Growth in early life stages of fishes: an explanatory model

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Although fish larvae grow very fast, little is known about to which extent their metabolism and nutritional requirements differ from larger fish. Modelling can be a powerful tool to promote understanding and optimisation of growth in fish larvae. The main objective of this study was to develop an explanatory model that can simulate growth in fish larvae. The study was conducted in three steps, i.e., a design, an experimentation and a modelling step. In the first step, the framework of the model was developed using yolk-sac larvae of the African catfish, *Clarias gariepinus*. The model is nutrient driven, and based on the biochemical reactions underlying the growth process. The model predicted growth rather accurately until complete yolk absorption, and suggested what information was needed for its further development. In the second step, experimental data on protein metabolism and the energetics of growth were collected. They constituted the basis for the design and parameterisation of a model for fed larvae. In the African catfish, the cost of growth decreases with increasing growth rates, down to the theoretical minima for protein synthesis. Fast growing larvae of turbot, *Scophthalmus maximus*, also have a cost of growth close to the theoretical minimum for protein synthesis. The amino acid (AA) profile of the free pool in turbot larvae is highly variable, being very sensitive to AA coming from the diet and protein turnover. Both in the African catfish and turbot, the larval AA profile changed during ontogeny, especially before the start of exogenous feeding. The AA profiles of the diets differed considerably from the larval ones, suggesting high unavoidable AA losses. In yolk-sac larvae of catfish, there is little regulation of catabolism of AA, and no sparing of essential AA. In contrast to this, turbot larvae may be able to spare essential AA towards the end of the larval stage. In the third and final stage, the model was parameterised and validated for African catfish and turbot. With a sensitivity analysis of the model the key parameters governing larval growth were identified. These results are discussed in view of elucidating the dietary requirements of fish larvae, evaluating the present feeding practices, and proposing research trends for the future.
PhD thesis, Department of Fish Culture and Fisheries, Wageningen Institute of Animal Sciences, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands.

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Stellingen

1. Fish larvae grow fast because they can synthesise proteins at metabolic costs close to the theoretical minimum.

   This Thesis.

2. The metabolic costs for body mass growth decrease asymptotically with increasing growth rates towards the minimum theoretical biochemical cost of protein synthesis.

   W. Wieser (1994) Cost of growth in cells and organisms: general rules and comparative aspects. *Biological Reviews* 68, 1-33; *This Thesis.*

3. Despite what is often assumed, *Artemia* nauplii are not an optimal food for larval fish, based on their amino acid profile.

   This Thesis.

4. Researchers should give more attention to dietary protein and amino acid requirements in relation to problems in growing fish larvae.

5. The power of modelling techniques in the understanding of biological systems will be undermined by premature attempts to use models to predict the behaviour of the systems.

6. Il n'existe pas de sciences appliquées, mais seulement des applications de la science.

   L. Pasteur (1872). *Comptes rendus des travaux du congrés viticole et séricole de Lyon*, 9-14 septembre 1872, p. 49.

7. When the sustainability of biological production systems is evaluated by politicians the importance of economic criteria is often exaggerated at the expense of social aspects. "Environmental" criteria are often used as an alibi.

8. In the market society, public financing of research tends to be strongly biased by the urge of the industry for solutions to current problems, at the expense of research which would give a better basis for solving future problems and create new technologies.

9. Despite the far better organisation of the Dutch University system compared to the Portuguese one, there is no obvious difference in the quality of the graduates from the two systems. The compensating factor seems to be the effort the students have to make.

10. ... I marvel how the fishes live in the sea. — Why, as man do a-land — the great ones eat up the little ones.

    W. Shakespeare. *Pericles* II. i. 27.

11. ...the gap between the swimming fish and the scientist is closing, but the fish is still well ahead.

    C.C. Lindsey (1978). Form, function and locomotory habits in fish. In: *Fish Physiology* (W.S. Hoar and D.J. Randall, Eds.) Vol. VII. Academic press, New York, p. 8.

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Contents

General Introduction

Larviculture

The world aquacultural production is steadily growing at a yearly rate of 7 to 10%. For many fish species, a further increase in production volume is hampered by an irregular and sometimes insufficient fingerling supply. This is usually the case when new species are brought into cultivation. Especially in marine fish species, high mortalities during the larval period are common. These mortalities are often related to feeding practices which do not meet the nutritional requirements of the larvae. Diets with nutrient imbalances can also result in depressed growth, which in turn can have consequences for fitness and viability in later stages of development, e.g., the quality of the live food during start-feeding influences the later acceptance of artificial diets (Bromley 1978; Bromley and Howell 1983).

Cultivation of fish larvae relies largely on feeding strategies based on live food. Because live food organisms also have to be cultured , these strategies pose extra strains on hatchery management, leading to increased fingerling production costs. Development of suitable dry larval diets, together with the determination of the earliest weaning time, would alleviate these problems and increase the reliability of the fingerling production. Despite some progress in the development of artificial diets for fish larvae, few species can be reared exclusively on them (Kamler 1992; Watanabe and Kiron 1994). Furthermore, live food production is in practice restricted to a few organisms, the most important being the brine shrimp *Artemia franciscana* and the rotifer *Brachionus plicatilis.* Despite considerable progress in production procedures for *Artemia* and rotifers (e.g., Léger et al. 1987; Sorgeloos et al. 1988; Rainuzzo et al. 1994; Watanabe and Kiron 1994) considerable difficulties remain unsolved in the manipulation of larval diets composition.

Most published work on larval dietary requirements focuses on the essential polyunsaturated fatty acid (PUFA) requirements for survival and growth (e.g. Watanabe 1982, 1993; Léger et al. 1986; Koven et al. 1990; Rainuzzo et al. 1994). If survival is dependent on micro-nutrients, e.g., polyunsaturated fatty acids and vitamins, growth is essentially protein deposition (Jobling 1985; Houlihan et al. 1993a). Therefore, optimisation of growth is closely linked to the understanding of protein and amino acid (AA) metabolism in order to supply dietary protein with an appropriate quality in the right amounts.

Growth and growth modelling

Fish larvae have the highest growth rates among vertebrates. In both freshwater and marine species growth rates may easily exceed 50% body weight.day^{-1}. Such high growth rates are probably a result of selective pressures for bigger size. In the natural environment, larger larvae

have higher chances of survival, as they are more competitive in capturing prey and have a higher chance of escaping predators (Blaxter 1988). Although larvae grow very fast, little is known about their metabolism and nutritional requirements, and to which extent this differs from larger fish.

Modelling can be a powerful tool to promote understanding and optimisation of growth in fish larvae. In a model, sound hypotheses are integrated with information from different sources, in order to produce simulations of growth under different conditions. A growth model describes mass deposition in an organism based on nutrient inputs. Models can be considered as descriptive or mechanistic. In a descriptive model, the organism is regarded as a black box converting nutrients (input) into body mass (output). The parameters of the equations used in the model are determined by fitting experimental data using statistical procedures. The use of such models is limited: although they can accurately describe experimental data, they cannot predict growth well when conditions are changed , e.g. for a different diet or another species. Mechanistic models have the advantage of being able to predict growth under conditions others than those under which they were developed. An explanatory, or mechanistic, model predicts growth based on the underlying biochemical processes. In addition to its predicting power, a mechanistic model may promote understanding of the growth process and may reveal gaps in knowledge and can be used to design experiments for further research. Yet, mechanistic models usually contain descriptive elements. Developing a growth model is always a compromise between an accurate representation of the processes involved and the information available to define their equations and to determine the respective parameters. Modelling can be seen as a stepwise process of combining existing knowledge and designing new experiments. As knowledge on larval growth increases it will be possible to increase the complexity of the model, and remove more and more of the descriptive elements.

The principles for the development of mechanistic models, an overview of the existing models in animal nutrition and their possible uses, were reviewed by Gill et al. (1989) and Baldwin and Sainz (1995). An explanatory model for growth of juvenile African catfish, *Clarias gariepinus*, was developed and tested by Machiels and co-workers (Machiels and Henken 1986, 1987; Machiels and van Dam 1987; Machiels 1987). This model is capable of predicting growth and changes in body composition with time, under different feeding levels and feed compositions. Recently, it was slightly modified, and validated for juveniles of rainbow trout, *Oncorhynchus mykiss*, and tilapia, *Oreochromis niloticus* (van Dam and Pauly 1995; van Dam and Penning de Vries 1995). The latter version includes a module that allows for simulation of the limitation that oxygen availability imposes on food intake and growth.

Fish larvae are developing animals, and particularly in the early stages they do not have all their physiological functions present or fully developed. Therefore, before a simulation

model for growth in early life stages of fish is developed, the possible differences in physiological processes between larval and post-larval fish should be evaluated. The studies of Machiels and co-workers and van Dam and co-workers have stressed the importance of an accurate definition of the relative proportions of protein and non-protein nutrients used in energy production in order to simulate growth. This energy resource partitioning seems to be closely related to the quantity and quality of the protein consumed. These studies also pointed to the importance of the definition of the energetic costs of maintenance and growth, and the representation of the regulation of food intake for growth modelling. In the following sections these processes will be presented in more detail.

Food intake and assimilation

In order to grow, a larva must ingest nutrients. Knowledge about the factors controlling food intake in larvae is scarce. Food intake depends on the size, type and density of the prey. The size of the prey a larva can ingest is limited by the size of the mouth, e.g., in many species the size of the mouth at first feeding is too small for *Artemia* nauplii. Furthermore, with increasing size fish larvae select for larger prey size (e.g., van der Meeren 1991; Gulbrandsen 1993; Cunha and Planas 1995). When fed a mixture of plankton organisms, turbot (*Scophthalmus maximus*) larvae are known to prefer copepod nauplii over rotifers (Kuhlmann et al. 1981; van der Meeren 1991) and to select for some species of copepods (Danielssen et al. 1990; van der Meeren 1991). Feeding rate increases asymptotically with increased prey density to reach a maximum level, the satiation level (Kamler 1992). However, for most species little is known about how this satiation level is affected by factors like larval density, size structure of the larval population and temperature. Determination of food intake in larvae is difficult due to the small size of both the larvae and the food particles (see Kamler 1992 for review of methods).

Before the ingested food is available to anabolic and catabolic processes, it must be digested and absorbed from the gut. The two processes together determine assimilation efficiency, although the term digestibility, in a broad sense, is also used. Estimation of food digestibility in fish larvae is difficult due to the small size of the animals and the difficulties in collecting faeces. Assimilation efficiencies have been determined indirectly, by dividing the sum of energy retained as growth and spent in metabolism by the energy in the food consumed (Houde 1989; Day et al. 1996). Direct estimation of assimilation efficiencies has been done by faeces collection (Pedersen and Hjelmeland 1988; Conway et al. 1993) or by using radioactive tracers (Sorokin 1966; Govoni et al. 1986; Kolkovski et al. 1993; Rust 1995). The use of different techniques, each with their own experimental errors, may explain part of the high variability in published values on assimilation efficiencies. Digestibility tends to increase with larval age (Rust 1995; Day et al. 1996). This is probably associated with the incomplete

development of the capacity to hydrolyse food by the digestive tract of most fish larvae at first feeding (Govoni et al. 1986; Segner et al. 1993). There is also variation in the speed of development of the digestive tract between species. In the African catfish the digestive tract seems to be functionally completed at about 20 mg wet weight (Verreth et al. 1992). In turbot, a functional stomach only appears at the end of metamorphosis (Segner et al. 1994). Furthermore, in fish larvae the digestive efficiencies seem to decrease at high food intakes due to increased passage rates (Govoni et al. 1986; Houde 1989; Day et al. 1996). The importance of factors such as food type and temperature on digestibility in fish larvae remains to be established.

Energetics of growth

Growth is an energy demanding process and it accounts for a large portion of the total energy expenditure (Jobling 1985; Wieser 1994). Besides the energy needed for growth, a fish larvae also needs energy for maintenance functions and activity. Fish larvae grow very rapidly in comparison with older fish. It has been suggested that either the cost of depositing each unit of body mass or the cost involved in maintenance functions are reduced to allow fish larvae to accommodate both the costs of growth and maintenance (Wieser and Medgyesy 1990; Wieser 1994).

The definition of growth and maintenance costs is not uniform in literature. In this thesis, the total energy expenditure is considered to be divided into the cost of maintenance and the cost of growth. The cost of maintenance is defined as the energy expenditure of a non-growing larvae, in terms of energy contents. In this study it was approximated by the metabolism of a (short-term) fasting larvae. As such, this term also includes the energy spent on activity in a fasting larvae. Routine activity, protein turnover and ion transport are believed to be the main components of the cost of maintenance. In general, the cost of protein turnover accounts for around 40% of the cost of maintenance (Houlihan et al. 1995b). In vitro estimates of the contribution of the cost of ion transport to the cost of maintenance are widely variable, ranging from 3 to 41% (Reeds et al. 1985; Pannevis and Houlihan 1992). The cost of growth is defined as the energy expenditure above the maintenance metabolism. It includes the cost of deposition of protein and lipids, the cost of protein turnover above its maintenance level, and also the costs of food search, capture and assimilation. Nutrient intake will surely involve costs of absorption, transport and metabolism of the nutrients, but it is unlikely that these processes will have a major contribution to the total costs of growth (Reeds et al. 1985). In common carp (*Cyprinus carpio*) larvae, the cost of capturing prey was only 1% of the prey energy content (Drost and van den Boogaart 1985). Protein degradation is normally associated with protein synthesis, but although the importance of its cost is unknown it is considered to be negligible (Waterlow and Millward 1989). The cost of transcription and processing of RNA (or of RNA

turnover) is not well established, but probably only mRNA turnover has a significant cost (Waterlow and Millward 1989). Reeds et al. (1985) suggested that a significant part of cost of growth may be due to energy spent in control processes involved in the regulation of metabolism in general and of protein synthesis in particular. These authors pointed out that some of the regulatory energy demanding processes could be the activity of adenyl cyclase, Na^+-K^+ ATPase and protein kinases, and the metabolism of arachidonates and phospho-inositides. However, Waterlow and Millward (1989) believe that the costs of these processes are negligible.

The cost of growth has been largely attributed to the cost of protein deposition. Stimulation of protein synthesis by feeding (McMillan and Houlihan 1988) or by infusion of AA (Brown and Cameron 1991) leads to an increase in oxygen consumption comparable to the increase in postprandial metabolism. Furthermore, the allometry equations ($Y=aX^b$) for the change of protein synthesis with body size in fish seem to have weight exponents similar to those for oxygen consumption (Houlihan 1991), suggesting a close relation between protein synthesis and energy expenditure. Therefore, the biochemical cost of protein synthesis might be expected to set the lower limit for the cost of growth. When increasing rates of protein synthesis are associated with an increase in protein degradation, the cost of protein growth (mmol $ATP.g^{-1}$protein deposited) will be greater than the cost of protein synthesis (mmol $ATP.g^{-1}$protein synthesised). However, if the rate of protein degradation remains constant the cost of protein deposition will be equal to the cost of protein synthesis.

The minimum, or theoretical, cost of protein synthesis is 50 mmol $ATP.g^{-1}$protein synthesised, or 5 mol ATP per mol of AA incorporated into protein (Waterlow et al. 1978; Reeds et al. 1985). This includes the cost of tRNA activation (2 ATP per AA), the cost of peptide elongation (2 ATP per peptide bond), and the cost of AA transport (1 ATP per AA). However, the estimated costs of protein synthesis normally exceed the theoretical costs. Differences between estimated and minimum theoretical cost are highly variable both in fish and in mammals (Reeds et al. 1985; Waterlow and Millward 1989; Houlihan et al. 1992, 1995a,d). In general, estimated costs are three to five-fold higher than the theoretical cost. Juvenile tilapia, *Oreochromis mossambicus*, is an exception as it appears to synthesise protein at a cost close to the minimum theoretical cost (Houlihan et al. 1993b).

This wide variation in the cost of protein synthesis may be due to differences in rates of protein synthesis. The cost of protein synthesis seems to decrease with increased rates of protein synthesis (Adeola et al. 1989; Pannevis and Houlihan 1992; Houlihan et al. 1992). Pannevis and Houlihan (1992) proposed that this can be due to the existence of a fixed component (independent of synthesis rate) and a variable component in the cost of protein synthesis.

Protein synthesis and turnover

The efficiency of absorbed AA utilisation will depend on the rates of protein synthesis and protein turnover. Higher protein synthesis rates may lead to a higher AA utilisation efficiency as absorbed AA will be drawn faster from the free pool, reducing the probability of being catabolised. On the other hand, the synthesis of protein requires energy. Therefore, increased protein synthesis may also lead to an increased mobilisation of AA for energy production. Increased protein turnover usually decreases AA utilisation efficiency, as a higher amount of AA will be available for catabolism.

Protein turnover is the dynamic balance between protein synthesis and protein degradation. It is therefore the process of renewal of tissue proteins. Protein deposition (or protein growth) is dependent on this balance, and can be achieved by increasing the rate of protein synthesis and/or by decreasing the rate of protein degradation. Protein turnover equals protein degradation in a growing organism, is the same as both protein synthesis and degradation at maintenance, and is equivalent to protein synthesis during starvation (Wiesner and Zak 1991). Studies on protein synthesis and turnover in fish larvae are scarce, but most of the general principles known for mammals and fish seem to be valid for larvae as well (Houlihan et al. 1995c).

Protein turnover can be divided in two components (Reeds 1989): an unavoidable component associated with the maintenance of cell functions and a variable turnover related to growth. Schimke (1977) suggested that the main functions of protein turnover would be: (1) the ability to change rapidly the amounts (and activities) of specific enzymes; (2) the mobilisation of protein during fasting, either to provide AA as precursors for protein synthesis or as substrates for energy; (3) the removal of abnormal proteins; and (4) the pre-condition for restructuring cells during cell development combined with changes in cell functions.

Different proteins have different rates of turnover, and also different tissues have different rates of protein turnover with: liver>kidney>heart>brain>muscle (Waterlow et al. 1978). Moreover, the turnover rate of a particular protein may differ among tissues. How these different turnover rates are regulated is still largely unknown (Simon 1989).

Protein synthesis is a complex process and its mechanism is described in several textbooks (e.g., Waterlow et al. 1978; Bender 1985; Stryer 1995). The different methodologies to determine rates of protein synthesis have been described and extensively discussed (see reviews by: Waterlow et al. 1978; Wolfe 1992; Houlihan 1995b,c). The rate of protein synthesis is directly proportional to growth rate (Houlihan et al. 1988, 1995a,b,c). Protein synthesis has normally a positive correlation with feeding level and with protein intake at a certain feeding level (e.g., Simon 1989; McMillan and Houlihan 1988; Fauconneau et al. 1986b; Houlihan et al. 1992). Fractional rates of protein synthesis (g protein synthesised.g^{-1}body protein content.day^{-1},

%.day^{-1}), protein turnover and protein degradation decrease with body size and with age. This was demonstrated for mammals (Simon 1989), juvenile and adult fish (Houlihan et al. 1986, 1993b, 1995a, b) and fish larvae (Fauconneau et al. 1986a; Houlihan et al. 1995c). The decrease in protein synthesis with age may be related to the increase in the proportion of white muscle tissue to the tissues with high synthetic rates, as the liver and the digestive tract (Dabrowski 1986). Fractional protein synthesis rates vary considerably among different tissues. In adult fish (Fauconneau 1985; Houlihan and Laurent 1987; Houlihan et al. 1988), as in mammals (Simon 1989), protein synthesis rates are about one order of magnitude higher in liver, gill, digestive tract, kidney, and spleen than in heart and muscle. Within a given organ or tissue the synthesis rates of individual proteins will also change (Houlihan et al. 1995a).

Growth rates can be increased by an increase in protein synthesis, by a reduction in protein degradation, or by both. In liver, and perhaps in other proliferative tissues, growth is mainly achieved by a reduction in protein degradation, whereas increases in protein deposition in muscle appear to be related to a co-ordinated increase in both protein synthesis and protein degradation (Houlihan et al. 1988, 1993a; Reeds 1989). Therefore, any stimulation of muscle growth implies that the concomitant increase in protein synthesis must be enough to allow both the deposition of new protein and satisfy the raise in protein turnover. However, in larval nase (*Chondrostoma nasus*) rates of protein synthesis increased with growth rates while the rate of protein degradation remained constant (Houlihan et al. 1992). It is unknown whether this is a general pattern for fish larvae.

Amino acid metabolism

Growth of fish larvae depends on the amount, relative proportion, and utilisation of the different AA supplied by the diet. Amino acids are present in living organisms either polymerised in protein, or free in the body fluids. Dietary AA are mostly absorbed as free AA (FAA), and the size of the FAA pool is kept within narrow limits (Houlihan et al. 1995a). Free AA pools are small in larvae as in older fish and higher vertebrates. Larval FAA pools do not exceed a few percent of the total AA in the whole larval body (Rønnestad and Fyhn 1993; Finn et al. 1995). Nevertheless, FAA are the currency of nitrogen metabolism (see Fig. 1). Absorbed dietary AA are either used for the synthesis of proteins or used otherwise. Amino acids (AA) which are not polymerised into proteins can be used for energy production (catabolised), transaminated into another AA, used in gluconeogenesis or lipogenesis, or used in the synthesis of other nitrogen-containing molecules (e.g. purines, pyrimidines, hormones). Furthermore, there is a dynamic relationship between the FAA and the protein pools, as protein is in permanent turnover.

An efficient use of the available AA resources is assured by the higher affinity for AA of the enzymes involved in protein synthesis compared to the enzymes involved in AA catabolism

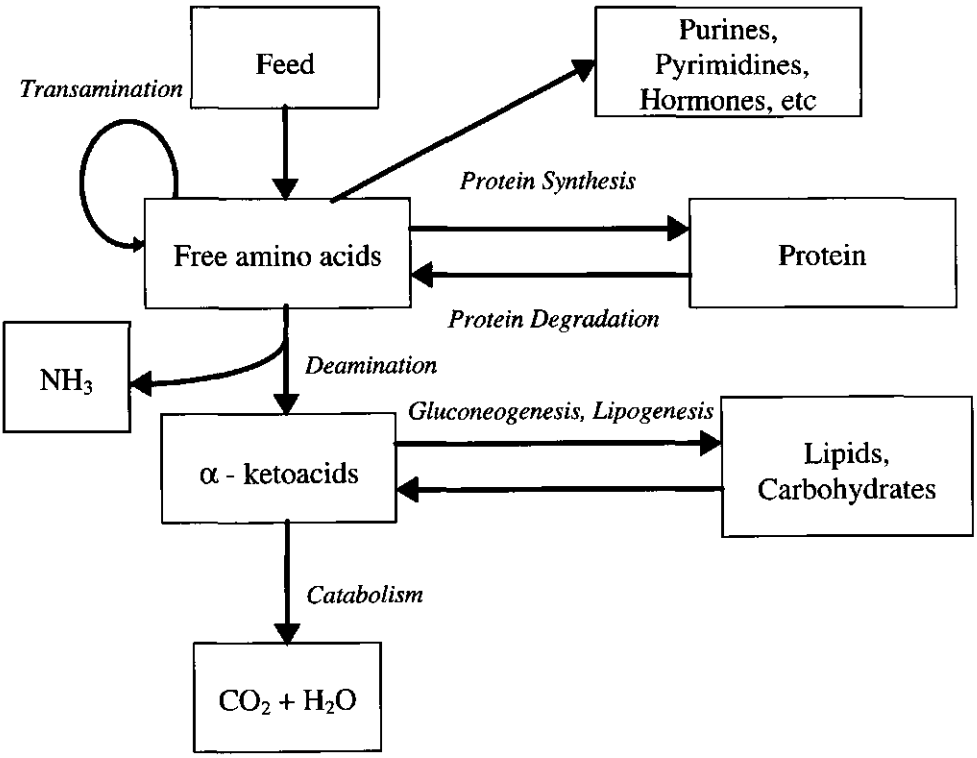

Fig. 1. Overview of the metabolism of amino acids.

(Cowey and Walton 1989). The absorption of individual AA in the gut depends on different transport systems (Jürss and Bastrop 1995) and may proceed at different rates (Dabrowski 1983). Variation in the rates of absorption of individual AA may lead to transitory AA imbalances, and thus to an increase in AA catabolism. Different enzymes are involved in transamination and catabolism of AA (Cowey and Walton 1989; Jürss and Bastrop 1995) allowing for the differential use of individual AA in these processes.

