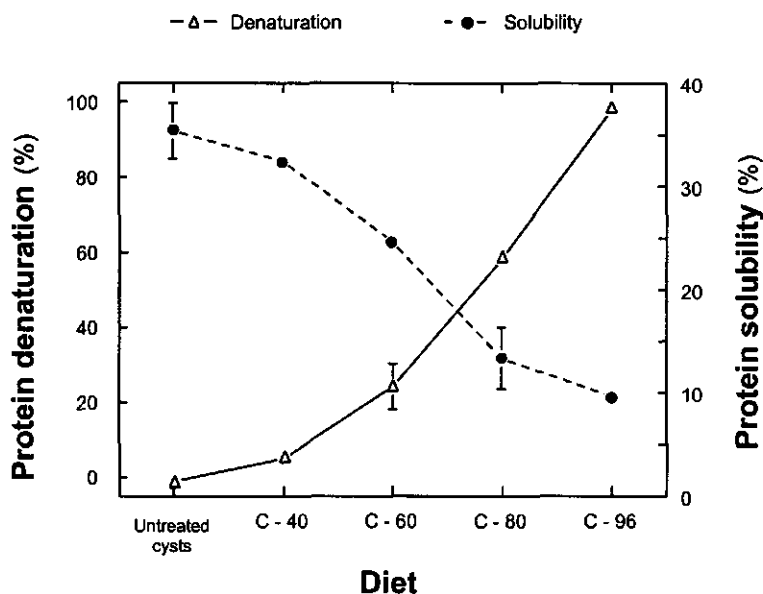
### ***Protein quality of cyst diets***

The DSC values show that at higher processing temperature more protein in the cysts is denatured (Fig. 1). At temperatures above 60°C, the protein denaturation was significantly stronger compared to the cysts heated at 40°C. In comparison to untreated cysts most of the protein in the cysts at 96°C is denatured. The denaturation of protein comprises modifications in the conformation of the secondary, tertiary and quaternary structures, but not in the primary structure (Boye et al., 1997). This spatial re-arrangement in the protein structure could lead to beneficial or detrimental effects in the protein nutritional quality depending on the severity of heating. The heating of cysts also had a significant effect on the protein solubility. The solubility of protein in the cyst diets decreased with increasing temperature of treatment (Fig. 1).

### ***Larval fish growth***

The weight of catfish larvae differed according to the food treatment (Table 1). During the first four days of exogenous feeding catfish larvae fed the cyst heated at 40°C had the highest growth. However, from day 7 onwards the control diet *Artemia* nauplii yielded the highest catfish weight which was significantly higher than in the larvae receiving any of the cyst

diets. Among the inert diets, cysts heated at 40°C produced higher growth than the other cyst preparations, including the untreated cysts. Cysts heated at 60°C and above yielded lower weight gains. Both DSC results and fish growth suggest a positive effect on the nutritional quality of the cysts because of the moderate protein denaturation induced by heating at 40°C. Contrary to this, higher heating temperatures decrease the protein quality in cysts and a lower growth of catfish larvae was obtained.



**Figure 1.** Protein denaturation and protein solubility of decapsulated cysts of *Artemia* subjected to a five-minutes heat-treatment. The temperature of treatment corresponded to 40, 60, 80 and 96°C.



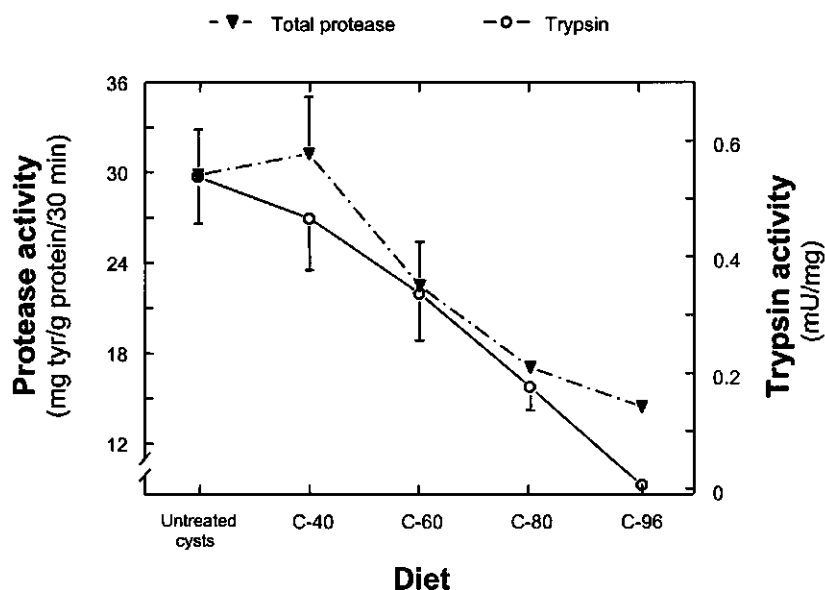
Table 1. Weight of *Clarias gariepinus* larvae fed live *Artemia* nauplii and different heat-treated preparations of decapsulated cysts. The indicated time refers to days after the start of exogenous feeding.

Diet	Wet weight (mg)				Dry weight (mg)			
	day 1	day 4	day 7	day 9	day 1	day 4	day 7	day 9
Untreated cysts	4.3 <sup>ab</sup> ± 0.3	16.2 <sup>b</sup> ± 1.6	47.8 <sup>a</sup> ± 1.6	77.5 <sup>c</sup> ± 3.9	0.63 <sup>ab</sup> ± 0.02	2.8 <sup>b</sup> ± 0.1	7.8 <sup>b</sup> ± 0.1	12.5 <sup>c</sup> ± 0.8
C-40*	4.7 <sup>a</sup> ± 0.6	19.7 <sup>a</sup> ± 0.9	54.3 <sup>a</sup> ± 4.0	86.8 <sup>b</sup> ± 2.7	0.68 <sup>a</sup> ± 0.06	3.2 <sup>a</sup> ± 0.2	9.0 <sup>a</sup> ± 0.5	14.4 <sup>b</sup> ± 0.6
C-60	3.8 <sup>bc</sup> ± 0.1	14.3 <sup>bc</sup> ± 3.0	38.4 <sup>b</sup> ± 1.8	64.1 <sup>d</sup> ± 2.7	0.56 <sup>c</sup> ± 0.02	2.3 <sup>c</sup> ± 0.1	6.4 <sup>c</sup> ± 0.3	10.4 <sup>d</sup> ± 0.2
C-80	3.7 <sup>c</sup> ± 0.2	10.6 <sup>d</sup> ± 1.4	33.9 <sup>b</sup> ± 6.9	60.2 <sup>d</sup> ± 3.0	0.54 <sup>c</sup> ± 0.06	1.9 <sup>d</sup> ± 0.2	5.3 <sup>d</sup> ± 0.5	9.8 <sup>d</sup> ± 0.6
C-96	3.5 <sup>bc</sup> ± 0.7	11.8 <sup>cd</sup> ± 1.8	35.1 <sup>b</sup> ± 2.5	61.9 <sup>d</sup> ± 5.4	0.53 <sup>c</sup> ± 0.03	2.1 <sup>cd</sup> ± 0.1	6.0 <sup>cd</sup> ± 0.3	10.4 <sup>d</sup> ± 0.9
Nauplii	3.8 <sup>bc</sup> ± 0.2	13.2 <sup>bcd</sup> ± 0.8	50.4 <sup>a</sup> ± 0.2	102.7 <sup>a</sup> ± 7.0	0.58 <sup>bc</sup> ± 0.01	2.7 <sup>b</sup> ± 0.1	8.6 <sup>a</sup> ± 0.5	17.8 <sup>a</sup> ± 1.3

\* The temperature of treatment (in °C) is indicated for each of the cyst diets. Means in the same column with the same superscript are not significantly different (P>0.05). Means and standard deviations values were rounded to decimals.

### Enzyme activity

The total alkaline protease activity and the specific trypsin activity in decapsulated cysts diets decreased with higher processing temperature (Fig. 2). Enzymes are proteins and their properties are also affected by the effect of heating. At a heat treatment of 96°C the trypsin activity reached almost zero. However, total proteolytic activity in cysts was not completely halted, not even at 96°C. Apparently cyst enzymes are very tolerant to severe environmental conditions. The reduction of enzyme activity in cysts heated at high temperature implies that less exogenous digestive enzymes are available for the digestion of food. When heated cysts were fed to the fish larvae, the reduced enzyme activity in cysts was not evident in the total proteolytic activity and specific trypsin activity measured in fish samples. Enzyme activities in fish were similar in all food treatments during larval development (Table 2).



**Figure 2.** Total proteolytic activity and specific trypsin activity in decapsulated cysts of *Artemia* subjected to a five-minutes heat-treatment. The temperature of treatment corresponded to 40, 60, 80 and 96°C.

**Table 2.** Total protease and trypsin activities in *Clarias gariepinus* larvae from day 1 to day 9 after the start of exogenous feeding. The larvae were fed live *Artemia* nauplii and different heat-treated preparations of decapsulated cysts.

Diet	Protease activity (mg tyr g <sup>-1</sup> sample 30 min <sup>-1</sup> )				Trypsin activity (mU mg <sup>-1</sup> )			
	day 1	day 4	day 7	day 9	day 1	day 4	day 7	day 9
Untreated cysts	17.8 <sup>a</sup> ± 2.3	15.5 <sup>a</sup> ± 0.9	14.7 <sup>a</sup> ± 0.6	13.8 <sup>a</sup> ± 1.4	0.46 <sup>a</sup> ± 0.03	0.44 <sup>b</sup> ± 0.04	0.65 <sup>ab</sup> ± 0.09	0.48 <sup>b</sup> ± 0.08
C-40*	16.8 <sup>a</sup> ± 0.9	16.3 <sup>a</sup> ± 0.6	13.0 <sup>b</sup> ± 0.6	12.8 <sup>a</sup> ± 1.5	0.40 <sup>a</sup> ± 0.06	0.60 <sup>a</sup> ± 0.07	0.66 <sup>ab</sup> ± 0.02	0.60 <sup>a</sup> ± 0.04
C-60	17.6 <sup>a</sup> ± 1.9	13.8 <sup>b</sup> ± 0.9	14.6 <sup>a</sup> ± 0.7	14.1 <sup>a</sup> ± 0.5	0.30 <sup>b</sup> ± 0.03	0.46 <sup>b</sup> ± 0.02	0.54 <sup>ab</sup> ± 0.07	0.53 <sup>ab</sup> ± 0.04
C-80	14.1 <sup>b</sup> ± 1.0	13.0 <sup>b</sup> ± 0.5	15.0 <sup>a</sup> ± 0.4	12.7 <sup>a</sup> ± 0.7	0.28 <sup>b</sup> ± 0.05	0.33 <sup>c</sup> ± 0.01	0.51 <sup>b</sup> ± 0.05	0.54 <sup>ab</sup> ± 0.03
C-96	11.9 <sup>c</sup> ± 0.7	15.1 <sup>a</sup> ± 1.1	13.2 <sup>b</sup> ± 0.9	13.7 <sup>a</sup> ± 1.6	0.28 <sup>b</sup> ± 0.03	0.30 <sup>c</sup> ± 0.03	0.50 <sup>b</sup> ± 0.06	0.49 <sup>b</sup> ± 0.02
Nauplii	12.5 <sup>c</sup> ± 0.6	15.9 <sup>a</sup> ± 1.8	13.1 <sup>b</sup> ± 0.8	13.9 <sup>a</sup> ± 1.5	0.47 <sup>a</sup> ± 0.08	0.48 <sup>b</sup> ± 0.04	0.68 <sup>a</sup> ± 0.17	0.49 <sup>b</sup> ± 0.06

\* The temperature of treatment (in °C) is indicated for each of the cyst diets.  
Means in the same column with the same superscript are not significantly different (P>0.05)  
Means and standard deviations values were rounded to decimals

## Discussion

The final weight of catfish larvae (Table 1) obtained in the present study was higher than in other previously reported studies on *C. gariepinus* larvae fed decapsulated *Artemia* cysts (Verreth et al., 1987; Pector et al., 1994). A high weight gain when using decapsulated cysts might be partially related to the salt ions present in the cysts after being brine dehydrated, which have a positive effect on the fish osmoregulatory system (Pector et al., 1994). Moreover, the feeding frequency as carried out in the present study involved feeding the fish during the day and night every four hours. This implies a constant supply of food for the fish and allows sufficient time between meals for digestion of food.

The effect of heat on the nutritional quality of cyst diets has several implications. The reduced growth of fish fed cysts treated at higher temperatures could indicate that the destruction of cyst enzymes, mainly trypsin, had a negative effect on the digestion and absorption of food by the larvae. However, the decrease in dietary enzymes was not reflected in the proteolytic activity measured in the larval samples. Thus it is assumed that the contribution of enzymes from *Artemia* to the total proteolytic activity measured in the larval fish is small. This was also demonstrated in larvae of marine fish species fed live food organisms (Cahu and Zambonino Infante, 1997; Díaz et al., 1997; Kurokawa et al., 1998). Additional information on food intake, enzyme inhibition and post-feeding proteolytic activity in the larval gut is necessary to present a more definitive conclusion for catfish larvae.

Heat treatment is the most important procedure for denaturation of protein during the processing of food (Boye et al., 1997). The denaturation of proteins in food involves the disruption of inter and intra-molecular bindings which lead to alterations in the structural properties of the protein molecule. These changes in the native structure of the protein have effects in some of its functional properties. In fishmeal proteins, heating above 50°C caused the oxidation of the sulfhydryl groups and the formation of disulfide bonds. These changes were associated with a decline in protein digestibility (Opstvedt et al., 1984). Heating *Artemia* cysts at 50°C for 60 min resulted in the loss of viability and total cyst mortality (Miller and McLennan, 1988). Thus, heating at temperatures above 50°C seems to have deleterious effects on the quality of cyst protein. The results of the present study corroborate this conclusion, as is shown by the data on protein denaturation and solubility (Fig. 1) and on the catfish larvae growth (Table 1). The losses in protein solubility and enzymatic activity are

probably caused by the change in the structure of the polypeptide chains in *Artemia* cysts after the strong heat treatments. In contrast, it seems that heating at temperatures under 40°C does not have a negative effect on the nutritional quality of cyst protein. The differences in fish growth among the cyst diets might be related to modifications in the conformational state of the protein during heating. Those modifications have an effect in one or several functional properties of the protein (Mulvihill and Donovan, 1987). Due to the lack of a functional stomach and consequently of acid denaturation in catfish larvae, a beneficial effect can be derived from the denaturation caused by heating cysts at 40°. Possibly the change in the spatial arrangement of dietary protein treated at this temperature allowed a more effective hydrolysis by the fish trypsin. The mechanisms that induce the beneficial effect of heating cysts at 40°C require further study. Fish larvae fed cysts heated at 40°C had higher growth than those fed untreated cysts (Table 1).

The reduction in protein solubility is another important effect of the thermal denaturation (Boye et al., 1997). Although the solubility of the proteins can be either favorably or adversely affected by heating during feed manufacturing (Morr et al., 1985), in this study no negative effect on the fish growth was observed with the small decrease in solubility in cysts heated at 40°C.

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## Chapter 3

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### **A new method for the quantification of *Artemia* consumption in nutrition studies with fish larvae**

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**Abstract** 

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A new method for the estimation of food consumption and gut evacuation in fish larvae was evaluated. The method consists of measuring the temporary accumulation of ascorbic acid 2-sulfate (AAS) in fish larvae after feeding decapsulated cysts of *Artemia*. A first experiment with larvae of African catfish served to verify whether biosynthesis of AAS by the fish larvae did occur or not. In a second experiment the gut contents in larvae fed *ad libitum* were calculated as they changed during fish development. In a third experiment the gut evacuation rate was determined during continuous and discontinuous feeding regimes. The experimental period covered the first five days after the start of exogenous feeding in the larvae. The daily food consumption in catfish larvae increased from 46.5% of their body dry weight (BDW) at day 1 to 53.8% (BDW) at day 3. Thereafter, the food consumption decreased to 27.8% (BDW) at day 5. A similar pattern was observed for the gut evacuation rate. The reported method for the determination of food consumption enables an accurate and precise estimation of the gut contents and the evacuation rate of *Artemia* cysts in fish larvae.

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**Keywords:** food consumption, gut evacuation rate, fish larvae, ascorbic acid sulfate, *Artemia* cysts.

## Introduction

Estimation of food consumption in fish is difficult because of a lack of accurate methods to evaluate both the ingestion and evacuation of food. In ecology and fisheries biology food consumption has been extensively studied using mathematical models (Elliott and Persson, 1978; Jobling, 1981). Bioenergetic models are widely used and some of them were specifically adapted for larval fish (Marmulla and Rösch, 1990; Haylor, 1993; Madon and Culver, 1993; Karjalainen et al., 1997). However, the accuracy of the food consumption determinations from bioenergetic models has been questioned (Ney, 1993). Errors are associated to the estimations of respiration, consumption and excretion (Bartell et al., 1986). Other methods to estimate food consumption in fish larvae and juveniles include the use of gravimetric techniques (Kamler et al., 1986), the direct count of food particles in the fish digestive tract (Pedersen, 1984) or in the experimental tanks after feeding (Keckis and Schimer, 1992), the use of radio-isotopes (Kolkovski et al., 1997) and X-radiography with metallic markers (Hossain et al., 1998). The type of diet used, e.g. zooplankton organisms or artificial diets, can affect the estimations of food consumption. Zooplankton organisms are easily broken into pieces thus complicating the individual counts. On the other hand, some artificial diets lack natural attractants that are important for the acceptability of the food and the content of the marker substance may vary among individual food particles.

In the present study, feeding experiments were carried out to test a new method for the direct measurement of food consumption in larval fish. Basically this method relies on the accumulation of ascorbic acid 2-sulfate (AAS) in larval fish when fed an AAS containing diet. AAS constitutes a stable derivative of ascorbic acid (vitamin C) which is naturally occurring in the encysted embryos of the brine shrimp *Artemia* (Mead and Finamore, 1969) and is converted into ascorbic acid only during the embryonic development and until emergence of the nauplii (Golub and Finamore, 1972). The larval and juvenile stages of teleost fish are unable to utilize AAS as a precursor for ascorbic acid and no natural occurrence of AAS has been detected in fish (Dabrowski et al., 1990a; Dabrowski et al., 1990b). Cysts of *Artemia* can be successfully used as food for the larvae of several fish species (Drouin et al., 1986; Verreth et al., 1987; Vanhaecke et al., 1990). Hence the AAS in *Artemia* cysts can be used as natural marker to estimate the food consumption in fish larvae. *Artemia* cysts present the advantages of constituting a well-accepted food source for fish larvae. Additionally, cysts have a constant particle size and biochemical composition, as well an impermeable membrane that avoid the leaching of the marker substance or other nutrients and thus reducing the variations of their content in each individual food particle.

## Materials and Methods

### *Artemia* cysts

Cysts of the brine shrimp *Artemia* (EG, INVE Aquaculture N.V., Baasrode, Belgium) originating from Great Salt Lake, USA, were submerged in aerated freshwater during 1 h to achieve full hydration. Once the cysts were hydrated, their shell was chemically removed (decapsulation) with a chlorine solution according to the method of Sorgeloos et al. (1986). After decapsulation, the cysts were dehydrated again in saturated brine ( $>330 \text{ g NaCl L}^{-1}$ ) for 24 h and stored in fresh brine at  $4^{\circ}\text{C}$  until used to feed the fish larvae. Decapsulation of *Artemia* cysts was done one day prior to the start of each feeding experiment. The same batch of *Artemia* cysts was used in all experiments. Before being offered to the fish larvae the cysts were immersed in freshwater during five min to rinse the brine and allow cysts hydration. The particle size of this particular batch of decapsulated cysts corresponded to  $236 \mu\text{m} (\pm 16)$ .

### *Fish larvae rearing*

Larvae of African catfish (*Clarias gariepinus*) were obtained by artificial reproduction of controlled broodstock. Fertilized eggs were placed in plate incubators with running water at  $30^{\circ}\text{C}$ . Hatching of larvae occurred after approximately 23 hours. Two days after hatching, when the absorption of yolk sac was almost complete, the larvae were counted and transferred to a freshwater recirculation system with 17 L aquaria. Larvae were reared at a constant temperature of  $28^{\circ}\text{C}$  with a light regime from 08:00 to 20:00 h. The same procedure for the production and culture of fish larvae was performed in all experiments described here. The weight of the larvae was recorded daily and the amount of food supplied was calculated following the predicted maximum growth (Verreth and Den Bieman, 1987). In this way the feeding level was daily adjusted according to the changes in the relative growth rate. To avoid variations in the estimations of gut contents and gut evacuation due to the meal size (Bromley, 1994), the total amount of food per day was divided in five equal portions. Feeding started two days after hatching and it was done every four hours from 08:00 to 24:00. The experimental protocol in the present study was approved by the Ethical Committee Judging Animal Experiments from the Wageningen Agricultural University to ensure that appropriate animal management was applied.

## Experimental design and procedures

In three consecutive experiments a series of questions was addressed which all were related to the reliability of the new method and the amount of food consumed by the fish larvae under various conditions.

### *Experiment 1*

The first experiment was designed to verify whether the basic prerequisite for the new method, e.g., accumulation of AAS in the fish larvae as a result of feeding with *Artemia* cysts, was applicable. At the same time this experiment was used to obtain some baseline data on the relation between AAS content and number of cysts found in the gut of the fish larvae. Six aquaria with 1000 larvae each were used to test two feeding treatments. In treatment one, decapsulated cysts of *Artemia* were fed to estimate the gut contents calculated as the number of cysts present in the digestive tract. In the second treatment a dry artificial starter diet for catfish (9011, Provimi B.V., Zwolle, The Netherlands; particle size 300-500  $\mu\text{m}$ ) was fed to the larvae to verify the biosynthesis of AAS in the fish larvae. According to the manufacturer this artificial diet contained no AAS as source of vitamin C. This was further verified by measuring the AAS in the diet. Fish samples were taken at day 1 after the start of exogenous feeding.

### *Experiment 2*

The second experiment was used to verify whether the ingested AAS was retained or broken down. For this reason, the ingestion of food as measured by the AAS content in the fish larvae, was followed in time. In this way the experiment would provide also indications on how consumption changed during development while the fish larvae were increasing size. Catfish larvae were kept at an initial density of 1200 larvae per aquarium. Four diet treatments were tested. In treatment one catfish larvae were fed exclusively decapsulated *Artemia* cysts to measure the content of AAS present in fish that is derived from cysts (gut contents). In treatment two a combined feeding of live instar I nauplii of *Artemia* and decapsulated cysts was carried out to estimate the retention of AAS in the fish. In this treatment live nauplii were offered to the fish larvae and decapsulated cysts were fed exclusively during the sampling days. In treatment 3 (control 1) only live *Artemia* nauplii were fed to the fish larvae during the entire experiment. Comparison between treatment 1 and 3 should give an indication how daily food consumption changed during development. To reassure that AAS biosynthesis in fish

larvae did not occur, even when other sources of ascorbic acid are provided, a fourth diet treatment was included using a dry artificial diet (same as Experiment 1) with a source of ascorbic acid different than AAS. Fish samples were taken on days 1, 3 and 5 after the start of exogenous feeding.

### ***Experiment 3***

Besides AAS accumulation, the determination of AAS evacuation rate in the fish is necessary to obtain an accurate estimation of food consumption. Therefore, the gut evacuation rate of catfish larvae during development was estimated at two different feeding treatments. Gut evacuation was measured by feeding the larvae with AAS containing cysts only on the sampling days and exclusively at the first meal (at 08:00). By measuring the AAS decline in fish samples afterwards, we can obtain a picture of the evacuation rate. Because the gut evacuation rate might be affected by the meal frequency, two feeding strategies were applied during the sampling days. In the first treatment, a continuous feeding regime was applied. After the first meal with cysts, subsequent feeding was done with live instar I nauplii of *Artemia* which contained no AAS. In the second treatment, a discontinuous feeding strategy was performed. After the first feeding with cysts, no additional food supply was done during the rest of the day. As control diet *Artemia* nauplii were offered to the fish during the entire experiment. In all three diet treatments the fish were fed with nauplii in between sampling days. Fish larvae samples were taken at days 1, 3 and 5 after the start of exogenous feeding.

### ***Sample collection and preparation***

In all experiments samples of fish were taken 15 minutes before the first daily meal and three hours after each meal during the sampling days for all diet treatments. However, in treatment two from Experiment 1 and the control diets from Experiments 2 and 3, fish samples were taken only three hours after the fourth daily meal to account for the accumulation of AAS during the day. Additionally, in the discontinuous feeding treatment in Experiment 3 the samples were taken before the daily first meal and at 3, 7, 11 and 15 h after that first meal. The fish larvae samples were rinsed with tap water, the excess water was removed and the weight and number of fish in each sample was determined. The samples were shock frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until AAS determination. For all experiments, the number of fish larvae in each sample for AAS analysis ranged from 80-120 on day 1, 40-50 on day 3 and 15-20 on day 5 depending on the fish weight. The fish weight during the sampling days was determined before first feeding and after every feeding with

exception of the last feeding. The dry matter ( $\pm 10 \mu\text{g}$ ) of *Artemia* cysts, artificial diet and fish during the sampling days in all experiments were determined by drying the samples in an oven at  $60^\circ\text{C}$  during 24 h. Samples of decapsulated cysts of *Artemia*, hydrated during 1 and 3 hours after brine storage, and instar I nauplii were also prepared for determination of AAS content.