Therefore, the dietary AA profile that will allow for a maximum conversion efficiency (or growth) will depend on the efficiency of absorption of each AA, on the AA profile of proteins being synthesised as well as on the use of individual AA for energy or other purposes. This optimal AA profile may change between species, and also within species depending on environmental conditions (e.g., temperature), age and physiological state.

Amino acid catabolism is a major source of energy in fish (van Waarde 1983; Walton 1985; Cowey and Walton 1989). In adult fish it covers almost completely the maintenance needs (Brett and Zala 1975), and contributes usually more than 40% of the energy expenditure during routine activity (van Waarde 1983). Amino acids are also an important source of energy in eggs, in yolk-sac larvae (e.g., Dabrowski et al. 1984; Fyhn and Serigstad 1987; Fyhn 1989; Rønnestad et al. 1992, 1993; Rønnestad and Fyhn 1993; Verreth et al. 1995; Finn et al. 1995, 1996) and in fed larvae (Fauconneau et al. 1986a; Dabrowski et al. 1987; Terjesen et al. 1997).

Amino acid catabolism involves removal of the amino group (deamination). After deamination, the resulting α-ketoacids can be oxidised to carbon dioxide and water via the tricarboxylic acid cycle, but can also be used in lipid or carbohydrate synthesis. The pathways through which the carbon back-bones of the different AA enter the tricarboxylic acid cycle are complex, and sometimes multiple (for details see: Cowey and Walton 1989; Stryer 1995).

Fish larvae seem to have an even higher AA catabolism than older fish. The high usage of AA for energy in fish larvae has been attributed to a reduced catabolic adaptability, related to their strictly carnivorous nature (Dabrowski 1986).

In the rat, alanine, glutamate, glutamine and aspartate are preferentially used for energy production, while the other non-essential AA (NEAA) and the essential AA (EAA) are spared for the synthesis of proteins, in particular when dietary protein is limiting or when there are AA imbalances (Tanaka et al. 1995). In juveniles of turbot (Cowey and Sargent 1979) and rainbow trout (Kim et al. 1992), oxidation of NEAA was higher than the oxidation of EAA. However, while trout has the ability to conserve EAA better than NEAA when fed protein deficient diets (Kim et al. 1992), dietary protein level has no effect on AA catabolism rates in common carp (Nagai and Ikeda 1972, 1973) and in turbot (Cowey and Sargent 1979). Little information is available on selective catabolism of AA in fish larvae, but in turbot embryos and yolk-sac larvae, depletion rates of FAA from the egg yolk were comparable for EAA and NEAA (Rønnestad et al. 1993), indicating an absence of selectivity.

Amino acids are used for the synthesis of a number of non-proteinic N-containing molecules (Table 1). The losses of AA through these pathways are generally considered quantitatively of little significance, especially for the EAA (Simon 1989).

In fish AA are the best precursors for lipid and carbohydrate synthesis (Nagai and Ikeda 1972, 1973). The α-ketoacids resulting from AA deamination can be used for this purpose through gluconeogenesis and/or lipogenesis. Whether a given AA can be used as a precursor for carbohydrates (glucogenic) and/or lipids (ketogenic) depends on its carbon back-bone (for details see: Stryer 1995).

Gluconeogenesis is believed to be a minor pathway of AA metabolism in fish, and alanine is the most important gluconeogenic AA in fish (Walton 1985). This suggests that

Table 1. Some non-proteinic compounds which need amino acids to be synthesised.

Compound	Amino acid
Taurine	Cysteine
Purines, Pyrimidines	Aspartate, Asparagine, Glycine, Glutamine
Glutathione	Glutamate, Cysteine, Glycine
Cathecolamines, Dopamine, Melanin, T3, T4	Tyrosine
Serotonin, Nicotinamide (NAD, NADP)	Tryptophan
Carnitine	Lysine, Methionine
Heme	Glycine
Histamine, Carnosine, Anserine	Histidine
Ornitine	Arginine, Glutamate
Creatine	Glycine, Arginine, Methionine
GABA	Glutamate

Based on: Bender (1985) and Stryer (1995).

lactate recycling is the main function of gluconeogenesis. In addition to this role, gluconeogenesis is also involved in synthesis of glucose from dietary AA, and the production of glycerol for lipogenesis. In fish larvae, gluconeogenesis is only significant after the onset of exogenous feeding (van Waarde 1988).

NEAA can be synthesised *de novo* from α-ketoacids or through transamination and other reactions from both EAA and NEAA. Glutamate has a pivotal role, being involved in the synthesis of most of the other NEAA either as a precursor or as an amino group donor (see Bender 1985; Stryer 1995). Cysteine and tyrosine are special cases, since although being NEAA they can only be synthesised from one EAA, respectively, methionine and phenylalanine. Synthesis of NEAA from glucose has been demonstrated in fish, although it is unknown whether this *de novo* synthesis of AA is of quantitative significance (Cowey and Walton 1989).

Transaminases and other AA converting enzymes have been found in juvenile and adult fish (for review see: Cowey and Walton 1989), but their importance in the AA flux is largely unknown. They can be important to increase protein utilisation as they may compensate for imbalances of NEAA in dietary protein. In the African catfish larvae transaminase activities changed both with development and with diet type (Segner and Verreth 1995).

Amino acid requirements of fish larvae

Growth and food conversion efficiencies can be optimised through manipulation of the dietary AA composition. AA imbalances in the diet cause an increased AA oxidation and lead to decreased food conversion efficiencies (growth / food intake) (Tacon and Cowey 1985; Fauconneau et al. 1992). The dietary supply of the EAA in the right amounts and balance is of particular importance as fish cannot synthesise them (Tacon and Cowey 1985; Wilson 1994).

In fish the same 10 AA as in other animals are considered as essential: arginine, histidine, leucine, isoleucine, valine, threonine, lysine, methionine, phenylalanine and tryptophan (Wilson 1989). Usually tyrosine and cysteine are grouped together with the EAA as they can only be synthesised from the EAA.

In addition to this specific requirement in EAA, fish also have a non-specific protein requirement, which can be fulfilled either with EAA or with NEAA. The common non-essential AA are: glutamate, glutamine, aspartate, asparagine, serine, alanine, glycine, proline, (cysteine and tyrosine). The protein requirements in fish vary between species, in function of the feeding habits. It can also change within the same species, depending on several factors (Wilson 1989): (1) size and age, with younger fish having normally higher requirements; (2) water temperature, in some species, with the requirement increasing with temperature; (3) protein to energy balance; (4) dietary AA profile; (5) digestibility of the diet; and (6) amount of non-protein energy sources in the diet. The estimated protein requirements for juvenile fish are normally between 30 and 55% of the diet (Wilson 1989). Little is known about the AA requirements of larval fish and how these change during ontogeny. Compared to older fish, fish larvae have a higher protein requirement (Dabrowski 1986). Fiogbé and Kestemont (1995) found that goldfish, *Carassius auratus,* larvae have much higher EAA requirements (g $AA.g^{-1}$protein) than juvenile and adult fish.

Although fish require a higher dietary protein concentration when compared to other vertebrates, fish in general do not require more dietary protein for growth or for maintenance. At maximum growth, both protein intake (g protein $ingested.g^{-1}$body $weight.day^{-1}$) and protein retention efficiency (g protein $retained.g^{-1}$protein ingested) are comparable in fish and other vertebrates (Bowen 1987). Thus, fish should not be considered as poor protein utilisers, although they use a high proportion of their protein for energy purposes.

In juvenile fish, a more efficient use of dietary protein (protein-sparing action) can be achieved by increasing dietary lipid, carbohydrate or both (e.g., Bromley 1980; Kaushik and Oliva Teles 1985; Henken et al. 1986; Andersen and Alsted 1993). However, it is unknown whether dietary lipids and carbohydrates also exert a protein-sparing action in larval fish.

This Thesis

The aim of this thesis has been to develop an explanatory model that can simulate growth during the early life stages of fish. It should contribute to the understanding of growth and growth metabolism in larval fish, and therefore contribute to elucidate their nutritional requirements and develop appropriate feeding strategies. Protein metabolism and the energetics of growth received particular attention during the experimental phase of the thesis, as these processes are believed to be determinant for growth and its modelling.

Two fish species with commercial importance were used as biological models in this thesis. The African catfish (*Clarias gariepinus* Burchell) is a freshwater species with a wide natural distribution in Africa, which has recently also been introduced in Europe, Asia and Latin America. Its natural history (Bruton 1979), rearing practices (Verreth et al. 1993) and importance to aquaculture (Verreth 1994) have been reviewed. The turbot (*Scophthalmus maximus* L.) is a highly appreciated European marine species, being important for both commercial fisheries and aquaculture. Its biology and rearing practices have been reviewed by Person-Le Ruyet (1991). In some of the chapters of this thesis yolk-sac larvae were used as a biological model as they constitute a semi-closed system where food intake (yolk absorption) can be easily monitored, and where there is no defecation.

In **Chapter 1** a growth simulation model for endogenously feeding larvae of the African catfish is presented. It confirms the importance of a correct definition of the energetics of growth and of protein metabolism, if growth of fish larvae is to be understood and predicted.

Chapters 2 to 6 supply experimental data to parameterise and support the design and the assumptions of a simulation model for growth of fed fish larvae. **Chapter 2** deals with the question of how fish larvae manage to accommodate the costs of high growth rates with cost of maintenance within their limited energy budget. In **Chapter 3** the cost of growth is estimated at different stages of development in the African catfish, and related to food intake and growth rate. The cost of growth in larval turbot is estimated in **Chapter 4**, and its biochemical composition is studied in relation to development and feeding regime. In **Chapter 5** growth and AA utilisation of turbot larvae are related to the composition of the free AA pool, the rate of protein turnover, and the flux of AA. In **Chapter 6** differences in utilisation efficiency of the individual AA in yolk-sac larvae of the African catfish are analysed, and related to differences between dietary and larval body AA profiles. Changes in larval body AA profiles during ontogeny are also investigated in Chapters 5 and 6.

In **Chapter 7** the model is described and validated for feeding African catfish and turbot. A sensitivity analysis is also performed in order to pin-point the key parameters governing larval growth. In the **general discussion** the main factors determining fish larval growth are discussed, together with the prospects to of using and improving the present model.

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Chapter 1

A simulation model for the metabolism of yolk-sac larvae of *Clarias gariepinus*

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A simulation model for the metabolism of yolk-sac larvae of the African catfish, *Clarias gariepinus* (Burchell)

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Abstract

In fish larvae there is a strong natural selection for high yolk utilisation efficiencies and high yolk absorption rates. Larvae with higher absorption and utilisation will be larger at the onset of exogenous feeding, leading to higher survival and growth rates in the following stages of development. Modelling can be a powerful tool in clarifying the processes of fish larvae metabolism, providing the model is as explanatory as possible. The present model considers the larvae as a two-compartment system: the yolk-sac and the larval body. This system exchanges oxygen, carbon dioxide, water, ammonia and heat with the environment. Yolk is absorbed through the syncytium, digested, and the resulting nutrients are released into the blood circulation. Circulating amino acids and fatty acids are considered to be used first for respiration, and what is left will be used for growth. From the moment that absorbed nutrients do not satisfy the energy needs, embryo tissue starts to be catabolised. Temperature is the only environmental factor which was assumed to affect significantly the metabolic processes. From the comparisons between simulations and experimental data, the simulations would appear to be accurate until complete yolk absorption, losing accuracy afterwards. Simulation outputs suggest that fat is the main energy substrate during yolk absorption, with protein becoming progressively more important, being predominant during starvation. Ideal culture conditions for African catfish yolk-sac larvae seem to include: the highest temperature that combines with acceptable mortality rates; selection of broodstock towards obtaining the largest egg size, insofar as viability is not affected; the start of feeding as close as possible to 144 physiological day-degrees.

Introduction

Eleuthero-embryos of fish use their yolk reserves for energy supply and growth. High efficiencies of yolk utilisation result in larger larvae at the onset of exogenous feeding. These are believed to be less affected by competition, more resistant to starvation, less susceptible to predation, and are able to commence feeding earlier (Blaxter 1988). Obviously, the rate of yolk absorption and the efficiency of yolk utilisation are important determinants of early growth and survival. Several factors, such as egg weight, yolk composition, temperature, oxygen, and salinity, affect the rate of yolk absorption and the efficiency of yolk utilisation (Heming and Buddington 1988). A detailed knowledge of the relation between these factors and embryonal

metabolism may be very instrumental in defining ideal conditions and strategies for larval rearing. To study these processes in early life stages of fish and to elucidate their relations with the mentioned biotic and abiotic factors, many technically difficult experiments would be required. Because of the required detail, this approach may lead to a 'reductionist' focus towards certain biochemical and/or physiological aspects of embryonal metabolism, forgetting thereby the interactions occurring at the system level of the organism, i.e. the whole larva or embryo.

Modelling may help to overcome these problems. When a model is based on the underlying biochemical processes, it may be considered as explanatory. The quantitative equations used in an explanatory model should describe the biochemical processes, allow extrapolation to other conditions than the ones used to calibrate the model, and should reveal gaps in the present knowledge of the underlying processes (Machiels and Henken 1986). Such a biochemical model already exists for the growth of juvenile and adult stages of African catfish, *Clarias gariepinus* (Burchell) (Machiels and Henken 1986; Machiels and Henken 1987; Machiels and van Dam 1987). In the present study, a similar model will be developed for the growth and metabolism of the early life stages of the same species, *C. gariepinus*, focusing thereby on the eleuthero-embryonal period and on larvae that are st ed after complete yolk-sac absorption.

Model derivation and description

The main elements of the model and their relations are shown in Figure 1. The larvae are considered as a two-compartment system: the yolk-sac and the larval body (hereby also referred as embryo). After being absorbed, yolk is digested in the syncytium, and the resulting nutrients are released into the blood circulation (Heming and Buddington 1988). According to the model assumption, circulating amino acids and fatty acids are first used for energy purposes, and what is left will be used for growth. When absorbed nutrients do not satisfy the required energy needs, embryonal tissue will be broken down to compensate for the energy shortage. As a matter of fact, in starving larvae body tissue will be the only energy source. O_2 is taken up from the environment, while CO_2 and NH_3 are released. Water and heat are exchanged in both directions. Temperature is the only environmental factor taken into account in the model. Other environmental factors (e.g. oxygen) have threshold levels, above which they do not limit the metabolic processes. Under fish culture conditions, these factors are usually maintained above these thresholds, and therefore were neglected in the model.

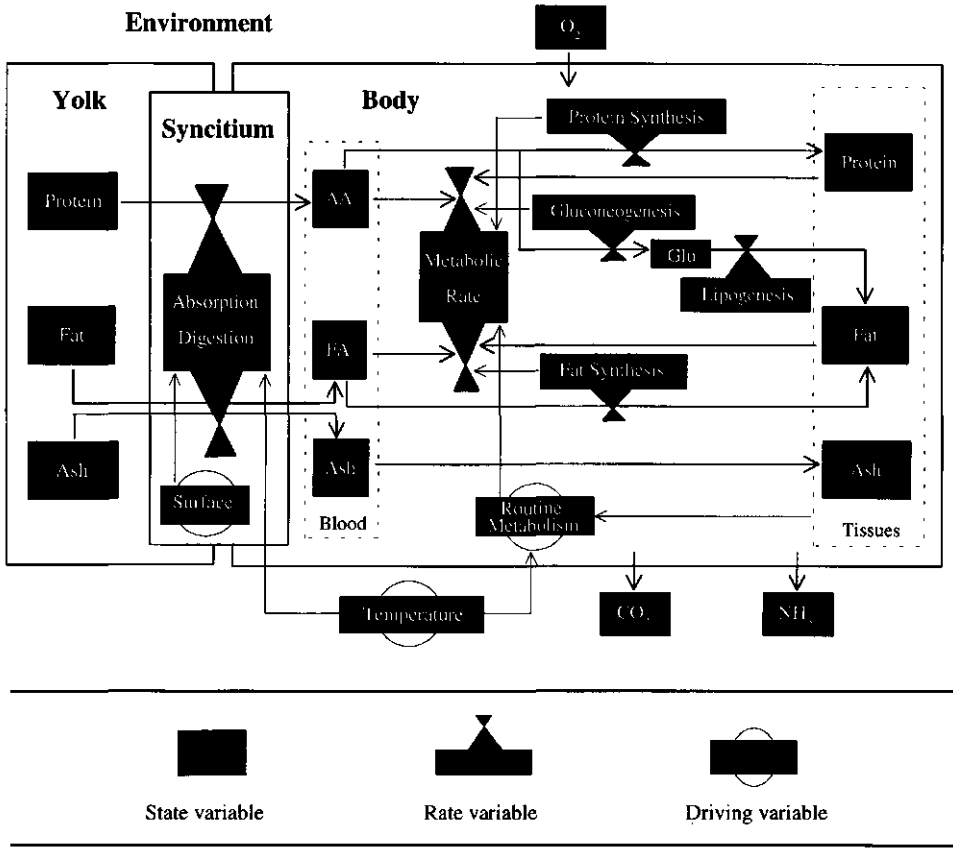

Figure 1. Model relational diagram. Flux of nutrients (thick arrows) between state variables is determined by the rate variables. Driving variables regulate (thin lines) the activity of the rate variables.

Yolk composition

Yolk was assumed to consist of three fractions: protein, fat and ash. The ash fraction includes all the materials not included in the protein and fat fractions. These three yolk fractions are assumed to be absorbed non-selectively, which means that the relative yolk composition remains constant during absorption. Carbohydrates were neglected as a yolk component. Fish eggs usually have a relatively low carbohydrate content, and even this is probably depleted to a large extent prior to hatching (Boulekbache 1981; Heming and Buddington 1988).

Rate of yolk absorption

According to Heming and Buddington (1988), the rate of yolk absorption is a function of: (1) the absorptive surface (i.e. the syncytium surface area); and (2) the metabolism of the syncytium, which depends on the temperature.

A logistic curve was chosen to simulate the amount of absorbed yolk during the yolk-sac period. Four parameters, defining the start- and end-point of the curve are required to fit it: time at hatching (t_{ha}), time at apparent yolk absorption (t_{acya}) and the yolk weight at these moments. Based on the experience with *Clarias gariepinus*, the duration of yolk absorption was considered to be independent of initial yolk weight (hence, a 1:1 relationship between yolk volume and yolk absorption was assumed). The rate of yolk absorption (RYA), obtained by derivation of the equation of the logistic curve, increases with temperature.

To avoid empirical assessment of t_{ha} and t_{acya} for different temperatures, in the model these parameters were introduced with the dimension of physiological day-degrees (PD°), which can be assessed from a single set of data. They are calculated by correcting the amount of day-degrees for the curvilinear relation between temperature and the rate of physiological processes, using the q correction factors of Winberg (1956). For each temperature, the computer programme converted these PD° values into hours after fertilisation before starting simulation.

Energy expenditure

Energy expenditure is calculated as the sum of energy needed for routine metabolism and the energy cost of growth.

Routine metabolism is expressed following the Winberg equation (Winberg 1956). To account for temperature effects, a Q_{10} factor was included:

$$T = \alpha * WW^{\beta} * Q_{10}^{\frac{(Temp-RTemp)}{10}}$$

where T stands for routine metabolism (mol $ATP.fish^{-1}.h^{-1}$), α for metabolic level (mol $ATP.g^{-\beta}.h^{-1}$), WW for wet weight of the embryonal tissue (g), ß is the weight exponent of routine metabolism, Temp is the water temperature (°C), and RTemp the reference temperature for α (°C).

The wet weight used to estimate the routine metabolism refers only to the embryo tissue as the metabolism of the yolk-sac is assumed to be zero.

Following the empirical relation for *C. gariepinus* derived by Machiels and Henken (1986), the relative amounts of amino acids and fatty acids catabolised are assumed to be determined by their proportion in the blood pool, according to the equation:

$$CFA = (1.298 + 1.130 * \text{Log}(RBP)) * 100$$

where CFA is the contribution of fatty acids (%) to respiration and RBP the ratio fatty acids/amino acids in the blood pool.

Intermediary metabolism

The stoichiometry for the reactions of intermediary metabolism are given in Table 1, assuming that: (a) oleic acid and tri-oleylglycerol (TOG) are typical for combusted fatty acids and oxidised fat, respectively (Love 1980); (b) a fixed fraction (5%) of the absorbed (and not combusted) amino acids is non-selectively converted into glucose and ATP by gluconeogenesis, and all the glucose produced is converted into fat through lipogenesis (Machiels and Henken 1986). The stoichiometry of amino acid metabolism was based on experimental data regarding the selective combustion of different amino acids during the yolk-sac phase of *C. gariepinus* (Polat and Verreth, unpublished data).

The cost of biosynthesis refers only to synthesis from nutrients absorbed from the yolk-sac. Tissue renewal (i.e., protein and fat degradation followed by re-synthesis) was not taken into account.

Growth rate

In the model, larval growth results from three processes: (1) the synthesised protein minus the body protein used in respiration; (2) the sum of the synthesised fat and the fat produced through lipogenesis, minus the combusted fat; (3) the ash absorbed from the yolk-sac, which is assumed to be converted into body tissue with an efficiency of 100%. The whole model is processed in dry weight. Dry weights were converted into wet weights, assuming a water content of 90% in the body tissue and 70% in the yolk, according to unpublished experimental information.

Conversion Efficiency

The cumulative and instantaneous conversion efficiency of the yolk (CUMCEFF and ICEFF) are calculated using the following equations:

$$CUMCEFF_t = \frac{Embryo\ DW_t - Embryo\ DW_{ha}}{Yolk\ DW_{ha} - Yolk\ DW_t}$$

$$ICEFF_t = \frac{Growth\ rate_t}{Rate\ of\ Yolk\ Absorption_t}$$

Table 1. Stoichiometric equations of intermediary metabolism as used in the model.

Protein breakdown:

$1g\ AA + 1.34g\ O_2 \longrightarrow 1.70g\ CO_2 + 0.17g\ NH_3 + 0.33mol\ ATP$

Fat breakdown:

$1g\ TOG + 2.9g\ O_2 \longrightarrow 2.8g\ CO_2 + 1.1g\ H_2O + 0.51mol\ ATP$

Gluconeogenesis:

$1g\ AA + 0.80g\ O_2 \longrightarrow 0.96g\ CO_2 + 0.20g\ NH_3 + 0.51g\ Glucose + 0.23mol\ ATP$

Lipogenesis:

$1g\ Glucose + 0.22g\ O_2 \longrightarrow 0.63g\ CO_2 + 0.29g\ H_2O + 0.29g\ TOG$

Protein synthesis:

$1g\ AA + 0.051mol\ ATP \longrightarrow 0.85g\ Protein + 0.15g\ H_2O$

Fat synthesis:

$1g\ Olein + 0.10g\ Glycerol + 0.014mol \longrightarrow 1g\ TOG + 0.06g\ H_2O$

where DW stands for dry weight (mg), t for time after fertilisation (hours), and ha for hatching time (hours after fertilisation).

Exchanges with the environment

Before complete yolk absorption, both oxygen consumption and carbon dioxide production result from gluconeogenesis, lipogenesis, fat breakdown and protein breakdown, according to the biochemical reactions given above. During starvation, only the two last processes are considered.

Ammonia was assumed to be the only nitrogenous excretion product (Machiels and Henken 1986; Kiørboe 1989). It is mainly produced during protein breakdown, but before complete yolk absorption, also gluconeogenesis contributes to ammonia production.

Parameterisation

Basically, simulation models consist of a set of equations describing the processes involved in the studied system. The model can only use these equations when starting for the parameters values of the equations are established.

Table 2. Species specific parameters and values for *C. gariepinus*, as introduced into the model.

Parameter	Value	Unit
(Yolk at apparent complete yolk absorption/yolk at hatching)* 100	8[a]	%
Fraction of absorbed amino acids used in gluconeogenesis	5[b]	%
Routine metabolism:		
Metabolic level	0.05[c]	mmol ATP/$g^{0.80}$/h
Weight exponent	0.8[c]	-
Q10 constant	1.6702[a]	-
Q10 slope	0.0058[a]	-
Reference temperature	25[a]	° C
Yolk composition at Hatching:		
Protein	69.3[a]	%
Fat	20.0[a]	%
Ash	10.7[a]	%
Time at:		
Hatching	52[a]	PD°
Apparent complete yolk absorption	190[a]	PD°
End of simulation	450	PD°

Sources: (a) our laboratory, unpublished; (b) assumed; (c) Machiels and Henken 1986

The present model has three basic parameters as input variables: temperature, larval weight at hatching and the fraction of this weight that represents yolk. Further, it contains a set of species-specific parameters, or variable parameters. All temperature-related parameters were introduced in PD°, as explained for the yolk absorption. A set of values for these species specific parameters relating to *C. gariepinus* was derived from proper laboratory studies, from literature, or based on assumed values. This set of values, as is shown in Table 2, was defined in the programme.