## Quantification of AAS

### *Preparation and purification of crude extracts*

The preparation of crude extracts of *Artemia* cysts and catfish larvae for AAS analysis was done according to Nelis et al. (1994). Approximately 100 mg dry weight (DW) of *Artemia* cysts were homogenized in 2 ml acetate buffer (60 mM, pH 5.0) using an all-glass Potter Elvehjem tube. For the preparation of crude extracts of catfish larvae, approximately 500 mg wet weight (WW) of sample was used. After centrifugation of the homogenate at 1500 g during 5 min the supernatant was transferred to a polypropylene tube and the homogenization procedure was repeated on the residue. The final residue was rinsed twice with 1 ml of acetate buffer and the supernatants were combined. For the purification of the crude extracts by anion exchange chromatography, the crude extract (6 ml) was quantitatively transferred to a Bond Elut DEAE cartridge (Varian, Harbor City, CA, USA) which has been preconditioned with methanol (1 ml), water (1 ml) and acetate buffer (1 ml; pH 5.0). After the cartridge was washed twice with 1 ml of water, elution was carried out with 1 ml of 0.1 M sodium salicylate. The pH of the elute was adjusted to 2.0 with 0.1 ml of 1 M HCl, and the solution was extracted twice with 5 ml of diethyl ether. The organic layer was removed and the aqueous solution was purged with nitrogen to remove the last traces of ether. Subsequently, the pH was readjusted to 5 with 1 M sodium hydroxide and the volume was brought to 5.0 ml in a volumetric flask. A 100  $\mu\text{l}$  aliquot was injected in the column.

### *Chromatographic conditions*

A reversed-phase, ion-paired, high-performance liquid chromatography (HPLC) system was developed for the quantification of AAS in fish and *Artemia* cysts samples. The HPLC system consisted of a LKB 2249 pump (LKB, Bromma, Sweden), an N-60 valve injector fitted with a 100  $\mu\text{l}$  loop (Valco, Houston, TX, USA), a Philips PYE Unicam PU 4025 UV detector (PYE Unicam Ltd., Cambridge, UK) and a Merck Hitachi D-7500 integrator (Hitachi Ltd., Instrument Division, Japan). A 3  $\mu\text{m}$  Adsorbosphere HS C18 (150 X 4.6 mm; Alltech Ass. Inc., Deerfield, IL., USA) column was used with a mixture of acetonitrile and

ammonium phosphate buffer (60 mM pH 5.0) containing 32 mM TBAP (1.5:8.5 (v/v)) as mobile phase. The flow rate was 1 ml/min and detection was performed at 254 nm.

### ***Quantification and method validation***

The linearity, recovery and detection limits of the method were evaluated by analyzing buffer solutions supplemented with AAS (A-3758, Sigma, Belgium). Standard curves were constructed by plotting the absolute peak heights obtained with increasing concentrations of AAS against their respective concentration. A linear regression was performed to obtain a correlation coefficient that expresses the relationship between the AAS concentration and the response of the HPLC system. When a linear correlation was obtained, the quantities of AAS in the unknown extracts could be calculated from the standard curve and extrapolated per gram of dry weight. Recovery was determined by comparing the peak heights with and without (direct injection) solid phase extraction (SPE).

### **Estimation of food consumption**

The gut content (GC) expressed as total number of cysts present in the digestive tract of catfish larvae was estimated with two different formulas:

$$GC_{dwf} = [AAS_s / (DW_s / BDW)] * 1/AAS_c \quad (1)$$

$$GC_{nf} = (AAS_s / AAS_c) / n \quad (2)$$

Where  $GC_{dwf}$  (number of cysts per fish) is the gut content calculated with the DW of fish in the sample;  $AAS_s$  is the total AAS content measured in the fish sample ( $\mu\text{g}$ );  $DW_s$  is the dry weight of the sample (mg);  $BDW$  is the individual body dry weight of the fish in the sample (mg) and  $AAS_c$  is the calculated AAS content for one *Artemia* cyst ( $\mu\text{g}$ ).  $GC_{nf}$  is the gut content (number of cysts per fish) as calculated with the number of fish in the sample, and "n" corresponds to the exact number of fish larvae in the sample. The AAS content of one cyst was calculated by dividing the mean content of AAS in cyst samples by the number of individuals (292 398) in 1 g (dw) of decapsulated cysts. The individual cyst dry weight of this particular batch of cysts was 3.42  $\mu\text{g}$  (García-Ortega et al., 1998). The gut evacuation (GE) rate was calculated from data of Experiment 3 as:

$$GE_{tx} = (GC_{nt})_{tx} * 100 / (GC_{nt})_{ti} \quad (3)$$

Where  $GE_{tx}$  is the gut evacuation (% from initial number of cysts) at time X (7, 11 or 15 h after food deprivation),  $(GC_{nt})_{tx}$  is the gut content after 7, 11, or 15 h after deprivation of food with AAS and  $(GC_{nt})_{ti}$  is the initial gut content at 3 h after the first feeding (100% of ingested food at first feeding). The food consumption (FC) was calculated by subtracting the GE from the GC and is expressed as percent of fish body weight.

### Statistical analysis

Three replicates per diet treatment were used to determine the fish weight, AAS content and dry matter. Three replicates were also tested for dry matter and AAS content of *Artemia* cysts and nauplii. All data were tested for normal distribution and homogeneity of variance before analysis of variance was performed. Data on fish weight and gut content from Experiments 2 and 3 were tested separately for the effect of feeding time and diet treatment using a two-way ANOVA according to the model:

$$Y_{ijk} = \mu + F_i + T_j + (F * T)_{ij} + e_{ijk}$$

Where  $Y_{ijk}$  corresponds to either the fish weight or the gut content;  $F_i$  relates to the feeding time effect;  $T_j$  to the diet treatment effect;  $(F * T)_{ij}$  to the interaction effect;  $e_{ijk}$  represents the error term. When effects were found ( $P < 0.05$ ), Duncan multiple range test was used to test the differences between feeding time levels, and least squares means were calculated to test for differences between diet treatment within feeding groups. The statistical analysis was done separately for each of the sampling days. A t-test was done to evaluate if there were significant differences ( $P < 0.05$ ) in the GC as calculated by formula (1) and (2), and in the AAS content between cysts hydrated at 1 and 3 hours. The required number of replicates per treatment and the power of ANOVA for the gut content estimations were tested with the evaluation of the quantity  $\phi$  according to Sokal and Rohlf (1995) with the formula:

$$\phi = (n\delta^2 / 2as^2)^{1/2}$$

Where  $n$  = number of replications;  $\delta$  = unit (number of cysts) difference between the most different means;  $a$  = number of treatments;  $s^2$  = mean squares among groups (among treatments). The analysis was performed to test the ANOVA for a power of at least 80% at  $\alpha = 0.05$  and with df ( $\nu$ ) among treatments of  $\nu_1 = a - 1$  and  $\nu_2 = a(n - 1)$ .



## Results

### *Fish growth*

The catfish larvae displayed a high feeding activity every time the food was offered. Approximately one hour after feeding all the food particles were consumed and the fish larvae were resting on the bottom of the aquarium until one hour before the next feeding when they started to swim slowly. These observations are in accordance with the satiation time of 30 min found for African catfish larvae (Haylor, 1993). The transparent gastrointestinal tract of the larvae at this stage of development allowed visual verification of the gut filled with food particles after every feeding. This indicated that the decapsulated cysts and nauplii of *Artemia* were well accepted and ingested by the catfish larvae. In contrast, the formulated artificial diet was not well accepted. Although the fish were able to ingest this type of food, their growth was lower than when cysts and live nauplii were fed (Table 1). The catfish larvae fed with cysts and live nauplii showed a higher growth during the first days after the start of exogenous feeding (days 1 to 5), gaining approximately 50% of their initial daily weight within 12 h of normal feeding. The dry matter percentage of fish larvae increased towards the end of the larval stage (Table 1). At all sampling days the growth was similar for fish fed with cysts and for fish fed cysts plus nauplii (Experiment 2: treatments one and two).

### *Accuracy and precision of the method*

The described new method for AAS quantification in cysts and fish larvae yielded a linear response over a low (2.6–11.4 ng) and over a high (2.4–33.6 µg) level of AAS in the sample with correlation coefficients exceeding 0.999. Concerning the accuracy of the analysis, the recovery of AAS from the buffer solutions was 93.1 % ( $\pm 1.3$ ;  $n=8$ ) for a 2.25 µg AAS per sample and 90.8 % ( $\pm 2.1$ ;  $n=8$ ) for an 11.25 µg AAS per sample. The reproducibility of the AAS measurements was determined eight times in the same batch of *Artemia* nauplii. The nauplii contained 87.7 µg AAS g<sup>-1</sup> DW with SD = 3.0 µg g<sup>-1</sup> and CV = 3.5%. This small amount of AAS found in nauplii was probably due to some cyst contamination in the nauplii suspension during sample taking. With regard to the precision in the estimation of gut contents in terms of number of cysts, the CV was 6.0% at day 1, 10.1% at day 3 and 8.5% at day 5 (Experiment 2). The analysis of number of replicates and the power of ANOVA yielded a quantity  $\phi$  of 3.1 at day 1, 7.3 at day 3 and 9.5 at day 5 for  $n = 3$ . Those represented a power of 99.9% in each case, which indicates a good reproducibility of the data mainly due to the small within-group mean squares. The minimum detectable difference ( $1 - \beta = 0.80$ ,  $\alpha = 0.05$ )

between two means in different treatments was estimated at 13 cysts per fish at day 1, 85 at day 3 and 130 at day 5. These numbers represent relative detectable differences of 17, 36 and 34 % of the daily average food consumption (% body dry weight) during days 1, 3 and 5 respectively. To increase the resolution of the method and enable detection of differences of at least 10 % of the daily food consumption, the number of replicates per treatment should be increased to 7, 22 and 20 for days 1, 3 and 5 respectively ( $1 - \beta = 0.80$ ,  $\alpha = 0.05$ ).

**Table 1.** Mean individual weight and dry matter content of African catfish larvae fed decapsulated cysts and nauplii of the brine shrimp *Artemia* and an artificial starter diet. The indicated time corresponds to days after the start of exogenous feeding.

	Weight (mg)			
	Experiment 1	Experiment 2		
	Day 1	Day 1	Day 3	Day 5
<i>Before first feeding (F0)</i>				
Cysts		3.80 ± 0.1	9.8 ± 0.5	22.7 ± 0.5
Nauplii+cysts		3.59 ± 0.1	8.9 ± 1.0	24.0 ± 1.6
<i>First feeding (F1)</i>				
Cysts	3.84 ± 0.0	4.05 ± 0.1	10.8 ± 0.6	26.1 ± 0.7
Nauplii+cysts		3.87 ± 0.2	10.4 ± 0.8	28.0 ± 2.6
<i>Second feeding (F2)</i>				
Cysts	4.33 ± 0.1	4.57 ± 0.3	12.3 ± 0.8	27.5 ± 1.6
Nauplii+cysts		4.41 ± 0.3	11.4 ± 1.2	29.8 ± 1.1
<i>Third feeding (F3)</i>				
Cysts	4.64 ± 0.1	4.81 ± 0.4	13.5 ± 0.4	29.5 ± 2.5
Nauplii+cysts		4.54 ± 0.0	12.2 ± 1.9	31.2 ± 1.7
<i>Fourth feeding (F4)</i>				
Cysts	5.47 ± 0.1	5.27 ± 0.6	15.0 ± 0.8	36.0 ± 2.2
Nauplii+cysts		5.03 ± 0.2	14.3 ± 2.3	34.2 ± 3.1
Nauplii		4.75 ± 0.3	8.8 ± 0.2	22.1 ± 1.4
Artificial diet	3.82 ± 0.1	4.80 ± 0.0	6.9 ± 0.2	11.6 ± 0.7
<i>Dry matter (%)</i>				
Cysts	11.4 ± 0.5	11.9 ± 0.2	11.8 ± 0.1	14.6 ± 0.1
Nauplii+cysts		12.0 ± 0.1	12.5 ± 0.5	15.3 ± 0.3
Nauplii		11.0 ± 0.2	12.7 ± 0.1	15.1 ± 0.1
Artificial diet	11.0 ± 0.2	12.9 ± 0.4	13.0 ± 0.1	12.4 ± 0.1

### *AAS content in cysts and artificial diet*

The mean AAS content of cysts samples after one hour hydration was about  $380 \mu\text{g g}^{-1}$  DW in Experiment 1;  $470 \mu\text{g g}^{-1}$  DW in Experiment 2; and  $314 \mu\text{g g}^{-1}$  DW in Experiment 3. The AAS content of one cyst was calculated to be 0.0013, 0.0016 and  $0.0011 \mu\text{g}$  for Experiments 1, 2 and 3 respectively. This difference in AAS content for the same batch of cysts is not attributed to the hydration time in cysts because between 1 and 3 hours of hydration time, no significant difference was found ( $P>0.05$ ). Instead, variations in the content of AAS in *Artemia* cysts might be expected after 5-6 hours hydration (Dabrowski 1991). The AAS content of the artificial starter diet was  $2.7 \mu\text{g g}^{-1}$  DW.

### *AAS retention in fish larvae*

The retention of AAS in fish at the end of each sampling day for all experiments is shown in Table 2. With regard to the control diets in Experiment 2, the fish fed exclusively *Artemia* nauplii contained an average AAS content of  $33.5 \mu\text{g g}^{-1}$  DW for the three sampled days. This amount represents a small part of what was measured for other fish samples in the diet treatments one and two, and is probably due to the presence of some cysts in the nauplii suspension during feeding. The difference in the content of AAS in *Artemia* cysts among the different experiments partially explains the difference in AAS content between the fish with the same feeding treatment (cysts) in Experiments 1 and 2 (Table 2). Moreover, the fish larvae from this diet treatment in Experiment 1 had a higher weight than that in Experiment 2 for the same day. This higher growth accounts for a higher accumulation of AAS. The small amount of AAS in the fish samples taken before first feeding in Experiment 2 (Table 3) indicates that the AAS is not retained for a long time in the fish. The acquired AAS from cysts was almost completely evacuated after one day of nauplii feeding as observed in the fish from diet treatment two (Table 3). This provides further evidence that fish would not incorporate AAS into their body when feeding *Artemia* nauplii because the AAS in live nauplii is transformed to ascorbic acid during the embryonic development of cyst into nauplii (Dabrowski, 1991). In the fish fed the dry artificial diet without AAS, just a small amount was detected (Table 2), and probably it was derived from the AAS measured in the artificial diet. The results in the AAS content of the two control diets in Experiment 2 confirm that no AAS biosynthesis occurs in the larvae of the African catfish.

**Table 2.** Ascorbic acid sulfate content of catfish samples taken 15 h after the first daily meal. The indicated time corresponds to days after the start of exogenous feeding. The experimental diets consisted of decapsulated cysts and live nauplii of the brine shrimp *Artemia* and an artificial starter diet without AAS.

Diet	Ascorbic acid sulfate ( $\mu\text{g g}^{-1}$ dw)		
	Day 1	Day 3	Day 5
<i>Experiment 1</i>			
Cysts	243.6		
Artificial diet	1.3		
<i>Experiment 2</i>			
Cysts (treatment 1)	192.4	257.6	189.8
Nauplii + cysts (treatment 2)	249.6	287.2	183.0
Nauplii (control diet)	39.4	38.3	22.8
Artificial diet (control diet)	0	0.3	0.5
<i>Experiment 3</i>			
Continuous feeding	12.0	13.5	24.6
Discontinuous feeding	17.8	18.5	31.2
Nauplii	13.7	11.8	13.8

### *Gut contents and meal frequency*

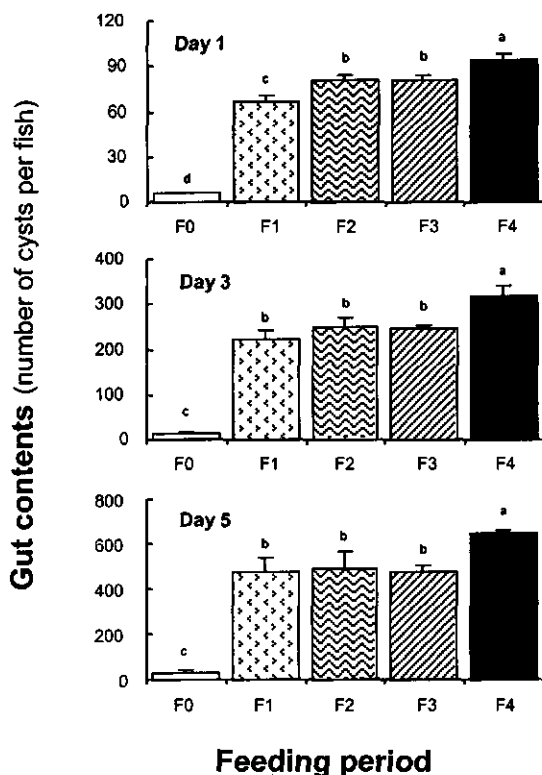
The amount of AAS measured in fish samples from diet treatment one (Experiment 1) and diet treatments one and two (Experiment 2) were used to calculate the number of cysts in the fish larvae at the various sampling periods. The amount of cysts found in the fish samples was considered as the gut contents. With regard to the formula used for the calculation of the gut contents, in the second experiment and for all sampling days, the number of cysts in the gut of catfish larvae were not significantly different ( $P > 0.05$ ) regardless whether it was calculated with the fish DW (1) or with the exact number of fish in the sample (2). When the gut contents were expressed as number of cysts per individual fish (Experiment 2), a diet effect ( $P < 0.05$ ) was detected in most of the feeding periods in days 1 and 5 (Table 3). In the F0 samples (before first meal in each sampling day), the number of cysts per fish was the lowest among the different feeding times studied because during overnight the fish were deprived of food for at least 8 hours. During this time the fish digested and evacuated the nauplii. Thus, the larvae fed with live nauplii have almost an empty gastrointestinal tract in the morning before the first meal. On the other hand, in the case of the fish fed with cysts, some food particles from the last daily meal were retained until the next morning. This indicates that for catfish larvae it takes longer time to digest and evacuate cysts than nauplii. With regard to the

amount of food ingested during each meal in Experiment 2, this is similar for the two treatments in day 1 (Table 3). During day 3 there is no significant difference ( $P>0.05$ ) in the gut contents among diet treatments with exception of the sample taken before the first daily feeding (F0). The number of food particles ingested during the first meal and during the day is very close to the total number of cysts measured at the end of the day (Fig. 1). This indicates a tendency in the larvae to fill completely the gut with food particles during each meal. The same observation was made for African catfish larvae (Haylor, 1993) and fingerlings (Hossain et al., 1998). As the fish grows, the volume available to store food increases. In this way the fish is able to ingest an increasing amount of food with each meal. Once the gut is filled, no additional ingestion of food occurs until more space is available in the gut. Subsequent food ingestion every 4 h might occur at a similar or a higher rate than in the first meal.

**Table 3.** Daily gut contents in African catfish larvae fed decapsulated cysts and nauplii of the brine shrimp *Artemia* from day 1 to day 5 after the start of exogenous feeding (Experiment 2). In the diet treatment cysts, decapsulated cysts were fed to the larvae during the entire experiment. For the diet treatment nauplii+cysts, decapsulated cysts were fed exclusively during the indicated sampling days.

Diet	Number of cysts per fish			
	Experiment 1	Experiment 2		
	Day 1	Day 1	Day 3	Day 5
<i>Before first feeding (F0)</i>				
Cysts		26.1 $\pm$ 0.9*	159.5 $\pm$ 4.4*	206.2 $\pm$ 2.4*
Nauplii+cysts		6.0 $\pm$ 0.1*	13.3 $\pm$ 2.5*	29.6 $\pm$ 9.5*
<i>First feeding (F1)</i>				
Cysts	78.4 $\pm$ 9.1	39.3 $\pm$ 1.2*	175.5 $\pm$ 30.3	378.3 $\pm$ 12.1*
Nauplii+cysts		66.5 $\pm$ 4.5*	221.8 $\pm$ 22.5	474.2 $\pm$ 64.7*
<i>Second feeding (F2)</i>				
Cysts	81.7 $\pm$ 2.8	55.9 $\pm$ 1.6*	227.7 $\pm$ 15.5	405.4 $\pm$ 12.4*
Nauplii+cysts		81.1 $\pm$ 3.5*	248.8 $\pm$ 21.8	545.9 $\pm$ 6.5*
<i>Third feeding (F3)</i>				
Cysts	103.9 $\pm$ 9.4	59.7 $\pm$ 2.1*	246.6 $\pm$ 13.8	451.3 $\pm$ 28.2
Nauplii+cysts		80.6 $\pm$ 3.2*	244.5 $\pm$ 7.9	473.5 $\pm$ 28.0
<i>Fourth feeding (F4)</i>				
Cysts	115.6 $\pm$ 1.8	79.6 $\pm$ 0.8*	282.5 $\pm$ 4.3	573.4 $\pm$ 27.9*
Nauplii+cysts		94.1 $\pm$ 4.7*	316.5 $\pm$ 24.5	649.3 $\pm$ 11.4*

Means with an asterisk in the same feeding period and for the same day are significantly different ( $P<0.05$ )

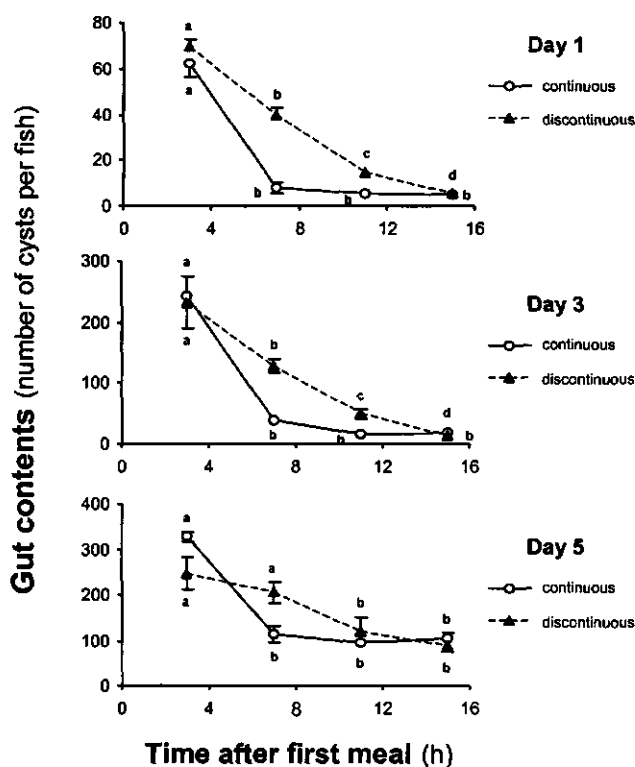


**Figure 1.** Daily variation in gut contents in African catfish larvae fed cysts of the brine shrimp *Artemia* at days 1, 3 and 5 after the start of exogenous feeding (Experiment 2). The number of cysts per fish was determined 3 h after each feeding. F0 = before the first daily feeding, F1= first feeding, F2= second feeding, F3= third feeding, F4= fourth feeding. The time between feedings was 4 hours. Bars in the same graph with the same letter are not significantly different ( $P > 0.05$ ). Note the difference of the scale in the gut contents axe between plots.

### Gut evacuation

The food evacuation rate differed significantly ( $P < 0.05$ ) between the continuous and discontinuous feeding treatments (Fig. 2). After 7 h of ingestion of the first meal at day 1 after the start of exogenous feeding, the fish larvae evacuated approximately 87% of the amount of food ingested in that meal compared to 43% when discontinuous feeding was applied (Table 4). Hence, 3 h after the second meal ingestion, the newly ingested food has a significant effect on the evacuation rate of the previous meal. During the continuous feeding regime the food

evacuation occurred faster than when a discontinuous feeding was applied. The results from Experiment 3 further provide an indication of the time required for emptying the gastrointestinal tract in catfish larvae. In days 1 and 3, more than 90% of the food ingested in the first meal was evacuated after 15 h (Table 4). During the first days after the start of exogenous feeding, i.e. from day 1 to 3, the gut evacuation rate of *C. gariepinus* appears to be independent of the fish body weight. A similar observation was done for *Coregonus lavaretus* juveniles (Rösch, 1987).



**Figure 2.** Gut evacuation in African catfish larvae during days 1, 3 and 5 after the start of exogenous feeding (Experiment 3) under continuous and discontinuous feeding regimes. The fish were fed decapsulated cysts and live nauplii of the brine shrimp *Artemia*. In both treatments only cysts were offered in the first daily meal, subsequent feedings were done with nauplii in the continuous feeding, and no additional food was supplied in the discontinuous feeding. Symbols with the same letter in each graph and in the same feeding treatment are not significantly different ( $P > 0.05$ ).