To convert the biological model into a computer programme, it was written in the PC language TURBO PASCAL 6.0. The programme was made menu-driven by using a library of routines (IOLIB) developed by Klompmaker and Oosters (unpublished).

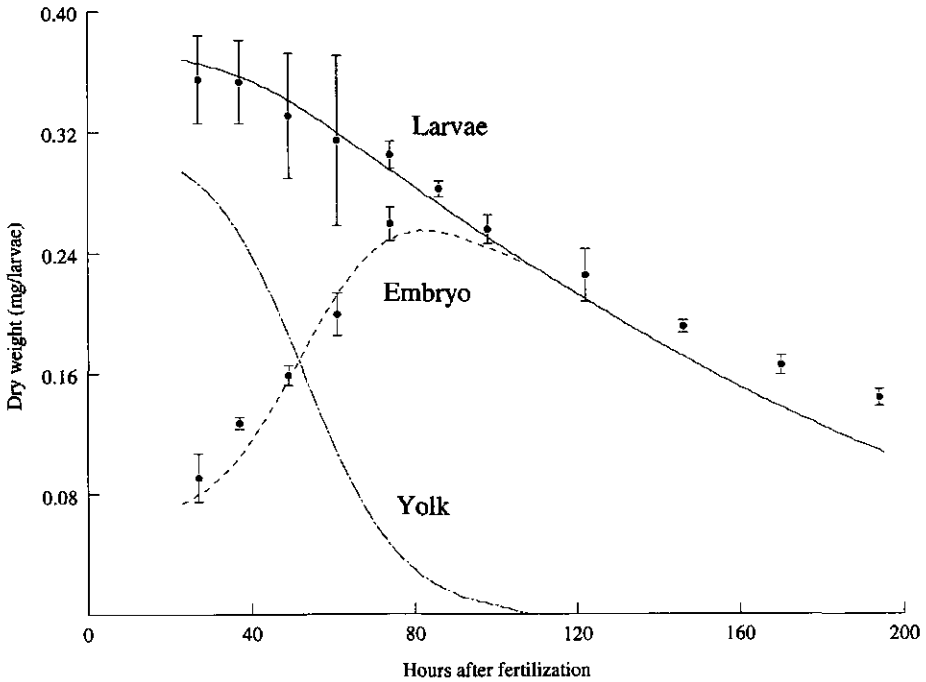

Figure 2. Simulations of embryonic (---), yolk (—·) and larval (—) dry weight of *C. gariepinus* (28°C; 0.368 mg at hatching, with 80% as yolk). Real experimental data (Polat and Verreth, unpublished) are also displayed, together with the 95% confidence limits.

Results

Model validity

To validate the model, simulations were performed and compared with (unpublished) data from Polat and Verreth (Figures 2 and 3), who investigated changes in dry weight, protein, lipid and energy content of yolk and body tissue in eggs, eleuthero-embryos and starving larvae of *C. gariepinus*. These data were independent from those used during parameterisation of the model. The simulation seems rather accurate for larval dry weight until complete yolk absorption, but underestimates the experimental data in starving larvae. The same conclusion holds for data regarding the protein and lipid content of the larvae. The protein content of the larvae increases slightly until maximum embryonal dry weight is reached, and then decreases. The lipid content, in turn, declines continuously, especially prior to maximum embryonal dry weight.

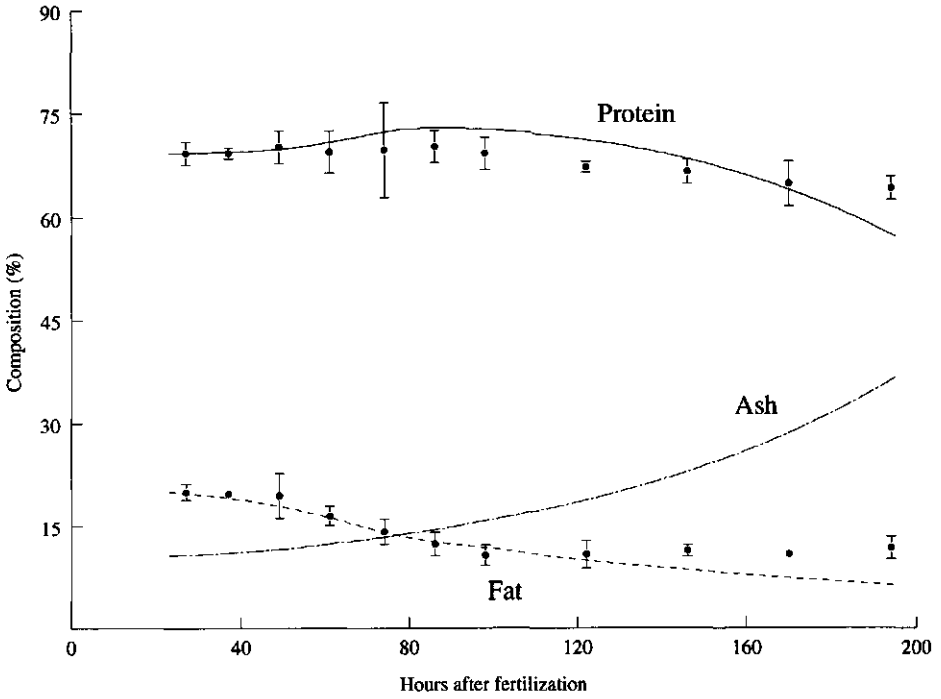

Figure 3. Simulation of larval composition (%) in protein (—), fat (---) and ash (·—) for *C. gariepinus* (28°C; 0.368 mg at hatching, with 80% as yolk). Real experimental data (Polat and Verreth, unpublished) are also displayed, together with the 95% confidence limits.

Energy expenditure

Both O_2 consumption and CO_2 production increase strongly following hatching, and reach a peak just before the point of maximum embryonal dry weight (Figure 4). Afterwards, they decrease steadily until the end of the simulation. The respiratory quotient declines from hatching to the aforementioned peak, rising from then onwards.

The model simulations (Figure 5) show further that the cost of routine metabolism exceeds the cost of biosynthesis throughout the yolk-sac stage. The costs of biosynthesis are largely represented by the cost of protein synthesis.

Temperature and size effects on conversion efficiency

A simulated experiment was performed to assess the sensitivity of the model for effects of temperature and initial larval weight. As a criterion, yolk conversion efficiency was used. Simulations were performed for larvae of 0.3 and 0.4 mg dry weight at hatching, at three

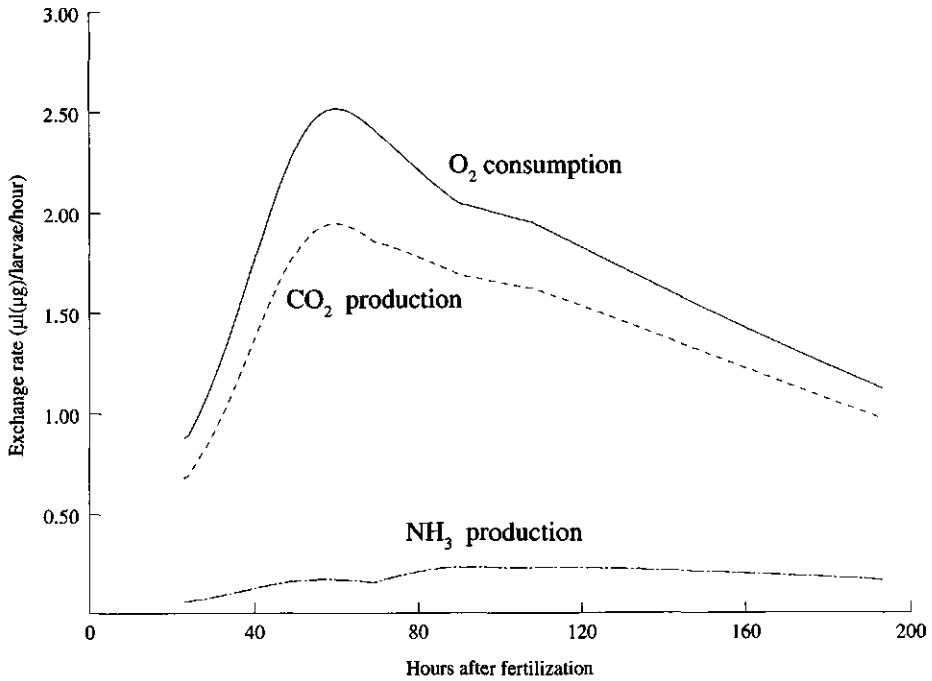

Figure 4. Simulation of larval exchanges with the environment, O_2 consumption (µl/h) (—), CO_2 production (µl/h) (---) and NH_3 production (µg/h) (-·-) for *C. gariepinus* (28°C; 0.368 mg at hatching, with 80% as yolk). No real experimental data were available for comparison.

different temperatures: 20, 25, and 30 °C. The yolk weight at hatching was fixed by assuming, at hatching, that 85 % of the larval dry weight consisted of yolk.

Due to the initial high rate of yolk absorption, cumulative conversion efficiency (Figure 6) increases until a maximum is reached, after which it decreases until yolk absorption is complete. Conversion efficiency is positively correlated with higher temperatures and higher initial weights.

Recalculating the results of simulations from hours to PD° for the three temperatures, complete yolk absorption was found to occur around 248 PD°. Similarly, maximum embryonal dry weight occurs around the time of apparent complete yolk absorption (190 PD°). Time of maximum cumulative conversion efficiency and maximum energy expenditure coincide at about 144 PD°.

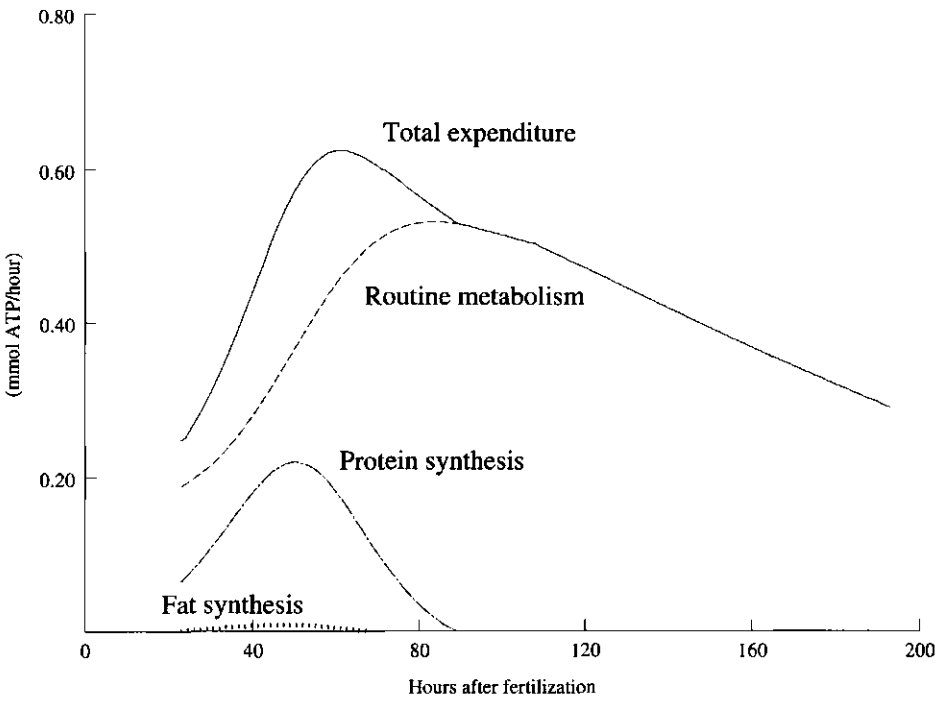

Figure 5. Simulation of total energy expenditure (—), routine metabolism (---), cost of protein synthesis (·—·), and cost of fat synthesis (···) for *C. gariepinus* (28°C; 0.368 mg at hatching, with 80% as yolk). No real experimental data were available for comparison.

Discussion

Model description

Modelling is a powerful tool in clarifying the processes of fish larvae metabolism. Nevertheless, simulated data are never as accurate as experimental data, and do not represent the entire biological reality. Models are always a simplification of this reality because they must consist of a set of quantifiable variables linked to one another by quantifiable rates and fluxes. Not all information on biological processes is quantifiable yet, and is therefore unsuitable for explanatory modelling. In these situations the model must use a descriptive approach. In the present model, two processes were introduced according to such a descriptive approach: yolk absorption and routine metabolism.

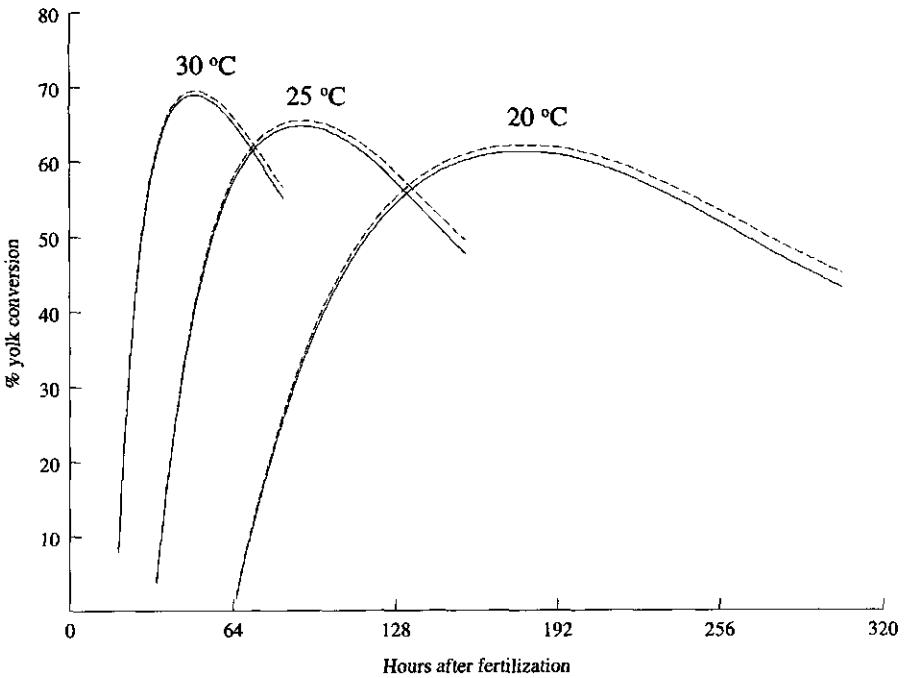

Figure 6. Simulated cumulative conversion efficiency (%) at 20, 25 and 30 °C, with larval dry weight at hatching of 0.3 mg (—) and 0.4 mg (---) (85% as yolk). No real experimental data were available for comparison.

Routine metabolism consists mainly of the costs of maintenance. In fish larvae, the costs of tissue renewal, in particular protein turnover, are a major fraction of these maintenance costs (Kiørboe 1989). Therefore, to replace the (descriptive) equation of routine metabolism by a more explanatory approach, a key process to study seems to be the protein turnover rate.

Growth

The comparisons with experimental data reveal that in general the model would appear accurate until complete yolk absorption. After that, the simulated values underestimated the empirical data. Several hypotheses may explain this phenomenon.

A first hypothesis refers to an eventual uptake of nutrients from exogenous sources, possibly due to either cannibalism or direct utilisation of dissolved organic compounds from the water. Cannibalism was only observed sporadically among starving larvae (Polat pers. communication), and therefore cannot explain the observed underestimation. The uptake of dissolved compounds has been demonstrated for fish larvae (Fauconneau et al. 1989). However,

it is unlikely that this process contributes significantly to the needs of developing fish (Heming and Buddington 1988).

The inaccuracies also may result from an overestimated routine metabolism in the model. This may have resulted from an overestimation of α (metabolic level) or an underestimation of ß (weight exponent) in the routine metabolism equation. In fact, the values used in the model refer to juvenile fish and were derived from Machiels and Henken (1986). Further, fish larvae may have lower maintenance costs than juvenile fish (Kiørboe et al. 1987), because of a reduced turnover of body components (especially protein).

Routine metabolism also depends on the wet weight of the body tissues. The estimations for wet weight are by far the least accurate of the model. This probably results from inaccurate assumptions concerning the water contents for both yolk and embryonal tissue in the model. This hypothesis is further supported by Heming and Buddington (1988) who refer to conflicting reports concerning the variation of water content during development of both the embryo and the yolk, even within the same species.

Obviously, the accuracy of the model is largely dependent on accurate predictions of routine metabolism. The Winberg formula, a descriptive approach, was used to overcome the lack of quantifiable data regarding the underlying processes of maintenance in larvae. Parallel measurements on (1) protein turnover and (2) respiration, could be a step forward towards a more explanatory approach of quantifying maintenance costs.

Energy substrates

The model output regarding the respiratory quotient and the chemical composition of the larvae indicates that lipid is the most important energy substrate during yolk absorption, mainly during the initial phase. Protein becomes progressively more important, being the major energy source in starving larvae. This agrees with the results of several studies in *C. gariepinus* (our laboratory, unpublished), *Pleuronectes platessa* (Ehrlich 1974) and *Scophthalmus maximus* (Rønnestad 1989). In the present study, at the end of the yolk-sac period protein and lipid are equally important energy sources. This seems to contradict the hypothesis that lipid, often present as an oil globule, is the main energy source at this stage (Eldridge et al. 1982; Heming and Buddington 1988). However, the sequence of nutrient consumption varies between species, being affected by temperature, access to food during early life (Dabrowski et al. 1984) and/or by the reproductive strategy (Heming and Buddington 1988). From an evolutionary point of view, it may be advantageous for yolk-sac larvae to combust first lipid, saving thereby protein for deposition. When larvae are starved after yolk absorption, protein rather than energy is the first commodity to be depleted (Rogers and Westin 1981). Therefore, larvae with higher protein content are likely to have increased probabilities of survival.

Effect of temperature and egg size

The increase in conversion efficiency with temperature and initial weights contradicts what is generally mentioned in literature. According to Kamler and Kato (1983), larvae with greater initial weight have lower conversion efficiencies because their larger size entails higher maintenance costs. Conversion efficiency is said to reach a maximum within the range of thermal tolerance of a given species (Heming and Buddington 1988). Nevertheless, the effect of temperature on yolk utilisation efficiency may vary among species, probably in relation to differences in reproductive strategy and rearing habitat (Heming and Buddington 1988). Temperature may act along two lines: higher temperatures (1) accelerate the occurrence of developmental events, and also (2) increase the metabolic rate (Kamler and Kato 1983; Arul 1991). Both actions work in opposite directions and may have a different balance for different species.

When trying to determine the optimum values of temperature and initial weight to select for fish culture, the survival rates also will have to be considered (Kamler and Kato 1983).

Exogenous feed should be available as close as possible to 144 PD°, as this is the moment at which cumulative conversion efficiency is a maximum, thus ensuring an optimum use of the original yolk reserves.

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Chapter 2

Fast growth, protein turnover and costs of protein metabolism in yolk-sac larvae of the African catfish (*Clarias gariepinus*)

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Fish Physiology and Biochemistry (in press)

Fast growth, protein turnover and costs of protein metabolism in yolk-sac larvae of the African catfish (*Clarias gariepinus*)

Luis E.C. Conceição, Dominic F. Houlihan and Johan A.J. Verreth

Abstract

In fish larvae the costs of rapid growth may be accommodated by a decrease in the rate of protein turnover or by a reduction in the costs of protein synthesis. Protein growth, synthesis and degradation were measured in yolk-sac larvae of *Clarias gariepinus* and the costs of protein synthesis and protein growth were estimated. Growth rates were over 100% protein weight.day^{-1}. Protein synthesis retention efficiency (retained protein per unit of synthesis) was estimated to be 69.6%, a value comparable to that of larger fish. The larvae used 43% of their oxygen consumption for protein synthesis. Nevertheless, protein synthesis costs were close to theoretical minima. Therefore, the high growth rates of catfish yolk-sac larvae seem to be possible through minimisation of the costs of protein synthesis. These low costs are associated with high rates of protein synthesis (138%protein weight.day^{-1}), and elevated RNA concentrations (107 µg RNA.mg^{-1}protein), which together suggest very high RNA efficiencies (12.9 g protein synthesised.g^{-1}RNA.day^{-1}).

Introduction

Growth is primarily protein deposition, i.e., the increase in protein mass in a given time interval, and growth is an energetically expensive process. Jørgensen (1988) estimated the costs of growth in fast growing juvenile fish to be around 87% of their total energy expenditure. The costs of growth are related to the costs of protein deposition (Jobling 1985; Brown and Cameron 1991; Houlihan 1991) with protein growth being the net result of protein synthesis and protein degradation. The costs of protein turnover include the costs of both protein synthesis and protein degradation, and turnover of proteins may account for 11 to 25% of the basal metabolic rate (Reeds et al. 1985; Hawkins et al. 1989; Waterlow and Millward 1989). Protein synthesis retention efficiency, defined as the quotient between the rates of protein growth and protein synthesis (Houlihan 1991), allows the comparison of turnover rates of animals with widely different protein growth rates.

Fish larvae may grow at rates of 20 %protein weight.day^{-1} or more (Wieser et al. 1988b; Houlihan et al. 1995a), but as fish larvae have a limited capacity for oxygen uptake the metabolic scope for growth is limited (Wieser et al. 1988a). In comparison to larger fish, larvae

require specific energetic solutions to accommodate the costs of their high growth: Kiørboe et al. (1987) and Kiørboe (1989) suggested that fast growth was possible because larval fish may have a reduced protein turnover in comparison to juvenile and adult fish. Houlihan et al. (1995a) challenged this view because larval nase *Chondrostoma nasus* (Houlihan et al. 1992) and larval herring *Clupea harengus* (Houlihan et al. 1995b) were found to have levels of protein turnover (as measured by the protein synthesis retention efficiencies) comparable to those observed in older fish.

An alternative strategy to reduce the costs of growth would be to reduce the costs of protein synthesis (Kiørboe 1989). On theoretical grounds, the minimal costs of protein synthesis can be estimated to be 40 mmol ATP.g^{-1}protein synthesised or 50 mmol ATP.g^{-1}protein synthesised when transport costs are included (Reeds et al. 1985). The costs of protein synthesis were estimated to be close to this theoretical minimum in herring embryos and larvae (Kiørboe et al. 1987; Kiørboe and Møhlenberg 1987) and in juvenile tilapia, *Oreochromis mossambicus* (Houlihan et al. 1993), but much higher costs of protein synthesis were reported for both larval nase (Houlihan et al. 1992) and larval herring (Houlihan et al. 1995b).

The objective of the present work was to investigate how larval African catfish *Clarias gariepinus* (Burchell) manage to grow rapidly and to accommodate the associated costs. The study focused on yolk-sac larvae in which growth rates may reach more than 100% body protein.day^{-1} (Conceição et al. 1993; Kamler et al. 1994). Rates of protein synthesis, degradation and growth were estimated, and their roles in the energy expenditure of the larvae were assessed.

Material and methods

Eggs of the African catfish, *Clarias gariepinus*, were obtained by artificial fertilisation (Hogendoorn and Vismans 1980) of eggs obtained from a broodstock held under standardised conditions (Richter et al. 1995) at the hatchery of the Department of Fish Culture and Fisheries, Wageningen Agricultural University. Five independent batches of eggs were produced, each using eggs from two females (individual wet weight ranging from 0.66 to 1.50 kg) and sperm from two males (0.47 to 1.01 kg). Fertilised eggs were incubated in a recirculation system at 28°C. After hatching, yolk-sac larvae were collected in 16 l aquaria receiving water from the same recirculating system. Larvae hatched from three batches of eggs were used for measurements of protein synthesis, those from a fourth batch were used to measure the effect of cycloheximide on protein synthesis and oxygen consumption, and the larvae hatched from a fifth batch of eggs were used for measurements of oxygen consumption and protein growth rates during development.

Protein synthesis and turnover

Two samples of about 300 larvae were taken and transferred to 100 ml bottles 34 h after fertilisation (i.e., 12 h after hatching). Each bottle had 50 ml tap water which was continuously aerated. The bottles were kept in a water bath at 28.0±0.1°C. A solution of 24 mM phenylalanine containing L-[2,6-^{3}H]phenylalanine at a concentration of 9.47 Mbq.ml^{-1} was prepared. From this solution, 2 ml were added to one of the bottles and 3 ml were added to the other to test the effect of isotope concentration on the measurement of protein synthesis. This experiment was repeated three times, using yolk-sac larvae from the three independent batches of eggs.

In a fourth test, two groups of larvae from the fourth batch of eggs were prepared as above, except that only one concentration of labelled phenylalanine (2 ml per 50 ml^{-1} tap water) was used. Cycloheximide was added to one of the bottles 3.5 h after starting the incubation, to give a concentration of 2 mg cycloheximide.ml^{-1} incubation solution.

In each of the four tests described above, three samples of 15 larvae were taken from each bottle at various times (0, 1, 2, 4, 6 or 8 h) after the addition of phenylalanine. Sampled larvae were rinsed in tap water and placed in water at 1-2°C until yolk-sac dissection (maximum of 20 min). Yolk-sacs were dissected under a binocular microscope, and the larval bodies kept at -20°C until further analysis. The samples were analysed within three weeks after sampling.

Each sample of 15 larvae was homogenised in 0.5 M perchloric acid and centrifuged to separate the free amino acid (AA) pool from the precipitated proteins. Following removal of the free pool, the pellet was washed, solubilised in 0.3 M NaOH and protein content determined (Lowry et al. 1951). Phenylalanine content in protein was estimated considering that one mg of larval protein contains 275 nmoles of phenylalanine (Houlihan et al. 1992). Phenylalanine contents of the free AA pool were determined by a fluorometric assay after conversion to ß-phenylethylamine (Suzuki and Yagi 1976). Phenylalanine standards were included in all determinations and the efficiency of phenylalanine recovery was measured (Houlihan et al. 1988). Liquid scintillation counts (dpm, disintegrations per minute) were measured both in the solubilised protein and in the free AA pool, using Hionic Fluor (Packard Instrument) as scintillation liquid. Specific radioactivity of protein-bound phenylalanine (Sb) and free pool phenylalanine (Sa) was determined by dividing liquid scintillation counts by phenylalanine contents (Houlihan et al. 1986). RNA contents were measured using the dual wavelength method (McMillan and Houlihan 1988) and results were expressed as RNA/protein ratio (µg RNA.mg^{-1}protein).

Fractional rates of protein synthesis (k_s, %protein weight (pw) per day) were estimated using two methods, the flooding dose method and the phenylalanine flux method developed in this study. To ensure that the labelled phenylalanine was homogeneously distributed amongst

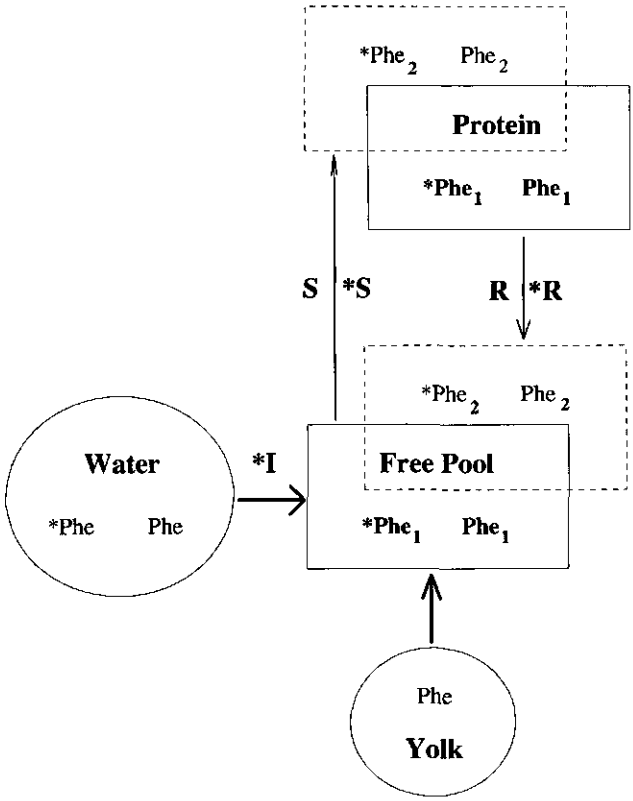

Figure 1. Model for the phenylalanine flux method. Four compartments are shown: body proteins, free AA pool, incubation water and yolk-sac. Except for the yolk-sac, two distinct pools are considered per compartment: (^{3}H) labelled phenylalanine (*Phe, dpm) and total phenylalanine (Phe, nmoles). Protein and free AA compartments are represented at two different times during an incubation ($_1$ and $_2$). Phenylalanine may be absorbed from the yolk-sac and both labelled (I*) and unlabelled phenylalanine may be taken up from the incubation water. Labelled (S*) and unlabelled (S) free phenylalanine are incorporated into protein. Protein degradation also releases labelled (R*) and unlabelled (R) phenylalanine into the free pool.

the different metabolic pools, rates of protein synthesis were estimated in the interval of 6 to 8 h of incubation.

In the flooding dose method the fractional rate of protein synthesis is calculated according to the equation (Garlick et al. 1980; Houlihan et al. 1988):

$$k_s = (Sb_2 - Sb_1) / (SA) \cdot 1/t \cdot 1440 \cdot 100$$

Where Sb_2 and Sb_1 are specific activities of protein-bound phenylalanine at two times of incubation, SA is the mean specific activity of the free pool phenylalanine in a time interval, t is this time interval in minutes and 1440 refers to the number of minutes per day. Protein degradation (k_d, %protein weight.day^{-1}) can be calculated by subtracting protein growth from protein synthesis:

$$k_d = k_s - k_g$$

The phenylalanine flux method was developed in an attempt to directly estimate both protein synthesis and degradation. Four compartments are considered (see Figure 1): body proteins, free AA pool (plasma and intracellular fluids), incubation water and yolk-sac. The data from the water and yolk compartments are not needed for further calculations. In the protein and free AA compartments, two pools of phenylalanine can be measured: (tritium) labelled phenylalanine (*Phe, dpm) and total phenylalanine (Phe, nmoles, labelled plus unlabelled). If these four pools are measured at two sampling times during the incubation, the rates of protein synthesis and degradation can be estimated. All data are expressed as dpm or nmoles per larva.

Within a certain time interval, the change in the amount of tritiated phenylalanine in the free pool (ΔF^*Phe, dpm) is the net result of the amounts of tritiated phenylalanine that enter the larva (I^*, dpm), plus that which re-enters the free pool due to protein degradation (R^*, dpm) and the amount that is incorporated into protein (S^*, dpm):

$$\Delta F^*\text{Phe (dpm)} = I^* + R^* - S^*$$

Similarly, the change in the amount of total phenylalanine in protein (ΔPPhe, nmoles) depends on the amount of phenylalanine incorporated into protein (S, nmoles) and that entering the free AA pool due to protein degradation (R, nmoles):

$$\Delta\text{PPhe(nmol)} = S - R$$

The specific activities (dpm.nmoles^{-1}phenylalanine) of the phenylalanine incorporated into protein (Ss) and the phenylalanine re-entering the free pool (Sr) can be assumed to equal the mean specific activities of phenylalanine in the free pool (SA) and in protein (SB), respectively:

$$Ss = SA = S^*/S$$

$$Sr = SB = R^*/R$$

The amount of tritiated phenylalanine (dpm) that entered the larva (I^*) can be calculated as the sum of the increases in the amounts of tritiated phenylalanine in the free pool (ΔF^*Phe, dpm) and in protein (ΔP^*Phe, dpm):

$$I^* (dpm) = \Delta F^*Phe + \Delta P^*Phe$$

With five equations and four unknowns (S^*, S, R^* and R) an algebraic solution is possible. The equations were solved using the program MERCURY (Real Software, P.O.Box 1680, Soquel, CA 95073, USA) to provide estimates of the four unknown parameters. These estimates were subsequently used to calculate the rates of protein synthesis (k_s, %pw.day^{-1}) and protein degradation (k_d, %pw.day^{-1}) using the following equations:

$$k_s = (S/275)/([Pro_1 + Pro_2]/2) .1/t .1440.100$$

$$k_d = (R/275)/([Pro_1 + Pro_2]/2) .1/t .1440.100$$

Where Pro_1 and Pro_2 are the protein contents of the larvae (mg) at the two sampling times, t is the time interval between the two samples, and 275 refers to the nmoles of phenylalanine in one mg of larval protein.

Protein growth rates (k_g, %pw.day^{-1}) were calculated assuming linear growth:

$$k_g = [(Pro_2 - Pro_1) / Pro_1] . 1/t . 1440 . 100$$

RNA efficiency (k_{RNA}, g protein synthesised.g^{-1}RNA.day^{-1}) was calculated as the ratio between k_s and the RNA to protein ratio, multiplied by 10 (Millward et al. 1973).

Oxygen consumption and cycloheximide

Oxygen consumption was measured using a flow-through respirometer. A constant flow (12 ml.min^{-1}) of 28.0±0.1°C tap water was pumped through two 150 ml chambers containing about 400 larvae each. A third chamber without fish was employed as a blank and used as a reference for the inlet water in the other chambers. The oxygen electrode (WTW TriOxmatic 300 sensor) received a water flow that was alternated from chamber to chamber at intervals of six minutes. This alternating water flow was automatically regulated by magnetic valves connected to a timer. Oxygen concentrations were measured by a precision oxygen meter (WTW Microprocessor-Precision-Oxygen Meter Oxi 3000, Weilheim, Germany). Oxygen concentration in outlet water was never less than 70% of air saturation. Measurements of oxygen consumption were started on larvae 12 h post-hatch and continued for 5 h. To determine the effect of cycloheximide, a solution of 2 mg cycloheximide.ml^{-1} tap water was fed through

one of the chambers during the first hour of measurements. Changes in oxygen consumption due to cycloheximide addition were compared with changes in protein synthesis rates as measured in parallel incubations of larvae from the same batch in labelled phenylalanine.

Oxygen consumption (VO_2 , nmol O_2 $larva^{-1}.day^{-1}$) in the cycloheximide and control chambers was calculated as:

$$VO_2 = ({}_bO_2 - {}_cO_2) \,.\, Flow \,.\, 1 \,/\, (n \,.\, 32) \,.\, 10^6$$

Where ${}_bO_2$ and ${}_cO_2$ are the oxygen concentrations (mg l^{-1}) in the outlet of the blank chamber and of a chamber with larvae respectively, Flow is the flow through the chambers ($l.day^{-1}$), n is the number of larvae in the chamber, and 32 the molecular weight of oxygen.

The costs of protein synthesis (Ck_s , mmol $ATP.g^{-1}$protein synthesised) were estimated as:

$$Ck_s = [({}_{co}VO_2 - {}_{cyc}VO_2) \,/\, (k_s \,.\, Pro)] \,.\, 100 \,.\, 6$$

Where ${}_{co}VO_2$ and ${}_{cyc}VO_2$ are the oxygen consumption (nmol O_2 $larva^{-1}.day^{-1}$) of the control and cycloheximide chamber, respectively, k_s the fractional rate of protein synthesis (%pw.day^{-1}), Pro the protein content (µg $larva^{-1}$) and one mol of oxygen consumed is assumed to be equivalent to the synthesis of 6 mol ATP (Reeds et al. 1985). The costs of protein turnover (Ck_t , mmol $ATP.g^{-1}$protein.h^{-1}), considering only the synthesis costs of the renewed protein, were calculated as:

$$Ck_t = (k_d \,.\, Ck_s) \,/\, (24 \,.\, 100)$$

Where k_d the fractional rate of protein degradation (%pw.day^{-1}).

Oxygen consumption and protein growth

Oxygen consumption measurements were made from hatching until complete yolk absorption, and for one day after this. Three 150 ml chambers containing 250 to 300 larvae each were monitored in the flow-through respirometer as described above. An extra chamber was used as a blank. In parallel to these oxygen measurements, larvae from the same batch of eggs were kept in 16 l aquaria at 28.0±0.2°C and sampled regularly for analysis of protein content. These data were collected to estimate growth rates and mass-specific rates of oxygen consumption (both in terms of protein mass). Pooled samples of 50-150 individuals were taken in triplicate every 12 hours, yolk-sacs were dissected and the larval bodies were stored at -20 °C. Following freeze-drying, the samples were analysed for nitrogen content using the Kjeldahl method, and protein content was estimated as N x 6.25.

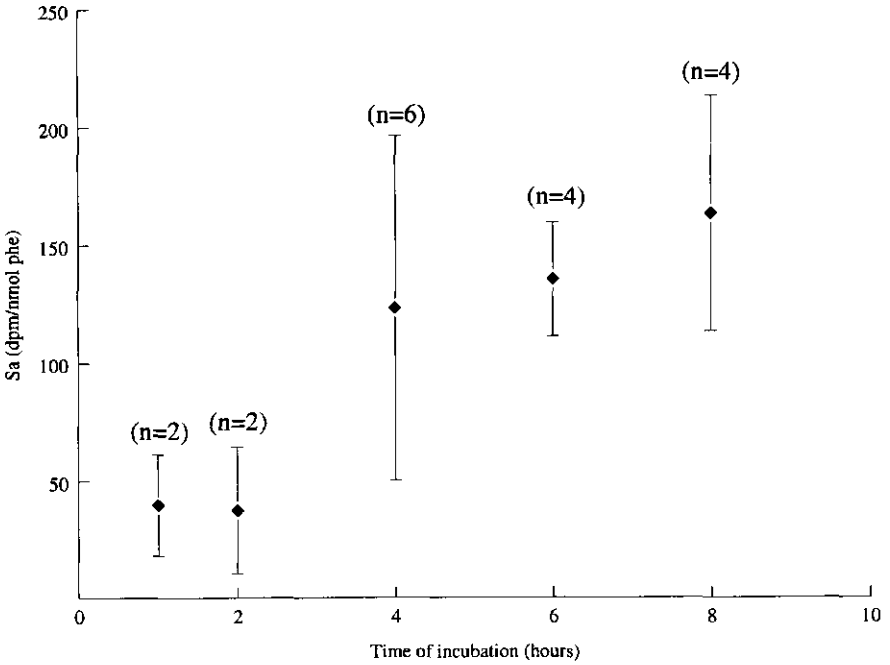

Figure 2. Specific activity of free phenylalanine (Sa) in yolk-sac larvae of *C. gariepinus* at various times after the addition of phenylalanine to the water (Means ± SD). Phenylalanine was added 34 h after fertilisation.

Statistics

Data were analysed using the SAS Statistical Software package (SAS Inc. 1995). For the protein synthesis measurements, the effects of phenylalanine concentration (CONC, 1 df), experimental tests (EXP, 2 df) and time of incubation (TIME, 5 df) were studied for the different parameters (Y) by analysis of variance (Proc GLM) using the model:

$$Y = \mu + EXP + CONC + TIME + EXP.CONC + EXP.TIME + CONC.TIME + EXP.CONC.TIME + e$$

The three samples of 15 larvae taken in each sampling point (sub-replicates) were used for the partitioning of the variance, but were not used to test differences between components. Both the effect of concentration and experimental test were tested against the interaction term EXP.CONC. When a significant effect of time of incubation was detected for a given parameter, a linear regression was performed (Proc REG). Student's t-test (Proc TTEST) was used to test pair-wise differences when required. Extreme values of the analytical sub-replicates were not considered for analyses when justified by the Dixon's test for outliers (Snedecor and Cochran 1989). Differences were considered significant when $p<0.05$.

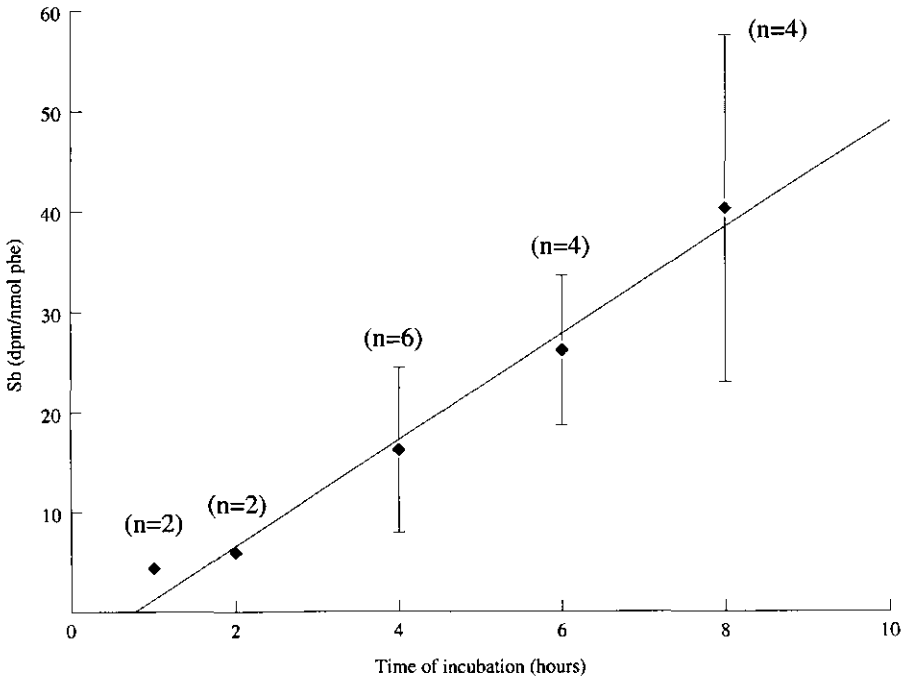

Figure 3. Specific activity of protein-bound phenylalanine (Sb) in yolk-sac larvae of *C. gariepinus* at various times after the addition of phenylalanine to the water (Means ± SD). Sb = -4.14 + 5.21 . Time of incubation (r^2=0.65, p<0.0001, n=18). Phenylalanine was added 34 h after fertilisation.

Results

Protein synthesis and turnover

No significant differences were found between the separate experimental tests and between the two phenylalanine concentrations in the incubation solution (ANOVA) in terms of the protein, free phenylalanine and RNA contents and in terms of specific radioactivity in the protein and free AA pool. Therefore, data from the three experimental tests and the two concentrations were pooled and considered as replicates of a single time course. The effect of time course was significant (ANOVA) for all the parameters except the specific radioactivity in the free AA pool. During the period of incubation (8 h), the phenylalanine content of the free pool (FP Phe, nmol.larva^{-1}) almost doubled:

$$FP\ Phe = 33.55 + 3.27 \cdot t \qquad (r^2=0.54,\ p<0.0001,\ n=24)$$

Table 1. Phenylalanine pools in yolk-sac larvae of *C. gariepinus* 6 and 8 h after addition of phenylalanine to the water, together with the estimates derived from the phenylalanine flux method.

Observed			Calculated	
	6 hours	8 hours		Interval 6 - 8 hours
Pro Phe (nmol)	210.3 (8.4)	229.2 (8.0)	S (nmol)	28.4
FP Phe (nmol)	48.2 (15.5)	62.5 (26.6)	R (nmol)	9.5
Pro *Phe (dpm)	5693.9 (35.3)	9137.7 (39.4)	S^* (dpm)	4290.5
FP *Phe (dpm)	6278.4 (35.5)	9776.3 (25.0)	R^* (dpm)	312.7
Sa (dpm.nmol^{-1})	135.4 (17.8)	164.3 (30.7)	I^* (dpm)	7475.7
SA (dpm.nmol^{-1})	151.2		k_s (%pw.day^{-1})	137.8
Sb (dpm.nmol^{-1})	25.5 (28.6)	40.5 (43.0)	k_d (%pw.day^{-1})	41.9
SB (dpm.nmol^{-1})	33.0		k_g (%pw.day^{-1})	95.9
Pro (µg)	764.0 (8.4)	832.7 (8.0)	t (min)	135

Means per larva and coefficients of variation (CV, sd / mean x 100) are given. Values (n = 4) are based on the pooled results of the three incubations. Pro - protein; FP - free AA pool; Phe - total Phenylalanine (labelled plus unlabelled); *Phe - labelled phenylalanine; Sa - specific activity of phenylalanine in free pool; Sb - specific activity of phenylalanine in protein; SA - mean specific activity of phenylalanine in free AA pool in the time interval; SB - mean specific activity of phenylalanine in protein in the time interval; S^* - labelled phenylalanine entering the protein compartment in the time interval; R^* - labelled phenylalanine re-entering the free pool in the time interval; S - total phenylalanine entering the protein compartment in the time interval ; R - total phenylalanine re-entering the free pool in the time interval; I^* - labelled phenylalanine entering the larvae in the time interval; t - duration of the time interval; k_s - fractional rate of protein synthesis; k_d - fractional rate of protein degradation; k_g - fractional rate of protein growth.

Where t is the time of incubation. The specific activity of phenylalanine in the free pool (Figure 2) increased with incubation time during the first 4 hours of incubation. Although it continued to increase in the period between 4 and 8 h, the specific activities between 6 and 8 h were not statistically different from each other (t-test $p<0.05$). The specific activity of protein-bound phenylalanine (Sb) increased linearly (Figure 3) from 0 to 8 h of incubation:

$$Sb = -4.14 + 5.21 \cdot t \qquad (r^2=0.65, p<0.0001, n=18)$$

Where t is the time of incubation. The values obtained for the different phenylalanine pools, together with the estimates obtained from the phenylalanine flux equations are shown in Table 1.

Protein growth (k_g) was 95.9%pw.day^{-1}. Fractional rates of protein synthesis (k_s) were 105.9%pw.day^{-1} when estimated by the flooding dose method or 137.8%pw.day^{-1} when estimated using the phenylalanine flux equations. Similarly, protein degradation (k_d) was calculated as 10.0%pw.day^{-1} or 41.9%pw.day^{-1} when using the flooding dose equation or the phenylalanine flux equations, respectively. In the further calculations, only the phenylalanine flux method values were considered because, unlike the flooding dose method, they account for recycling of labelled phenylalanine. Protein synthesis retention efficiency ($k_g.100.k_s^{-1}$) was 69.6%.

Both the larval RNA content (RNA, µg larva^{-1}) and RNA to protein ratio (RNA/Pro, µg RNA.mg^{-1}protein) increased linearly with time as expressed by the following regression equations:

$$RNA = 56.35 + 3.98 \,.\, t \qquad (r^2=0.92,\ p<0.0001,\ n=24)$$

$$RNA/Pro = 86.65 + 2.98 \,.\, t \qquad (r^2=0.40,\ p=0.0009,\ n=24)$$

Where t is the time of incubation. The RNA to protein ratio increased slowly and averaged 106.7 (±12.4) µg RNA.mg^{-1}protein between 6 and 8 h of incubation. RNA efficiency could be estimated as 12.9 g protein synthesised g^{-1}RNA.day^{-1} in this interval.

Costs of protein synthesis

After one hour, larvae exposed to cycloheximide showed a reduction in oxygen consumption of 43% when compared to the control group (80.6 nmol O_2 larva^{-1} h^{-1} versus 45.8 nmol O_2 larva^{-1} h^{-1} for a larva with 65.8 µg of protein content). This reduction was stable during the following 4 h of measurement. Rates of protein synthesis measured in larvae exposed to cycloheximide were zero. The costs of protein synthesis were estimated as 55.3 mmol ATP.g^{-1}protein synthesised. Costs of protein turnover were calculated as 1.06 mmol ATP.g^{-1}protein.h^{-1}.

Costs of protein growth

Oxygen consumption per larva increased until around 75 h after fertilisation, and then decreased (Figure 4). While the larvae were growing, larval protein content (Pro, µg) increased linearly with time (HPF, hours post-fertilisation):

$$Pro = -58.38 + 3.279 \,.\, k_g \qquad (r^2 = 0.99,\ p<0.0001,\ n=6)$$

Figure 4. Oxygen consumption (VO_2) per larva during development of yolk-sac and starved larvae of *C gariepinus*. Solid lines are means and dashed lines means ± SD. Complete yolk absorption occurred around 100 h after fertilisation.