**Table 4.** Gut evacuation in catfish larvae fed decapsulated cysts of the brine shrimp *Artemia* (Experiment 3). The amount of food present in the fish 3 h after the ingestion of the first meal was considered as the total and subsequent presence of food was subtracted from this total.

Time (h)	Gut evacuation (%) of total food ingested at the first daily meal		
	Day 1	Day 3	Day 5
<i>Continuous feeding</i>			
3	0	0	0
7	87.0	84.1	65.6
11	91.4	93.4	71.2
15	92.1	92.7	68.3
<i>Discontinuous feeding</i>			
3	0	0	0
7	42.7	44.3	17.1
11	78.9	78.3	71.2
15	92.4	94.0	64.4

### ***Food consumption***

The estimated daily food consumption (FC) and gut evacuation expressed as percentage of the body weight of African catfish larvae are presented in Table 5. Until day 3 the fish larvae ingested more than 45% of their body weight daily. In that day the ingestion of food per unit dry body weight reaches its maximum. After this time the relative ingestion rate decreases during the larval development towards the juvenile stage. This decrease continues during the juvenile stage from 27.9% of their body weight at a size of 0.8 g down to 6.2% in juveniles of 37.7 g (Hogendoorn 1983; Conceição et al. 1998). The gut evacuation rate presented a similar pattern as the food consumption, it increased during the first days of exogenous feeding and decreased as the fish growth continued (Table 5).



**Table 5.** Food consumption and gut evacuation rate in *Claras gariepinus* larvae during the first days of exogenous feeding with decapsulated cysts of the brine shrimp *Artemia*. The gut contents in Experiment 2 and the gut evacuation rates from Experiment 3 were used to obtain the food consumption and gut evacuation per feeding time and per day.

Feeding time	Food consumption (% body dry weight)			Gut evacuation (% body dry weight)		
	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
First feeding (F1)	40.0 ± 2.2	51.4 ± 3.6	35.0 ± 3.3	-	-	-
Second feeding (F2)	48.1 ± 3.1	52.6 ± 5.6	22.4 ± 0.8	33.9 ± 2.2	42.6 ± 4.5	22.9 ± 0.8
Third feeding (F3)	46.0 ± 0.4	52.6 ± 7.3	24.4 ± 1.3	47.0 ± 0.4	52.9 ± 7.4	23.4 ± 1.2
Fourth feeding (F4)	52.2 ± 1.5	58.7 ± 8.5	29.4 ± 2.8	41.9 ± 1.2	44.8 ± 6.5	21.2 ± 2.0
Daily average	46.5	53.8	27.8	40.9	46.8	22.5

## Discussion

### *Reliability of the method*

The methodology for AAS quantification in samples of *Artemia* cysts and fish larvae allowed an accurate and precise estimation of the AAS contents in fish samples. This permits repetition of the experiments and comparison of results. Because the recovery of AAS by the present method was high (91-93%), the differences in AAS content found in cysts from the same batch among the experiments might be related to a combined effect of analytical errors and biological effects. The first is related to the stability of AAS standards and storage of cysts. When the activity of the AAS standards changes with time, the calibration curves derived from such standards will reflect those changes. Moreover, although AAS is a very stable compound, it has not been investigated yet if the content of AAS in cysts changes during storage. This stresses the need to determine the AAS of the cysts used in the feeding experiments at every analysis with HPLC. Other sources of variation in the food consumption estimations by the proposed method are related to biological aspects. Variations in food consumption by individual fish, due to differences in size, were reduced because of the high fish number in each sample. Thus, a small SD was obtained in the weight measurements. The number of replicates in the present study was appropriate for the statistical analysis of the results with a power of ANOVA close to 100%. However, the precision to detect significant differences in the number of cysts in the gut between diet treatments was rough. The method

to calculate the gut contents can be improved by increasing the number of replicates per feeding treatment. Further studies are required to improve the precision in the calculations of the number of cysts in individual fish. The use of AAS as a natural marker in the method is enforced because no biosynthesis of AAS was found in the larvae of *C. gariepinus*. Therefore, the AAS contained in *Artemia* cysts constitute a suitable natural marker for food consumption studies in fish larvae.

### *Food consumption in fish larvae*

When estimating daily food consumption and gut evacuation rates in the larval stages of fish, their high growth rate during the first days of exogenous feeding must be considered. The larvae of *C. gariepinus* have higher growth rates and food conversion efficiencies than the larvae of other fish species (Wieser and Medgyesy, 1990; Keckeis and Schiemer, 1992; Conceição et al., 1998). Within the same day and from one meal to the next the fish is growing at a high rate, consequently the gut size increases and more capacity to store food becomes available. Evidently, the amount of food ingested by the fish larvae increases with their body weight. However, if we express the daily food consumption as a percentage of the body weight after the start of exogenous feeding, in *C. gariepinus* larvae the amount of ingested food decreases with growth from day 3 onwards. Similar observations were made in the larvae of three cyprinid species where a continuous decline in the food consumption from 50 to 30% of the body weight was found as the fish weight increased from 10 to 100 mg (Keckeis and Schiemer, 1992). The determination of the AAS content in the gut enables to estimate food consumption accurately. However, the AAS-based estimations differ from those calculated by the exponential model of Elliot and Persson (1978). If a constant evacuation rate during the larval period is assumed, the relative food consumption (% body weight) would increase with fish size, as occurs with the gut content. Haylor (1993), following this approach stated that the food consumption in African catfish larvae increases from 26.6 to 36.3% of the body weight when the larvae grew from 5 to 60 mg. However, these food consumption data could have been overestimated. Several factors affect the food evacuation rate in fish (Persson, 1981; Karjalainen et al., 1991; Bromley, 1994), and thus it is unlikely to have a constant gut evacuation under natural conditions. Moreover, the stomach in *C. gariepinus* larvae is not functional at the start of exogenous feeding (Stroband and Kroon, 1981) and only becomes functional at day 5 after the start of exogenous feeding (Verreth et al., 1992). Therefore, in fish larvae with a digestive system that is still in development, differences in food consumption and evacuation might be expected because of the influence from developmental and physiological processes during food digestion. In the case of catfish

larvae, before the stomach is completely functional, its role during food digestion is reduced to storage of food. The ingestion and evacuation of food as measured by the new proposed method indicated differences during the fish larvae development. The food consumption in catfish larvae was high during the first days of exogenous feeding with a maximum level found on day 3 at a fish weight of 10-15 mg, then the food intake decreased as the fish approached the end of the larval period. In fish larvae, after the yolk sac absorption and during the first days of exogenous feeding, high food consumption rates might be expected to support the high growth rates the fish achieve during this stage of development. These high food consumption rates probably occur also in the larvae of many other fish species. In the larvae and juveniles of five cyprinids and perch, high rates of food consumption (>50% body weight per day) have similarly been found (Marmulla and Rösch 1990).

With a radio-isotope technique, the maximum daily food consumption in *C. gariepinus* larvae was estimated to be 51.5% for fish at days 2 and 8 of exogenous feeding (Conceição et al., 1998). The value for day 2 is close to the result in the present study for the same fish size. However, in the former study the gut evacuation was not determined and possibly that might account for the high food consumption rate found at day 8. Such differences in the estimations of food consumption, using different methods, might be related to the number of assumptions and calculations and the procedures to obtain them in each particular method. In the present study mathematical models were not used. Only direct measurements were made and used to calculate the gut contents and gut evacuation under normal rearing conditions in the laboratory. Possible variations from the factors that affect gut evacuation in fish i.e. temperature, light regime, fish size, periodicity of feeding, stomach fullness, and food quantity, quality and size (Grove and Crawford, 1980; Persson, 1981; Rösch, 1987) can be controlled in the proposed new method. The feeding strategy also has an effect on the food consumption in fish larvae. The frequency of feeding and the type of food can affect the digestibility of the food with effects on the food consumption and evacuation rates. The evacuation rate is significantly higher under multiple meal conditions than under a single meal condition in the juvenile stage of carp and turbot (Rösch, 1987; Grove et al., 1985). Shorter times of gut emptying are expected in a continuous feeding regime. A rapid evacuation allows the fish to consume food at a faster rate (Johnston and Mathias, 1996). Similarly, *C. gariepinus* larvae fed multiple meals showed a faster evacuation rate than when only a single meal was given. However, caution should be taken when comparing food evacuation results due to differences in the evacuation times when the analysis is done in individual fish or by groups of fish. A curvilinear decrease in gut contents is expected when analyzing a group of fish larvae in comparison of a linear evacuation in individual fish (Karjalainen et al., 1991).

This stresses the importance of considering all the conditions under which the food consumption and the food evacuation rate are estimated.

The AAS method provides a useful tool for the determination of daily food consumption and gastric evacuation studies in fish larvae and permits adequate standardization. It avoids the individual count of food particles or marker substance by the direct analysis of the total amount of AAS in the fish and in the diet samples. Other methods might underestimate the food consumption by missing particles during counting. The described method further avoids the variations in the content of the marker substance in the food particles that might bias the food consumption estimations. The negative effects on the nutritional condition of the fish that are associated with a low food intake and poor diet digestibility, are reduced by the use of a natural food source that is well accepted and digested by the fish larvae. The only specific requirement in the method is that the fish larvae under study should be able to ingest the decapsulated cysts of *Artemia* or any artificial diet that might include cysts in the formulation.

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## Chapter 4

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### **Post-prandial protease activity in the digestive tract of African catfish *Clarias gariepinus* larvae fed decapsulated cysts of *Artemia***

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**Abstract**

The alkaline proteolytic activity in the gut of African catfish larvae was studied during short time ranges from 30 min to 4 h after ingestion of decapsulated *Artemia* cysts. The variation in total protease and trypsin activities during the day was monitored during starvation, after one single meal ingestion, and during continuous feeding. In starved larvae the enzymatic activity was low and did not change in time. No significant endogenous secretion of digestive enzymes was detected. The level of alkaline proteolytic activity found in starved larvae was further considered as the basal level. In larvae fed only one meal during the day, the enzyme activity significantly increased from 3 h post-feeding up to a maximum level found 12 h after feeding. In the larvae receiving a meal every 4 h, the effect of feeding on the proteolytic activity was significantly different from the one in fish fed only once a day. The total protease activity in this dietary treatment changed according to the feeding time and fluctuated around a constant level, which was intermediate between the maximum and the basal level. No rhythmic cycle of enzyme production in the fish was observed when the proteolytic activity was studied during a cycle of 24 h. When specific trypsin activity was measured, a similar pattern was found as with the total protease. The contribution of digestive enzymes from *Artemia* to the total digestion of food by the catfish larvae was calculated to be less than 1% of the total amount of the proteolytic activity measured in the larval gut.

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**Keywords:** digestive enzymes, enzymatic response, feeding, fish larvae, live food, protease, trypsin.



## Introduction

In contrast to fish species which can be reared with artificial diets from the start of exogenous feeding onwards (e.g., salmonid fishes), the larval stages of some other fish species require live food organisms for optimal nutrient utilization. Although artificial diets have been used with satisfactory results in the rearing of African catfish, *Clarias gariepinus*, larvae (Uys and Hecht, 1985; Appelbaum and Van Damme, 1988) the growth rate and survival were lower than those obtained in fish fed live *Artemia* nauplii. To explain the success of live food over artificial diets in the larviculture of some freshwater and many marine fish species several hypotheses have been proposed. It was suggested that fish larvae have a low enzyme production and a reduced digestive capacity which make the enzymes in live zooplankton organisms necessary for the digestion of food (Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984; Munilla-Moran et al., 1990; Kolkovski et al., 1993). On the other hand, it was demonstrated that larvae of *C. gariepinus* (Verreth et al., 1992), whitefish *Coregonus lavaretus* (Segner et al., 1989), sea bass *Dicentrarchus labrax*, (Vu, 1983; Cahu and Zambonino Infante, 1994) and herring *Clupea harengus* (Pedersen et al., 1987) possess the digestive capabilities and the necessary enzymes for the digestion of live food. Moreover, recent data suggest that in some marine fish larvae dietary enzymes do not contribute significantly to the digestion of food (Zambonino Infante and Cahu, 1994; Moyano et al., 1996; Kurokawa et al., 1998). However, due to the variety of methods used in the quantification of dietary enzymes and due to differences in the development of the digestive tract between fish species, definitive conclusions can not be made yet for all fish species.

In fish larvae the digestive processes are fast and occur within few hours after the ingestion of food (Govoni et al., 1986; Hofer and Bürkle, 1986; Pedersen et al., 1987). However, the majority of studies on proteolytic activity during larval development measured the enzyme activity during a time span of several days as an indication of the long-term nutritional condition of the larvae. Only few studies present data on short-term variation of proteolytic activity in fish larvae in periods smaller than one day (Pedersen and Hjelmeland, 1988; Ueberschär, 1993). Information on the short-term variations in proteolytic activity under different feeding regimes, in comparison to starvation, are necessary to provide insights on the capacity of the fish larvae for the digestion of every single meal offered during a day. The aim of the present study was to monitor the variations of alkaline proteolytic activity in the digestive tract of African catfish larvae for short time ranges from 30 min to 4 h after food ingestion. Additionally, the contribution of exogenous enzymes to the larval digestion of food was estimated and discussed.

## Materials and Methods

### *Artemia* cysts

Decapsulated cysts of *Artemia* were used as experimental diet because they combine the practical advantages of a dry diet with the balanced nutritional composition of live nauplii. Additionally, they have been previously tested successfully as starter food in the rearing of African catfish (Verreth et al., 1987). Cysts of *Artemia* (EG type, INVE Aquaculture) from Great Salt Lake, USA, were hydrated during 1 h in aerated freshwater and decapsulated with a solution of NaOCl, NaOH and water in accordance to the method of Sorgeloos et al. (1986). After decapsulation, the cysts were dehydrated with saturated brine ( $>330 \text{ g L}^{-1}$ ) during 24 h and stored at 4°C in fresh brine until their use to feed the fish larvae. The cysts were decapsulated and prepared the day before the start of each feeding experiment.

### *Fish larvae rearing*

Eggs and larvae of African catfish *Clarias gariepinus* were obtained by artificial reproduction of broodstock kept at the hatchery in Wageningen. Two days after hatching, the larvae were counted and allocated to a recirculation system with 17 L aquaria at a density of 1000 larvae per aquarium. The larvae were reared at a constant temperature of  $28 \pm 0.2 \text{ }^{\circ}\text{C}$  and a light regime from 08:00 to 20:00 h. The same procedure for the production and rearing of larvae was carried out in each feeding experiment. Larval feeding started two days after hatching, i.e., after the yolk sac absorption. In all feeding experiments the fish weight was determined every day and the amount of food was calculated daily according to the predicted maximum growth (Verreth and Den Bieman, 1987). Feeding was done every four hours from 08:00 to 23:00 h (i.e. five times a day) and the food consisted exclusively of decapsulated *Artemia* cysts. Before being fed to the larvae, the cysts were immersed in freshwater during five min to rinse the brine and to allow cyst hydration. Approximately one hour after each food addition, the fish larvae had ingested all food particles in the aquaria. When some food particles remained, they were siphoned out the aquarium one hour after each feeding. In all experiments and independently of the diet treatment, the fish larvae were fasted for 20 h before each sampling day in order to empty the intestine and reduce the protease activity to a low level. The proteolytic activity after this starvation period was considered the basal level of enzyme activity.

### *Short-term experiments*

Two separate feeding experiments were designed to measure the short-term post-prandial proteolytic activity in the digestive tract. In the first experiment gut samples were taken every 30 min during the first 3 h after food ingestion, and in the second experiment every hour during 7 h after food ingestion. In both experiments only one meal was presented to the larvae during the sampling days. The initial weight ( $w_i$ ) of catfish larvae at day 0 (i.e. start of exogenous feeding) was 2.1 mg (Experiment 1) and 2.0 mg (Experiment 2). At days 3 and 7 after the start of exogenous feeding, samples of fish larvae were taken for the determination of the total protease activity. Samples were also taken before the first feeding.

### *Feeding experiments*

Two other experiments were carried out to determine the time of maximum proteolytic activity after the ingestion of one single meal (Experiment 3) and to measure the variations in proteolytic activity in a 24 h cycle (Experiment 4). In each of the latter experiments, three different feeding treatments were tested during the sampling days and gastrointestinal tract samples were taken exclusively at day 3 and day 7 after the start of exogenous feeding in Experiment 3, and at day 7 in Experiment 4. For both experiments the first diet treatment consisted of depriving the fish of food during the entire day, in the second treatment the fish were offered only one first daily food, and in the third treatment the food was offered every four hours. In Experiment 3 ( $w_i = 1.7$  mg), total protease and trypsin activities were measured during 16 h after the first daily feeding. Samples were taken every 2 and 4 h depending of the diet treatment. For all diet treatments, the number of dissected fish and the weight of gut samples were recorded for calculation of the percentage of gut weight on total body weight. In Experiment 4 ( $w_i = 2.5$  mg), the gut samples were taken every 2 and 4 h, depending on the feeding treatment, during a complete period of 24 h. When the time of feeding and gut sampling coincided, the samples were taken before feeding in all the experiments.

### *Sample preparation*

Fish larvae were collected from each aquarium, rinsed with tap water and excess water was removed with tissue paper. The fish were placed in a glass dish constantly maintained at 0-2°C for dissection of the entire digestive tract under a stereoscopic microscope. The number of fish per sample ranged from 30 to 40 during day 3, and from 10 to 15 during day 7. The dissected gastrointestinal tracts from each aquarium were pooled and placed in a plastic

Eppendorf cup kept on ice. Immediately thereafter the gut samples were shock-frozen with liquid nitrogen. The samples were stored at  $-80^{\circ}\text{C}$  until enzyme analysis. The elapsed time between fish sampling and shock-freezing did not exceeded 15 min. The same procedure for the preparation of digestive tract samples was done in all experiments. Samples of decapsulated cysts were also prepared for the determination of total protease activity.

### *Enzyme analyses*

To measure total protease activity, 15 to 30 mg of digestive tract tissue of fish larvae samples was ground and homogenized in a potter blender with 750  $\mu\text{l}$  of 0.1 M Tris buffer at pH 8. After homogenization the samples were sonified during 30 seconds to break the cell wall and centrifuged at 12000 g during 10 min at  $4^{\circ}\text{C}$ . During preparation the homogenates were constantly kept on ice. Total protease activity in fish homogenates was measured by a casein method modified from Walter (1984) using casein (10 mg  $\text{ml}^{-1}$ ) dissolved in 0.1 M Tris buffer at pH 8. A buffer composed of 0.2 M Tris and 0.1 N HCl was used to detect alkaline protease activity at pH 8 using L-tyrosine as standard. A mixture of 400  $\mu\text{l}$  casein solution, 400  $\mu\text{l}$  buffer and 200  $\mu\text{l}$  supernatant of homogenate was incubated at  $30^{\circ}\text{C}$  during 40 min, and then the reaction was halted with trichloroacetic acid. The assay mixtures were left cooling on ice during 15 min and were centrifuged at 1700 g during 10 min at  $4^{\circ}\text{C}$ . The absorbance of the supernatant was measured at 280 nm. The results are expressed as mg tyrosine liberated  $\text{g}^{-1}$  gut sample  $40 \text{ min}^{-1}$ .

For the determination of trypsin activity the gut homogenates were prepared similarly as for the total protease analysis. The trypsin activity was determined using a modified method from Hofer and Köck (1989) and Bergmeyer and Graßl (1983), with BAPNA ( $\text{N}\alpha$ -benzoyl-DL-arginine-4-nitroanilide, Merck 1670) as substrate. The assay cocktail consisted of 0.42 mM BAPNA in a 0.1 M Tris buffer at pH 8 with 0.2 M  $\text{CaCl}_2$ . By the addition of BAPNA solution to a mixture of homogenate and buffer the reaction started. The change in absorbance during 5 min was measured at 405 nm. Trypsin activity is reported as Units  $\text{g}^{-1}$  of gut sample (1 U = 1  $\mu\text{mol}$  BAPNA converted per min).

### *Data analysis*

In all experiments the total proteolytic and trypsin activities at every sampling time were determined with three replicates each. Data on enzyme activity were tested for normal distribution and homogeneity of variance before ANOVA (analysis of variance) was

performed to detect differences ( $P < 0.05$ ) in enzyme activity between diet treatment during time. When significant effects were found, the differences of the means were compared with Duncan multiple range test and least squares means, for equal and unequal sample size respectively. The analyses were done with the statistical software package SAS 6.12 (SAS Institute, USA).

## Results

The relative weight of the dissected gut decreased with fish body size (Table 1). The size of the digestive tract at this age depends on the food ingestion by the larvae, i.e. the more food is offered and ingested at a normal rate, the bigger the gut size is, consequently higher growth rate is obtained. This is an indication that for the fish larvae it is very important to have the capabilities for digestion of food in terms of food storage and enzyme secretion if a high growth rate is to be achieved.

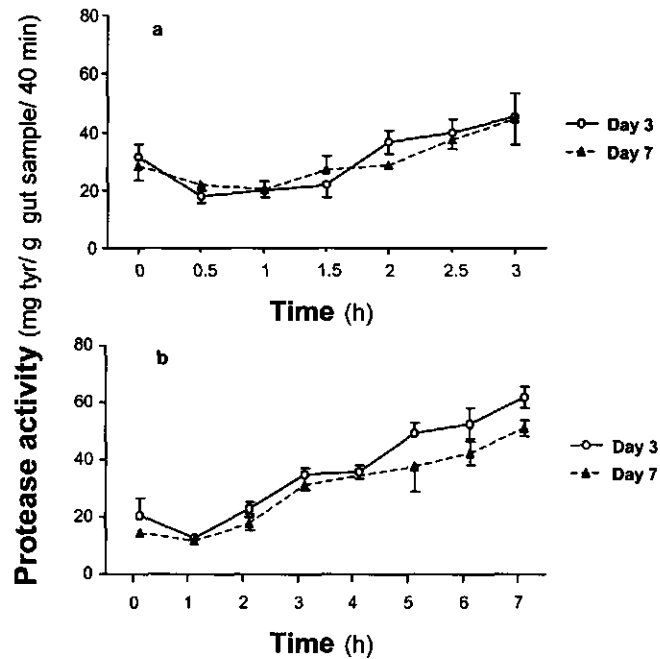
**Table 1.** Body weight, gut weight and proportion of gut weight in total body weight in catfish larvae fed decapsulated *Artemia* cysts at different diet treatments (Experiment 3). The feeding treatments were performed only during the indicated days after the start of exogenous feeding.

Treatment	Individual weight (mg)		Gut weight per larvae (mg)		% gut of body weight	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
Starved	4.8 ± 0.2	20.4 ± 0.9	0.7 ± 0.0	2.4 ± 0.4	14.2	11.8
One feeding	5.4 ± 0.2	29.7 ± 0.2	1.6 ± 0.3	5.1 ± 0.2	29.3	17.1
Four feedings	5.6 ± 0.1	33.1 ± 1.2	2.0 ± 0.1	6.1 ± 1.8	34.8	18.5

### *Post-prandial protease activity*

The protease activity measured every 30 min during the first 3 h after feeding is presented in Figure 1a. Total protease activity changed significantly during time after food ingestion. Immediately after the food was offered, the proteolytic activity in the gut of the fish larvae decreased significantly. This time corresponds to the period in which the fish are actively swimming and ingesting food particles. After approximately 1 h, when the fish ingested all

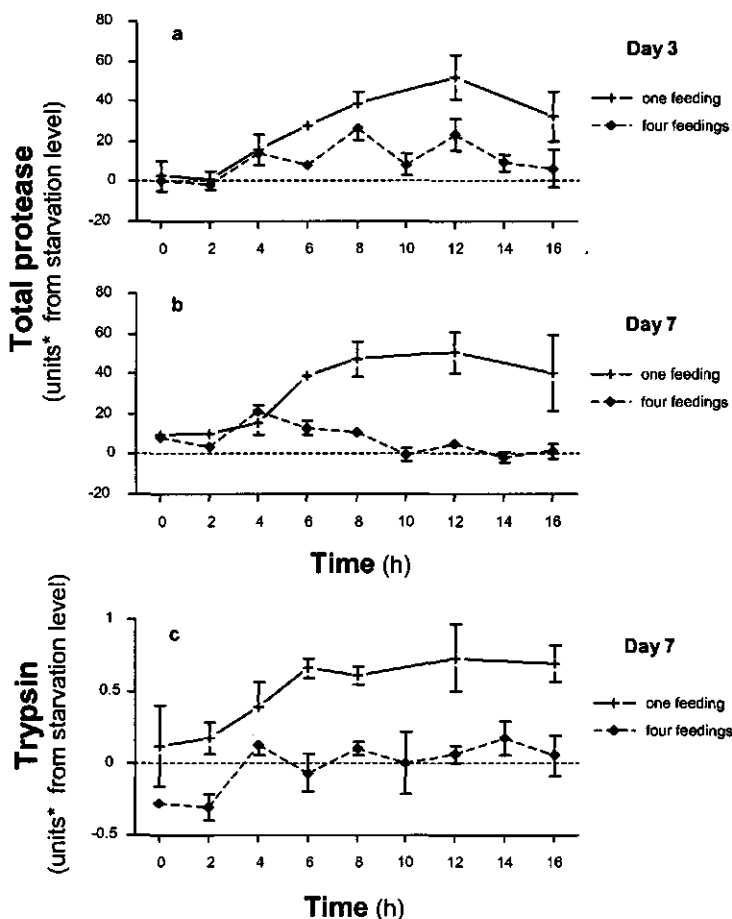
the food that can be stored in the digestive tract, the protease activity started to increase. After 3 h the enzyme activity increased significantly above the basal level measured before feeding. This increase continued during 7 h after one single meal ingestion (Figure 1b). The pattern in the post-prandial protease activity was very similar for days 3 and 7 after the start of exogenous feeding.