Protein growth rate decreased as the larva increased in age (Figure 5). Mass-specific oxygen consumption (MO_2, nmol oxygen.mg^{-1}protein.h^{-1}) was correlated with the positive values of protein growth rate (k_g, %pw.day^{-1}):

$$MO_2 = 655.81 + 3.63 \,.\, k_g \qquad (r^2 = 0.98, p=0.0011, n=5)$$

The slope of this equation gives the costs of protein growth provided that differences in dimensions (hours *vs.* days, mg *vs.* %) are taken into account. In the present study, the costs of growth were 3.63 nmol O_2.mg^{-1}protein 24 100 or 52.3 mmol ATP.g^{-1}protein deposited. The intercept provides an estimate for the costs of maintenance (655.81 nmol oxygen.mg^{-1} protein.h^{-1}, i.e., 3.94 mmol ATP.g^{-1} protein.h^{-1}). Apparently protein turnover accounts for 27% (1.06 / 3.94 * 100) of the costs of maintenance in yolk-sac larvae. After complete yolk absorption, when the larvae were not fed and thus subjected to starvation stress, mass-specific oxygen consumption decreased when protein was lost (Figure 5).

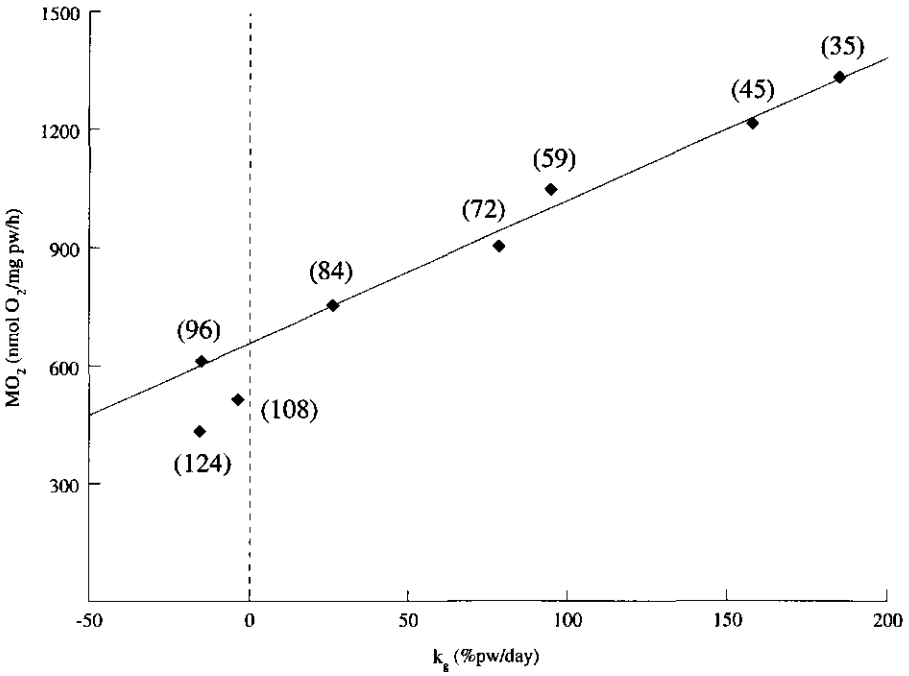

Figure 5. Mass-specific oxygen consumption (MO_2) against protein growth rate (k_g) during development of yolk-sac and starved larvae of *C. gariepinus*. Only the positive values for k_g were used to calculate the regression line (see text). Time (hours post-fertilisation) of the measurements are also given (values in brackets). Complete yolk absorption occurred around 100 h post-fertilisation. Vertical dashed line stands for zero protein growth.

Discussion

Methodology

Larval uptake of phenylalanine from the incubation medium was relatively slow, and an incubation time of 8 h was required to double the size of the free phenylalanine pool. The final concentrations of phenylalanine in the bathing solution (0.96 or 1.44 mM) were lower than used in previous studies (2.7 mM) (Houlihan et al. 1995a,b), but results of preliminary experiments (not shown) showed that there was no additional phenylalanine uptake when higher concentrations were used. Considering the linear increase in the specific activity of the protein compartment, and the high metabolic activity (e.g., fast growth) of the larvae, we assumed that the labelled phenylalanine was homogeneously distributed amongst the different metabolic pools. This assumption was corroborated by the continued, but slow, increase in specific activities of the free pool during the interval of 4 to 8 h of incubation. Therefore, the procedure

to measure protein synthesis and turnover rates only in the interval of 6 to 8 hours of incubation can be considered as appropriate. However, it did not seem reasonable to assume that no labelled phenylalanine incorporated into protein would re-enter the free pool during the measurement period as required by the flooding dose (Garlick et al. 1980; Houlihan et al. 1988) and other protein synthesis determination methods (Waterlow et al. 1978; Wolfe 1992; Toffolo et al. 1993). The phenylalanine flux method, developed in the present study, allows for recycling of labelled phenylalanine and permits a direct estimation of both protein synthesis and degradation. The conventional flooding dose equation underestimated protein synthesis rate by about 25% when compared to the phenylalanine flux equations. This new method may be of value in studies of fast growing organisms, when the assumptions of no-recycling of labelled phenylalanine and/or steady state of the protein pool may not be met. A major limitation of the method is its deterministic nature; calculation of error terms is not possible.

The costs of protein synthesis were estimated by measuring oxygen consumption and protein synthesis in the absence and presence of a protein synthesis inhibitor. Cycloheximide which has been widely used to inhibit protein synthesis in previous studies on whole fish (Nichols and Fleming 1990; Brown and Cameron 1991; Houlihan et al. 1992, 1995b) and on fish cell cultures (Kent and Prosser 1980; Saez et al. 1982; Pannevis and Houlihan 1992; Krumschnabel and Wieser 1994; Smith and Houlihan 1995) was also used in the present study. This approach assumes that cycloheximide has little or no effect on energy-dependent reactions other than protein synthesis (Aoyagi et al. 1988). Smith and Houlihan (1995) used a second protein synthesis inhibitor, actinomycin-D, in addition to cycloheximide and obtained similar inhibition results. In the present study, the similar estimates for the costs of protein synthesis through cycloheximide inhibition (55.3 mmol $ATP.g^{-1}$protein synthesised) and for the costs of protein growth by regression (Figure 5) of mass-specific oxygen consumption with protein growth rate (52.3 mmol $ATP.g^{-1}$protein deposited) gives a good support to the use of cycloheximide. The use of protein synthesis inhibitors is still the only method for direct estimation of the costs of protein synthesis, and if cycloheximide has an inhibitory effect on other metabolic processes, the costs of protein synthesis would be overestimated. In this study however, the costs of protein synthesis were close to the theoretical minima, hence the uncertainties of the method should not affect the final conclusions.

Protein synthesis and turnover

The whole body rate of protein synthesis (137.8%$pw.day^{-1}$) observed in the present study is amongst the highest measured in any vertebrate, although comparable values (90%$pw.day^{-1}$) were measured in larval tilapia (Houlihan et al. 1993). High values have also been reported for carp, *Cyprinus carpio* (Fauconneau 1984) and sturgeon, *Acipenser baeri* (Fauconneau et al.

1986b), but these probably represent overestimates due to the methodology used to measure protein synthesis (Houlihan et al. 1995a). In adult fish whole body rates of protein synthesis are usually between 1 and 3%pw.day^{-1} (Fauconneau 1985; Houlihan 1991), although tissues with high metabolic activity, e.g., liver, may display rates as high as 30%pw.day^{-1} (McMillan and Houlihan 1988; Houlihan 1991). In the present study the rate of protein degradation was quite high (41.9%pw.day^{-1}), and the protein synthesis retention efficiency was slightly higher (69.6%) than that of other fast growing fish larvae and juveniles (Houlihan et al. 1995a).

RNA concentrations and efficiencies

RNA concentrations (106.7 µg RNA.mg^{-1}protein) were considerably higher than previously reported for fish larvae (45 µg RNA.mg^{-1}protein) (Houlihan et al. 1995a). Comparable values were found in trout liver (McMillan and Houlihan 1988). The high RNA efficiency (12.9 g protein synthesised g^{-1}RNA.day^{-1}) observed in the present study is comparable to values found in 10 mg tilapia (Houlihan et al. 1993) and in mammals (Reeds and Davies 1992). In growing fish values typically range from 3 to 6 g protein synthesised g^{-1}RNA.day^{-1} (Houlihan et al. 1995a).

Costs of protein synthesis

Higher protein growth rates are usually associated with increased rates of protein synthesis and also with higher protein turnover rates, both in fish (Houlihan et al. 1986, 1988) and in mammals (Waterlow et al. 1978). Consequently, it can be expected that the rate of protein turnover will be higher in a growing organism than in an organism at maintenance (zero) growth. Therefore, in growing fish the costs of growth include the energy needed for protein turnover above the costs of protein turnover at maintenance. In fact, the costs of protein growth are usually higher than the costs of protein synthesis (Houlihan 1991). However, in the present study the estimates for the costs of protein synthesis (by cycloheximide inhibition) and for the costs of protein growth (by regression of mass-specific oxygen consumption on growth rate) were identical. This indicates that costs associated with protein deposition other than the synthesis costs are insignificant, i.e., that the rate of protein turnover is fixed, and independent of the rate of protein synthesis. Unchanged rates of protein turnover at different protein synthesis rates have been observed before in larval nase (Houlihan et al. 1992). So, the costs of protein growth in yolk-sac larvae of *C. gariepinus* are almost entirely attributable to the costs of protein synthesis because protein turnover remains fixed, although at a high rate.

Both direct (cycloheximide inhibition) and indirect (regression of mass-specific oxygen consumption on growth rate) evidence indicate that yolk-sac larvae of *C. gariepinus* synthesise

protein at a cost very close to theoretical minima. These results agree with data for tilapia (Houlihan et al. 1993), but contrast with the estimates for larval nase (Houlihan et al. 1992) and for larval herring (Houlihan et al. 1995b), and also differ from the general value for the costs of growth calculated by Wieser (1994).

The low estimate for the costs of protein synthesis may be related to the high protein synthesis rates measured because costs of protein synthesis have been reported to be inversely related with rates of protein synthesis in trout hepatocytes (Pannevis and Houlihan 1992), larval nase (Houlihan et al. 1992) and in different cell lines (Smith and Houlihan 1995). Using data for seven species of aquatic ectothermic metazoans, Wieser (1994) calculated that the costs of growth decrease asymptotically with increasing growth rate. Costs of growth were estimated to be 66 mmol ATP.g^{-1}dry weight for animals growing faster than 16.8% body dry weight.day^{-1}. Growth rates show a positive correlation with rates of protein synthesis (Fauconneau et al. 1986b; Houlihan et al. 1992). The decreased costs of protein synthesis at higher growth rates may also explain the lack of correlation between oxygen consumption and growth rates in yolk-sac larvae of rainbow trout, *Oncorhynchus mykiss*, (Rombough 1994) and in larval herring (Houlihan et al. 1995b), as well as the independence of oxygen consumption and growth rate at high growth rates in larval whitefish, *Coregonus wartmanni*, and roach, *Rutilus rutilus* (Wieser and Medgyesy 1990a,b). Rates of protein synthesis decrease with size in a similar pattern to growth rates (Fauconneau et al. 1986a; Houlihan et al. 1993). This may explain the higher costs of protein synthesis found in larger fish. Costs of growth in juvenile *C. gariepinus* (growing up to 7.5%pw.day^{-1}) have been calculated as 186 mmol ATP.g^{-1}protein (Jobling 1985). The high protein synthesis costs for larval nase (Houlihan et al. 1992) and larval herring (Houlihan et al. 1995b) are also probably explained by the relatively low growth rates observed (0 to 15%pw.day^{-1}).

How do fish larvae grow so fast?

According to the results of the present study, the hypothesis that larval fish have a low protein turnover to save energy for fast growth (Kiørboe 1989) does not hold. Protein turnover in yolk-sac larvae of *C. gariepinus* appears to be comparable to that of older fish. Similar conclusions were drawn from studies with larvae of other fish species (Houlihan et al. 1995a). The fast growth observed in the present study was apparently attained through high rates of protein synthesis at minimal costs. High rates of protein synthesis may be associated with high temperatures (28°C in the present study, 26.5°C for tilapia as reported by Houlihan et al. 1993), which probably allow for a very high production of protein per unit of RNA. However, they may also be correlated with high RNA contents.

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Chapter 3

Cost of growth in larval and juvenile African catfish (*Clarias gariepinus*) in relation to growth rate, food intake and oxygen consumption

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Abstract

The effect of fish size on the cost of growth, growth rates, food intake and oxygen consumption was studied in *Clarias gariepinus*. Measurements were performed on larvae reared at 28°C, and results compared to those for juveniles (recalculated from literature). As *C. gariepinus* grows (0.07 mg to 38 g BDW), the cost of growth increases from 64 to 149 mmol ATP.g^{-1}DW deposited. A higher cost of growth is associated with reduced growth rates (from 141 to 2.4% BDW.day^{-1}), and is also reflected in lower gross food conversion efficiencies (93 to 24%). Decreasing growth rates are a result of reductions in relative rates of food intake (152 to 6.2 % BDW.day^{-1}) and are accompanied by a reduction in oxygen consumption (1.0 to 0.033 µmol O_2.mg^{-1}DW.h^{-1}). Fish size was allometrically related to growth rate, food intake and oxygen consumption. The absence of any distinct breakpoint in these relations suggests a gradual transition from the larval to the juvenile pattern of fish growth and metabolism. Providing that optimal conditions are met (e.g., temperature, oxygen availability and feeding) the pattern of fast growth at low cost might be extended for a longer time span during the transition period from the larval to the juvenile stage, resulting in improved growth and food conversion efficiency.

Introduction

Growth rates (per unit of body mass) decrease as animals increase in size. Increasing body mass gives rise to a diminishing mass-specific oxygen consumption (Oikawa and Itazawa 1984; Wieser et al. 1988). Growth is an energy demanding process and it accounts for a large portion of the total energy expenditure (Jobling 1985; Wieser 1994). Fish larvae grow very rapidly in comparison with older fish. It has been suggested that either the cost of depositing each unit of body mass or the cost involved in maintenance functions are reduced at high growth rates allowing fish larvae to accommodate both the costs of growth and maintenance (Wieser and Medgyesy 1990; Wieser 1994). Wieser (1994) suggested that the cost of growth decreases asymptotically as growth rate increases: the cost of growth was estimated to be 66 mmol ATP.g^{-1}dry weight (DW) deposited for animals growing faster than 16.8% body dry weight (BDW).day^{-1}. However, there is no evidence that the cost of growth change in such a pattern for individuals within a given species growing at different rates.

The cost of growth has been largely attributed to the cost of protein deposition (Jobling 1985; Brown and Cameron 1991; Houlihan 1991). Therefore, the biochemical cost of protein synthesis might be expected to set the lower limit for the cost of growth. When increasing rates of protein synthesis are associated with an increase in protein degradation above its rate at zero growth, the cost of protein deposition (mmol ATP.g^{-1}protein deposited) (and thus the cost of growth) will be greater than the cost of protein synthesis (mmol ATP.g^{-1}protein synthesised). However, if the rate of protein degradation remains constant the cost of protein deposition will be equal to the cost of protein synthesis. Interestingly, the value of 66 mmol ATP.g^{-1}DW deposited estimated by Wieser (1994) is quite close to the 50 mmol ATP.g^{-1}protein synthesised (based on 5 mol ATP needed per peptide bond) estimated to be the minimum biochemical cost of protein synthesis (Reeds et al. 1985).

The primary goal of this study was to investigate the relationship between growth rates and the cost of growth, within a single species. Growth rates, food intake and oxygen consumption were measured in yolk-sac larvae and in fed larvae of the African catfish *Clarias gariepinus* (Burchell), and results were compared with values recalculated from published data for juveniles of the same species (Hogendoorn 1983). The relationships between growth rates, estimated cost of growth and food conversion efficiencies at different fish sizes were analysed, and the findings were discussed in the perspective of growth optimisation in fish culture operations.

Material and methods

Fish

Eggs of the African catfish, *C. gariepinus*, were obtained by artificial fertilisation (Hogendoorn and Vismans 1980) of eggs from a broodstock held under conditions described by Richter et al. (1995). Eggs produced by two females (individual wet weight ranging from 0.70 to 1.58 kg) were mixed with the sperm from two males (0.72 to 1.96 kg) and were incubated in a recirculation system at 28.0±0.2 (daily fluctuation)°C. Offspring originating from one batch of eggs were used for measurements during the yolk-sac stage. Growth and oxygen consumption of exogenously fed larvae were measured on offspring from a second egg batch. Offspring from three additional batches of eggs were used for estimation of the consumption of *Artemia* during larval development.

Rearing, sampling and body weight

Yolk-sac larvae hatched about 22 hours after fertilisation, and were reared at 28.0±0.2°C in 16 l aquaria until complete yolk absorption. The aquaria had a continuous water flow (0.2 to 0.5

$l.min^{-1}$) from a recirculation system, and gentle aeration. The larvae were not fed. Larvae were sampled at 26, 45, 59, 72 and 84 hours post-fertilisation for determination of dry matter content. At each sampling point, three samples of 50-150 individuals were taken both for whole larvae and for larval bodies (whole larvae minus yolk-sac). Samples were stored at -20 °C until dry matter determination. Samples were freeze dried, and dry weights determined (±10µg) using a Mettler balance (AE160).

For the experiments conducted on fed larvae, groups of 1200 larvae were transferred to each of three 16 l aquaria when yolk absorption was almost complete (48h after hatching). Larvae were reared in a recirculation system, with 5 to 10% water renewal per day and UV filtration. The aquaria had a flow rate of 1 to 1.5 $l.min^{-1}$, and were kept at 28.0±0.3°C with the water continuously aerated. During 10 days, the larvae were fed *Artemia* nauplii 5 times a day (equal portions) at intervals of 3.5h between 8.30 to 22.30h. Daily ration was calculated to be near satiation according to Verreth and den Bieman (1987), allowing for maximum growth. *Artemia* cysts (strain Great Salt Lake, Sanders Co.) were incubated for 24 hours in 35‰ salt water at 28°C. Newly hatched nauplii were stored in salt water at 4°C. A sample of 10 to 50 larvae was taken from each of the three aquaria for dry matter determination at 0, 2, 3, 4, 5, 6, 8, and 9 days of exogenous feeding, before the first daily meal. Samples were stored at -20°C until analysed. Larvae were dried overnight at 70°C, then kept at 103°C for 4h, and DW measured (±10µg) with a Mettler balance (AE160).

Body DW was used to estimate growth rates and mass-specific rates of oxygen consumption. Relative growth rates (RGR, $\%BDW.day^{-1}$) were calculated as (Ricker 1958):

$$RGR = (e^{g}-1).100 \qquad \text{with} \quad g = (\ln BDW_2 - \ln BDW_1) / (t_2 - t_1)$$

where BDW_2 and BDW_1 are the body DW in samples taken at t_2 and t_1.

Food intake measurements

Food intake (FI, $\%BDW.\ day^{-1}$) for yolk-sac larvae was estimated based on the absorption of yolk material between two sampling points:

$$FI = (YDW_1 - YDW_2) / [(BDW_1 + BDW_2) / 2] / (t_2 - t_1) \,.\, 100$$

where BDW_2, BDW_1, YDW_1 and YDW_2 are the body DW and yolk DW ($mg\ .\ individual^{-1}$) at two sampling times (t_2 and t_1). Yolk DW was calculated as the difference between the whole larvae DW and the body DW at each sampling point.

Food intake of fed larvae was estimated on days 2 and 8. Decapsulated *Artemia* cysts were hatched as previously mentioned, but they were held in salt water containing L-[2,6-

^{3}H]phenylalanine at a specific activity of 2.04 Tbq.mmol^{-1}phenylalanine, to give a final concentration of 12.6 kbq.ml^{-1}. During hydration *Artemia* cysts take up considerable amounts of dissolved amino acids (D'Agostino 1980). Newly hatched nauplii were fed to groups of approximately 400 (day 2) or 180 (day 8) larvae held in bottles containing 200 ml of continuously aerated tap water. The bottles were kept in a water bath at 28.0±0.1°C. After 30 min 6 samples of 5 larvae (day 2) or 2 larvae (day 8) were taken by siphoning and well rinsed with tap water. Satiation time for *C. gariepinus* fed *Artemia* is about 30 min, and within that period no material is defecated (Haylor 1993). The experiment was repeated three times, always at the first meal of the day. Samples were placed into scintillation vials to which one ml tissue solubiliser was added (Soluene 100, Packard Instrument). Vials were incubated for 2h at 50°C to aid solubilisation. After cooling, 4 ml scintillation liquid (Hionic Fluor, Packard Instrument) was added and samples were counted for radioactivity (dpm, disintegrations per minute). Samples of newly hatched *Artemia* were also prepared in the same manner and counted. Food intake (FI, , %BDW. day^{-1}) was estimated as:

$$FI = [(R_{larva} / SR_{Artemia}) / BDW_{larva}] . 5. 100$$

where R_{larva} is the total radioactivity per larva (dpm.larva^{-1}), $SR_{Artemia}$ is the specific radioactivity in *Artemia* samples (dpm.mg^{-1}DW), BDW_{larva} the body DW (mg DW.larva^{-1}) and 5 the number of meals in one day. Although we only measured the first meal of the day, we assumed that the food intake in the 5 meals of the day was identical.

Oxygen consumption measurements

Oxygen consumption was measured using a flow-through respirometer. A constant flow (12 ml.min^{-1}) of 28.0±0.1°C tap water was pumped through 150 ml chambers containing the larvae. The flow was maintained by a peristaltic pump, and measured gravimetrically every hour after collection of outlet water for 1 min. One chamber without fish was always used as blank and as a reference for the inlet water in the other chambers. The oxygen electrode (WTW TriOxmatic 300 sensor) received a continuous water flow alternating from chamber to chamber at intervals of 6 min. This alternating water flow was automatically regulated by magnetic valves connected to a timer. Oxygen concentrations were measured by a precision oxygen meter (WTW Microprocessor-Precision-Oxygen Meter Oxi 3000, Weilheim, Germany). Oxygen concentration in outlet water was never less than 70% of air saturation.

Oxygen consumption of yolk-sac larvae, was monitored in three chambers containing 250 to 300 larvae each. Measurements were taken from hatching until complete yolk absorption. For the fed larvae, oxygen consumption was measured on days 2, 5, and 8 of exogenous

feeding. Measurements were performed at the time of the first meal of the day (about 10 hours after the preceding meal), both in larvae fed a single meal (fed group) and in unfed larvae (fasted group). The fed group was allowed to feed for 30 min in the growing tanks, and rapidly transferred to the measurement chambers. Two chambers containing 41 to 213 larvae were used for each group. Measurements of oxygen consumption were taken for 8 h, and no food was given during the measurement period. To estimate the daily oxygen consumption of fed larvae, only the first 3.5h after the meal were considered, and extrapolated to the following four meals.

Oxygen consumption (MO_2, nmol $O_2.mg^{-1}DW.h^{-1}$) was calculated as:

$$MO_2 = ({}_bO_2 - {}_cO_2) \,.\, Flow \,/\, (n \,.\, BDW \,.\, 32) \,.\, 10^6$$

where ${}_bO_2$ and ${}_cO_2$ are the oxygen concentrations ($mg.l^{-1}$) in the outlet of the blank chamber and of a chamber with larvae, respectively, Flow is the flow through the chambers ($l.h^{-1}$), n is the number of larvae in the chamber, BDW the mean body DW (mg), 32 the molecular weight of oxygen and 10^6 is a factor to correct for units.

Estimation of the cost of growth

The cost of growth was estimated assuming that total oxygen consumption (MO_2, nmol $O_2.mg^{-1}DW.day^{-1}$) is composed of two parts: the energy needed for maintenance and the energy required for growth and associated processes (Smith 1957; Wieser 1994; Rombough 1994). This can be formalised as:

$$MO_2 = COM + COG \,.\, RGR$$

where COM (nmol $O_2.mg^{-1}DW.day^{-1}$) represents the cost of maintenance, COG (nmol $O_2.mg^{-1}DW$ deposited) the cost of growth, and RGR (%BDW.day^{-1}) the relative growth rate.

The cost of growth (COG, mmol $ATP.g^{-1}DW$ deposited) for yolk-sac larvae was calculated by regression of mass-specific oxygen consumption (MO_2, nmol $O_2.mg^{-1}$ $DW.day^{-1}$) on relative growth rate (RGR, %BDW.day^{-1}). The slope of this equation estimates the cost of growth (Wieser 1994; Rombough 1994) provided that differences in units are taken into account. The unit "(nmol $O_2.mg^{-1}DW.day^{-1}$) / (%BDW.day^{-1})" can be transformed to "mmol $ATP.g^{-1}DW$ deposited" if one mol of oxygen consumed is taken to be equivalent to the synthesis of 6 mol ATP (Reeds et al. 1985). The intercept of the regression provides an estimate for the metabolic rate at zero growth (cost of maintenance). Also here units can be converted to "mmol $ATP.g^{-1}DW$ deposited. day^{-1}".

For fed larvae, the cost of growth was estimated by subtracting the oxygen consumption of fed larvae from the oxygen consumption of fasted larvae, and dividing this by RGR.

Metabolism of the fasted larvae was assumed to be indicative of maintenance metabolism. Oxygen consumption of fed larvae ($_{fed}MO_2$, nmol O_2.mg^{-1}DW.day^{-1}) was calculated as:

$$_{fed}MO_2 = 5.\int^{3.5}{}_{fed}MO_2 + \int^{6.5}{}_{fast}MO_2$$

where 5 is the number of meals in a day, $\int^{3.5}{}_{fed}MO_2$ the cumulative oxygen consumption in the first 3.5h after a meal in fed larvae, and $\int^{6.5}{}_{fast}MO_2$ is the cumulative oxygen consumption in the 6.5h after start of the measurements in the fasted group. The interval between meals was 3.5h, and it was assumed that oxygen consumption in the five meals of the day was identical. Similarly, oxygen consumption for unfed larvae ($_{fast}MO_2$, nmol O_2. mg^{-1}DW.day^{-1}) was calculated as:

$$_{fast}MO_2 = 4 .\int^{6}{}_{fast}MO_2$$

The cost of growth (COG, mmol ATP.g^{-1}DW deposited) was then estimated as:

$$COG = (\,_{fed}MO_2 - {}_{fast}MO_2) / RGR \,.\, 6$$

where 6 is the factor to convert moles of oxygen consumed to moles of ATP synthesised (Reeds et al. 1985).

The cost of growth for juveniles of *C. gariepinus* (data from Hogendoorn 1983) were calculated as for fed larvae, using oxygen consumption measurements of growing fish and fish at maintenance.

Literature experiments

The data presented for *C. gariepinus* juveniles studied by Hogendoorn (1983) were analysed. In that study, growth, food intake, and oxygen consumption were measured for four size groups of *C. gariepinus*, with initial sizes of approximately 1.5, 7.5, 45 and 95 g fresh weight. Fish were reared for a period of 21 to 42 days at 25°C. For each size group, fish were reared at five feeding levels, i.e., deprivation, maintenance (zero growth), optimal (maximum conversion efficiency), and two and three times optimal, as estimated by preliminary results. In the present study only the data referring to fish growing at maximum rates in each of the size groups are presented. Oxygen consumption data of fish at maintenance were also used for estimation of the cost of growth.

Table 1. Mass-specific oxygen consumption (MO_2), food intake (FI), relative growth rate (RGR) and gross conversion efficiency (K_1) at different body DW (BDW) of *C. gariepinus*.

Stage	Age[1]	BDW (mg)	MO_2 (nmolO2.mg^{-1} DW.h^{-1})	FI (%BDW.day^{-1})	RGR (%BDW.day^{-1})	K_1 (%)
Yolk-sac	35 hpf	0.07 (0.003)	990.6 (37.8)	152.1	141.0	92.7
larvae	45 hpf	0.12 (0.009)	823.9 (54.9)	-	124.7	-
	59 hpf	0.18 (0.005)	722.1 (38.2)	95.0	78.1	82.2
	72 hpf	0.26 (0.012)	581.2 (35.2)	73.0	69.4	95.2
	84 hpf	0.31 (0.002)	464.6 (29.9)	38.4	28.2	73.9
Fed	2 def	0.56 (0.005)	438.6 (46.7)	51.5 (2.8)	36.4	70.6
Larvae	5 def	1.77 (0.097)	341.8 (16.1)	-	67.2	-
	8 def	5.02 (0.030)	317.4 (3.55)	51.5 (1.0)	40.8	79.3
Juveniles[2]		851	106.9	27.9	8.6	30.8
		6449	57.7	14.8	7.6	51.1
		17833	54.1	15.7	3.7	23.8
		37685	32.8	6.2	2.4	37.9

Standard deviations (pooled samples) are given in brackets. n = 3 for BDW and FI; n = 2 for MO_2.

[1] Age is given in hours post-fertilisation (hpf) or days of exogenous feeding (def).

[2] Recalculated from Hogendoorn (1983). MO_2 values were adjusted from 25 to 28°C using a Q_{10} of 2.0 (Machiels and Henken, 1986).

Statistics

Student's t-tests (Proc TTEST), and linear regressions (Proc REG) were performed with the SAS Statistical software package (SAS Inc. 1995). To calculate allometric relations a logarithmic transformation was applied to the data.

Results

Weight gain, food intake, oxygen consumption, and gross food conversion efficiencies (K_1, mg growth / mg food intake .100) of the fish of different sizes are shown in Table 1. Yolk-sac larvae had higher food intakes, growth rates and (mass-specific) rates of oxygen consumption than both fed larvae and juvenile *C. gariepinus*. Highly significant correlations were found

Table 2. Cost of growth (COG) and cost of maintenance (COM) at different body DW (BDW) of *C. gariepinus*.

Stage	Age[1]	BDW (mg)	COM (μmol ATP.mg^{-1}DW.h^{-1})	COG (mmolATP.g^{-1}DW)
Yolk-sac larvae		0.07 to 0.31	1.964 (0.346)	63.5 (9.2)
Fed Larvae	2 def	0.56	1.744 (0.099)	56.1
	5 def	1.77	1.264 (0.058)	51.3
	8 def	5.02	1.202 (0.088)	49.4
Juveniles[2]		851	0.364	148.9
		6449	0.238	95.8
		17833	0.148	121.0
		37685	0.121	94.4

Standard deviations (pooled samples) are given in brackets. n = 2 for COM of fed larvae.

[1] Age is given in hours post-fertilisation (hpf) or days of exogenous feeding (def). [2] Recalculated from Hogendoorn (1983). COM values were adjusted from 25 to 28°C using a Q_{10} of 2.0 (Machiels and Henken, 1986).

between BDW (mg) and relative growth rate (RGR, %BDW.day^{-1}), food intake (FI, %BDW.day^{-1}) and mass-specific oxygen consumption (MO_2, nmol O_2.mg^{-1}DW. h^{-1}):

$$RGR = 57.62 \,.BDW^{-0.276} \qquad (r^2 = 0.95,\ n = 10,\ p < 0.001)$$

$$FI = 70.76 \,.BDW^{-0.185} \qquad (r^2 = 0.91,\ n = 8,\ p < 0.001)$$

$$MO_2 = 469.8 \,.BDW^{-0.236} \qquad (r^2 = 0.99,\ n = 10,\ p < 0.001)$$

These regressions refer to a range of BDW from 70 μg to 37.7 g. Only the first three sampling points in the yolk-sac stage were considered. The latter two were not included since yolk supply was limiting growth (Conceição et al. 1993). From the data on juveniles (Hogendoorn 1983) only the groups growing at maximum rates were used in the allometric relations.

As *C. gariepinus* got bigger the relative growth rate decreased faster than did food intake and this was reflected in a decrease in K_1 (Table 1). Food conversion efficiencies were approximately 90, 65, and 30% for yolk-sac larvae, fed larvae and juvenile fish, respectively.

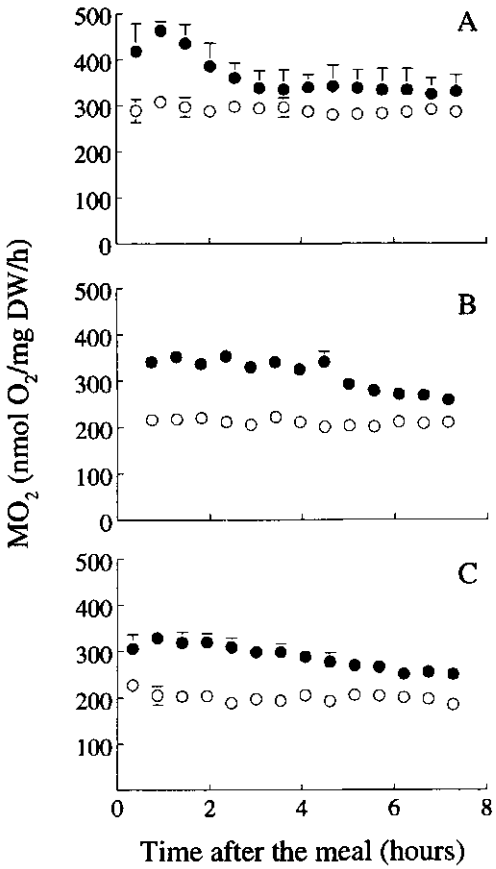

Figure 1. Mass-specific oxygen consumption (MO_2) in larvae of *C. gariepinus* after a single meal of *Artemia* nauplii at (A) day 2, (B) day 5 and (C) day 8 of exogenous feeding. Values are given for fed (●) and fasted larvae (○). Error bars are standard deviations of two measurements in groups of larvae.

Estimates of the cost of growth and the cost of maintenance at different sizes for the three stages are given in Table 2. The cost of growth in yolk-sac larvae was calculated as 63.5 mmol ATP.g^{-1}DW deposited from the relation:

$$MO_2 = 327.3 + 4.407 \,.\, RGR \qquad (r^2 = 0.94,\ n = 5,\ p < 0.001)$$

For larvae fed *Artemia* the cost of growth was estimated as 56.1 (day 2), 51.5 (day 5) and 49.4 (day 8) mmol ATP.g^{-1}DW deposited, based on the rates of oxygen consumption in Figure 1.

The cost of growth estimated for juveniles growing at maximum rates ranged from 94.4 to 148.9 mmol ATP.g^{-1}DW deposited.

Concerning the cost of maintenance, two distinct phases during ontogeny can be recognised. The difference between the cost of maintenance in yolk-sac larvae and in larvae on day 2 of exogenous feeding was not significant (t-test, $P>0.05$). After day 2 the cost of maintenance (COM, µmol ATP.mg^{-1}DW.h^{-1}) decreased. The COM of juvenile fish was calculated by adjusting measured values (Hogendoorn 1983) to 28°C by using a Q_{10} of 2.0 (Machiels and Henken 1986). From day 2 of exogenous feeding onwards, the following allometric relationship was obtained:

$$COM = 1.586 \cdot BDW^{-0.211} \qquad (r^2 = 0.98, n = 7, p < 0.001)$$

Based on the cost estimates given in Table 2, the partitioning of the total oxygen consumption between growth and maintenance was predicted (Figure 2). The two smaller juvenile size groups suggest that the fish would need considerably more oxygen than that measured to satisfy maintenance and growth. Yolk-sac larvae seem to allocate more energy to growth than older fish.

Discussion

Larvae of *C. gariepinus* have high growth rates (36.4 to 141%BDW.day^{-1}) and high food conversion efficiencies (70.6 to 95.2%, dry matter basis) when compared with the larvae of other fish species (Houde 1989; Wieser and Medgyesy 1990; Keckeis and Schiemer 1992; Rombough 1994), in which growth rates range from 1 to 35%BDW.day^{-1}, and conversion efficiencies from 10 to 66%. Growth rates (2.4 to 8.6%BDW.day^{-1}) and food conversion efficiencies (23.8 to 51.1%, dry matter basis) of juvenile *C. gariepinus* are comparable to those in juveniles of other fish species. Bowen (1987) listed a series of species growing close to maximum rates, where growth rates ranged from 1.2 to 6.6%BDW.day^{-1}, and food conversion efficiencies (protein basis) ranged from 21 to 48%.

Accurate estimation of the cost of growth relies on the assumption that the costs of growth and maintenance are additive, i.e., that the cost of maintenance will not change with different sizes and/or feeding levels, and that the cost of growth is constant (Rombough 1994). In at least some of the groups of juvenile fish the cost of maintenance may have been reduced with increasing feeding level to accommodate the cost of growth. This is suggested by the sums of the costs of maintenance and growth exceeding 100% in some groups (Figure 2), and also by the considerable variation in the estimates of the cost of growth (Table 2). Such a reduction in the cost of maintenance to accommodate the cost of growth has been suggested before (Kiørboe

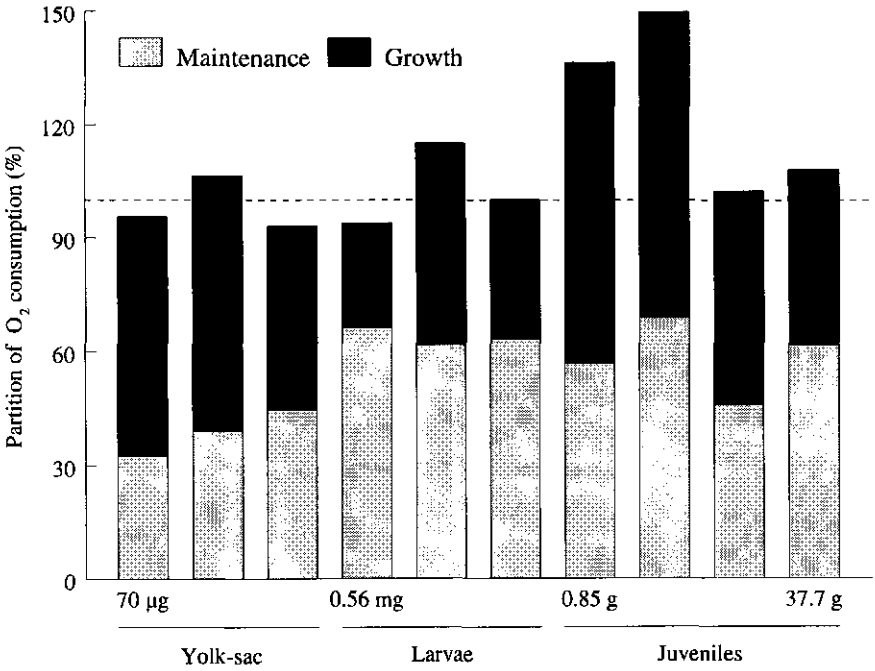

Figure 2. Partition of oxygen consumption between maintenance (grey bars) and growth (black bars) at the different larval sizes of *C. gariepinus*. Based on the COM and COG estimates from Table 2.

1989; Wieser and Medgyesy 1990; Wieser 1994; Rombough 1994). If this happens to be the case in juvenile *C. gariepinus*, then at least some of the estimates for the cost of growth in the present study are underestimated. The estimates for the cost of growth in fed larvae were obtained by extrapolating to the five meals of the day the postprandial oxygen consumption of one meal. This could bring some bias to the estimates if the different meals would be of different sizes. However, the measurements were done after the first meal of the day which would tend to be the larger (so would oxygen consumption), as the larvae were unfed for 10 hours. Thus, if an error exists it should be an overestimation of the cost of growth, which seems unlikely as these values are already close to the minimum theoretical value (see below).

In larval *C. gariepinus* the estimates for the cost of growth (49.4 to 63.5 mmol $ATP.g^{-1}DW$ deposited) were close to the minimum biochemical cost of protein synthesis (50 mmol $ATP.g^{-1}$protein synthesised). A comparable low cost of growth has been found for herring embryos and larvae (Kiørboe and Møhlenberg 1987; Kiørboe et al. 1987) and turbot larvae growing between 15.4 and 22.8%$BDW.day^{-1}$ (Conceição et al., unpublished data). The cost of

protein synthesis was also close to the biochemical minimum in yolk-sac larvae of *C. gariepinus* (Conceição et al., unpublished data) and in fast growing juvenile tilapia (Houlihan et al. 1993). However, in juvenile *C. gariepinus* growing at maximum rates, the cost of growth seems to be at least two to three times the minimum biochemical cost of protein synthesis. Fish larvae growing at slower rates (Wieser and Medgyesy 1990; Houlihan et al. 1992,1995b) have a cost of growth several times higher than this minimum theoretical value. Wieser (1994) based on literature data for a wide range of species, proposed that in general the cost of growth in aquatic ectotherms would be about three times the biochemical cost of protein synthesis. The present study shows that *C. gariepinus* has a variable cost of growth, the cost of growth being reduced at high growth rates. We recalculated data for Japanese whiting, *Sillago japonica* (Oozeki and Hirano 1994) and estimated that the cost of growth was 60 mmol $ATP.g^{-1}DW$ deposited for larvae growing at $34\%BDW.day^{-1}$, while in larvae growing at $18\%BDW.day^{-1}$ the cost of growth was 134 mmol $ATP.g^{-1}DW$ deposited. Thus, it seems that individual species follow the pattern that was previously suggested based on an inter-specific comparison (Wieser 1994). The cost of growth is variable, decreasing towards the minimum biochemical cost of protein synthesis at high growth rates.

It is hypothesised that it is the concurrent high rates of protein synthesis and not the high growth rates that are the cause of the reduced cost of growth. The cost of protein synthesis was shown to decrease with augmenting rates of protein synthesis in larval nase (*Chondrostoma nasus*)(Houlihan et al. 1992), in trout hepatocytes (Pannevis and Houlihan 1992) and in different cell lines (Smith and Houlihan 1995). It has been proposed that this would be due to the existence of a fixed component of this cost, independent of the rate of protein synthesis (Pannevis and Houlihan 1992). Houlihan et al. (1995a) suggested that a constant activation of tRNA and a constant production of rRNA will contribute to this fixed energy expenditure. At low growth rates this fixed component of the cost of protein synthesis will have a large contribution to the cost of growth when compared with the cost of peptide-bond formation.

High growth rates in fish larvae seem to be associated with a low cost of growth and high food conversion efficiencies. The present paper suggests that the transition from larval to juvenile patterns of fish growth and metabolism is a gradual process. If growth rates reach levels that allow for a reduced cost of growth, food conversion efficiencies will increase, allowing for higher growth. Thus, higher growth may bring an amplification effect on growth itself. Once fish are reared under optimal conditions, e.g., oxygen levels, feeding regime, diet quality and water temperature, the larval pattern of high growth rates / low cost of growth / high food conversion efficiencies might be extended for a longer time span in the larval / juvenile transition period. Conversely, sub-optimal conditions will probably lead to increasingly restrained growth rates and food conversion efficiencies.

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Chapter 4

Growth and cost of growth in turbot larvae (*Scophthalmus maximus*) fed natural zooplankton or *Artemia*

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Abstract

The present study analysed the influence of different food regimes on growth, body composition, and the cost of growth during the larval stage of turbot (*Scophthalmus maximus*). Costs of growth and maintenance were estimated, and discussed in terms of their importance in the energy budget. In general, body composition in terms of protein, total lipids and ash contents was stable throughout the larval period. The contribution of glycogen to body composition increased slightly, and the contribution of free amino acids was small but variable. Larvae fed enriched *Artemia* grew less and had a higher lipid content than larvae eating natural zooplankton. When using standard enrichments, *Artemia* may cause sub-optimal larval growth due to their high lipid content. High growth rates in turbot larvae are associated with the deposition of new tissues at a cost close to the theoretical minimal cost of protein synthesis.

Introduction

High mortalities and low growth rates are common during the larval stage of many fish species, including the turbot (*Scophthalmus maximus* L.). These mortalities and growth depressions are often related to feeding regimes which do not meet the nutritional requirements of the larvae (e.g. Støttrup and Attramadal 1992; Reitan et al. 1993). Larviculture of marine species relies on feeding regimes based on live food (Watanabe and Kiron 1994). This makes manipulation of diet composition more difficult, despite considerable progress in enrichment procedures for the brine shrimp *Artemia* and the rotifer *Brachionus plicatilis* (e.g., Léger et al. 1987; Sorgeloos et al. 1988; Rainuzzo et al. 1994; Watanabe and Kiron 1994). An alternative to these cultured live organisms, is the use of collected natural zooplankton. Turbot larvae are known to prefer copepod nauplii to rotifers (Kuhlmann et al. 1981) and to select for larger prey size with increasing larval size (van der Meeren 1991b; Cunha and Planas 1995).

Besides an adequate feeding regime, larval growth also depends on food conversion efficiency (growth / food intake). The food conversion efficiency decreases if the energetic cost of growth increases. Growth is expensive; a minimum of 50 mmol ATP is needed per gram of protein synthesised (Reeds et al. 1985). The cost of protein synthesis can be used as a minimum

estimate for the cost of growth (Conceição et al. 1997a). The cost of protein synthesis in larval fish ranges from the minimum value mentioned above (Houlihan et al. 1993; Conceição et al. 1997a; Conceição et al. 1997b) to values several times higher (Houlihan et al. 1992; Houlihan et al. 1995). Besides the cost of protein synthesis, the cost of growth includes the cost of protein turnover above the maintenance level, the cost of lipid deposition, and the costs of food search, capture, and assimilation. In addition to the cost of growth, larval fish need energy for maintenance functions. In fast growing fish larvae maintenance and growth may compete for the (limited) amount of energy available (Wieser et al. 1988). The relative importance of the various energy substrates to energy expenditure has been extensively studied in turbot and other species during the egg and yolk-sac stage (e.g. Rønnestad et al. 1992, 1994; Finn et al. 1995, 1996). However, little is known about the energetics in feeding stages of marine fish larvae.

The present study analysed the influence of different food regimes on growth and body biochemical composition of larval turbot. In addition, the costs of growth and maintenance in larval turbot were estimated after measurements of oxygen consumption, and their importance in the larval energy budget was discussed. In another paper, growth rates of the same batch of turbot larvae will be related to the fluxes of amino acids in the larvae, and to the larval amino acid profile in both protein and the free pool.

Materials and methods

Fish, rearing and sampling

One batch of turbot (*Scophthalmus maximus* L.) eggs was obtained by artificial fertilisation of 3 females and 3 males from the broodstock kept at the Austevoll Aquaculture Research Station, Norway. Eggs and larvae were incubated at 15.1±0.5°C in conical plastic tanks (70 litre) with intermittent aeration and a limited water flow in order to maintain a gentle water circulation. Two groups of 15000 larvae were transferred to two 1500 litre tanks 2 days after hatching. The larvae were reared in these tanks until day 26 after hatching, under natural photoperiod (August, 60°N), at a temperature of 18.0±0.3°C and a salinity of 34.3±0.2‰. Water flow was progressively increased from 0.3 (day 2) to 1.4 $l.min^{-1}$ (day 26), giving a daily exchange rate of the rearing volume of 28.8 to 134.4%. To optimise rearing conditions, green water was used. The algae *Isochrysis galbana* and *Tetraselmis* sp. were continuously added to the water supply to maintain a concentration between 50 and 100 million cells $litre^{-1}$ in the tanks.

During the experiment, the larvae were sampled for analysis of gut content, oxygen consumption and body composition. Up until day 11 larvae were sampled from the two tanks in a 1:1 basis. After day 10 samples from each tank were kept separate, as the two tanks were then fed different live food organisms (see below). Samples for body composition were taken in the

morning, before or shortly after the first daily addition of live food to the tanks, on days 3, 7, 10, 16, 20, 23 and 26 after hatching. At each sampling point, pooled samples of 20 (until day 20) or 10 (after day 20) larvae were taken for body composition. For each parameter (dry weight and ash, total lipids, protein, free amino acids, and glycogen) four different samples were taken. Samples were rinsed in tap water, dried on a sieve, placed into Nunc cryo tubes and stored at -20°C until further analysis.

The samples for determination of gut content were taken in the evening between 19:00 and 21:00 h, on days 4, 6, 7, 10, 11, 13, 16, 17, 21, 23, 24 and 26 after hatching. The collected larvae were fixed in 4% formaldehyde buffered with hexamethylenetetramine. In each sample the gut of 10 larvae was removed and opened under a dissecting microscope. The gut content was analysed with a 40 to 80X magnification, with both light and dark field illumination.

Larvae were transferred to a laboratory for oxygen consumption measurements on day 24 after hatching. Before measurements started, the larvae were allowed to acclimate for two hours in a glass beaker (5 litres sea water) at 18.0±0.05°C with gentle aeration.