**Figure 1.** Total proteolytic activity in the digestive tract of African catfish larvae after a single meal ingestion (at 0 h). The variation in proteolytic activity was first measured within a fine time range of every 30 min (a), and in a second experiment at every hour (b). In both experiments the enzyme activity was measured at days 3 and 7 after the start of exogenous feeding. Note the difference in time scale between the two graphs.

*Protease activity at a wide range*

The post-prandial protease activity in catfish larvae during starvation and at different feeding regimes is presented in Figures 2 and 3. In general, the fluctuations of proteolytic enzyme activity were significantly dependent on food ingestion. During starvation a low and constant



**Figure 2.** Total proteolytic activity (a, b) and trypsin activity (c) in the digestive tract of African catfish larvae after one single meal ingestion (at 0 h), and during continuous feeding every four hours (four feedings a day) as compared to the proteolytic activity during starvation. The horizontal dotted line at zero corresponds to the basal level of proteolytic activity found during starvation (see results section). The indicated days correspond to days after the start of exogenous feeding. \*mg tyr per g gut sample in 40 min.

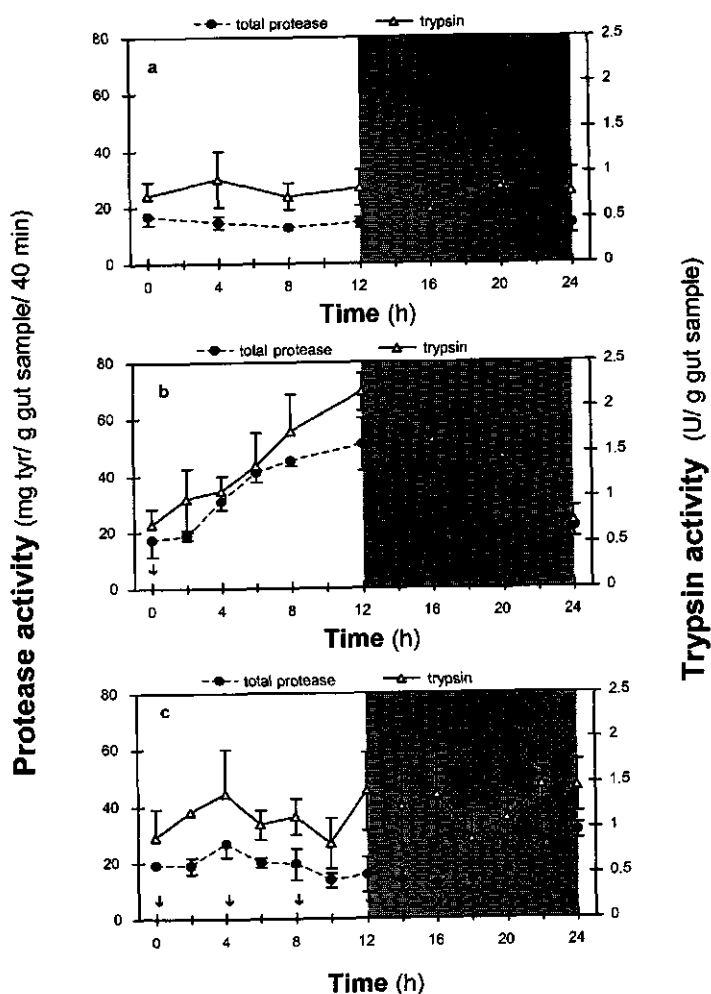
level of proteolytic activity was observed and no significant variations in the starved group were detected in both days 3 and 7 after the start of exogenous feeding. The mean values of proteolytic activity during starvation were  $29.9 (\pm 5.8)$  and  $13.8 (\pm 2.1)$  mg tyrosine liberated per g of gut tissue in 40 min, at day 3 and day 7 respectively. For trypsin activity, the starvation level at day 7 was  $0.52 (\pm 0.0)$  units per g of gut tissue. The average protease activity during starvation was considered as the basal level zero and was subtracted from each individual value measured in the other two food treatments (Figure 2). This was done to observe the variation of enzyme activity caused by the presence of food in the digestive tract. With regard to the fish larvae fed only one meal in the morning, the protease activity significantly increased from 3 h up to a maximum level at 12 h after feeding, regardless no more food was supplied (Figure 2a,b). The peak in proteolytic activity was similarly observed at 12 h after food ingestion in the two sampling days. After this time, the total protease activity started to decline. In the larvae fed every 4 h, the effect of feeding on the proteolytic activity in the gut was significantly different from the one in fish fed only once during the day (Figure 2a,b). The total protease activity changed according to the feeding schedule and fluctuated around a constant level, which was intermediate between the maximum and the basal level. The protease activity slightly decreased after the food was ingested then increased again until the next feeding. This pattern of proteolytic activity in the gut was repeated during the experimental period in this diet treatment. The specific trypsin activity for both diet treatments at day 7 followed similar patterns as with the total protease activity (Figure 2c).

#### ***Proteolytic activity in a 24 h cycle***

During a 24 h period no significant change in enzymatic activity was found for starved catfish larvae (Figure 3a). Similar to Experiment 3, in the fish fed only one ration of food during the day the total protease and trypsin activities increased up to a maximum level found at 12 h after the food was ingested (Figure 3b). After this time the proteolytic activity decreased towards the basal pre-feeding level. The digestion of decapsulated cysts by the catfish larvae took more than 4 h and after approximately 9 h post-ingestion the cysts in the gut appeared completely digested as visually observed with a microscope. Interestingly, the peak of proteolytic activity and the start of the decrease in both total protease and trypsin activities in catfish fed only once coincided with the start of the dark period. However, the pattern of enzyme activity in the gut was constant along the 24 h cycle in the starved larvae (Figure 3a). Moreover, in the larvae fed every 4 hours there was a building up of protease activity towards the end of the dark phase as a result of the presence of food in the digestive tract (Figure 3c). It was assumed that light regime had no influence in the larval proteolytic



activity during the 24 cycle. Further, no rhythmic cycle of enzyme production was observed. The total protease enzyme activity in the fish larvae fed every 4 h had small significant differences during the 24 h period and no significant differences were found in the trypsin activity (Figure 3c).



**Figure 3.** Variation of proteolytic activity during a cycle of 24 h in the larvae of African catfish during starvation (a), after ingestion of one single meal (b), and after feeding every 4 h (c). The vertical arrows indicate the time of feeding and the dark area indicates 12 h of darkness period during the light regime. The samples were taken at day 7 after the start of exogenous feeding.

## Discussion

In the present study the first changes in proteolytic activity in the gut of catfish larvae were observed within 30 min after feeding. These rapid changes in protease activity indicate a fast enzyme secretion and utilization in response to food ingestion. Similar observations were made for pancreas enzymes in larval herring (Pedersen et al., 1987; Ueberschär, 1993). The fast response of digestive enzymes to food intake is in accordance with the hypothesis that larval digestion and assimilation of nutrients must be highly efficient in order to ensure high growth rates as occur in fish larvae. In the larvae of African catfish the specific growth rate increased asymptotically up to 63.9% of the body weight per day between days 0 and 3 after the start of exogenous feeding, and on average, it corresponds to 41% for the first 10 days (Verreth and Den Bieman, 1987). Although the digestive system in African catfish larvae is only fully developed at day 5 after the start of exogenous feeding, the larvae have sufficient capacity to digest and absorb nutrients from live food organisms at the start of exogenous feeding (Verreth et al., 1992). African catfish larvae grow efficiently on live food and this coincides with a pronounced responsiveness of the proteolytic activity as revealed by the results on protease activity in the larval gut. On the other hand, this is in disagreement with the observation that the digestive system in some fish species is not fully functional during early life (Lauff and Hofer, 1984). It remains to be studied how the proteolytic activity changes when dry diets are fed to the catfish larvae.

The high trypsin activity after ingestion of one single meal might be related to a continuous enzyme secretion as a response to the presence of food in the gut. Pedersen and Hjelmeland (1988) found a significant rise in trypsin following ingestion of food in herring larvae. The same authors attribute the high trypsin content to the retention of the enzyme in the digestive tract, assuming that despite food evacuation, the trypsin activity was not significantly affected. Although in catfish larvae fed one single meal the high content of trypsin might be related to the retention of the enzyme, in the fish fed every 4 h the retention effect was not fully evident since trypsin activity in the gut fluctuated. The variations in the proteolytic activity in the fish fed every 4 h coincide with the time of food ingestion. The decrease in enzyme activity in the first 60 min after each feeding is probably due to the immediate utilization of enzymes present in the larval intestine at the moment of food ingestion for protein hydrolysis. Although the enzyme secretion probably starts at the moment of food ingestion, it is only reflected by the increase in proteolytic activity 2 h after feeding. A similar observation was reported for herring larvae (Ueberschär, 1993). Nevertheless, an extensive re-use of trypsin for the digestion of several meals might be also considered as

occurs in herring larvae (Pedersen and Hjelmeland, 1988). In the present study it was further demonstrated that despite of lacking a functional stomach at the start of exogenous feeding (Stroband and Kroon, 1981) the larvae of *C. gariepinus* have sufficient enzymatic capacity for digestion of several meals during the day. The optimal period between meals is to be determined from the digestibility of the diet and the corresponding induced secretion of digestive enzymes. The time when the peak of proteolytic activity occurs is probably related to the type of food consumed. In catfish larvae fed decapsulated cysts of *Artemia* only once a day this peak was observed 12 h after food ingestion. The decapsulated cysts were completely digested approximately 9 h after ingestion. For other types of food with high protein digestibility, e.g. *Artemia* nauplii (Watanabe et al., 1978; Lan and Pan, 1993) the peak of proteolytic activity might occur earlier.

In nutritional studies with fish larvae the assessment of food intake provides valuable information on the importance of the ingested diet on the growth of fish. To estimate the effect of dietary nutrients and their value for the fish larvae in terms of their quantity, the cyst uptake by catfish larvae was previously studied (García-Ortega et al., unpublished data). According to those authors the daily food intake in African catfish larvae ranges from 46 to 54% of the fish body weight during the first days of exogenous feeding. The total protease activity at pH 8 of *Artemia* cysts was calculated at  $14.7 \pm 1.0$  mg tyrosine liberated per g of cysts in 40 min. Thus, if we calculate the protease activity in the determined amount of ingested cysts, the contribution of the enzyme activity from cysts to the total activity measured in the larval gut is estimated to be lower than 1%. A low contribution of enzymes from live food was also demonstrated for the larvae sea bass of (Zambonino Infante and Cahu, 1994), sea bream (Moyano et al., 1996) and sardine (Kurokawa et al., 1998). Most of the protease activity in *Artemia* cysts was related to a cysteine protease (Warner et al., 1995) which is not active at alkaline pH (Warner and Shridhar, 1985). In fish without a functional stomach at the larval stage, the importance of the contribution of digestive enzymes from *Artemia* is therefore reduced. No significant differences in the nutritional composition and proteolytic activity were found between decapsulated cysts and newly hatched nauplii of *Artemia* (García-Ortega et al., 1998). Hence, in fish larvae the superior performance of live food over inert diets might be more related to food consumption and ingredient digestibility than to the dietary enzyme supply. Moreover, the action of biomolecules present in the ingested live food induces pancreatic enzyme secretion (Hjelmeland et al., 1988), thus when the fish larvae are fed artificial diets, the stimulus for enzyme secretion might be insufficient. The same authors suggest a hormonal induced secretion of digestive enzymes produced by active substances released from the live food. Due to the high protein digestibility of *Artemia*

cysts and nauplii, their autolytic capacity and protein solubility must be also taken into consideration when explaining their suitability as food for larval fish. Functional differences in the digestive tract among fish species might be the cause for the differential protein utilization from the food and the use of endogenous proteolytic enzymes (Kawai and Ikeda, 1973; Hofer and Nasir Uddin, 1985; Baragi and Lovell, 1986; Segner et al., 1989; Cahu et al., 1999). The poor growth in fish larvae fed artificial diets might be related to other factors such as the inadequacy of the formulated diet to meet the digestive specifications of the fish during the larval stage (Cahu and Zambonino Infante, 1994). The water content of live food organisms is high, this can induce certain spatial arrangements in the protein molecule than might differ from the one in dry artificial diets. Possibly the structure of the proteins in live food enables a better digestion by the fish larvae, in contrast to the structure of proteins in artificial diets (Verreth, 1994).

The present results further indicate that, when studying physiological processes of food ingestion and digestion in fish larvae, care should be taken in selecting sampling times. This because of the significant variations in proteolytic activity after feeding, e.g., low activities might be expected immediately (within 1 h) after food ingestion with a subsequent increase in activity until the next feeding. The time to reach maximum and minimum proteolytic activities after feeding in fish larvae is probably related to the type of food and is possibly age and species dependent. Nevertheless, low proteolytic activities are expected in fish that is constantly feeding or submitted to starvation in comparison to larvae fed one meal a day or when long periods exists between meals. In the latter case, high activity could occur after few hours of food intake.

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## Chapter 5

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### ***In vitro* protein digestibility of *Artemia* decapsulated cysts, nauplii and microbound diets for larval fish**

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## Short communication

### Abstract

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The protein digestibility of *Artemia* cysts and nauplii, and microbound diets made of decapsulated cysts with diverse binders was estimated by an *in vitro* method. The conditions in the *in vitro* digestion of food were adapted to simulate the situation of protein digestion in fish larvae without a functional stomach at the start of exogenous feeding. The method consisted of incubating food samples in a multi-enzyme system with trypsin as the main proteolytic enzyme. Different incubation times and two sources of enzymes were tested. The maximum level of protein digestion of decapsulated cysts of *Artemia* was reached between 4 and 8 hours of incubation. High protein digestibility coefficients were obtained for decapsulated cysts and nauplii. From the microbound diets tested, the highest digestibility, was observed in the diets with carboxymethyl-cellulose as binder. When commercial purified enzymes were used the digestibility coefficients of cysts and nauplii were 87.1% and 86.2% respectively. When intestinal extracts of catfish were used, the digestibility coefficients were 81.6% and 82.0% for cysts and nauplii respectively. The *in vitro* technique permits accurate estimations of the protein digestibility in food for fish larvae and constitutes an alternative to feeding experiments with live animals.

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**Keywords:** *in vitro* method, protein digestibility, fish larvae, live food, *Artemia*



## Introduction

The protein digestibility in feed for farmed animals has been extensively studied *in vivo*. However, digestibility experiments with live animals are laborious, time consuming, and expensive. Thus, much attention has been given to the development of *in vitro* methods for a fast, reproducible and reliable evaluation of protein quality in animal feeds (Boisen and Eggum, 1991). *In vitro* methods basically rely on the use of purified or extracted enzymes for the digestion of feed samples. Purified enzymes which are commercially available have been used to evaluate the digestibility of pig feeds (Babinszky et al., 1991). In fish feeds, extracts from the entire gut or from the pyloric caeca of rainbow trout have been used for *in vitro* digestibility tests yielding good correlation with digestibility experiments *in vivo* both in juvenile and adult fish (Grabner, 1985; Dimes and Haard, 1994; Bassompierre et al., 1998). *In vitro* techniques might also be very interesting for the development of artificial diets for fish larvae given the problem of assessing protein digestibility *in vivo* in small sized fish. Recently, *in vitro* methods have been used for the preliminary evaluation of protein used in microcapsulated diets for fish larvae (Ozkizilcik and Chu, 1996; Alarcón et al., 1999). Those studies used whole larval body extracts as source of digestive enzymes. However, using whole body homogenates has the disadvantage that the proteolytic enzymes from the gut might interact with other enzymes in the fish body tissue, and consequently, the results could be less representative for intestinal digestion. Moreover, in those studies the incubation period for the complete *in vitro* digestion of the microcapsules was not mentioned. This information can be useful to determine the optimal feeding frequency in nutritional studies with fish larvae, because the digestion of different types of feed particles might change with incubation period and with fish species. Marine fish and some freshwater fish species do not have a functional stomach at the start of exogenous feeding (Govoni et al., 1986). As a consequence, during the larval stage, the fish mostly rely on an alkaline and trypsin-type of digestion. The aim of this work is to develop a simple *in vitro* method for the determination of protein digestibility of live food and artificial diets simulating the conditions in fish larvae without a functional stomach using both purified and fish intestinal extracts as source of digestive enzymes. Additionally, the time for optimal *in vitro* digestion of food for fish larvae was determined.

## ***In vitro* technique**

For the determination of protein digestibility in food for fish larvae, a filtration method with a multi-enzymes system was developed with trypsin as the main proteolytic enzyme. Two sources of enzymes were tested in the method, a mix of commercial purified enzymes and an extract from the anterior intestine from adult African catfish *Clarias gariepinus*. The commercial enzymes mix consisted of a solution of 0.1 ml cod (*Gadus morhua*) trypsin (type XX-S, T-9906), 1ml chymotrypsin (type II, C-4129), 0.1 ml leucine-aminopeptidase (type V, L-1503), 1ml  $\alpha$ -amylase (type L, Termamyl 120L, Novo Nordisk, Denmark), 40 mg lipase (type I, L3001), 80 mg bile salts (Sigma B-8756), 1 ml  $\text{CaCl}_2$ , 0.1 ml  $\text{MgCl}_2$  and 0.1 ml  $(\text{NH}_4)_2\text{SO}_4$ . All the enzymes were obtained from Sigma-Aldrich, The Netherlands, or otherwise indicated. With the exception of trypsin, the commercial enzymes had bovine, porcine or wheat germ origin. The enzymes were dissolved in a buffer made with  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ . The pH was 8 and the temperature of incubation 30°C. After incubation during 4 h in the multi-enzymes system or in the intestinal homogenate, the digestion was stopped by adding HCl (1mol L<sup>-1</sup>). The solution was filtrated over a glass filter (porosity 2) with a layer of ashless floc (nitrogen free, 1704-010, Whatman). The amount of undigested crude nitrogen (N) after the digestion *in vitro* was determined by the Kjeldahl method in the ashless floc filter + residu. From the N content of the original diet samples and of the undigested protein after the *in vitro* method, the digestible protein ( $\text{N} \times 6.25$ ) was calculated.

## **Method development**

In a first set of two trials, the *in vitro* protein digestibility of decapsulated cysts of *Artemia* was tested with purified enzymes and different cod trypsin concentrations in the enzyme mix: 0, 0.05, 0.1 and 0.5 ml, and at four different incubation times: 1, 2, 4 and 8 h. In the second trial, catfish intestinal homogenate (1 ml) was used instead of the commercial enzyme mix to evaluate the protein digestibility in samples of *Artemia* decapsulated cysts and nauplii during 4 h of incubation. Cysts of *Artemia* originating from Great Salt Lake, USA were decapsulated with a hypochlorite solution. One part of the decapsulated cysts was ground with a mortar and the other part was left intact for further hatching. The intact decapsulated cysts were hatched by incubation in salt water ( $\text{NaCl}$ , 33 g L<sup>-1</sup>) at 28°C with constant illumination and aeration during 20 h. The decapsulated cysts and instar I nauplii of *Artemia* were frozen at -20°C and freeze-dried.

The effect of trypsin concentration and incubation time was tested ( $P < 0.01$ ) by a two-way ANOVA. The highest digestibility of cysts was achieved with 0.1 and 0.5 ml of cod trypsin in the solution (Fig. 1). Small but significant ( $P < 0.01$ ) differences were found in protein digestibility when cysts and nauplii were incubated with intestinal homogenate instead of cod trypsin (Fig. 2). The protein digestibility was significantly higher after 4 h of incubation. However, no further increase was found after 8 h of incubation (Fig. 1).

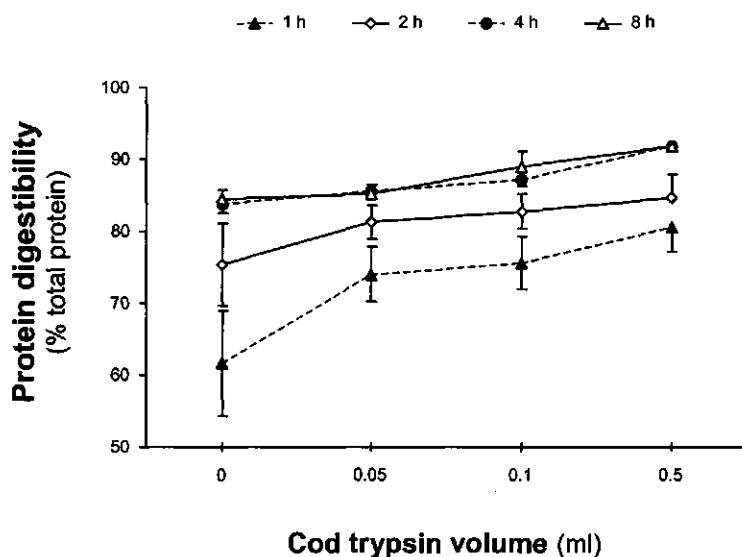


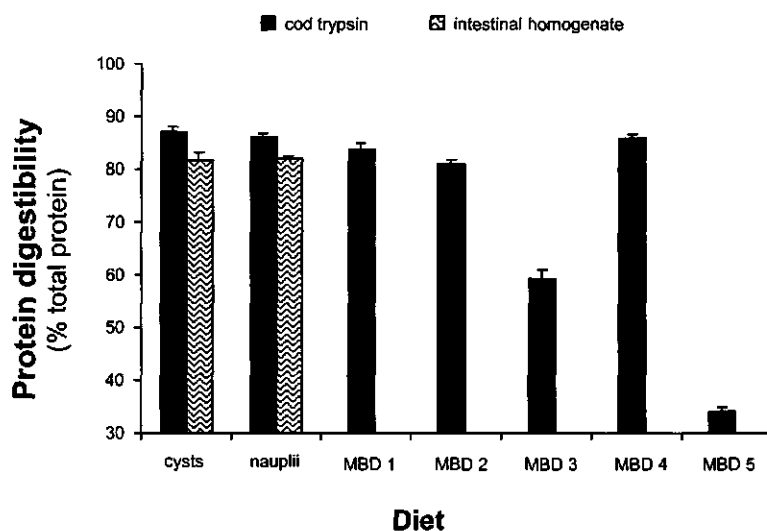
Figure 1. Protein digestibility *in vitro* of *Artemia* decapsulated cysts at different incubation times and increasing concentration of cod trypsin in the enzyme mix.

## Verification tests

In a third trial, the described method was used to evaluate the protein digestibility *in vitro* of microbound diets (MBDs) for larval fish made of *Artemia* decapsulated cysts. Five MBDs were prepared by mixing freeze-dried cysts with a binder (5% dw) according to the following scheme: MBD 1: ground cysts + carboxi-methylcellulose (CMC; 21900, Fluka Chemie, Switzerland); MBD 2: ground cysts + durabond (Provimi BV, The Netherlands); MBD 3: ground cysts + alginate (A-2033, Sigma-Aldrich); MBD 4: intact cysts + CMC; MBD 5: intact cysts + carrageenan (Type I, C-1013, Sigma-Aldrich). In MBD 5, heated water at 80°C was added during mixing. After mixing, the paste was milled, dried, ground and sieved to a particle size of 300-500 µm. Samples ( $\pm$  400 mg DW) of *Artemia* cysts and nauplii and MBDs were incubated during 4 h in a one-step procedure with the purified enzymes and cod trypsin at a concentration of 0.1 ml (4200 BAEE units ml<sup>-1</sup>). The protein digestibility of the MBDs was significantly different ( $P < 0.01$ ) among the diets (Fig. 2). The highest digestibility was found for MBD 1 and 4 which had CMC as binder. Interestingly MBD 5, which was made with a binder which requires water at 80°C during mixing with cysts, produced the lowest digestibility.

The high *in vitro* digestibility of *Artemia* cyst and nauplii partially explains their suitability as food for fish larvae. The MBDs made with cysts and CMC further indicates that the use of decapsulated cyst as feed ingredient in combination with a cold binder produces diets with high digestibility coefficients. In contrast, the low digestibility of MBD 5 in the present experiment can be attributed to the negative effect of strong heating on the protein quality of cysts (García-Ortega et al., in press). When using *Artemia* in digestibility experiments, high digestibility coefficients might be expected due to the capacity of zooplanktonic organisms for autolysis (Hjelmeland et al., 1993). Also, the processing of live food by freeze-drying might have an effect on the nutrient digestibility. Thus, further studies are required to differentiate between the digested protein by the *in vitro* method, the soluble protein and the digested protein product of the autolysis of *Artemia*.

The precision in the estimations of protein digestibility in trial 3 yielded a coefficient of variation of 1.4 %. The results of the analysis of the power of ANOVA (Sokal and Rohlf, 1995) produced a power of 99.9 %, which indicates a high reproducibility of the data. The proposed *in vitro* technique permit accurate estimations of the protein digestibility in food for larval fish and constitutes an alternative to feeding experiments with fish larvae where different protein sources or ingredients are tested.



**Figure 2.** Protein digestibility *in vitro* of *Artemia* decapsulated cysts and nauplii and microbound diets made of cysts. Two different sources of enzymes were tested in cysts and nauplii.

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## Chapter 6

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### **Evaluation of protein quality in microbound starter diets for fish larvae made with decapsulated cysts of *Artemia* and fishmeal as protein source**

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## Abstract

The protein quality in microbound diets (MBDs) made with decapsulated cysts of *Artemia* and/or fishmeal as protein source was used as indicator of their suitability as starter food for fish larvae. Studies on the amino acid composition, the *in vitro* protein digestibility, the diet solubility and protein structure were combined with an *in vivo* feeding experiment with African catfish larvae to obtain a more complete picture of the protein quality in the MBDs. The growth of catfish larvae was higher when fed *Artemia*-based MBDs than with fishmeal-based MBDs, despite the higher protein and amino acid content of the latter. The *in vitro* protein digestibility was high for all the MBDs in comparison to a commercial diet. Differences were found on the protein molecular weight among the diets. Most of the proteins in the fishmeal-based diets had low molecular weight in the range between 7.4 and 49.2 kDa. The *Artemia*-based MBDs had bigger protein fractions between 29.4 and 82 kDa. Decapsulated cysts improved the utilization of the MBDs when used in combination with fishmeal. Besides the effect of chemical attractants, the explanation to the positive effect of *Artemia* has yet to be elucidated. However, attention should be given to interactions between nutrients (e.g. protein-lipid) in live food which might have an effect on the functional properties of food proteins.

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**Keywords:** Feeding and nutrition-fish; *in vitro* protein digestibility, protein quality; *Artemia* cysts; fish larvae; SDS-PAGE; microbound diets



## Introduction

The first feeding of many fish larvae relies on the use of live food organisms (e.g. rotifers and *Artemia*). The numerous attempts to replace live food by artificial starter diets had limited success so far, i.e. the highest growth in larval fish is still obtained with live food (Jones et al., 1993; Watanabe and Kiron, 1994). One of the hypotheses that have been proposed to explain this suggests that because of the high content of free amino acids (FAA) in live food organisms (Dabrowski and Rusiecki, 1983; Fyhn et al., 1993), the supply of FAA and low molecular weight peptides in starter diets would facilitate their intestinal absorption by the fish larvae (Fyhn, 1989; Walford and Lam, 1993; Rønnestad et al., 1999). Similarly, it can be suggested that the structure of proteins in live food, which are easily digested by the fish larvae, might differ from that of the protein used in the manufacturing of artificial diets. Additional support for this hypothesis is found in the different growth results when fish larvae are fed with either decapsulated cysts or nauplii of *Artemia*. On individual weight basis, the decapsulated cysts and nauplii of *Artemia* have similar biochemical composition in all the major nutrients (García-Ortega et al., 1998). Thus, with regard to the amount of nutrients, there is no difference in feeding *Artemia* cysts or nauplii to fish larvae. However, in some species higher fish growth is achieved with nauplii. Protein is the major component of the dry matter in *Artemia*. Because the interaction of proteins with water has an effect on the functional properties of the protein (Damodaran, 1997), the protein structure might differ between cysts and nauplii due to the high water content in the latter.

The aim of the present study is to evaluate the quality of different protein sources in microbound diets (MBDs) for fish larvae and to relate the differences in performance to the protein structure. In addition, the suitability of decapsulated cysts of *Artemia* as protein source in starter diets for fish larvae is evaluated. MBDs were selected as experimental diet because they were used successfully in the larval rearing of African catfish (Pector et al., 1994), red sea bream and Japanese flounder (Kanazawa et al., 1989). Decapsulated cysts of *Artemia* were used as protein source in experimental MBDs for fish larvae because cysts have similar nutritional composition as nauplii (the control diet in the present study), they are well accepted by the fish larvae and have high digestibility coefficients (Pector et al., 1994; García-Ortega et al., 1998).

The quality of protein in fish feeds has been previously studied by diverse *in vitro* techniques (Grabner, 1985; Dimes and Haard, 1994), in combination with electrophoretic methods (Bassompierre et al., 1998; Alarcón et al., 1999). *In vitro* techniques permit simple,

fast and reproducible methods for the evaluation of nutrients digestibility (Boisen and Eggum, 1991). However, only a few studies utilized *in vitro* techniques for the determination of protein digestibility in larval fish feeds (Ozkizilcik and Chu, 1996; García-Ortega et al., 1998; Alarcón et al., 1999). None of the previous studies used *in vivo* growth experiments to test the quality of their larval diets. For a more complete picture of the protein quality in fish feeds, *in vivo* growth trials can be combined with studies on amino acid composition, the *in vitro* digestibility, diet solubility and studies on the protein structure. This combined approach was followed in our investigations.

## Materials and Methods

The present study is composed of two parts. In the first part a feeding experiment was done to test the growth response of African catfish (*Clarias gariepinus*) larvae to four experimental MBDs made of decapsulated cysts of *Artemia* or fishmeal as protein source, one commercial starter feed and *Artemia* nauplii. In the second part, a set of analyses to evaluate the quality of protein in the tested diets was done. The criteria to determine the protein quality in the diets were the proximate and amino acid composition, the *in vitro* digestibility and the analysis of the protein structure or molecular weight by electrophoresis.

### Diet preparation

Cysts of *Artemia* from Great Salt Lake, USA were decapsulated with a hypochlorite solution (Sorgeloos et al., 1986), dehydrated in brine ( $>330 \text{ g NaCl l}^{-1}$ ) during 24 h and stored in fresh brine at 4°C for one day until further processing. Decapsulated cysts of *Artemia* and fishmeal were used alone or in combination as protein source in the MBDs. Because cysts and fishmeal have other nutritional components besides protein, the formulation of the experimental MBDs was done to obtain a similar protein and lipid composition among the different diets (Table 1). Each MBD was prepared by mixing all the ingredients with carboxymethyl-cellulose CMC (10% dw) and water. An advantage of the use of CMC as binder is that the application of heat during diet manufacturing is avoided. This facilitates the use of *Artemia* as diet ingredient because processing food protein with heat might produce negative effects on the protein quality (Boye et al., 1997). After thorough mixing of the ingredients, the paste was milled and dried at 40°C, then ground and sieved to a particle size of 300-500  $\mu\text{m}$ . In MBDs 1 and 2, decapsulated cysts of *Artemia* were included as the only feed ingredient with CMC. Previous to preparation of MBD 2, the decapsulated cysts were

hydrated and then submitted to a heat treatment in water at 80°C during 5 min to reduce the protein quality (García-Ortega et al., 2000a). For MBD 3, approximately 48% of the protein originated from decapsulated cysts and 52% from fishmeal. In MBD 4 the total of the protein originated from fishmeal.

**Table 1.** Formulation and proximate composition (g 100g<sup>-1</sup> dw) of experimental microbound diets made with decapsulated cysts of *Artemia* and fishmeal as protein source, and proximate composition of a commercial starter diet and instar I nauplii of *Artemia*.

	Diet					
	MBD 1	MBD 2	MBD 3 <sup>*</sup>	MBD 4	Nippai	Nauplii
<b>Formulation</b>						
<i>Artemia</i> cysts <sup>1</sup>	90		47.2			
<i>Artemia</i> cysts heated at 80°C		90				
Fishmeal <sup>2</sup>			34	74.2		
Fish oil <sup>2</sup>			6.3	13.3		
Mineral and vitamin premix <sup>2</sup>			2.5	2.5		
CMC <sup>3</sup>	10	10	10	10		
<b>Proximate composition</b>						
Protein	46.4	52.6	52.7	56.4	60.6	60.6
Lipid	14.4	15.8	18.3	23.2	14.3	18.3
Ash	9.3	5.1	9.4	10.4	12.3	2.2
Dry matter	91.6	94.1	93.9	96.1	96.4	17.4

<sup>1</sup> INVE Aquaculture NV, Baasrode, Belgium. <sup>2</sup> Provimi, BV, Rotterdam, The Netherlands. <sup>3</sup> Carboxymethylcellulose Sodium salt, Fluka Chemie, Buchs, Switzerland.

\* Total protein in MBD 3 was approximately 48% from cysts (47 g protein in 100 g dw of cysts) and 52% from fishmeal (71 g protein in 100 g dw of fishmeal).

### Feeding experiment

Eggs and larvae of African catfish were obtained by artificial reproduction of controlled broodstock. Two days after hatching, the larvae were counted and transferred to a recirculation system with 17 l aquaria at a density of 66 larvae l<sup>-1</sup>. Fish larvae rearing was done at a constant temperature of 28°C and a light regime from 08:00 to 20:00. Four experimental MBDs, one commercial starter feed (Nippai, Japan), and live *Artemia* nauplii as control diet were tested in triplicate. The amount of food was calculated according to the predicted maximum growth (Verreth and Den Bieman, 1987), and the fish weight was determined daily for every diet treatment. Two days after hatching, once the yolk sac was completely absorbed, exogenous feeding was initiated. Feed was administered five times a day (every 4 h) from 08:00 to 23:00 for the total duration of the experiment, i.e. nine days.

### ***Diet quality parameters***

#### **Proximate composition and water stability**

The protein, lipid, ash, individual amino acid and fatty acids compositions of the four MBDs, the commercial feed and instar I nauplii of *Artemia* were determined as previously reported by García-Ortega et al. (1998). The dry matter of diets and fish larvae were estimated by drying the samples in an oven at 60°C during 24 h. The water stability of the MBDs was determined by exposing approximately 0.25 g of the feed to 50 ml distilled water in a rotating tube during 60 min. The dissolved matter was then separated by centrifugation at 2000 X g during 3 min and the dry weight was then determined. The protein content of the insoluble part after the water stability test was also determined.

#### **Protein digestibility *in vitro***

A multi-enzyme system to simulate the conditions in which food digestion occurs in fish larvae without a functional stomach (García-Ortega et al., submitted) was used to estimate the *in vitro* protein digestibility of larval fish diets. MBDs samples ( $\pm 400$  mg) and instar I nauplii of *Artemia* were incubated at 30°C and pH 8 during 4 h in a solution of purified enzymes. The enzyme mix consisted of cod (*Gadus morhua*) trypsin (T-9906, Sigma-Aldrich, Zwijndrecht, The Netherlands), chymotrypsin from bovine pancreas (C-4129, Sigma), leucine-aminopeptidase from porcine kidney (L-1503, Sigma),  $\alpha$ -amylase (Termamyl 120L, Novo Nordisk, Denmark), lipase from wheat germ (L-3001, Sigma) and bile salts (B-8756, Sigma) dissolved in a solution of  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ . To stabilize the enzymes in the solution,  $\text{CaCl}_2$  and  $\text{MgCl}_2$  with  $(\text{NH}_4)_2\text{SO}_4$  were added. After incubation in the multi-enzyme solution, the digested fraction was vacuum-filtrated and the undigested protein fraction was determined by the Kjeldahl method. The protein digestibility *in vitro* was estimated from the original nitrogen content of the MBD and the undigested fraction after incubation.

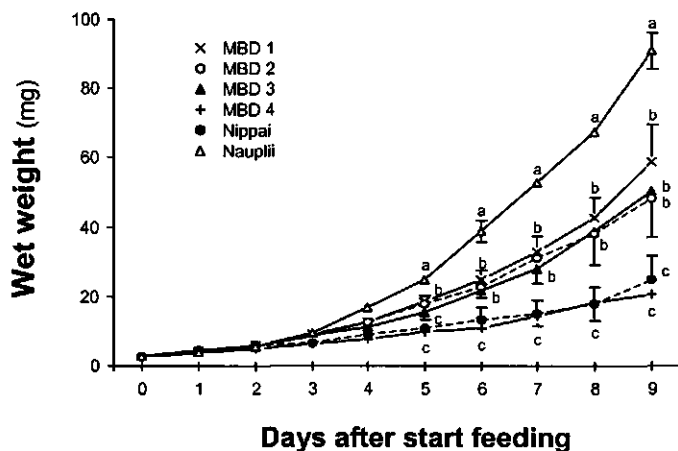
#### **SDS-PAGE and molecular weight determination**

Protein in the diet samples and instar I nauplii of *Artemia* was extracted with 6 M urea in a phosphate buffer saline (PBS, 0.9%) at pH 7.3 and subsequently ground for 2 min. The mixture was centrifuged for 3 min at 2000 rpm and the supernatant collected and diluted with distilled water (1:1). The sample was heated at 100°C for 3 min in the presence of 2-mercaptoethanol sodium dodecyl sulfate (SDS) (1:3) to dissociate the proteins into their component polypeptides. Separation of proteins in the diets was done by SDS-polycrarnide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli (1970). A running gel containing 10% polycrarnide and a stacking gel containing 4% polycrarnide were used.

The protein standards (broad range 161-0318, Bio-Rad Laboratories, CA, USA) were myosin (200 kiloDaltons),  $\beta$ -galactosidase (113 kDa), bovine serum albumin (82 kDa), ovalbumin (49.2 kDa), carbonic anhydrase (34.8 kDa), soybean trypsin inhibitor (29.4 kDa), lysozyme (20.9 kDa) and aprotinin (7.4 kDa). The protein in samples and the protein standards were run in a Bio-Rad Mini-Protean II cell in two programs, with 80 V applied during 40 min and 120 V during 90 min respectively. The gel was stained with Coomassie Brilliant Blue (R-250, Sigma) in a solution of methanol, acetic acid and water (5:1:5). Destaining was done with a solution of methanol, acetic acid and water (3:1:10). The molecular weight estimation of the proteins in the gel was done with a laser densitometer (UltroScan XL, Pharmacia LKB Biotechnology, Uppsala, Sweden) and the Gel Scan XL software (Pharmacia LKB Biotechnology). After scanning the gel, the equation of Ferguson (1964) was used to determine the relative mobility ( $R_f$ ) of the polypeptides in the acrylamide. By plotting the logarithmic  $R_f$  values from the standard proteins against the known acrylamide concentration, straight lines and slopes of  $K_R$  (retardation coefficient) were obtained. The  $K_R$  values were plotted against the molecular weight of the standard proteins. A linear relationship was obtained which was used to estimate the molecular weight of unknown proteins. The distribution of the protein polypeptides from the diets among the molecular weight of each standard protein is expressed as percentage of the relative area found for each band in the gel.

### ***Data analysis***

Three samples (each sample  $n=20$ ) per diet treatment were used to determine the fish weight. The individual amino acid content was determined with four samples per diet, with exception of cystine, methionine, tyrosine, histidine and phenylalanine, which were determined with two samples. Fatty acids and diet solubility were determined in triplicate for each diet, *in vitro* protein digestibility was determined in duplicate. All data were tested for normal distribution and homogeneity of variance before ANOVA was done. To detect diet effects ( $P<0.05$ ) on fish weight, a one-way ANOVA was performed. It was also used to detect differences in the diets amino acid and fatty acid contents, in the diets solubility and on their protein digestibility. When significant effects were found, the means were compared with Duncan multiple range test and least squares means for equal and unequal sample size respectively. Statistical analyses were performed with SAS 6.12 (SAS Institute Inc., NC, USA).



**Fig. 1.** Daily growth of *Clarias gariepinus* larvae fed four experimental microbound diets (MBD), one commercial starter diet, and nauplii of *Artemia* during the first days of exogenous feeding. Means in the same day with different letter are significantly different ( $P < 0.05$ ).

## Results

### *Fish growth*

During the first 3 days after the start of exogenous feeding the catfish larvae showed similar growth in all the dietary treatments (Fig. 1). From that day onwards, the larval growth differed significantly ( $P < 0.05$ ) among the different diet treatments. Three groups of treatments can be recognized according to the larval growth (Fig. 1). The highest fish growth (90.9 mg) was achieved with live *Artemia* nauplii. The larvae fed with MBDs 1, 2 and 3, which were made with decapsulated cysts of *Artemia* as total or partial protein source, achieved lower growth (58.9, 50.05 and 48.4 mg respectively) than nauplii. However, their growth was significantly higher than the fish larvae fed MBD 4 (20.5 mg), which was made with fishmeal as sole protein source, and also higher than the larvae fed the commercial diet (24.8 mg). In general, the MBDs made with decapsulated cysts of *Artemia* were well accepted by the catfish larvae. The transparent gastrointestinal tract of the larvae allowed visual verification of the gut fullness after every feeding. All the feed particles were ingested 30 min after each meal. The fish ingested most of the particles from MBD 4 and the commercial feed. However, some of those food particles were not eaten.

## Diet quality

### Proximate composition

The protein, lipid and ash contents of the MBDs and *Artemia* instar I nauplii are presented in Table 1. More protein was contained in the nauplii, in the MBDs with fishmeal as protein source and in the commercial diet. The amino acid composition had small variations among the diets (Table 2). No significant differences ( $P>0.05$ ) were found for cystine and histidine. Notoriously, MBD 4 and the commercial diet contained more total amino acids. However, a higher total amino acid content did not yield higher fish growth. In the *Artemia*-based MBDs, the difference of the protein content and total amino acids between MBD 1 and MBD 2 might be related to the strong heating of the cysts which were used in the latter.

**Table 2.** Amino acid composition (g 100 g<sup>-1</sup> protein) of experimental microbound diets made with decapsulated cysts of *Artemia* and fishmeal as protein source, a commercial starter diet, and instar I nauplii of *Artemia*.

Amino acid	Diet					
	MBD 1	MBD 2	MBD 3	MBD 4	Nippal	Nauplii
Aspartic acid	7.3 <sup>d</sup> ± 0.2	7.9 <sup>c</sup> ± 0.1	8.4 <sup>b</sup> ± 0.1	9.8 <sup>a</sup> ± 0.4	9.6 <sup>a</sup> ± 0.5	8.2 <sup>bc</sup> ± 0.2
Threonine	4.2 <sup>c</sup> ± 0.1	4.5 <sup>b</sup> ± 0.0	4.6 <sup>b</sup> ± 0.0	4.8 <sup>a</sup> ± 0.0	4.5 <sup>b</sup> ± 0.1	3.9 <sup>d</sup> ± 0.1
Serine	5.4 <sup>b</sup> ± 0.2	5.7 <sup>a</sup> ± 0.1	5.1 <sup>c</sup> ± 0.1	4.8 <sup>d</sup> ± 0.1	4.5 <sup>e</sup> ± 0.1	5.2 <sup>bc</sup> ± 0.2
Glutamic acid	11.1 <sup>c</sup> ± 0.6	11.5 <sup>c</sup> ± 0.3	12.3 <sup>b</sup> ± 0.2	13.3 <sup>a</sup> ± 0.4	12.9 <sup>ab</sup> ± 0.6	9.8 <sup>d</sup> ± 0.5
Proline	3.9 <sup>b</sup> ± 0.1	4.2 <sup>b</sup> ± 0.0	4.0 <sup>b</sup> ± 0.0	4.2 <sup>b</sup> ± 0.3	4.2 <sup>b</sup> ± 0.4	4.9 <sup>a</sup> ± 0.2
Glycine	3.9 <sup>a</sup> ± 0.1	4.1 <sup>d</sup> ± 0.1	5.0 <sup>c</sup> ± 0.1	6.0 <sup>a</sup> ± 0.1	5.7 <sup>b</sup> ± 0.2	4.3 <sup>d</sup> ± 0.1
Alanine	4.0 <sup>j</sup> ± 0.1	4.2 <sup>a</sup> ± 0.0	5.2 <sup>d</sup> ± 0.1	6.2 <sup>b</sup> ± 0.1	5.8 <sup>c</sup> ± 0.1	6.4 <sup>a</sup> ± 0.2
Cystine	1.2 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.0	1.2 ± 0.0
Valine	4.9 <sup>d</sup> ± 0.1	5.3 <sup>bc</sup> ± 0.1	5.4 <sup>abc</sup> ± 0.2	5.6 <sup>a</sup> ± 0.1	5.2 <sup>c</sup> ± 0.1	5.4 <sup>ab</sup> ± 0.2
Methionine	2.4 <sup>c</sup> ± 0.2	2.6 <sup>bc</sup> ± 0.1	2.8 <sup>ab</sup> ± 0.2	3.2 <sup>a</sup> ± 0.2	2.9 <sup>ab</sup> ± 0.1	2.3 <sup>c</sup> ± 0.0
Isoleucine	4.4 <sup>c</sup> ± 0.1	4.7 <sup>ab</sup> ± 0.1	4.6 <sup>bc</sup> ± 0.1	4.8 <sup>a</sup> ± 0.2	4.7 <sup>ab</sup> ± 0.3	4.6 <sup>bc</sup> ± 0.1
Leucine	6.3 <sup>a</sup> ± 0.2	6.8 <sup>d</sup> ± 0.1	7.1 <sup>c</sup> ± 0.1	7.9 <sup>a</sup> ± 0.1	7.4 <sup>b</sup> ± 0.4	6.3 <sup>a</sup> ± 0.1
Tyrosine	3.2 <sup>b</sup> ± 0.1	3.5 <sup>b</sup> ± 0.1	3.3 <sup>b</sup> ± 0.0	3.6 <sup>b</sup> ± 0.4	3.3 <sup>b</sup> ± 0.5	4.9 <sup>a</sup> ± 0.1
Phenylalanine	3.7 <sup>b</sup> ± 0.1	4.0 <sup>ab</sup> ± 0.0	4.0 <sup>ab</sup> ± 0.0	4.3 <sup>a</sup> ± 0.3	4.0 <sup>ab</sup> ± 0.3	3.7 <sup>b</sup> ± 0.1
Histidine	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.6 ± 0.5	2.5 ± 0.4	2.8 ± 0.0
Lysine	6.8 <sup>d</sup> ± 0.2	7.2 <sup>c</sup> ± 0.1	7.7 <sup>b</sup> ± 0.0	8.4 <sup>a</sup> ± 0.1	7.8 <sup>b</sup> ± 0.0	7.2 <sup>c</sup> ± 0.2
Arginine	6.3 <sup>b</sup> ± 0.2	6.7 <sup>a</sup> ± 0.1	6.2 <sup>bc</sup> ± 0.1	6.0 <sup>c</sup> ± 0.4	6.0 <sup>c</sup> ± 0.4	5.4 <sup>d</sup> ± 0.1
Total amino acids	81.0 <sup>d</sup> ± 2.0	86.4 <sup>c</sup> ± 1.0	88.9 <sup>bc</sup> ± 0.8	96.2 <sup>a</sup> ± 2.6	91.6 <sup>b</sup> ± 3.3	86.6 <sup>c</sup> ± 2.1

Means in the same row with the same superscript are not significantly different ( $P>0.05$ ).

Means and standard deviation values were rounded to decimals

Heating at 80°C has detrimental effects on the protein quality of *Artemia* cysts (Garcia-Ortega et al., in press). Strong heating also reduces the amount of non-protein nutrients in the cysts, resulting in elevated levels of protein and total amino acids in MBD 2 when compared to MBD 1 (Tables 1 and 2). The lipid content was higher in MBD 3 and MBD 4 than in the other diets, probably due to the inclusion of fish oil in their formulation (Table 1). Similarly, the levels of individual fatty acids differed significantly ( $P < 0.05$ ) between diets (Table 3). In general, MBD 3 and 4 had significantly higher content for most fatty acids with exception of the C-18 group.

**Table 3.** Fatty acid composition (mg g<sup>-1</sup> dw) of experimental microbound diets made with decapsulated cysts of *Artemia* and fishmeal as protein source, a commercial starter diet, and instar I nauplii of *Artemia*.