Food supply

Live food was added to the rearing tanks twice a day, in the morning between 9:00 and 11:00, and in the afternoon between 16:00 and 18:00. Daily ration was calculated from a theoretical model of larval bioenergetics for turbot (van der Meeren 1991b), and corrected for temperature with a Q_{10} factor of 2.3. Daily energy requirements were calculated according to an expected relative growth rate (RGR) of 20% body weight day^{-1}. To simplify the feeding procedure, values used for average energy content of the different food categories were 3.3, 5.4, 4.4, 37.0, and 370.0 $mJ.ind^{-1}$ for rotifers, copepod nauplii, *Artemia* nauplii, copepodites, and copepods, respectively (calculated from Schauer et al. 1980; Emmerson 1984; Böttger and Schnack 1986; Blom et al. 1991; van der Meeren 1991b). Since mortality could not be estimated during rearing, the larval population size in a tank was only corrected for the number of larvae sampled. However, the amount of live food added to the tanks was sometimes limited by availability, leading to daily rations below the calculated requirements (Figure 1).

During the first two weeks post-hatch all the larvae received both rotifers and natural zooplankton (Table 1). On days 12 to 14, one of the tanks (group ART) received both natural zooplankton, rotifers and *Artemia* nauplii, and after that only enriched *Artemia* nauplii. The other tank (group ZOO), continued to receive natural zooplankton until the end of the experiment (Table 1).

Rotifers (*Brachionus plicatilis*) were raised in continuous cultures in 250 litre tanks with 34‰ sea water at 20°C. Rotifers were fed bakers yeast and the alga *Isochrysis galbana*.

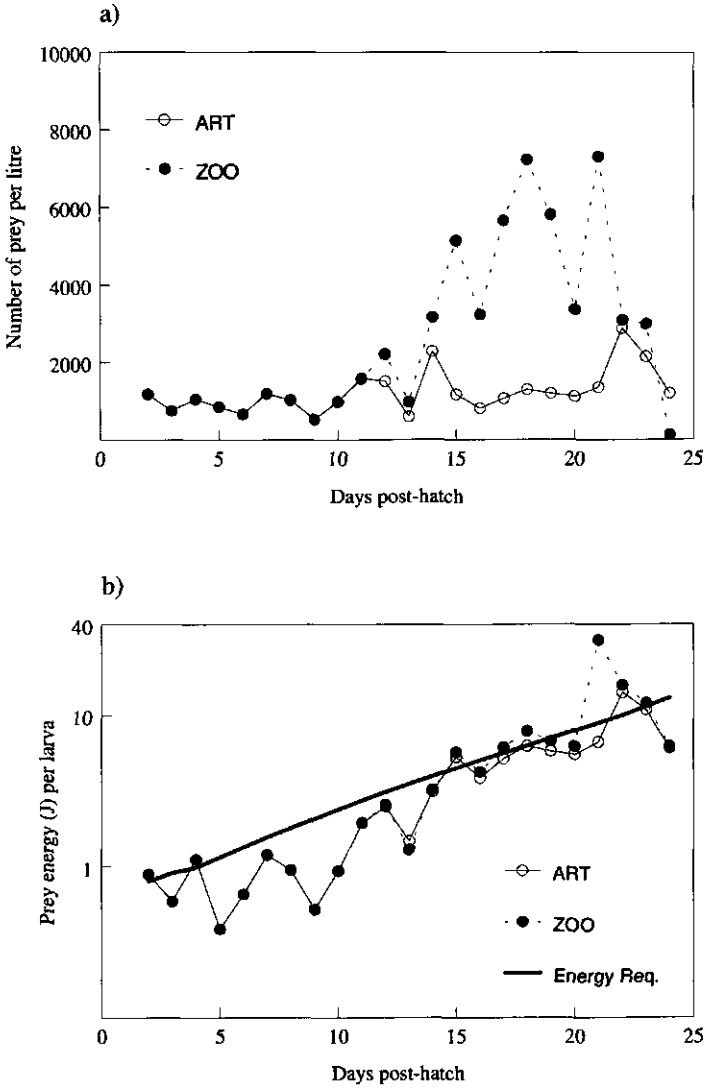

Figure 1. Live food presented daily to turbot larvae fed natural zooplankton or *Artemia* nauplii. a) prey per litre of tank volume. b) Prey energy per larvae together with the estimated requirements by the model of van der Meeren (1991b) for a relative growth rate of 20% body weigh.day^{-1}.

Table 1. Feeding regime of turbot larvae fed natural zooplankton (ZOO) or *Artemia* (ART).

Group	ZOO	ART
Feeding regime:		
Brachionus plicatilis	day 2-14	day 2-14
Zooplankton (80-180µm)	day 2-11	day 2-11
Zooplankton (80-250µm)	day 11-17	day 11-14
Zooplankton (80-350µm)	day 17-19	
Zooplankton (80-500µm)	day 19-23	
Zooplankton (>350µm)	day 23	
Zooplankton (>80µm)	day 24	
Artemia		day 12-24

Zooplankton composition and size fraction are explained in the text.

Natural zooplankton was collected from a large salt water basin of 20000 m^3 (Svartatjønn) close to the Austevoll Aquaculture Research Station. To enhance the zooplankton production, the basin was fertilised and mixed as described by Naas et al. (1991). Zooplankton (mostly nauplii, juveniles and adult stages of copepods) were collected by a UNIK wheel filter which concentrated two different size fractions of the plankton (van der Meeren 1991a). About 90% (in number) of the zooplankton consisted of one species, the copepod *Acartia grani*. The remaining 10% was composed of a mixture of *Centropages hamatus*, *Eurytemora affinis* and Harpacticoids. To compensate for the increasing energy demand of the growing larvae, prey size was increased (Table 1) by expanding the higher mesh size of the collecting wheels in the filter. Towards the end of the rearing period, the cladoceran *Evadne normannii* also became abundant in the plankton filtrate. However, it was not accounted for in the ration calculations due to its low energy content (Blom et al. 1991).

After decapsulation, *Artemia franciscana* cysts (EG type, *Artemia* Systems) were hatched at 28°C in 50 litre conical plastic tanks containing 34‰ sea water diluted 1:1 with tap water. After hatching (24 hours) the nauplii were harvested on a plankton net, washed in sea water and transferred to an enrichment tank. Enrichment (16 hours) was conducted at 25°C in a 250 litre tank with an *Artemia* density not higher than 50 nauplii.ml^{-1}. An emulsion of 0.1 mg.ml^{-1} Superselco pre-enriched with vitamin C (*Artemia* Systems), and mixed with 0.1

$mg.ml^{-1}$ high grade fish meal (SSF-microfeed, The Norwegian Herring Meal and Oil Research Institute) was used.

Oxygen consumption

Oxygen consumption was measured by flow-through respirometry on day 24 in both group ART and group ZOO. A constant flow (7 $ml.min^{-1}$) of filtered sea water was pumped through cylindrical chambers (150 ml) containing 8 to 10 larvae, placed in a water bath at 18.0 ± 0.05 °C. Eight chambers were used simultaneously, two blanks and three for each group. An oxygen electrode (WTW TriOxmatic 300 sensor) received a continuous water flow alternating from chamber to chamber at intervals of six minutes. This alternating water flow was automatically regulated by magnetic valves connected to a timer. Oxygen concentrations were measured by a precision oxygen meter (WTW Microprocessor-Precision-Oxygen Meter Oxi 3000, Weilheim, Germany). Oxygen concentration in the outlet water was never below 70% of air saturation. Measurements started at 22:00 h, after 2 hours of acclimation in the chambers, and lasted for 14 hours. To estimate the oxygen consumption of fed and fasted larvae the readings from the first and last 2 hours (3 readings per chamber), respectively, were averaged.

Growth and Biochemical composition

Dry weight (DW) and ash contents were determined in the same samples using a Mettler M3 microbalance (accuracy ±0.1 µg). Samples were first freeze-dried, weighed, and then ashed in a muffle oven at 600°C for 12 hours. Relative growth rates (RGR, % day^{-1}) were calculated assuming exponential growth as:

$$RGR = (e^{g} - 1) * 100 \quad \text{with} \quad g = [\ln(DW_2) - \ln(DW_1)] / (t_2 - t_1)$$

where DW_2 and DW_1 are the larval dry weights at two sampling times t_2 and t_1.

Protein content (crude protein) was determined as total nitrogen determined by the Kjeldahl method multiplied by 6.094. It has been estimated that turbot larvae protein contains 6.094 grams of protein per gram of nitrogen (Conceição et al. 1997c). Total lipids were determined gravimetrically after extraction in chloroform / methanol using a modification (Rønnestad et al. 1995) of the method of Bligh and Dyer (1959). A Mettler balance (AT 261, ±10µg) was used to determine sample dry weight and total lipids.

Free amino acids (FAA) were extracted for about 24 hours in 6% (final concentration) trichloroacetic acid. After centrifugation (10 min at 15000 x g) the supernatants were analysed in an automatic amino acid analyser (Chromaspeck J180, Hilger Analytical) with fluorimetric detection (OPA reagent) and high pressure loading.

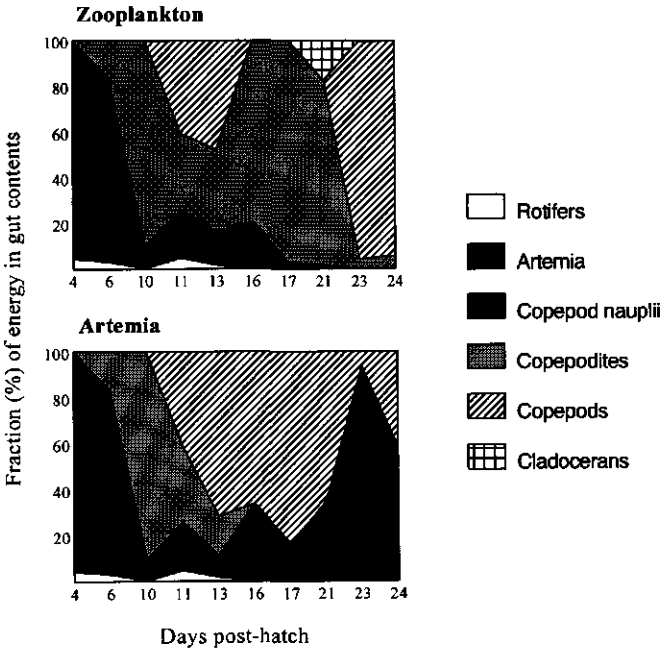

Figure 2. Fraction of each prey type (energy basis) observed in the gut contents of turbot larvae fed natural zooplankton until day 14, followed by natural zooplankton (top) or *Artemia* nauplii (bottom).

Glycogen (including free glucose) contents were analysed in freeze-dried samples using the enzymatic method of Hemre et al. (1989). After enzymatic hydrolysis, glucose was measured spectrophotometrically in an automatic analyser (Technicon RA-1000).

Larval contents ($\mu g.individual^{-1}$) of each biochemical fraction were calculated after dividing by the number of larvae in each sample. Biochemical composition (% of DW) was calculated by dividing sample weights by the respective dry weights. All determinations were done with four replicates.

Plankton samples were also analysed for dry weight, protein contents, free amino acids and total lipids, as above. These samples were prepared by filtration of known volumes, with known plankton concentrations, into pre-ashed fibre glass filters (1 µm).

Statistical analysis

Values are given as means ± standard deviations. Differences were considered significant when $p<0.05$. Results were analysed through the SAS package using one-way ANOVA (Proc GLM), linear regression (Proc REG) and Student's t-test (Proc TTEST) where appropriate.

Results

Gut contents

Initially, the larvae ate mostly copepod nauplii, but after day 10 copepodites and adult copepods were dominant in the gut contents (see Figure 2). The group which received exclusively *Artemia* after day 14 (group ART) also had a significant contribution of copepods in the gut contents until day 21.

Biochemical composition and growth

Between days 23 and 26 larvae fed *Artemia* had a considerable lower growth rate than larvae fed natural zooplankton. Ash contents were around 16% of dry weight throughout the larval period in both groups (Table 2). Protein contents seem were fairly constant at around 68% of dry matter; they were only significantly lower on days 23 and 26 for the *Artemia*-fed group (65 %). Free amino acids contributed only 1 to 4 % of the larval dry weight (Table 2). Total lipid contents fluctuated around 16.5% of larval dry weight. However, significantly higher lipid contents were found at first feeding (day 3) (30.9%), and at day 26 for the *Artemia*-fed group (19.5%). Glycogen contents increased with larval size (Table 2). Although *Artemia*-fed larvae had a decrease in glycogen content at day 16, by day 26 they had a value similar to the larvae fed natural zooplankton.

The biochemical composition of the natural zooplankton showed an increase in protein, FAA and total lipids contents with increasing size (Table 3). The protein/lipid ratio was lower for both enriched *Artemia* nauplii (3.2) and rotifers (4.1) than for natural zooplankton (5.4 to 8.9).

Oxygen consumption

Larvae which had been feeding consumed around 250 nmol $O_2.mg^{-1}DW.h^{-1}$ on day 24 (Table 4). Oxygen consumption of fasted larvae was somewhat lower, around 190 nmol $O_2.mg^{-1}$ $DW.h^{-1}$. The estimated cost of growth (deposition of dry matter) was around 42 mmol $ATP.g^{-1}DW$ (Table 4). Taking the energy expenditure of fasted larvae as an estimate for the cost of maintenance, 24 days old turbot larvae allocated 75% of their energy expenditure to maintenance and 25% to growth.

Discussion

Biochemical composition and growth

In general, the relative growth rates (RGR) were similar or slightly higher than values published elsewhere for turbot larvae (van der Meeren 1991b; Støttrup and Attramadal 1992; Planas et al.

Table 2. Relative growth rate (RGR) and composition of dry matter (DW) in larval turbot fed natural zooplankton (ZOO) or *Artemia* (ART). Fractions (%) measured were ash, protein, free amino acids (FAA) , total lipids and glycogen.

Group	Day	DW (µg)		RGR (%/day)	Ash (%)	Protein (%)		FAA (%)		Total Lipids (%)		Glycogen (%)	
ZOO	3	24.6 ± 4.4			24.0 ± 0.4 a	70.1 ± 4.6 a		4.2 ± 0.3 a		30.9 ± 3.4 a			
	7	74.1 ± 10.1		31.8	15.4 ± 4.8 b	69.9 ± 4.0 a		2.7 ± 0.3 bc		11.7 ± 1.5 b		2.9 ± 0.5 ab	
	10	134.7 ± 11.6		22.1	17.5 ± 1.9 b	69.9 ± 4.0 a		2.0 ± 0.1 bc		13.6 ± 1.5 b			
	16	672.8 ± 65.3		30.7	14.5 ± 1.3 b	71.4 ± 2.7 a		2.9 ± 0.5 b		14.0 ± 0.4 b		2.5 ± 0.2 b	
	20	2967.5 ± 276.5		44.9	16.7 ± 0.5 b	64.4 ± 0.7 a		2.0 ± 0.1 c		16.8 ± 1.6 b			
	23	6062.7 ± 510.8		26.9	16.3 ± 1.1 b	66.6 ± 0.9 a		2.3 ± 0.3 bc		15.0 ± 1.6 b			
	26	12003.6 ± 1359.9		25.6	16.2 ± 0.4 b	69.0 ± 0.2 a		2.0 ± 0.6 bc		14.1 ± 1.7 b		3.5 ± 0.2 a	
ART	16	827.4 ± 30.5	**	35.3	14.7 ± 1.0 b	70.4 ± 0.3 a		1.7 ± 0.2 b	**	14.9 ± 0.4 b	*	2.0 ± 0.1 b	**
	20	2925.5 ± 129.0		37.1	16.9 ± 0.5 a	62.7 ± 1.6 b		2.5 ± 0.2 a	**	15.5 ± 0.3 b			
	23	6800.7 ± 240.8	*	32.5	16.1 ± 0.6 a	64.1 ± 0.3 b	**	1.6 ± 0.2 b	*	17.0 ± 0.1 ab			
	26	10794.6 ± 474.8		16.6	16.3 ± 0.2 a	64.8 ± 1.0 b	**	1.3 ± 0.1 b		19.5 ± 3.0 a	*	3.5 ± 0.4 a	

Values are means ± sd of pooled samples of 10-20 larvae (n=4). Values with different letters within each column and group are significantly different (ANOVA, $p<0.05$). Significant differences between groups on the same day are shown as * ($p<0.05$) and ** ($p<0.01$).

Regression lines are: $DW_{ZOO} = 0.0099.e^{0.275 . Age}$ ($r^2 = 0.99$, $p < 0.0001$, $n = 28$); $DW_{ART} = 0.0103.e^{0.275 . Age}$ ($r^2 = 0.99$, $p < 0.0001$, $n = 28$).

Table 3. Composition of plankton dry matter (DM). Fractions (%) measured were protein, free amino acids (FAA) and total lipids.

Type	Day	DW (µg/ind)	Protein (%)	FAA (%)	Lipids (%)
Rotifers	10	0.73 ± 0.13	31.8 ± 0.9 bc	0.035 ± 0.003 cd	7.7 ± 0.8 ab
Zooplankton	9	0.60 ± 0.14	28.0 ± 5.6 c	0.024 ± 0.005 d	4.4 ± 0.7 c
Zooplankton	18	0.76 ± 0.03	37.6 ± 0.8 b	0.116 ± 0.016 bc	6.9 ± 0.1 b
Zooplankton[1]	25	1.25 ± 0.26	49.5 ± 2.1 a	0.193 ± 0.043 b	9.2 ± 1.5 a
Zooplankton[2]	25	14.81 ± 1.77	50.9 ± 6.1 a	0.426 ± 0.060 a	5.7 ± 0.2 bc
Artemia[3]	25	3.73 ± 0.51	31.1 ± 0.8 bc	0.145 ± 0.038 b	9.8 ± 0.3 a

Values are means ± sd. Significant differences within each column are shown by different letters (ANOVA, followed by a Bonferroni t-test, $p < 0.05$).

[1] small zooplankton (80 - 500 µm). [2] big zooplankton (> 350 µm). [3] enriched *Artemia* nauplii.

1993; Reitan et al. 1993; Rainuzzo et al. 1994). The marked decrease in growth rate in the *Artemia*-fed larvae between days 23 and 26 was associated with an increase in lipid contents. This may reflect a dietary nutrient imbalance, as food intake seemed comparable to the larvae fed natural zooplankton (Figure 3). Such high lipid contents were also observed in turbot larvae growing at similar (lower) growth rates (Planas et al. 1993), and it was suggested that this can be related to a high lipid content in enriched *Artemia*. Rainuzzo et al. (1994) also found that turbot larvae with the highest lipid content were the ones fed with *Artemia* containing the highest lipid concentration. In the present study a high lipid to protein content was found for enriched *Artemia* when compared to the natural zooplankton (Table 3). Diets with high lipid to protein content have been shown to increase lipid content in adult turbot with a concurrent protein sparing action (Bromley 1980; Andersen and Alsted 1993). The increase in lipid content and lower growth in *Artemia*-fed turbot larvae may be a result of impaired lipid metabolism. Fish larvae fed with *Artemia* displayed large intracellular lipid droplets in the gut epithelium, which was associated with an inadequate synthesis of lipoproteins due to cell immaturity (Loewe and Eckmann 1988; Deplano et al. 1991). Further, the use of other cultivated food items for fish larvae has also resulted in an overload of lipids in the gut epithelial cells (Kjørsvik and Opstad 1989; Deplano et al. 1991; Kjørsvik et al. 1991a). In contrast, such a lipid accumulation was not found in cod (*Gadus morhua*) larvae fed natural zooplankton (Kjørsvik et al. 1991b). The nutritional quality of *Artemia* may then be sub-optimal in terms of larval growth, as has

Table 4. Mass specific oxygen consumption (R) for turbot larvae fed natural zooplankton or *Artemia* and then fasted for 12 hours on day 24, together with relative growth rates (RGR) and cost of growth (COG).

Food		R (nmol O_2/mg DW/h)	RGR (%/day)	COG[1] (mmol ATP/g DW)
Zooplankton	Fed	255.8 ± 34.6	22.8	47.4
	Fast	180.8 ± 17.6		
Artemia	Fed	248.1 ± 47.2	15.4	44.1
	Fast	200.9 ± 23.3		

Values are means ± sd of measurements on groups of 8 to 10 larvae (n = 9).

[1] $COG = [(R_{fed} - R_{fast}) / (RGR / 100 / 24) / 1000] . 6$ (Conceição et al. 1997a). One mol of oxygen consumed is assumed to be equivalent to the synthesis of 6 mol ATP (Reeds et al. 1985).

been suggested before (Clemmesen 1987; Planas et al. 1993).

Growth depression in the *Artemia*-fed group was only observed at the end of the experiment, probably because copepods were a major contribution to the diet until day 21 (see Figure 2). This high fraction of adult copepods found in the gut of the larvae fed exclusively on *Artemia* after day 14 (group ART) can only be explained by the development of a population of copepods in the rearing tanks from uneaten zooplankton. Results referring to this «*Artemia*-fed group», until day 21, should therefore be viewed with caution as copepods contributed considerably to their food consumption.

Cost of growth

In spite of the high growth rates (16.6 to 25.6% body dry weight.day^{-1}), turbot larvae spend a considerable proportion (75%) of the energy expenditure in maintenance. This is possible as they deposit new tissues at low costs (see Table 4), close to the theoretical minimal cost of protein synthesis (50 mmol ATP.g^{-1}protein synthesised, Reeds et al. 1985). This supports the hypothesis that fast growing fish larvae can grow at this minimal cost as recently shown for larvae of the African catfish (*Clarias gariepinus* Burchell) (Conceição et al. 1997a,b). Fish larvae growing at lower rates (Wieser and Medgyesy 1990) as well as juvenile fish (Jobling 1985) and mammals (Reeds et al. 1985; Waterlow and Millward 1989) have costs of growth 3 to 5 times higher than the theoretical minimal value. A cost of growth similar to the cost of

protein synthesis implies that the energy expenditure in the other processes involved in growth is of little significance when compared to the energy needed to synthesise proteins.

There seems to be a close relationship between the cost of growth and growth rate. The cost of growth decreases asymptotically with increasing growth rates, towards the minimum theoretical cost of protein synthesis (Wieser 1994; Conceição et al. 1997a). The lowest growth rates found in this study still allowed for the minimum cost of growth. However, if growth rates would be further reduced due to qualitative or quantitative deficiencies in the feeding regime, this would probably lead to a higher cost of growth. A higher cost of growth would be associated with a lower food conversion efficiency, and thus even lower growth rates. Therefore, growth depression in turbot larvae will be amplified through an increase in the cost of growth.

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Chapter 5

Amino acid metabolism and protein turnover in larval turbot (*Scophthalmus maximus*) fed natural zooplankton or *Artemia*

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Amino acid metabolism and protein turnover in larval turbot (*Scophthalmus maximus*) fed natural zooplankton or *Artemia*

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Abstract

The present paper studied the influence of different food regimes on the free amino acid (FAA) pool, the rate of protein turnover, the flux of amino acids, and their relation to growth of larval turbot (*Scophthalmus maximus* L.) from first feeding until metamorphosis. The amino acid profile of protein was stable during the larval period although some small, but significant, differences were found. Turbot larvae had proteins which were rich in leucine and aspartate, and poor in glutamate, suggesting a high leucine requirement. The profile of the FAA pool was highly variable and quite different from the amino acid profile in protein. The proportion of essential FAA decreased with development. High contents of free tyrosine and phenylalanine were found on day 3, while free taurine was present at high levels throughout the experimental period. Larval growth rates were positively correlated with taurine levels, suggesting a dietary dependency for taurine and/or sulphur amino acids. Reduced growth rates in *Artemia*-fed larvae were associated with lower levels of free methionine, indicating that this diet is deficient in methionine for turbot larvae. Leucine might also be limiting turbot growth as the different diet organisms had lower levels of this amino acid in the free pool than was found in the larval protein. A model describing the amino acid flux in growing turbot larvae was presented. It was estimated that the daily dietary amino acid intake might be up to 10 times the larval FAA pool. In addition, protein synthesis and protein degradation might daily remove and return, respectively, the equivalent of up to 20 and 10 times the size of the FAA pool. In an early phase (day 11) high growth rates were associated with a relatively low protein turnover, while at a later stage (day 17), a much higher turnover was observed.