Fatty acid	Diet					
	MBD 1	MBD 2	MBD 3	MBD 4	Nippai	Nauplii
14:0	1.4 <sup>d</sup> ± 0.2	1.4 <sup>d</sup> ± 0.0	6.3 <sup>c</sup> ± 0.2	10.9 <sup>a</sup> ± 0.1	10.2 <sup>b</sup> ± 0.4	1.4 <sup>d</sup> ± 0.1
16:0	13.8 <sup>a</sup> ± 1.0	14.9 <sup>d</sup> ± 0.1	21.9 <sup>c</sup> ± 0.8	28.3 <sup>a</sup> ± 0.1	23.7 <sup>b</sup> ± 0.6	15.5 <sup>d</sup> ± 0.2
16:1 n-7	4.4 <sup>a</sup> ± 0.4	4.7 <sup>ab</sup> ± 0.1	7.7 <sup>b</sup> ± 0.3	10.6 <sup>a</sup> ± 0.1	6.0 <sup>c</sup> ± 0.2	5.0 <sup>d</sup> ± 0.1
18:0	5.4 <sup>c</sup> ± 0.3	6.1 <sup>b</sup> ± 0.1	5.0 <sup>d</sup> ± 0.2	4.2 <sup>e</sup> ± 0.1	2.9 <sup>f</sup> ± 0.2	6.6 <sup>a</sup> ± 0.1
18:1 n-9	21.5 <sup>b</sup> ± 1.3	24.4 <sup>a</sup> ± 0.3	18.2 <sup>c</sup> ± 0.7	12.6 <sup>d</sup> ± 0.1	11.8 <sup>d</sup> ± 0.2	25.1 <sup>a</sup> ± 0.2
18:1 n-7	7.4 <sup>c</sup> ± 0.5	8.4 <sup>b</sup> ± 0.1	5.8 <sup>d</sup> ± 0.2	3.1 <sup>e</sup> ± 0.2	5.8 <sup>d</sup> ± 0.4	9.8 <sup>a</sup> ± 0.1
18:2 n-6-t	0.2 <sup>b</sup> ± 0.1	0.3 <sup>a</sup> ± 0.0	0.2 <sup>b</sup> ± 0.0	0.0 <sup>d</sup> ± 0.0	0.1 <sup>c</sup> ± 0.0	0.3 <sup>a</sup> ± 0.0
18:2 n-6-c	6.3 <sup>b</sup> ± 0.4	7.0 <sup>a</sup> ± 0.1	5.4 <sup>c</sup> ± 0.2	3.7 <sup>e</sup> ± 0.0	4.6 <sup>d</sup> ± 0.5	7.4 <sup>a</sup> ± 0.1
18:3 n-3	34.1 <sup>c</sup> ± 2.0	37.3 <sup>b</sup> ± 0.3	20.4 <sup>d</sup> ± 0.9	2.7 <sup>e</sup> ± 0.2	2.0 <sup>e</sup> ± 0.4	41.2 <sup>a</sup> ± 0.6
18:4 n-3	5.7 <sup>c</sup> ± 0.4	6.1 <sup>b</sup> ± 0.0	6.5 <sup>a</sup> ± 0.3	6.8 <sup>a</sup> ± 0.0	2.3 <sup>d</sup> ± 0.1	6.5 <sup>a</sup> ± 0.1
20:1 n-9	0.4 <sup>a</sup> ± 0.1	0.5 <sup>ab</sup> ± 0.0	4.7 <sup>b</sup> ± 0.1	9.3 <sup>a</sup> ± 0.0	1.2 <sup>c</sup> ± 0.1	0.6 <sup>d</sup> ± 0.0
20:4 n-6	1.5 <sup>c</sup> ± 0.1	1.7 <sup>b</sup> ± 0.1	1.3 <sup>d</sup> ± 0.1	0.8 <sup>e</sup> ± 0.0	0.6 <sup>f</sup> ± 0.0	2.0 <sup>a</sup> ± 0.0
20:4 n-3	0.9 <sup>b</sup> ± 0.1	1.0 <sup>a</sup> ± 0.1	1.1 <sup>a</sup> ± 0.1	1.1 <sup>a</sup> ± 0.0	0.3 <sup>c</sup> ± 0.1	1.1 <sup>a</sup> ± 0.0
20:5 n-3	5.2 <sup>a</sup> ± 0.2	5.7 <sup>d</sup> ± 0.1	13.2 <sup>b</sup> ± 0.4	21.1 <sup>a</sup> ± 0.0	12.8 <sup>b</sup> ± 0.3	6.8 <sup>c</sup> ± 0.1
21:5 n-3	0.1 <sup>a</sup> ± 0.0	tr	0.4 <sup>b</sup> ± 0.0	0.8 <sup>a</sup> ± 0.0	0.3 <sup>c</sup> ± 0.0	0.2 <sup>d</sup> ± 0.0
22:6 n-3	0.2 <sup>d</sup> ± 0.1	0.2 <sup>d</sup> ± 0.0	11.6 <sup>c</sup> ± 0.4	23.7 <sup>a</sup> ± 0.2	12.4 <sup>b</sup> ± 0.3	0.3 <sup>d</sup> ± 0.0
Σ n-3 > or = 20:3 n-3	7.3	8.0	27.6	48.4	26.9	9.4
Σ n-6 > or = 18:2 n-6-t	8.9	9.9	7.5	5.1	5.6	10.6
Total FAME	123.6 <sup>d</sup> ± 6.4	136.2 <sup>c</sup> ± 0.8	151.2 <sup>b</sup> ± 5.4	166.8 <sup>a</sup> ± 0.4	106.6 <sup>e</sup> ± 2.6	147.3 <sup>b</sup> ± 1.5

Means in the same row with the same superscript are not significantly different ( $P > 0.05$ ).

Means and standard deviation values were rounded to decimals

tr = Trace



C-18 fatty acids, especially the linolenic acid (18:3(n-3)) was substantially higher in the diets containing *Artemia* cysts and in the nauplii than in the other diets. In MBD 1, 2 and 3, and in nauplii the 18:3(n-3) represented more than 20% of the total FAME (Table 3). 20:5 n-3 (EPA) and 22:6 n-3 (DHA) were significantly higher in MBD 3 and 4, and in the commercial diet.

### Water stability and protein digestibility

The water stability was significantly different among the diets (Fig. 2a). The commercial diet was significantly more water stable than the MBDs. However, in the case of the commercial diet, high water stability was inversely related to the diet digestibility as shown by its lower *in vitro* protein digestibility (Fig 2b).

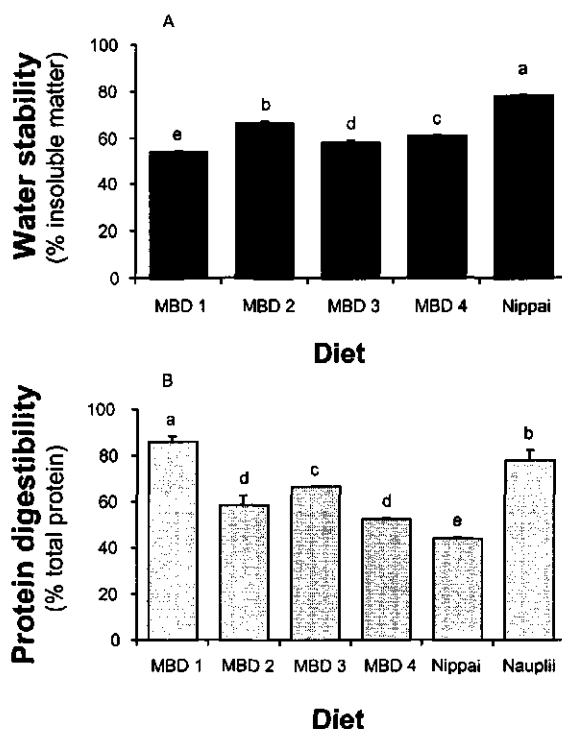
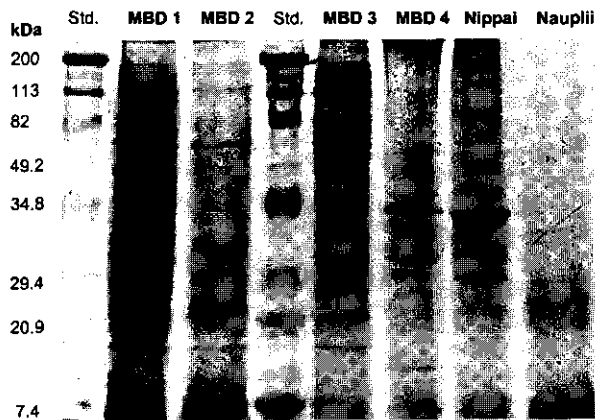


Fig. 2. Diet water stability (A) and protein digestibility *in vitro* (B) of four experimental microbound diets (MBD) and a commercial starter diet. The protein digestibility of instar I nauplii of *Artemia* was also determined. Bars with different letter are significantly different ( $P < 0.05$ ).

Although the water stability was lower in all MBDs compared to the commercial diet, leaching of protein into the water column was limited. The protein contents in the insoluble part after the water stability test for each diet were (in g 100 g<sup>-1</sup> dw): MBD 1 = 64.7; MBD 2 = 66.2; MBD 3 = 65.7; MBD 4 = 69.8; Nippai = 64.2. These protein values are higher than the original ones before the diet solubility test (Table 1). This indicates that there is a reduced leaching of protein in the water and that other diet components were dissolved in the water. The *in vitro* protein digestibility was significantly different ( $P < 0.05$ ) among the diets (Fig 2b). Higher protein digestibility was obtained with MBD 1 (86 %) and the nauplii (78 %), and the lowest protein digestibility was observed for the commercial diet (44 %). MBD 2, 3 and 4 presented intermediate digestibility values between the highest and the lowest, with 59, 66 and 53 % respectively.

### Molecular weight

The protein in the artificial diets, as analyzed by SDS-PAGE, showed different patterns between the *Artemia* cyst-based MBDs, the MBDs made with fishmeal as protein source, the commercial diet and *Artemia* nauplii (Fig. 3). The relative amounts of the different size fractions in the dietary protein are presented in Table 4. In the MBDs made with *Artemia* cysts most of the protein had a molecular weight between 29.4 and 82 kDa. The fishmeal-based MBDs, the commercial diet and the *Artemia* nauplii contained mainly small sized proteins between 7.4 and 49.2 kDa. In the case of the nauplii, almost half of the protein consisted of low molecular weight proteins between 7.4 and 20.9 kDa. Heating of cysts at 80°C prior to diet preparation apparently increased the low molecular weight protein fraction of MBD 2 compared to the MBDs made with non heated decapsulated cysts. However, in both diets the profile of the different protein fractions was very similar (Table 4). In MBD 3 containing 48% cysts and 52% fishmeal protein, the molecular weight profile of the proteins was more related to the profile of the cysts MBDs than to the fishmeal MBDs. The similarity of the protein bands in the gel (Fig. 3) and the protein distribution (Table 4) between the MBD 4 and the commercial diet suggest a strong similarity in the protein source used in the formulation of both diets.



**Fig. 3.** SDS-PAGE analysis of the protein pattern of four experimental microbound diets (MBD) made with *Artemia* decapsulated cysts and fishmeal as protein source, one commercial starter diet and instar I nauplii of *Artemia*. Std.= standard

**Table 4.** Molecular weight (MW) of protein fractions extracted from microbound diets made of decapsulated cysts of *Artemia* and fishmeal as protein source, a commercial starter diet, and Instar I nauplii of *Artemia*. The results are expressed as percentage of the total protein in the gel for each diet.

MW of protein standards* (kDa)	Diet					
	MBD 1	MBD 2	MBD 3	MBD 4	Nippai	Nauplii
113.0 – 200.0	3.7	1.3	13.9	0	0	0
82.0 – 113.0	14.4	8.9	22.4	4.6	15.8	0
49.2 – 89.0	28.2	15.9	30.7	30.3	27.7	10.6
34.8 – 49.2	31.3	28.2	24.1	20.7	21.9	9.6
29.4 – 34.8	3.8	36.7	6.1	3.3	5.6	7.8
20.9 – 29.4	11.4	9.1	1.4	2.8	1.7	23.6
7.4 – 20.9	7.3	0	1.5	38.3	27.4	48.7

\*SDS-PAGE standards, broad range 161-0316, Bio-Rad Laboratories, CA, USA.

## Discussion

The growth of *C. gariepinus* larvae in the present study was higher than that obtained in previous feeding tests with MBDs made of *Artemia* decapsulated cysts (Pector et al., 1994). Because the digestibility of the artificial diets might be lower than that of live food, in feeding experiments with fish larvae, the frequency of feeding should ensure enough time between meals for digestion of food. In the case of feeding MBDs made of *Artemia* cysts, a period of at least 4 h between meals is needed to allow a good digestion and absorption of nutrients (García-Ortega et al., 2000b). Despite the improvements in diet manufacturing techniques and nutrient quality, the highest growth in catfish larvae is still achieved with live *Artemia* nauplii. The use of CMC-MBDs as starter diet for catfish larvae allowed higher fish growth and protein digestibility than the commercial diet. Other forms of feed manufacturing than the microbound technique might have a negative effect on the protein quality due to severe conditions at which the feed components are processed (e.g. high temperatures and cooking pressures). CMC proved to be a good binder in the preparation of microbound diets that include decapsulated cysts of *Artemia* in their formulation. It allowed for a good water stability and high digestibility of the diet. Other binders (e.g. carrageenan) could reduce the digestibility of the protein and consequently limit their suitability as feed for fish larvae (Gawlicka et al., 1996). In the present study it was assumed that the fish acquired most of the protein in the diets. MBDs made with decapsulated cysts of *Artemia* as protein source were well accepted by catfish larvae and yielded higher growth and survival than fishmeal-based diets. The particle size was suitable for their ingestion and the insoluble matter was high enough to ensure that the fish larvae ingest all the protein contained in the diets. In a previous study with African catfish larvae (Pector et al., 1994) it was concluded that the differences in growth among fish fed MBDs was related to the differences in insoluble matter of the diets. However, in the present study the insoluble matter of the diets was similar among the experimental MBDs, and in the case of the commercial diet, it was even higher. Still, the commercial diet yielded the lowest larval growth. Thus, in the present study the low fish growth cannot be attributed to the water stability of the diet, which would be in any case a technological deficiency. Instead, it is assumed that the differences in growth and survival are mainly related to the nutritional quality and digestibility of the diets.

Marine fish larvae depend on the dietary supply of n-3 fatty acids to meet their requirement for essential fatty acids (Sargent et al., 1997). In contrast, it has been shown that freshwater fish larvae are able to survive and grow well with low levels of n-3 fatty acids in their diet (Radünz-Neto, et al., 1994). In the present study, the used nauplii and cysts of

*Artemia* were deficient in n-3 long-chain fatty acids (e.g. EPA and DHA). However, they contained a relatively high amount of linolenic acid (18:3n-3) which can be converted to EPA and DHA by freshwater fish larvae (Kanazawa, 1985). In MBD 4, the supply of essential fatty acids was covered by a high supply of EPA and DHA from the fish oil and fishmeal. Therefore, the differences in growth of catfish larvae can not be attributed to a deficiency of essential fatty acids in the diets. On the other hand, in freshwater fish high levels of EPA and DHA might cause too high levels of free radicals in freshwater fish, which could have negative effects on the growth of fish larvae. This might also contributed for the low larval growth obtained with MBDs 3 and 4, which had the higher content of EPA and DHA. With regard to the amino acids, high amino acid contents in MBD 4 and the commercial diet did not yield higher fish growth. Similarly, the higher amount of proteins with low molecular weight in those two diets did not ensure higher fish growth. This could be the result of a combined effect, from a reduced food intake due to the attractiveness of the diets, and from the quality of the diet protein. In the case of *Artemia* nauplii, which showed a high amount of low molecular weight proteins, the fish growth was the highest. This can be partially explained by the effect of visual and chemical stimuli from *Artemia* to the fish (Kolkovski et al., 1997). Furthermore, the quantity of protein in the diets apparently had no important effect on the catfish growth.

The structure of the protein molecules might have affected also the nutritional quality of *Artemia* cysts and nauplii. Although both have a similar nutritional composition, the nauplii might have a protein structure that differs from that in the cysts. *Artemia* nauplii have a high water content and this might have an effect on the molecular arrangement of the protein. The sampled protein from nauplii was composed by small peptides of molecular weight smaller than 89 kDa, with most of it under 29.4 kDa. In the case of the cysts-based MBDs, the majority of the protein had a higher molecular weight between 34.8 to 113 kDa in MBDs 1 and 3, and 29.4 to 89 in MBD 2. Fish larvae grew equally well on those three diets. Heating the cysts at 80°C reduced the *in vitro* protein digestibility of MBD 2 (from 86 % in MBD 1 down to 59 % in MBD 2). Yet, fish larvae receiving MBD 2 grew still better than the larvae receiving fishmeal-based MBD. The growth results with *Artemia* nauplii are consistent with the hypothesis that the use of low molecular weight proteins or free amino acids in artificial diets improves their utilization by the fish larvae (Fyhn, 1989; Walford and Lam, 1993). Moreover, it also has been suggested that the incorporation of small peptides or protein hydrolysates in the diets improved the developmental processes in the digestive tract of fish larvae (Cahu and Zambonino Infante, 1995; Cahu et al., 1999).

Decapsulated cysts improved the acceptability and digestibility of a dry artificial diet. In the present study *Artemia* cysts were used in combination with fishmeal (MBD 3). With this diet, larval growth was improved compared to the treatments where fishmeal was used as the sole protein source. Apparently minor amounts of cysts in the diet (50 % of the total protein) are enough to achieve this positive effect. The explanation for this positive effect has yet to be elucidated, but some factors can be mentioned. The cysts of *Artemia* might provide substances that might work as chemical attractants. This could be verified by assessing feed consumption in diets with varying concentrations of cysts as protein source according to the method described earlier (García-Ortega et al., submitted). Another explanation might be related to the effect of food processing on the protein-lipid complexes in the diet. Chobert and Hartlé (1997) stated that, while protein-lipid interactions are responsible for the functionality of the proteins, the structural complexes differ between those found in biological systems and those formed during food processing. Moreover, Rudloff and Lonnerdal (1992) demonstrated that the digestibility of dietary protein is reduced by the protein-lipid interactions after food processing. This is an aspect to consider further in the development of artificial diets for fish larvae. The *Artemia* embryos contained in the decapsulated cysts used represent a biological system in contrast to the fishmeal, which was used as an alternative protein ingredient. This difference might have affected the results. Thus, when developing artificial diets for fish larvae, the interactions between nutrients should be also studied in parallel to the dietary protein quality.

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## **General Discussion**

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## Introduction

The most critical period in fish larviculture is the start of exogenous feeding, after absorption of the yolk sac. For the larvae of fish species which have no functional stomach at the onset of exogenous feeding, live food is required for optimal growth. When instead, artificial diets are provided at first feeding, the growth and survival in these fish species are much lower than when *Artemia* is used (Jones et al., 1993; Watanabe and Kiron, 1994). Therefore, a key question in larval rearing is why live food is better than artificial diets. Three main hypotheses have been proposed to explain this: 1) differences in food intake induced by visual and chemical stimuli; 2) enzymes present in the live prey organisms contribute to the digestion of prey when ingested by the fish larvae; 3) differences in digestibility between live and artificial diets which are usually attributed to differences in protein digestibility. In this thesis the latter two aspects were considered. Possible bias from the first aspect was avoided by using a food source (e.g. *Artemia* cysts) that is well accepted by the fish larvae. Research focused on the nutritional composition of larval food and on the aspects related to food digestion and absorption. Hence, the main objectives of this thesis were to make a nutritional characterization of *Artemia*, to study how significant is the contribution of exogenous (dietary) enzymes to the digestion of food by fish larvae, and to study the importance of the protein structure in food for their digestion. The results of these studies can contribute to the development of experimental and practical microdiets which allow high food ingestion and digestion rates in fish larvae. The research in this thesis was divided in three phases. In the first phase, the nutritional composition of *Artemia* during the development from cyst to nauplii was analysed with emphasis on the fatty acids and amino acid composition. During the second phase of the research, we tested the hypothesis that dietary enzymes do not play a significant role in the digestion of food by fish larvae. Then, in a third phase of the research, the role of the structure of dietary protein for digestion and absorption received more attention.

## Nutritional composition of *Artemia* during development from cyst to nauplii

The most important live food organisms used in fish culture are the rotifer *Brachionus plicatilis* and the brine shrimp *Artemia* spp. Several studies have been dedicated to determine their nutritional value and to evaluate their use as food for fish larvae (Watanabe et al., 1983; Léger et al., 1986; Hagiwara et al., 1997). Despite the suitability of *Artemia* as starter diet for fish larvae, the nutritional quality among different batches of *Artemia* might vary considerably

(Léger et al., 1986). This high variation might even occur between *Artemia* batches from the same origin. In addition, in some cases the nutritional value of *Artemia* is not optimal and does not meet the nutritional requirements of some fish larvae (Watanabe et al., 1983). Therefore, in nutritional studies with fish larvae, it is important to determine the biochemical composition of the selected organisms used as live food. For the present research, this analysis is provided in Chapter 1. The nutritional composition of *Artemia* has been widely studied (see review by Léger et al., 1986). However, those studies focused mainly on the instar I nauplii, and on the macro-nutrients and fatty acids composition. Despite that the main component in the dry matter of *Artemia* is protein, the available information on the protein and amino acid composition in *Artemia* during development from cyst to nauplii is scarce.

Decapsulated cysts and live instar I nauplii of *Artemia* are used in the larviculture of several fish species. The use of decapsulated cysts of *Artemia* as food for the larvae of *C. gariepinus* has been successfully tested (Verreth et al., 1987; Pector et al., 1994). However, the fish growth achieved when cysts are fed to catfish larvae is significantly lower than the one obtained with live *Artemia* nauplii (Verreth et al., 1987). *Artemia* cysts consist of embryos that resume their reversibly interrupted metabolism upon hydration in saltwater at optimal temperature and light conditions (Sorgeloos et al., 1986). After hydration and decapsulation, the cysts containing the developing embryos provide a food item that might have a different biochemical and nutritional composition as compared to hatched nauplii. This might be due in part to the effect of the techniques of decapsulation and storage of cysts, in addition to the quality of hatching. Moreover, due to the differences in hatching time among *Artemia* cysts, a suspension of harvested nauplii might contain different stages, from non-hatched cysts, to "umbrella" stages, to live instar I nauplii. Those factors might explain the differences in growth as observed in catfish larvae when cysts and nauplii are fed. In Chapter 1, the nutritional quality of the different stages of *Artemia* from cysts to hatched nauplii was investigated. The results indicate that the individual protein and fat content decreased during development from cysts to nauplii. However differences in individual proximate composition were not statistically significant. Most nutritional constituents were used during the embryonal development, probably for energy purposes. To determine the nutritional quality of *Artemia* it is also important to study changes in proximate composition on an individual weight basis and not exclusively in relative terms (% dry weight). In general, decapsulated cysts constitute a food with a consistent biochemical composition. With regard to the amino acid and fatty acid composition of *Artemia*, it was concluded that the small changes observed during the development of *Artemia* cysts and early nauplii are not important for its nutritional value as food for fish larvae. The total amino acid content of cyst samples

and nauplii was not significantly different, and the total fatty acid content of nauplii was lower than in cysts. However, *Artemia* nauplii yielded higher growth than cysts when fed to catfish larvae as reported in Chapter 2. This suggests that the quantity of nutrients in cysts and nauplii is not the determining factor to explain the difference in fish growth when cysts and nauplii are compared as food for fish larvae. Probably, besides the quantity also the quality of nutrients plays an important role.

### ***The live food factor***

Beside the major nutrients in live food organisms, attention was paid to minor nutritional components to explain the poor growth obtained when artificial starter diets are used. It was proposed that a water insoluble substance or growth factor extracted from *Artemia* nauplii is essential for the metamorphosis of whitefish *Coregonus lavaretus* larvae (Flüchter 1982). However, this species has been successfully reared with starter diets without any live food component (Dabrowski et al., 1984). When tested for catfish larvae, the use of the same extracts from *Artemia* in a microdiet produced lower growth results than with decapsulated cysts (Verreth et al., 1987). These studies questioned the *Artemia* growth factors found by Flüchter, thus, directing the attention to other dietary factors, which might have a bigger relevance in the nutrition of fish larvae. Those include dietary components that inhibit or stimulate the action of hormones essential for the metamorphosis of fish (Lam 1980; Inui et al., 1989). Studying the role of micro-nutrients might help to understand why live food is a suitable starter diet for fish larvae in comparison to artificial diets. In addition, important information might be obtained from the physiological processes involved in the larval digestion. In the present study this information is provided in Chapters 3 and 4.

### **Proteolytic enzymes and digestive capacities of fish larvae**

The anatomical and physiological changes during the development of fish larvae are factors that define their nutritional requirements. Fish species without stomach functions during the larval stage are dependent on live food as starter diet. Considering that the major nutritional constituent in live food is protein, the proteolytic capacity for the digestion of food can be regarded as the most important one during the early life of fish. This aspect has important implications in nutritional studies with these fish larvae and was studied in detail in this thesis. Fish larvae without a functional stomach rely on an alkaline digestion of food of the trypsin-type (Table 1). When the stomach becomes fully functional the proteolytic activity

in fish larvae changes from mainly trypsin-like to pepsin-type or acid digestion. This aspect might have implications in the type of proteins the fish is able to digest as will be explained in the following paragraphs. In this thesis the attention focused on the alkaline proteolytic potential during the first days of exogenous feeding in fish larvae (see Chapters 2 and 4).

**Table 1.** Trypsin-type and pepsin-type enzymatic activities in freshwater and marine fish larvae without a functional stomach at the start of exogenous feeding, and once the stomach has become completely functional. (+) present, (++) moderately active, (+++) well active (-) not measured.

Fish species	Start exogenous feeding		With functional stomach		Reference
	Trypsin	Pepsin	Trypsin	Pepsin	
Whitefish <i>Coregonus lavaretus</i>	++	-	+	-	Lauff and Hofer, 1984
African catfish <i>Clarias gariepinus</i>	+++	+	++	+++	Verreth et al., 1992
Asian seabass <i>Lates calcarifer</i>	++	+	+	++	Wallford and Lam, 1993
European seabass <i>Dicentrarchus labrax</i>	++	-	-	+++	Zambonino Infante and Cahu, 1994; Nolting et al., 1999
Turbot <i>Scophthalmus maximus</i>	++	+	+	+++	Segner et al., 1994

### *Short-term capacity for food digestion*

The digestive processes in fish larvae are fast and occur within few hours after the ingestion of food (Govoni *et al.* 1986; Hofer and Bürkle, 1986; Pedersen *et al.* 1987). However, most studies on proteolytic activity during larval development measured the enzyme activity during a time span of several days, as an indication of the long-term nutritional condition of the larvae. Only few studies present data on short-term variation of proteolytic activity in fish larvae in periods smaller than one day (Pedersen and Hjelmeland 1988; Ueberschär 1993). The information in Chapter 4 of this thesis constitutes the first reported set of data on the changes in larval proteolytic activity in time ranges smaller than one hour. This information reveals the larval capacity for the digestion of each single meal. The first changes in proteolytic activity in the gut of catfish larvae were observed within 30

minutes after feeding. These are rapid changes in protease activity and indicate a fast enzyme secretion and utilization in response to food ingestion. These results confirm previous observations in herring larvae (Pedersen et al. 1987; Ueberschär 1993). The results further indicate that, when studying physiological processes of food ingestion and digestion in fish larvae, care should be taken in selecting sampling times. There are significant variations in proteolytic activity after feeding, e.g., low activities might be expected immediately (within 1 hour) after food ingestion with a subsequent increase in activity until the next feeding. The time to reach maximum and minimum proteolytic activities after feeding in fish larvae is probably related to the type of food and is possibly age and species dependent. Nevertheless, low proteolytic activities are expected in fish that is constantly eating. In larvae fed one meal a day or when long periods exist between meals, the proteolytic activity could reach much higher levels, hours after food intake. The fast response of the digestive enzymes to food ingestion supports the idea of a highly efficient digestion and assimilation of nutrients in the fish larvae, which is needed to ensure their high growth rates. In the larvae of *C. gariepinus* the specific growth rate increased asymptotically up to 63.9% of the body weight per day between days 0 and 3 after the start of exogenous feeding, and on average, it was 41% for the first 10 days (Verreth and Den Bieman 1987). Although the digestive system in African catfish larvae is only fully developed a few days after the start of exogenous feeding, the larvae have sufficient capacity to digest and absorb nutrients from live food organisms from the very start of exogenous feeding onwards (Verreth et al. 1992).

In the present research the catfish larvae digested *Artemia* nauplii catfish larvae very fast. In less than four hours the ingested food was almost completely digested. Obviously, when larval diets are formulated and prepared, highly digestible ingredients should be selected. Furthermore, the time between meals should allow enough time for the larvae to appropriately digest the food. The optimal frequency of feeding must be determined for every species under study. In some species part of the food could be evacuated before it is completely digested if the ingestion of new food particles occurs before the digestion of the previous meals is finished.

#### *Are exogenous enzymes necessary for digestion of food?*

In the larvae of *C. gariepinus* the digestive system becomes completely functional on day 5 after the start of exogenous feeding (Verreth et al., 1992). However, the larvae have the capacity to digest and assimilate the live food nutrients as of day 1. Similarly, the larvae of whitefish *Coregonus lavaretus* (Segner et al., 1989), seabass *Dicentrarchus labrax* (Cahu and

Zambonino Infante, 1994), herring *Clupea harengus* (Pedersen et al., 1987) and turbot *Scophthalmus maximus* (Segner et al., 1994) have the capacity for optimal digestion of live food at the start of exogenous feeding. Thus, the hypothesis that fish larvae have a low enzyme production and thus a reduced digestive capacity (Dabrowski and Glogowski, 1977, Lauff and Hofer, 1984; Munilla-Moran, et al., 1990; Kolkovski et al., 1993) might underestimate the capacity of the fish larvae to digest food. The former studies suggest that the deficiency in protein digestion can be compensated by the provision of digestive enzymes from live food organisms. The functional differences in the digestive tract among fish species might be the reason for a differential dietary protein utilization and the use of endogenous digestive enzymes in the larvae of different species (Kawai and Ikeda, 1973; Hofer and Nasir Uddin, 1985; Baragi and Lovell, 1986; Segner et al., 1989; Cahu et al., 1999). The hypothesis that exogenous enzymes are needed to support the digestive process in fish larvae has been challenged in several publications, including this thesis. It was demonstrated that for some fish species, the enzymes from live food do not have a significant contribution in the larval digestion of food (Zambonino Infante and Cahu, 1994; Moyano et al., 1996; Díaz et al., 1997; Kurokawa et al., 1998). To determine the importance of dietary enzymes in the digestion of food by African catfish larvae, the proteolytic activity in decapsulated cysts of *Artemia* (Chapter 1) and in larvae after feeding decapsulated cysts (Chapters 2 and 4) was determined. The proteolytic activity in decapsulated cysts and nauplii of *Artemia* was not significantly different. Thus, with regard to the hypothetical supply of exogenous enzymes to fish larvae, there is no difference in feeding cysts or nauplii to the fish larvae. Still, the highest fish growth is achieved with nauplii. To determine the importance of the exogenous enzymes, the amount of decapsulated cysts of *Artemia* ingested by the larvae needed to be quantified. This was done in Chapter 3. The contribution of exogenous enzymes from *Artemia* cysts to the total proteolytic activity measured in fish larvae was estimated to be lower than 1 %.

## Dietary protein quality

It was previously mentioned that most fish larvae possess the capacity for digestion of live food at first feeding (Pedersen et al., 1987; Segner et al., 1989; Verreth et al., 1992; Cahu and Zambonino Infante, 1994; Segner et al., 1994). This means that the suitability of artificial diets for fish larvae might depend more on their physical and nutritional properties than on the capacity of fish larvae to digest food. Artificial diets should meet the requirements set by the physiological processes in the larval fish during development. As shown earlier, during the early life stages most fish larvae do not have a functional stomach, and protein digestion relies

entirely on trypsin proteolytic activity. There is no pre-digestion of protein in the stomach by acid denaturation and pepsin breakdown. Under such circumstances, the digestion of dietary protein in larval feeds might vary according to the type of protein source, and to the processes the ingredients underwent during diet manufacturing. In this thesis these aspects were studied to elucidate the difference between protein structure and digestibility of live food and in the protein structure and digestibility of artificial diets (Chapters 2, 5 and 6).

### ***Importance of the dietary protein structure and amino acids***

To date, most of the nutritional research in fish larvae concentrated on the requirements and utilization of lipids and fatty acids (Watanabe and Kiron, 1994; Rainuzzo et al., 1997; Sargent et al., 1997). Nevertheless, also protein and amino acids are important for the developing fish larvae (Fyhn, 1989). Protein is the major component of the dry matter in *Artemia* and because the protein and amino acid composition in cysts and nauplii are not significantly different, some qualitative factors might have an important effect on the growth of fish larvae. Probably the protein is mainly present in small peptides or in a more easy-to-digest form in the nauplii than in the cysts. To obtain evidence for this hypothesis, the digestibility of the protein in *Artemia* and the characterization of its molecular size was investigated (Chapters 5 and 6). According to several authors (Fyhn, 1989; Walford and Lam, 1993; Segner et al., 1994; Rønnestad et al., 1999) the supply of free amino acids and low molecular weight peptides in starter diets would facilitate protein digestion and absorption in fish larvae. The high contents of free amino acids in zooplankton organisms (Dabrowski and Rusiecki, 1983; Fyhn et al., 1993) corroborated with this suggestion. Moreover, the amino acids pool in the eggs and yolk sac of marine fish species consists mainly of free amino acids, which are depleted during the yolk sac stage. This suggests a special role for the free amino acids in the metabolism of early life stages. After the yolk sac absorption, the fish might rely on a similar source of amino acids, which are then provided by the food organisms in their natural habitat. Studies on the proteolytic capacities of fish larvae have further suggested that the incorporation of small peptides or protein hydrolysates in the larval diets improve the developmental processes in the digestive tract of fish larvae (Cahu and Zambonino Infante, 1995; Zambonino Infante et al., 1997; Cahu et al., 1999). All this information supports the hypothesis that the structure of protein in the larval diets might be of high importance to determine its digestibility. The results in our research indicate that most of the protein in *Artemia* nauplii consists of small size proteins with a molecular weight between 7.4 and 49.2 kiloDaltons (kDa). The proteins in cysts, which have molecular weight between 29.4 to 82 kDa.



### *Protein digestibility in live food and artificial diets*

Better results might be achieved with artificial diets as starter food for fish larvae by including highly digestible components in the food. In this thesis a new method was developed to determine the protein digestibility of artificial microdiets for fish larvae (Chapter 5). The proposed method allowed the simulation of the conditions in which the digestion of food protein occurs in the alkaline environment of the larval gut. The results confirmed the high protein digestibility of *Artemia* decapsulated cysts and nauplii (87.1 % and 86.2 % respectively) and of microbound diets containing decapsulated cysts as protein source. The technique was further used to test whether processing of the ingredients affected the nutritional quality of larval diets.

During food processing, the nutritional properties of protein might be affected. These changes on the protein properties, might in turn have effects on the overall nutritional quality of the food. Some of those effects were studied in this thesis (Chapters 2 and 6). Low protein digestibility was found for microdiets made with fishmeal, which is a commonly used protein source in fish feeds. Microdiets made with *Artemia* decapsulated cysts had better digestibility. This might be the result of food processing on the protein quality. Food processing with heat changes the protein conformation, and consequently its nutritive quality (Boye et al., 1997). These changes in the protein structure can be either beneficial or detrimental depending on the conditions of heating (de Wet, 1983) and on the preferences of the larval digestive system. The effects of heating on the protein quality of *Artemia* cysts were also studied in this thesis (Chapters 2 and 6) and the results indicate that heat treatment of decapsulated cysts increased protein denaturation and decreased protein solubility. Heating to 80°C had a significant detrimental effect on the *in vitro* protein digestibility of decapsulated cysts compared to non-heated cysts. These findings stress the importance of food processing when larval diets are manufactured. Further microdiets prepared with fishmeal and a commercial diet yielded the lowest larval growth in feeding experiments with *C. gariepinus*. Interestingly, these same diets had the lowest *in vitro* protein digestibility compared to microdiets made with decapsulated cysts of *Artemia*. It is hypothesized that the high temperatures and cooking pressures used in preparing the commercial diet and/or fishmeal might be associated with these results.

Because of the high protein digestibility of *Artemia* cysts and nauplii, their autolytic capacity and protein solubility must also be taken into consideration when explaining their

suitability as food for larval fish. When using *Artemia* in digestion experiments, high digestibility coefficients might be expected due to autolytic activity in zooplanktonic organisms (Hjelmeland, Ugelstad & Olsen 1993). It was observed that herring *Clupea harengus* larvae secreted more pancreatic trypsin when offered live food organisms as compared to plastic spheres (Hjelmeland et al., 1988). This is probably due to an hormonal stimulation of pancreatic secretion caused by the exoskeleton of the prey or by degradation products derived from the ingested live food (Pedersen 1984; Hjelmeland et al., 1988). Recently, this hypothesis has also been considered to explain the higher trypsin activity in seabass *Dicentrarchus labrax* larvae fed on *Artemia* as compared to larvae fed artificial diets (Nolting et al., 1999). Only a few publications refer to the capacity for autolysis in live food organisms and its effect on digestion processes in fish larvae. Nevertheless, autolysis and the degradation products of live food organisms are factors that are absent in artificial diets. One approach to compensate for the lack of autolysis in artificial diets is to process the food ingredients in order to have a more digestible diet. Processes like pre-digestion of protein by enzymatic hydrolysis or the use of fish protein hydrolysates might improve the digestibility of the artificial larval diets.

### Food consumption in fish larvae

Besides the described research on the digestive capacity of the fish larvae, the consumption and evacuation of food by the fish larvae was also studied. The estimation of food consumption in fish larvae is difficult because of the lack of accurate methods to evaluate both the ingestion and evacuation of food. For this thesis a new method was developed to study food ingestion by fish larvae during their development (Chapter 3). The method consisted of measuring the temporary accumulation of ascorbic acid sulfate (AAS) in fish larvae after feeding decapsulated cysts of *Artemia*. Our experiments showed that no biosynthesis or degradation of AAS occurs in catfish larvae. This supported the hypothesis that the larvae and juvenile stages of teleosts are unable to utilize AAS as a precursor for ascorbic acid (Dabrowski et al., 1990a; Dabrowski et al., 1990b). The high food consumption rates found for catfish larvae (from 46.5 to 53.8 % of their body dry weight) during the first days of exogenous feeding are in accordance with the required input of nutrients to support their high growth rate at this developmental stage. These results were similar to those found in the larvae and juveniles of five cyprinids and perch, where consumption rates higher than 50 % body weight were have been reported (Marmulla and Rösch, 1990).

Probably, one of the most interesting aspects of our studies on food consumption is that an easy and accurate method is available to measure food consumption in early life stages of fish. By using this technique, more detailed nutritional studies in fish larvae will be possible because the intake of nutrients can be estimated with a high degree of precision. In the present thesis we used this approach to estimate the intake of exogenous enzymes by the fish larvae to determine their contribution to the total digestive capacity in fish larvae.

### Artificial diets for fish larvae

Nutritionally adequate starter diets are essential for the success of larviculture in many fish species. Several artificial diets for fish larvae have been tested with relative success. From the tested diets, better results were obtained with those which included yeast or single cell protein in their formulation, as demonstrated with the larvae of whitefish (Rösch and Appelbaum, 1985), common carp (Dabrowski and Poczczynski, 1988) and African catfish (Uys and Hecht, 1985). However, the growth and survival achieved with those diets was inferior than with live food. Higher growth rates can be obtained by a combined feeding of artificial diets with a live food supplementation or co-feeding (Person-Le Ruyet et al., 1993; Fernández-Díaz and Yúfera, 1997; Rosenlund et al., 1997). In this thesis, the use of decapsulated cysts of *Artemia* in microbound diets might possibly be compared with the latter approach. The improved diet utilization when decapsulated cysts are included in the formulation of microbound diets (Chapter 6), strengthen the proposal of using *Artemia* cysts as model diet in nutritional studies with fish larvae. It further permits the study of some factors in live food which might have an effect on the better utilization of food by the fish larvae. Recently, the use of fish protein hydrolysates in larval diets improved the diet utilization and growth of fish larvae compared to conventional protein sources (Cahu and Zambonino Infante, 1995; Berge and Storebakken, 1996; Carvalho et al., 1997; Cahu et al., 1999). Hydrolysates are protein that was pre-digested by enzymatic hydrolysis to obtain small sized peptides. However, the use of high amounts of protein hydrolysates might have undesired effects on the diet quality and the fish growth (Carvalho et al., 1997; Cahu et al., 1999). Additional research is needed in this field for an adequate diet formulation for different fish species.

## Final remarks and conclusions

The question of why live food is better for fish larvae than artificial diets received no definitive answer from this thesis. However, significant advances were obtained with regard to the knowledge of the digestive physiology in fish larvae, and new techniques were developed for nutritional studies with fish larvae.

What we learned is that the nutritional composition of decapsulated cysts and nauplii of *Artemia* is similar and both have high digestibility coefficients. A logical next step would be to simulate those properties in artificial microdiets. This was partially done in this thesis with encouraging results. However, more information is needed on the functional properties of dietary proteins as a factor that determines the nutritional quality in larval feeds. With this regard, it was emphasized that the effect of food processing must be considered in the elaboration of artificial diets, due to their positive or detrimental effects on the nutrient quality.

The present thesis also emphasizes the use of *Artemia* as model diet in studies on the development of artificial diets for stomachless fish larvae. The better performance of live food over artificial diets indicates that most of the required nutrients and factors are included in the live food itself. Thus, more can be learned by testing diets which include *Artemia* in their formulation than testing new ingredients or protein sources commonly used in the elaboration of juvenile or adult fish feeds.

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## Summary

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In Aquaculture, the production increase in many species is hampered by an insufficient and unpredictable supply of fish larvae and fingerlings for stocking on-growing farms. In most cultured fish species, the larvae require live organisms as first food.

Growth and survival in fish larvae are mainly influenced by nutritional aspects. During the larval stage, the fish pass through important anatomical and physiological changes that have effects on its nutrient requirements. In some freshwater and in most marine fish species, the major changes in the digestive system during the larval period are related to the onset of stomach functions. Those changes involve a shift from a trypsin-type protein digestion, to a combination of acid secretion and a pepsin-type of protein digestion once the stomach is completely functional. Fish species that rely on trypsin-type of food digestion cannot use dry artificial diets as efficiently as live food organisms at the start of exogenous feeding. At the same time, many species can be weaned successfully to a dry diet once the stomach has become functional. Apparently, the larval dependence on live food is related to the absence of the stomach functions. Several hypotheses have been proposed to explain the suitability of live food as starter diet of fish larvae. Those are mainly related to the nutritional value of food and to aspects dealing with the food ingestion and digestion. In this thesis, the main hypothesis was that the structure of the protein in live food would allow a better food digestion and absorption by the fish larvae. Decapsulated cysts of the brine shrimp *Artemia* were used as model diets because they combine the nutritional quality of live food and the physical properties of dry artificial diets.

The general objectives of this thesis were to evaluate the nutritional value of *Artemia* as starter food for fish larvae and to assess the effects of heat treatment on the nutritional quality of food protein. Furthermore, to determine the importance of proteolytic enzymes on the larval digestion of food and to evaluate the quality of *Artemia* proteins and its use as protein source in microdiets for larval fish.

For the evaluation of the nutritional quality of *Artemia*, the biochemical composition and proteolytic enzyme activity of decapsulated cysts and nauplii were determined during their development (Chapter 1). Cysts contain embryos of *Artemia* in a dormant stage. After cyst hydration and incubation in salt water, the embryo restarted its interrupted metabolism, and after approximately 20 hours of incubation a free-swimming nauplii was hatched. Samples of cysts and nauplii were taken every five hours from the start of incubation until 25 hours. The individual protein and lipid contents during the development of *Artemia* from cyst to nauplii remained constant until the time of hatching, after which they decreased slightly. However, no

significant incubation effect was found. Small changes in amino acid and fatty acid composition were found during development, but it was assumed that they are too small to be of nutritional importance to the fish larvae. During the first 25 hours of development in *Artemia*, no major changes were detected in protease activities measured at acid and alkaline pH. Likewise, no significant changes in trypsin activity were observed. It was concluded that, from a point of view of exogenous enzyme contribution, there is no difference in feeding *Artemia* cysts or nauplii to fish larvae since no difference in qualitative protease composition was found during the first 25 hours of *Artemia* development.

The effect of heat on the protein quality and protease activity in decapsulated cysts of *Artemia* as food for fish larvae was evaluated in **Chapter 2**. Decapsulated cysts of *Artemia* were subjected to different heat treatments (40, 60, 80 and 96°C) and were fed to African catfish *Clarias gariepinus* larvae. Heat treatment of cysts increased protein denaturation and decreased protein solubility. The protease activity in the cyst diets decreased with higher heating temperatures. The growth of catfish larvae differed according to the diet, higher fish growth was achieved with nauplii and cysts heated at 40°C. The digestive enzyme activity in larval samples remained similar in all dietary treatments during larval development. The differences in fish growth among the cyst diets might be related to modifications in the structure of the protein during heating. Due to the lack of acid denaturation in stomachless fish larvae, a beneficial effect can be derived from the denaturation caused by heating cysts at 40°C. It was hypothesised that the quality of food protein and the way this protein is processed might be more important for successful larval growth than an exogenous enzyme supply. This idea was further tested in Chapter 4.

The quantification of the ingested food is necessary to determine the contribution of enzymes from dietary sources to the total digestion of food by fish larvae. In **Chapter 3**, a new method for the estimation of food consumption and gut evacuation in fish larvae was developed and evaluated. The new method consists of measuring the temporary accumulation of ascorbic acid 2-sulfate (AAS) in fish larvae after feeding decapsulated cysts of *Artemia*. Larvae of *C. gariepinus* were used to verify whether biosynthesis of AAS by the fish larvae did occur or not. Furthermore, the gut contents in larvae fed *ad libitum* were calculated as they changed during fish development and the gut evacuation rate was determined during continuous and discontinuous feeding regimes. The experimental period covered the first five days after the start of exogenous feeding in the larvae. The daily food consumption in catfish larvae increased from 46.5% of their body dry weight (BDW) at day 1 to 53.8% (BDW) at day 3. Thereafter, the food consumption decreased to 27.8% (BDW) at day 5. A similar

pattern was observed for the gut evacuation rate. The reported method for the determination of food consumption enabled an accurate and precise estimation of the gut contents and the evacuation rate of *Artemia* cysts in fish larvae.

The post-prandial activity of alkaline protease in the gut of African catfish larvae was studied in **Chapter 4**. Different feeding regimes were studied, and the protease activity was monitored at different time ranges from 30 minutes to 4 hours after ingestion of decapsulated cysts of *Artemia*. In starved larvae the enzymatic activity was low and did not change in time. No significant endogenous secretion of digestive enzymes was detected. The level of alkaline proteolytic activity found in starved larvae was considered as the basal level. In larvae fed only one meal during the day, the enzyme activity significantly increased from 3 hours post-feeding up to a maximum level found 12 hours after feeding. In fish larvae receiving a meal every 4 hours, the total protease activity changed according to the feeding time and fluctuated around a constant level, which was intermediate between the maximum and the basal level. No rhythmic cycle of enzyme production in the fish was observed when the proteolytic activity was studied during a cycle of 24 hours. When specifically trypsin activity was measured, a similar pattern was found as with total protease. Together with the results on food consumption (Chapter 3) and the protease activities found in decapsulated cyst of *Artemia*, the contribution of digestive enzymes from *Artemia* to the total digestion of food by the catfish larvae was calculated. This was estimated to be less than 1% of the total amount of the proteolytic activity measured in the larval gut of the catfish.

To assess the importance of the protein structure as a factor to explain the suitability of live food as starter diet for fish larvae, in Chapters 5 and 6 the protein quality in *Artemia* and fishmeal in microdiets for fish larvae was evaluated by different techniques. In **Chapter 5** a method was developed for the determination of the *in vitro* digestibility of protein of *Artemia* cysts and nauplii and in microbound diets made with decapsulated cysts and various binders. This method proved useful as an alternative to feeding experiments with live fish and yielded accurate estimations of the protein digestibility in the diets. A microdiet consisting of *Artemia* decapsulated cysts and carboxymethyl-cellulose as binder gave the best results.

This combination was further tested in a microbound diet and used in a feeding experiment with catfish larvae as described in **Chapter 6**. It has been suggested that the supply of free amino acids and low molecular weight peptides in starter diets would facilitate their intestinal absorption by fish larvae. This hypothesis was tested in the present thesis. The quality of different protein sources in microbound diets (MBDs) for fish larvae was evaluated,

and the differences in diet performance were related to the protein structure. To obtain a more complete picture of the protein quality in fish feeds, studies on the diet amino acid composition, the *in vitro* protein digestibility, the diet solubility and protein structure were combined with an *in vivo* feeding trial. The protein quality of MBDs made with decapsulated cysts of *Artemia* and/or fishmeal as protein source was used as indicator of their quality as starter food for fish larvae. The growth of catfish larvae was higher when the *Artemia*-based MBDs were fed than when fishmeal-based MBDs were given despite the higher protein and total amino acid content of the latter. The *in vitro* protein digestibility was high for all the MBDs in comparison to a commercial diet. Differences were found in the molecular weight of the protein among the diets. Most of the proteins in the fishmeal-based diets had low molecular weight in the range between 7.4 and 49.2 kDa. The *Artemia*-based MBDs had bigger protein fractions between 29.4 and 82 kDa. Decapsulated cysts improved the utilization of the MBDs when used in combination with fishmeal. The explanation for the positive effect of *Artemia* has yet to be elucidated. However, it is recommended to investigate the interactions between nutrients (e.g. protein-lipid) in live food, because they might have an effect on the functional properties of food proteins.

The results of this thesis confirmed that for the larvae of African catfish *C. gariepinus*, the supply of exogenous enzymes through *Artemia* cysts has a small contribution to the total proteolytic activity measured in the gut after feeding. Moreover, it was confirmed that the food ingestion and evacuation in catfish larvae is not constant as previously reported. Our results confirm the high digestibility of *Artemia* decapsulated cysts and nauplii as food for fish larvae. Similarly, the use of decapsulated cysts as protein source in microdiets improved their performance as starter diet for fish larvae.

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## **Samenvatting**

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Productieverhoging en uitbreiding van de aquacultuur industrie wordt voor veel soorten geremd door een onvoldoende en onvoorspelbaar aanbod van vislarven en jonge vissen, die nodig zijn als pootgoed op de afmesterij-bedrijven. De larven van de meeste geteelde vissoorten, hebben levende organismen nodig als eerste voedsel.

Groei en levensvatbaarheid van vislarven worden in grote mate bepaald door nutritionele aspecten. Tijdens de larvale fase, ondergaan vissen belangrijke anatomische en fysiologische veranderingen, die effect hebben op de voedingsbehoefte. Bij bepaalde zoetwater- en bij de meeste zoutwatervissen, worden de grootste veranderingen in het verteringssysteem tijdens de larvale periode gerelateerd aan het op gang komen van maagfuncties. Die veranderingen houden een verschuiving in van eiwitvertering op basis van trypsine naar een combinatie maagzuurdenaturatie en eiwitvertering met behulp van pepsine als de maag volledig functioneel is. Vissoorten, die afhankelijk zijn van trypsine-vertering benutten droge diëten niet zo efficiënt als hun levende voedselorganismen. Niettemin, kunnen veel soorten succesvol worden gevoederd met een droog dieet, zodra de maag volledig functioneert. Blijkbaar valt de afhankelijkheid van larven voor levend voedsel samen met de afwezigheid van maagfuncties. Verschillende hypothesen werden geformuleerd om de geschiktheid van levend voedsel als startdieet voor vislarven te verklaren. In deze dissertatie was de belangrijkste hypothese, dat de structuur van eiwit in levend voedsel leidt tot een verbeterde voedselvertering en opname door vislarven. Gedecapsuleerde cysten van het pekelkreeftje *Artemia* zijn gebruikt als model-diëten, omdat zij de voedselkwaliteit van levend voedsel en de fysische eigenschappen van droge diëten combineren.

De algemene doelstellingen van dit verslag waren het evalueren van de voedingswaarde van *Artemia* als startvoer voor vislarven en het vaststellen van de gevolgen van hittebehandeling op de voedingskwaliteit van voedingseiwitten. Daarnaast werd het belang vastgesteld van proteolytische enzymen op de larvale vertering van voedsel en de kwaliteit geëvalueerd van *Artemia* eiwitten en haar gebruik als eiwitbron in microdiëten voor larvale vissen.

Voor de evaluatie van de voedingskwaliteit van *Artemia*, werden de biochemische samenstelling en proteolytische enzymactiviteit van gedecapsuleerde cysten en nauplii bepaald tijdens hun ontwikkeling (**Hoofdstuk 1**). Cysten bevatten embryo's van *Artemia* in een rustfase. Na hydratatie van de cyste en incubatie in zout water, herstart het embryo zijn onderbroken metabolisme. Na ongeveer 20 uur incubatie komen de vrij zwemmende nauplii uit. In dit onderzoek werden elke vijf uur monsters van cysten en nauplii genomen vanaf het

begin van de incubatie tot 25 uur daarna. De individuele eiwit- en vetinhoud van *Artemia* bleef tijdens de ontwikkeling van cyste tot nauplii constant en nam daarna licht af. Er werd geen significant incubatie effect gevonden. Kleine veranderingen in aminozuur en vetzuur samenstelling werden gevonden tijdens de ontwikkeling, maar er werd aangenomen, dat deze te klein waren om van nutritioneel belang te zijn voor de vislarven. Gedurende de eerste 25 uur van ontwikkeling van *Artemia* werden geen grote veranderingen gemeten in protease activiteit bij een zure en alkalische pH. Ook werden er geen significante verschillen waargenomen in de trypsine activiteit. Hieruit is geconcludeerd, dat vanuit het gezichtspunt van de exogene enzym bijdrage, geen verschil werd gevonden in het voeden van vislarven met *Artemia* cysten of nauplii met betrekking tot kwalitatieve protease samenstelling gedurende de eerste 25 uur van *Artemia* ontwikkeling.

Het effect van hittebehandeling op de eiwitkwaliteit en protease activiteit in gedecapsuleerde cysten van *Artemia* is geëvalueerd in **Hoofdstuk 2**. Gedecapsuleerde cysten van *Artemia* werden blootgesteld aan verschillende hittebehandelingen (40, 60 en 96°C). Daarna werden ze gevoerd aan larven van de Afrikaanse meerval, *Clarias gariepinus*. Hittebehandeling van cysten verhoogde de eiwitdenaturatie en verlaagde de eiwitoplosbaarheid. De protease activiteit in de cystediëten nam af bij hogere temperaturen. De groei van de meervallarven varieerde met het dieet; hogere visgroei werd bereikt met nauplii en cysten die verwarmd waren bij 40°C. De verteerbare enzymactiviteit in de larvale monsters bleef in alle dieetbehandelingen tijdens de larvale ontwikkeling gelijk. De verschillen in visgroei tussen de cystediëten kan worden gerelateerd aan modificaties in de structuur van het eiwit tijdens het verwarmen. Door het gebrek aan zure denaturatie in maagloze vislarven, kan een gunstig effect worden verwacht van de denaturatie veroorzaakt door het verwarmen van de cysten bij 40°C. Hierbij is de hypothese afgeleid, dat de kwaliteit van voedingseiwitten en de manier waarop dit eiwit wordt behandeld belangrijker zou kunnen zijn voor succesvolle larvale groei, dan een exogeen enzym aanbod. Dit idee is verder onderzocht in Hoofdstuk 4.

Om de bijdrage van enzymen uit dieetbronnen voor de totale vertering van voedsel door vislarven te bepalen is het nodig om precies vast te stellen hoeveel voedsel wordt opgenomen. In **Hoofdstuk 3** is een nieuwe methode voor de bepaling van voedselconsumptie en maagdarm-evacuatie door vislarven ontwikkeld en geëvalueerd. De nieuwe methode bestaat uit het meten van de tijdelijke accumulatie van ascorbine 2-sulfaat (AAS) in vislarven na het voeden met gedecapsuleerde cysten van *Artemia*. Larven van *C. gariepinus* werden gebruikt om te toetsen of de biosynthese van AAS door vislarven optrad of niet. De darminhoud van

larven die *ad libitum* werden gevoederd werd berekend tijdens de ontwikkeling van de larven en de evacuatie van de voedselbolus uit het maag-darmkanaal werd bepaald tijdens continue en discontinue voedselregimes. De experimentele periode omvatte de eerste vijf dagen na het begin van de exogene voeding in de larve. De dagelijkse voedselconsumptie in meervallarven nam toe van 46.5% van hun droog lichaamsgewicht op dag 1 tot 53.8% van hun lichaamsgewicht op dag 3. Hierna nam de voedselconsumptie af tot 27.8% van het lichaamsgewicht op dag 5. Een soortgelijk patroon werd waargenomen voor de snelheid van maagdarm-evacuatie. De beschreven methode voor de bepaling van consumptie geeft een nauwkeurige en precieze schatting van de darminhoud en de uitscheidingsnelheid van *Artemia* cysten in vislarven.

In **Hoofdstuk 4** is de post-prandiale activiteit van alkalische protease in de darm van Afrikaanse meervallarven bestudeerd. Verschillende voedingsregimes werden gebruikt en de protease activiteit is gemeten op verschillende tijdsintervallen van 30 minuten tot 4 uur na opname van de gedecapsuleerde cysten van *Artemia*. In uitgehongerde larven was de enzymactiviteit laag en veranderde niet in de tijd. Er werd geen significante endogene afscheiding van verteerbare enzymen waargenomen. Het niveau van alkalische proteolytische activiteit die werd gevonden in de uitgehongerde larven werd beschouwd als het minimum niveau. In de larven die één keer per dag werden gevoederd, nam de enzymactiviteit aanzienlijk toe van 3 uur na het voeden tot een maximumniveau, 12 uur na het voeden. In de vislarven, die iedere 4 uur werden gevoederd veranderde de totale protease activiteit met de voedertijd en fluctueerde rond een constant niveau, welke lag tussen het maximum en minimum niveau. Bij bestudering gedurende een cyclus van 24 uur werd geen ritmische cyclus van enzymproductie in de vissen gevonden. Bij specifieke meting van de trypsine activiteit werd hetzelfde patroon gevonden als bij de totale protease activiteit. Uit deze resultaten en de resultaten van de voedselconsumptie (Hoofdstuk 3) en de protease activiteit, die werd gevonden in gedecapsuleerde cysten van *Artemia*, werd de bijdrage van verteerbare enzymen van *Artemia* aan de totale voedselvertering door meervallarven berekend. Dit werd geschat op minder dan 1% van de totale hoeveelheid van de proteolytische activiteit gemeten in de larvale darm van de meerval.

Om het belang van de eiwitstructuur voor de geschiktheid van levend voedsel vast te stellen, werden in Hoofdstuk 5 en 6 de eiwitkwaliteit in *Artemia* en vismeel in microdiëten voor vislarven geëvalueerd met verschillende technieken. In **Hoofdstuk 5** is een methode ontwikkeld voor de bepaling van de *in vitro* eiwitverteerbaarheid van *Artemia* cysten en nauplii en van zogenaamde microbound diëten gemaakt met gedecapsuleerde cysten en



verschillende bindmiddelen. Deze methode bleek geschikt als alternatief voor voedingsexperimenten met levende vissen en gaf nauwkeurige schattingen van de eiwitverteerbaarheid in de diëten. Een microdieet bestaande uit gedecapsuleerde cysten van *Artemia* en carboxymethyl-cellulose als bindmiddel gaf de beste resultaten.

Deze combinatie werd verder onderzocht in een microbound dieet en gebruikt in een voedselexperiment met meervallarven, zoals beschreven in **Hoofdstuk 6**. Er is gesuggereerd, dat het aanbod van vrije aminozuren en peptiden met een laag moleculair gewicht, de darmopname door vislarven zou vergemakkelijken. Deze hypothese is getoetst in dit onderzoek. De kwaliteit van verschillende eiwitbronnen in microbound diëten voor vislarven is geëvalueerd. Verschillen in de resultaten werden gerelateerd aan de eiwitstructuur in het voeder. Om vollediger beeld te krijgen van de eiwitkwaliteit in visvoer, werden gegevens van de aminozuur-samenstelling, de *in vitro* eiwitverteerbaarheid, de oplosbaarheid en structuur van de eiwitten gecombineerd met een *in vivo* voederproef. De eiwitkwaliteit van microbound diëten (MBDs), gemaakt met gedecapsuleerde cysten van *Artemia* en/of vismeel als eiwitbron, werd gebruikt om de kwaliteit van het startvoeder te evalueren. De groei van meervallarven was groter bij het voeren met MBDs op *Artemia*-basis, dan met MBDs op vismeel-basis, ondanks het hogere eiwit en aminozuur gehalte van de laatste groep voeders. De *in vitro* eiwitverteerbaarheid was voor alle MBDs hoog in vergelijking met een commercieel dieet. De meeste eiwitten in diëten op vismeel-basis hadden een laag moleculair gewicht tussen 7.4 en 49.2 kDa. De MBDs op *Artemia*-basis hadden grotere eiwitfracties tussen 29.4 en 82 kDa. De kwaliteit van de MBDs op basis van vismeel werd verbeterd, als behalve vismeel, óók gedecapsuleerde cyste als eiwitbron in deze voeders werd verwerkt. Er is nog geen verklaring voor dit positieve effect van *Artemia* gevonden. In ieder geval wordt aangeraden om de interacties tussen nutriënten (b.v. eiwit-vet) in levend voedsel te onderzoeken, omdat deze effect kunnen hebben op de functionele eigenschappen van voereiwitten.

De resultaten van deze thesis bevestigen, dat voor de larven van de Afrikaanse meerval *C. gariepinus*, het aanbod van exogene enzymen door *Artemia* cysten een kleine bijdrage levert aan de totale proteolytische activiteit die in de darm wordt gemeten na het voeden. Daarnaast werd vastgesteld, dat de voedselopname en -evacuatie van meervallarven niet constant is zoals als eerder werd gerapporteerd. Onze resultaten bevestigen de hoge verteerbaarheid van gedecapsuleerde *Artemia* cysten en nauplii als voedsel voor vislarven. De resultaten van microdiëten als startdieet voor vislarven verbeterden door het gebruik van gedecapsuleerde cysten als eiwitbron.

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## **Resumen**

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En Acuicultura, el incremento de la producción en muchas especies esta limitado por un insuficiente e impredecible abastecimiento de larvas y juveniles de peces para su cultivo en granjas de engorda. En la mayoría de las especies cultivadas, las larvas requieren organismos vivos como primer alimento.

El crecimiento y sobrevivencia de las larvas de peces están principalmente influenciados por aspectos nutricionales. Durante el estado larvario, el pez atraviesa por importantes cambios anatómicos y fisiológicos que tienen efectos en los requerimientos nutricionales. En algunas especies de peces de agua dulce y en la mayoría de las especies marinas, los principales cambios en el sistema digestivo durante el periodo larvario están relacionados con el inicio del funcionamiento del estómago. Esos cambios incluyen el cambio de digestión de proteína por medio de enzimas tipo tripsina, a una combinación de secreciones ácidas y digestión con enzimas tipo pepsina, una vez que el estómago es completamente funcional. Aparentemente, la dependencia de las larvas por el alimento vivo esta relacionada con la ausencia de funciones estomacales. Varias hipótesis se han propuesto para explicar las ventajas del alimento vivo utilizado como alimento inicial para larvas de peces. Estas son relacionadas principalmente al valor nutricional del alimento y a aspectos sobre la ingesta y la digestión del alimento. En esta tesis, la hipótesis principal fue que la estructura de la proteína en el alimento vivo puede permitir una mejor digestión y absorción del alimento por parte de las larvas de peces. Quistes desencapsulados de *Artemia* fueron utilizados como dieta modelo ya que combinan la calidad nutricional del alimento vivo y las propiedades físicas de las dietas artificiales secas.

Los objetivos generales de esta tesis fueron evaluar el valor nutricional de *Artemia* como alimento inicial para larvas de peces y determinar los efectos de los tratamientos con calor en la calidad nutricional de la proteína en el alimento. Además, determinar la importancia de las enzimas proteolíticas en la digestión larval del alimento y evaluar la calidad de las proteínas en *Artemia* y su uso como fuente de proteína en microdietas para larvas de peces.

Para la evaluación de la calidad nutricional de *Artemia*, fueron determinadas la composición bioquímica y la actividad de las enzimas proteolíticas en quistes desencapsulados y nauplios de *Artemia* durante su desarrollo (**Capítulo 1**). Los quistes contienen embriones de *Artemia* en estado latente. Después de su incubación e hidratación en agua marina, el embrión reactiva su metabolismo interrumpido, y aproximadamente después de 20 horas de incubación, eclosiona un nauplio de movimientos libres. Muestras de quistes y nauplios fueron tomadas cada cinco horas desde el inicio de la incubación hasta 25 horas

después. Los contenidos individuales de proteína y lípidos durante el desarrollo de *Artemia* de quiste a nauplio permaneció constante hasta el tiempo de eclosión, después del cual, esos contenidos disminuyeron ligeramente. Sin embargo, no se encontró un efecto significativo de la incubación. Pequeños cambios en la composición de amino ácidos y ácidos grasos fueron encontrados durante el desarrollo, pero se asumió que estos fueron muy pequeños para ser de importancia nutricional para las larvas de peces. Durante las primeras 25 horas de desarrollo en *Artemia*, no se detectaron grandes cambios en las actividades de proteasas medidas a pH ácido y alcalino. De la misma manera, no se observaron cambios significativos en la actividad de tripsina. Se concluyó que, desde un punto de vista de contribución de enzimas exógenas, no hay diferencia en alimentar a las larvas de peces con quistes o nauplios de *Artemia* debido a que no se encontró diferencia en la composición de proteasas durante las primeras 25 horas del desarrollo de *Artemia*.

El efecto del calor en la calidad de la proteína y en la actividad de proteasas en quistes desencapsulados de *Artemia* utilizados como alimento para larvas de peces fue evaluado en el **Capítulo 2**. Quistes desencapsulados de *Artemia* fueron sometidos a diferentes tratamientos con calor (40, 60, 80 y 96°C) y fueron dados como alimento a larvas de bagre africano *Clarias gariepinus*. El tratamiento con calor incrementó la desnaturalización de la proteína y disminuyó la solubilidad de la misma. La actividad de proteasas en los quistes fue disminuyendo conforme se aumentaron las temperaturas de calentamiento. El crecimiento de las larvas de bagre fue diferente de acuerdo con la dieta, este crecimiento fue mayor con los nauplios y con los quistes sometidos a 40°C. La actividad de las enzimas digestivas en las larvas fue similar en todos los tratamientos alimenticios durante el desarrollo larvario. Las diferencias en el crecimiento de los peces entre las dietas puede estar relacionado a las modificaciones en la estructura de la proteína durante el tratamiento con calor. Debido a la falta de desnaturalización ácida en larvas de peces que no tienen estómago funcional, un efecto benéfico puede obtenerse de la desnaturalización cuasada por el tratamiento de los quistes calentados a 40°C. Se propuso que la calidad de la proteína en el alimento y la forma en que esta proteína es procesada, pueden ser factores mas importantes para un exitoso crecimiento larval que el suministro de enzimas exógenas. Esta idea fue adicionalmente examinada en el Capítulo 4.

La cuantificación del alimento ingerido es necesaria para determinar la contribución de enzimas derivadas de fuentes alimenticias a la digestión total del alimento por parte de las larvas de peces. En el **Capítulo 3**, un nuevo método para la estimación del consumo de alimento y la evacuación intestinal en las larvas de peces fue desarrollado y evaluado. El

nuevo método consiste en medir la acumulación temporal del ácido ascórbico 2-sulfatado (AAS) en las larvas de peces después de ser alimentadas con quistes de *Artemia*. Larvas de *C. gariepinus* fueron usadas para verificar si ocurre o no, la biosíntesis de AAS en las larvas de peces. Adicionalmente, los contenidos del intestino en las larvas alimentadas *ad libitum* fueron calculados durante su desarrollo a juvenil; así como la tasa de evacuación intestinal fue determinada durante dos regímenes alimentarios: continuo y discontinuo. El periodo experimental cubrió los primeros cinco días después del inicio de la alimentación exógena en las larvas. El consumo diario en las larvas de bagre se incrementó de 46.5% de peso seco corporal en el día 1 a 53.8% de peso seco corporal en el día 3. Después de este periodo, el consumo de alimento disminuyó a 27.8% en el día 5. Un patrón similar se observó para la tasa de evacuación intestinal. El método reportado para la determinación del consumo de alimento permitió realizar una estimación exacta y precisa del contenido del intestino y de la tasa de evacuación de quistes de *Artemia* en larvas de peces.

La actividad de proteasas alcalinas en el intestino de las larvas del bagre africano después de su alimentación fue estudiada en el **Capítulo 4**. Diferentes regímenes alimenticios fueron estudiados, y la actividad de proteasas fue monitoreada a diferentes rangos de tiempo de 30 minutos a 4 horas después de la ingestión de quistes desencapsulados de *Artemia*. En las larvas en ayuno la actividad enzimática fue baja y no cambió con el tiempo. No se detectaron secreciones endógenas significativas de enzimas digestivas. El nivel de actividad proteolítica alcalina encontrada en larvas en ayuno fue considerado como el nivel base. En las larvas alimentadas una sola vez durante el día, la actividad enzimática se incrementó significativamente desde 3 horas hasta un nivel máximo encontrado 12 horas después de la ingestión del alimento. En las larvas de peces que fueron alimentadas cada 4 horas, la actividad total de las proteasas cambió de acuerdo al periodo de alimentación y fluctuó alrededor de un nivel constante e intermedio entre el nivel base y el nivel máximo. No se observó un ciclo rítmico de producción de enzimas en las larvas de peces cuando la actividad proteolítica fue estudiada durante un ciclo de 24 horas. Cuando específicamente se midió la actividad de tripsina, un patrón similar fue encontrado como con el total de proteasas. Junto con los resultados de consumo de alimento (Capítulo 3) y las actividades de proteasas encontradas en quistes desencapsulados de *Artemia*; la contribución de las enzimas digestivas provenientes de *Artemia* a la digestión total del alimento por las larvas de bagre fue calculada. Esta se estimó en menos de 1% del total de la actividad proteolítica medida en el intestino de las larvas de bagre.

Para determinar la importancia de la estructura de la proteína como un factor para explicar el éxito del alimento vivo como dieta inicial para larvas de peces, en los Capítulos 5 y 6 se evaluó por medio de diferentes técnicas la calidad de la proteína de *Artemia* y de la harina de pescado en microdietas para larvas de peces. En el Capítulo 5 fue desarrollado un método para la determinación de la digestibilidad *in vitro* de la proteína de los quistes y nauplios de *Artemia* y de dietas microaglutinadas hechas con quistes desencapsulados y varios aglutinantes. Este método demostró ser útil como una alternativa a los experimentos con peces vivos, además de que produce estimaciones exactas de la digestibilidad de proteína en las dietas. Una microdieta con quistes desencapsulados de *Artemia* y carboxymetil-celulosa como aglutinante dio los mejores resultados.

Esta combinación fue adicionalmente puesta a prueba en una dieta microaglutinada y fue usada en un experimento con larvas de bagre como se describe en el Capítulo 6. Ha sido sugerido que el abasto de amino ácidos libre y de péptidos de bajo peso molecular en dietas iniciales puede facilitar su absorción intestinal en la larvas de peces. Esta hipótesis fue examinada en esta tesis. La calidad de las diferentes fuentes de proteína en dietas microaglutinadas (MBDs) para larvas de peces fue evaluada, y las diferencias en el desempeño de las dietas fueron relacionadas con la estructura de la proteína. Para obtener una visión mas completa de la calidad de proteína en los alimentos para peces, los estudios en la composición de amino ácidos de las dietas, la digestibilidad *in vitro* de la proteína, la solubilidad de la dieta y la estructura de la proteína fueron combinados con un experimento *in vivo*. La calidad de la proteína de MBDs hechas con quistes desencapsulados de *Artemia* y/o harina de pescado como fuente de proteína fue usada como indicador de su calidad como alimento inicial para larvas de peces. El crecimiento de larvas de bagre fue mas alto cuando las MBDs hechas a base de *Artemia* fueron proporcionadas a los peces que con las MBDs a base de harina de pescado, a pesar de los contenidos mas altos de proteína y amino ácidos de las últimas. La digestibilidad *in vitro* de proteína fue alta para todas las MBDs en comparación con el alimento comercial. Se encontraron diferencias en el peso molecular de la proteína entre las dietas. La mayoría de las proteínas en las dietas hechas a base de harina de pescado tuvieron bajos pesos moleculares en el rango entre 7.4 y 49.2 kDa. Las MBDs hechas a base de *Artemia* tuvieron fracciones de proteína mas grande entre 29.4 y 82 kDa. Los quistes desencapsulados de *Artemia* mejoraron la utilización de las MBDs cuando fueron utilizados en combinación con la harina de pescado. La explicación para el efecto positivo de *Artemia* todavía tiene que ser clarificado. Sin embargo, se recomienda que hay que investigar las interacciones entre nutrientes (e.g. proteína-lípidos) en el alimento vivo, porque estas pueden tener un efecto en las propiedades funcionales de las proteínas del alimento.

Los resultados de esta tesis confirman que para las larvas del bagre africano *C. gariepinus*, el suministro de enzimas exógenas a través de quistes de *Artemia* tiene una pequeña contribución al total de la actividad proteolítica medida en el intestino después de la alimentación. Así mismo, se confirmó que la ingesta y la evacuación del alimento en las larvas de bagre no es constante como ha sido reportado previamente. Nuestros resultados confirman la alta digestibilidad de los quistes desencapsulados y los nauplios de *Artemia* cuando son utilizados como alimento para larvas de peces. De manera similar, el uso de quistes desencapsulados como fuente de proteína en microdietas mejoró su desempeño como alimento inicial para larvas de peces.

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## List of publications

### Peer-reviewed articles

- García-Ortega, A., Verreth, J., Coutteau, P., Segner, H., Huisman, E.A. and Sorgeloos, P., 1998.** Biochemical and enzymatic characterization of decapsulated cysts and nauplii of the brine shrimp *Artemia* at different developmental stages. *Aquaculture* **161**, 501-514.
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### *Curriculum vitae*

Armando García Ortega, was born on the 11<sup>th</sup> of August 1963, in Mexico City, Mexico, as son of Angelina Ortega Olvera and Armando García Santos. After completing his high school studies he entered the Faculty of Sciences of the Universidad Nacional Autónoma de México (UNAM), in which he obtained the degree of Biologist in 1991. In the same year, he enrolled the University of Gent, Belgium, where in 1993 he was granted the diploma of Master of Science in Aquaculture with distinction. After one year of work as research assistant at the Insitute for Marine Sciences and Limnology of the UNAM, he started his PhD studies in October 1994 at the Wageningen Agricultural University, The Netherlands. During the time of his doctoral studies, he also did research at the Laboratory of Aquaculture and Artemia Reference Center, University of Gent under the supervision of Prof. Dr. Patrick Sorgeloos. In January 2000 he will start with a new position as researcher in fish and shrimp nutrition at CIAD (Centro de Investigación en Alimentación y Desarrollo) in Mazatlán, Mexico.