Introduction

Most published work on larval dietary requirements in turbot as in other species, focuses on the essential polyunsaturated fatty acid requirements for survival and growth (e.g. Witt et al. 1984; Planas et al. 1993; Reitan et al. 1993; Rainuzzo et al. 1994). However, growth is essentially protein deposition (Houlihan et al. 1993a), and thus an adequate supply of dietary protein is fundamental for growth optimisation. This protein requirement concerns the amount and the quality of the dietary protein, i.e. the balance of the different amino acids (AA), and in particular

the essential amino acids (Tacon and Cowey 1985; Wilson 1994; D'Mello 1994). Fish seem to have the same 10 essential amino acids (EAA) as other animals, but tyrosine and cysteine are usually considered as semi-EAA, as they can only be synthesised from EAA (Wilson 1994). Amino acid imbalances will lead to increased AA oxidation and thus to decreased food conversion efficiencies (growth / food intake) (Tacon and Cowey 1985). A poor dietary AA balance also increases the rates of protein synthesis and turnover (Langar et al. 1993). As protein synthesis is highly energy demanding (Jobling 1985; Houlihan 1991), this will result in lower food conversion efficiencies. Infused diets with an imbalanced essential AA composition also give a higher oxygen consumption than balanced diets (Kaczanowski and Beamish 1996). Since protein is the most costly component of fish diets, it is of paramount importance for aquaculture to determine the AA profile that will minimise the protein requirement of the cultured species. This "optimal" AA profile will depend on the AA requirement for protein synthesis and the use of individual AA for energy or other purposes (Rønnestad and Fyhn 1993; ten Doeschate 1995).

The EAA profile of fish carcass or muscle is considered to be a good index of EAA requirements in fish (Tacon and Cowey 1985; Wilson 1994; Mambrini and Kaushik 1995). Changes in the FAA levels after a meal have also been used as a criterion for determining AA requirements, based on the hypothesis that the free concentration of an individual AA will remain low until its requirement is met (Wilson 1994). Furthermore, the use of a model of AA fluxes has been proposed as an efficient tool for analysing protein and AA metabolism, as it can provide information on requirements under different dietary or environmental conditions (Houlihan et al. 1995a,b).

Fish larvae seem to have a higher protein requirement than older fish (Dabrowski 1986). However, little is known about AA metabolism and the specific AA requirements of fish larvae after the start of exogenous feeding. Fiogbé and Kestemont (1995) found that goldfish larvae have much higher EAA requirements (g AA.g^{-1}protein) than juvenile and adult fish. Rates of protein synthesis and turnover have been measured in larvae of some species in relation to ontogeny, temperature or feeding (Fauconneau 1984; Fauconneau et al. 1986a,b; Houlihan et al. 1992; Houlihan et al. 1995c; Conceição et al. 1997a). Houlihan et al. (1995c) constructed an AA flux model for larval herring which suggested that dietary AA have a limited impact on the profile of the FAA pool. Furthermore, due to the incomplete development of the digestive tract in the early larval stages (Govoni et al. 1986; Segner et al. 1993,1994), some effort has been made in recent years to study the importance of FAA in diet utilisation (Rønnestad and Fyhn 1993; Fyhn et al. 1993; Næss et al. 1995).

The present paper studies the influence of different food regimes on AA metabolism and protein turnover in relation to growth in larval turbot (*Scophthalmus maximus* L.). Special attention is given to the AA profile of both protein and the free pool, and to how AA imbalances

in the diet can affect food conversion efficiency and growth. The influence of the experimental food regimes on growth, feeding, cost of growth, and the body biochemical composition of the same batch of turbot larvae has been analysed (Conceição et al. 1997b).

Materials and methods

Fish and rearing

The turbot (*Scophthalmus maximus* L.) larvae used in this study were from the same two groups as described by Conceição et al. (1997b). Two groups of 15000 larvae each were transferred to two 1500 l tanks 2 days after hatching. The larvae were reared in these tanks until day 26 after hatching, under natural photoperiod (August, 60°N), at a temperature of 18.0±0.3 °C and a salinity of 34.3±0.2‰ . Water flow was progressively increased from 0.3 to 1.4 $l.min^{-1}$, giving a daily exchange rate of the rearing volume of 28.8 to 134.4%. To optimise rearing conditions green water was used (Naas et al. 1992). The algae *Isochrysis galbana* and *Tetraselmis* sp. were continuously added to the water supply in order to maintain a concentration between 50 and 100 million cells.l^{-1} in the tanks.

Feeding

Live food was added to the rearing tanks twice a day, in the morning between 9:00 and 11:00h, and in the afternoon between 16:00 and 18:00h (see Conceição et al. 1997b for details). Daily ration was calculated from a theoretical bioenergetic model for larval turbot (van der Meeren 1991). During the first two weeks post-hatch all the larvae received both rotifers and natural zooplankton. On days 12 to 14, one of the tanks (group ART) received both natural zooplankton, rotifers and *Artemia* nauplii, and after that only enriched *Artemia* nauplii. The other tank (group ZOO), continued to receive natural zooplankton until the end of the experiment. Natural zooplankton (mostly nauplii, juvenile and adult stages of copepods) was dominated (about 90%, in number) by one species, the copepod *Acartia grani*. The remaining 10% consisted of a mixture of *Centropages hamatus*, *Eurytemora affinis*, harpacticoids, and the cladoceran *Evadne normannii*.

Sampling

Samples for FAA measurements were taken in the morning on days 3, 7, 10, 16, 20, 23 and 26 post-hatch, before or shortly after the first daily addition of live food to the tanks. Samples for protein-bound amino acids (PAA) were taken in the evening (around 19:00h) on days 6, 11, and 23 post-hatch. On day 11 a second sample was taken in the morning (9.00h). At each sampling point (FAA and PAA) pooled samples of 20 (until day 20) or 10 (after day 20) larvae were

taken in four replicates. Samples were rinsed in tap water, dried on a sieve, placed into Nunc cryo tubes and stored at -20°C until further analysis.

Protein synthesis

Protein synthesis measurements were carried out on days 11 and 17 post-hatch. Larvae were gently transferred to aerated glass beakers (5 l) at 18.0±0.05 °C, and left to acclimatise for one to two hours before measurements started. A solution of 24 mM phenylalanine in filtered sea water containing L-[2,6-^{3}H]phenylalanine (Amersham) at a concentration of 7.4 Mbq.ml^{-1} was prepared, and diluted 1:50 in seawater. Three groups of 10 larvae (day 11), and 6 groups of 3 larvae (day 17), were incubated in light for 4 hours in about 6 ml of the phenylalanine solution in Nunc multi-well trays. In order to verify the dynamics of the uptake of labelled phenylalanine from the water and its incorporation into protein, a preliminary experiment was made on yolk-sac larvae close to complete yolk absorption (3 days after hatching) with a more extensive time course (0, 2, 4, 6 and 8 hours).

At the end of each incubation, larvae were put in a plankton net, rinsed well with distilled water, placed in Eppendorf tubes and frozen in liquid nitrogen. Each tube (sample) contained one group of incubated larvae (see above). Samples were kept at -20°C until further analysis.

The samples were homogenised in 0.5 M perchloric acid and centrifuged in order to separate the FAA pool from the precipitated proteins. Protein content was determined (Lowry et al. 1951) after solubilisation in 0.3 M NaOH. Specific activity of protein-bound phenylalanine (Sb, dpm.nmol^{-1}phenylalanine) was calculated as the quotient between liquid scintillation counts (dpm, disintegrations per minute) in solubilised protein and the mg of protein added to the scintillation vial divided by 271.4. The latter value refers to the nmoles of phenylalanine contained in one mg of turbot larval protein (see Results). Free pool phenylalanine specific activity (Sa, dpm.nmol^{-1}phenylalanine) was determined as described by Houlihan et al. (1986). Phenylalanine standards were included in all determinations and the efficiency of phenylalanine recovery was measured (Houlihan et al. 1988). RNA contents were measured using the dual wavelength method (McMillan and Houlihan 1988) and expressed as RNA/Protein ratio (mg RNA.mg^{-1} protein).

Fractional rates of protein synthesis (k_s, %.day^{-1}) were estimated using the flooding dose method equation (Garlick et al. 1980; Houlihan et al. 1988):

$$k_s = (Sb / Sa) \cdot (1/t) \cdot 1440 \cdot 100$$

where Sb is the specific activity of protein-bound phenylalanine, Sa is the specific activity of free pool phenylalanine, t is the time (minutes) from start of incubation, and 1440 is the number of minutes in one day. Protein degradation (k_d, %.day^{-1}) can be calculated by subtracting protein growth from protein synthesis. In a growing organism, the rate of protein turnover equals the rate of protein degradation (Wiesner and Zak 1991).

Protein growth rates (k_g, %.day^{-1}) were calculated assuming exponential protein growth (Ricker 1958):

$$k_g = (e^g - 1) .100 \qquad \text{with} \qquad g = [Ln(Pro_2) - Ln(Pro_1)] / (t_2 - t_1)$$

where Pro_2 and Pro_1 are the protein contents (mg.larva^{-1}) at the two nearest sampling points for biochemical composition (Conceição et al. 1997b).

RNA efficiency (k_{RNA}, g protein synthesised.g^{-1}RNA.day^{-1}) was calculated as the ratio between k_s and the RNA/Protein ratio (Millward et al. 1973).

Amino acid profiles

The FAA in the samples were extracted for 24 hours in 6% (final concentration) trichloroacetic acid. After centrifugation (10 min at 15000 x g) the supernatants were analysed in an automatic AA analyser (Chromaspeck J180, Hilger Analytical) with fluorimetric detection (OPA reagent) and high pressure loading. The amounts of PAA were determined similarly after hydrolysis in 6 M HCl as described by Finn et al. (1995a) on samples which had been extracted for FAA as described above. Plankton samples were also analysed for FAA. These samples were prepared by filtration of known volumes, with known plankton concentrations, into pre-ashed fibre glass filters (1 µm). All determinations were done with four replicates. Amino acid profiles were calculated as the percentage distribution of the mole contents.

Statistical analysis

Values were given as means ± standard deviations. Differences were considered significant when $p<0.05$. Results were analysed through the SAS package using one-way ANOVA (Proc GLM), Student's t-test (Proc TTEST), and linear regression (Proc REG) where appropriate.

Results

Amino acid profile of protein

The PAA profile seemed to be rather stable during the larval period (Table 1). Small, but significant, differences were only detected for leucine, lysine, phenylalanine and methionine.

Table 1. Comparison between the amino acid profiles of protein (PAA) and the free pool (FAA) of turbot larvae of different ages (days post-hatch) and feeding regimes.

Day	6 (pm)				11 (am)				11 (pm)				23 (pm)				23 (pm)			
Group	ZOO				ZOO				ZOO				ART				ZOO			
Pool	PAA		FAA		PAA		FAA		PAA		FAA		PAA		FAA		PAA		FAA	
leu	10.2 ±0.5	ab	7.4 ± 0.3	***	10.0 ±0.6	ab	7.3 ±0.1	**	10.4 ±0.3	a	7.4 ±0.5	***	9.5 ±0.4	ab	5.4 ±0.3	***	9.1 ±0.7	b	5.6 ±0.2	***
lys	7.6 ±0.6	a	9.0 ± 0.2	**	6.4 ±0.8	b	8.6 ±0.4	**	6.3 ±0.5	b	8.2 ±0.7	**	5.7 ±0.1	b	6.4 ±0.5		5.8 ±0.3	b	7.1 ±0.4	***
arg	5.2 ±0.2		8.8 ± 0.8	**	4.7 ±0.5		9.2 ±0.5	***	4.5 ±1.0		8.2 ±0.7	**	4.3 ±0.1		5.7 ±0.4	***	4.2 ±0.1		8.3 ±0.3	***
thr	5.0 ±1.4		4.2 ± 0.6		6.2 ±0.3		5.6 ±0.3	*	5.6 ±0.7		5.3 ±0.6		6.0 ±0.4		4.2 ±0.3	**	5.9 ±0.6		4.7 ±0.2	**
val	5.0 ±0.6		6.7 ± 0.6	**	6.0 ±1.6		6.6 ±0.5		6.1 ±0.4		6.4 ±0.8		5.0 ±1.1		5.4 ±0.4	*	6.0 ±0.2		5.3 ±0.8	
ile	4.0 ±0.6		4.0 ± 0.6		4.8 ±1.1		4.5 ±0.1		4.6 ±0.5		3.9 ±0.4		4.2 ±1.0		3.5 ±0.1		4.3 ±0.8		3.0 ±0.1	
phe	3.7 ±0.3	a	3.5 ± 0.4		3.6 ±0.6	ab	3.3 ±0.2		3.4 ±0.3	ab	3.4 ±0.2		3.1 ±0.1	ab	2.3 ±0.2	***	2.9 ±0.1	b	2.3 ±0.1	***
his	2.2 ±0.4		4.6 ± 1.1	**	2.0 ±0.4		3.2 ±0.1	**	2.1 ±0.3		4.5 ±0.4	***	1.8 ±0.3		2.9 ±0.3	***	1.7 ±0.2		3.5 ±0.2	***
tyr	2.1 ±0.4		4.4 ± 0.4	***	2.3 ±0.2		4.4 ±0.2	***	1.8 ±0.4		3.8 ±0.1	***	1.8 ±0.1		2.7 ±0.2	**	1.9 ±0.2		2.8 ±0.1	***
met	0.5 ±0.4	a	3.1 ± 0.2	***	2.2 ±0.8	b	3.3 ±0.2		1.4 ±0.8	ab	3.3 ±0.4	**	2.2 ±0.6	b	1.7 ±0.2		2.7 ±0.8	b	2.8 ±0.1	
asp	13.6 ±2.7		6.2 ± 0.6	*	13.3 ±3.1		4.4 ±0.4	*	13.7 ±3.2		5.6 ±0.8	*	15.9 ±0.7		4.5 ±1.3	***	15.0 ±0.5		3.7 ±0.6	***
gly	10.7 ±1.2		11.9 ± 1.9		9.6 ±0.9		8.5 ±1.4		10.1 ±0.8		6.0 ±0.4	***	10.7 ±0.5		9.0 ±0.8	*	10.4 ±0.6		12.8 ±0.2	***
ala	10.6 ±0.8		7.1 ± 0.7	***	9.5 ±0.4		8.7 ±1.4		11.0 ±1.8		9.1 ±0.5		10.0 ±0.5		17.4 ±1.9	**	10.4 ±1.6		13.3 ±1.2	**
ser	8.1 ±0.3		5.9 ± 0.3	***	7.6 ±1.3		10.1 ±0.7	*	7.8 ±0.4		10.5 ±1.6	*	8.5 ±1.6		6.6 ±0.8	*	8.4 ±0.9		7.5 ±1.0	
glu	6.6 ±1.8		10.7 ± 1.0	**	7.4 ±0.8		8.5 ±1.9		6.7 ±0.5		11.0 ±1.0	***	6.5 ±0.4		16.4 ±3.2	**	6.6 ±0.7		13.7 ±0.3	***
pro	4.9 ±0.5		2.4 ± 0.5	***	4.5 ±0.9		3.8 ±0.3		4.5 ±0.6		3.5 ±0.3	*	4.7 ±0.2		6.0 ±0.3	***	4.6 ±0.3		3.8 ±0.2	**
EAA	45.5 ± 2.5		55.8 ± 1.5	***	48.1 ± 4.7		56.1 ±0.8	*	46.3 ± 1.6		54.4 ±2.7	**	43.7 ± 2.8		40.1 ±1.7	*	44.5 ± 1.9		45.3 ±0.8	
NEAA	54.5 ± 2.5		44.2 ± 1.5	***	51.9 ± 4.7		43.9 ±0.8	*	53.7 ± 1.6		45.6 ±2.7	**	56.3 ± 2.8		59.9 ±1.7	*	55.5 ± 1.9		54.7 ±0.8	

Values are given in percentage (mole basis) as means±sd of pooled samples of 10-20 larvae (n = 4).Significant differences between PAA and FAA values of each amino acid at the different days are shown as: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Significant differences for the contents of each amino acid in PAA are shown by different letters (ANOVA, followed by a Bonferroni t-test, $p < 0.05$).

Table 2. Profiles of the FAA pool of turbot larvae fed natural zooplankton or Artemia from first feeding until metamorphosis.

Day	3	7	10	16	16		20	20		23	23		26	26	
Group	ZOO	ZOO	ZOO	ZOO	ART		ZOO	ART		ZOO	ART		ZOO	ART	
leu	6.8 ± 0.9 ab	6.3 ± 0.2 abc	4.4 ± 0.3 d	5.4 ± 0.3 cd	5.0 ± 0.7 a		7.0 ± 0.4 a	6.6 ± 0.8 a		5.9 ± 0.7 bc	4.9 ± 0.4 a		6.6 ± 0.7 abc	5.6 ± 1.1 a	
lys	3.0 ± 0.2 c	8.9 ± 0.2 b	4.9 ± 0.4 bc	3.6 ± 0.7 bc	3.1 ± 0.4 a		5.2 ± 0.2 b	3.9 ± 1.1 a		4.4 ± 0.8 b	2.8 ± 0.9 a	*	4.4 ± 0.9 bc	3.3 ± 1.0 a	
arg	1.4 ± 0.2 d	7.8 ± 0.5 a	5.0 ± 0.4 c	5.2 ± 0.9 c	6.2 ± 0.8 a		5.9 ± 0.7 bc	5.4 ± 0.7 ab		6.7 ± 0.4 ab	5.0 ± 0.6 ab	**	6.0 ± 0.4 bc	4.1 ± 0.6 b	**
thr	2.9 ± 0.5 b	5.0 ± 0.3 a	4.0 ± 0.9 ab	5.2 ± 0.9 a	4.9 ± 0.9 a		4.2 ± 0.2 ab	3.9 ± 0.5 ab		4.0 ± 0.2 a	2.7 ± 0.5 b	**	4.5 ± 0.6 a	3.8 ± 0.4 ab	
val	11.0 ± 0.5 a	5.6 ± 0.6 bc	4.1 ± 0.3 c	5.9 ± 0.6 b	4.5 ± 0.9 ab	*	6.2 ± 0.5 b	5.4 ± 0.6 a		5.2 ± 0.6 bc	3.9 ± 0.5 b	*	5.8 ± 1.1 b	5.1 ± 0.4 ab	
ile	4.2 ± 0.6 a	3.3 ± 0.2 bc	2.2 ± 0.2 d	3.0 ± 0.1 c	2.6 ± 0.4 b		3.9 ± 0.2 ab	3.7 ± 0.5 a		3.1 ± 0.5 c	2.5 ± 0.2 b	*	3.4 ± 0.3 bc	2.9 ± 0.4 ab	
phe	14.2 ± 0.5 a	3.1 ± 0.1 bc	2.2 ± 0.3 d	3.1 ± 0.5 bc	2.4 ± 0.2 a	*	3.3 ± 0.4 bc	2.6 ± 0.4 a		2.7 ± 0.2 cd	2.1 ± 0.3 b	**	3.3 ± 0.2 b	1.7 ± 0.1 b	***
his	4.2 ± 0.8 b	4.6 ± 0.7 b	7.1 ± 0.9 a	3.6 ± 0.5 bc	3.1 ± 0.6 a		3.5 ± 0.2 bc	2.9 ± 0.2 a	**	3.3 ± 0.1 bc	3.1 ± 0.5 a		2.8 ± 0.2 c	2.9 ± 0.4 a	
tyr	22.6 ± 1.0 a	3.9 ± 0.1 b	2.8 ± 0.3 c	3.4 ± 0.4 bc	3.0 ± 0.3 a		3.5 ± 0.1 bc	3.1 ± 0.4 a		2.8 ± 0.1 c	2.2 ± 0.3 b	*	3.3 ± 0.1 bc	2.5 ± 0.2 ab	***
met	1.4 ± 0.1 c	2.6 ± 0.2 ab	2.2 ± 0.3 bc	2.4 ± 0.4 b	2.3 ± 0.2 a		2.7 ± 0.3 ab	2.6 ± 0.3 a		2.6 ± 0.8 ab	2.4 ± 0.2 a		3.3 ± 0.3 a	1.3 ± 0.1 b	***
asp	3.0 ± 1.4 c	5.0 ± 0.6 b	7.9 ± 0.9 a	4.8 ± 0.9 bc	4.7 ± 0.5 a		3.4 ± 0.3 bc	3.2 ± 0.7 b		4.3 ± 0.3 bc	4.5 ± 0.3 a		4.4 ± 1.2 bc	3.6 ± 0.5 ab	
gly	5.5 ± 0.3 c	6.9 ± 2.7 bc	12.2 ± 0.9 a	9.8 ± 1.4 ab	11.6 ± 0.9 b		7.9 ± 0.3 bc	12.1 ± 1.3 b	**	12.0 ± 1.0 a	17.6 ± 1.4 a	***	11.2 ± 1.4 a	15.8 ± 1.3 a	**
glu	6.9 ± 0.9 c	9.4 ± 1.1 bc	13.0 ± 3.0 ab	14.1 ± 3.1 a	15.3 ± 2.2 a		10.9 ± 0.5 abc	9.8 ± 0.8 b	*	11.9 ± 2.0 ab	11.3 ± 1.0 b		10.2 ± 1.5 abc	14.9 ± 0.8 a	**
ala	4.4 ± 0.4 c	11.9 ± 1.4 a	8.5 ± 0.9 b	14.2 ± 1.8 a	12.2 ± 1.2 b		14.2 ± 1.0 a	18.7 ± 5.0 ab		13.7 ± 1.0 a	19.6 ± 2.3 a	**	12.0 ± 1.0 a	15.8 ± 1.7 ab	**
ser	4.9 ± 0.9 b	7.4 ± 0.3 ab	8.4 ± 2.1 a	7.1 ± 1.3 ab	7.7 ± 2.0 a		6.2 ± 0.2 ab	5.7 ± 1.0 a		7.4 ± 0.6 ab	5.2 ± 1.2 a	*	7.8 ± 1.6 ab	6.9 ± 0.7 a	
gln	1.5 ± 0.3 b	2.8 ± 0.1 a	2.9 ± 0.8 a	2.4 ± 0.4 ab	3.9 ± 0.9 a	*	2.6 ± 0.5 ab	1.8 ± 0.1 b		2.6 ± 0.5 a	3.1 ± 0.4 a		2.9 ± 0.7 a	3.7 ± 0.4 a	
pro	0.5 ± 0.1 c	3.3 ± 0.2 b	4.1 ± 1.0 ab	5.5 ± 1.1 a	5.7 ± 0.4 b		4.9 ± 0.5 a	6.9 ± 0.6 a	**	4.8 ± 0.3 ab	4.6 ± 0.4 c		4.1 ± 0.6 ab	2.9 ± 0.3 d	**
phs	1.8 ± 1.2 a	2.3 ± 1.4 a	4.1 ± 3.0 a	1.3 ± 2.7 a	1.7 ± 3.3 a		4.4 ± 0.2 a	1.7 ± 2.0 a		2.5 ± 1.7 a	2.4 ± 1.8 a		3.9 ± 1.1 a	3.2 ± 2.4 a	
EAA	71.6 ± 2.1 a	51.0 ± 1.6 b	38.9 ± 2.1 d	40.8 ± 1.6 cd	37.2 ± 3.6 ab		45.4 ± 0.8 c	40.1 ± 3.9 a		40.8 ± 2.1 cd	31.7 ± 2.2 b	**	43.5 ± 2.5 cd	33.1 ± 2.2 b	***
NEAA	28.4 ± 2.1 d	49.0 ± 1.6 c	61.1 ± 2.1 a	59.2 ± 1.6 ab	62.8 ± 3.6 ab		54.6 ± 0.8 b	59.9 ± 3.9 b		59.2 ± 2.1 ab	68.3 ± 2.2 a	**	56.5 ± 2.5 ab	66.9 ± 2.2 a	***
tau	25.1 ± 0.6 b	28.0 ± 2.3 b	42.8 ± 2.2 a	45.5 ± 3.7 a	49.7 ± 2.3 b		42.3 ± 1.0 a	32.9 ± 1.9 d	***	43.0 ± 4.3 a	43.6 ± 2.0 c		48.7 ± 6.7 a	59.3 ± 3.1 a	*
gaba	2.4 ± 0.2 a	2.6 ± 0.4 a	2.4 ± 0.1 a	1.6 ± 0.2 b	1.8 ± 0.3 a		1.0 ± 0.1 c	0.9 ± 0.1 b		0.9 ± 0.1 c	1.1 ± 0.1 b	**	0.8 ± 0.1 c	1.3 ± 0.1 b	**
FAA	0.37 ± 0.03	0.78 ± 0.09	1.07 ± 0.04	7.40 ± 1.04	5.7 ± 0.8	*	22.9 ± 0.8	30.1 ± 2.3	***	113 ± 16	91.7 ± 9.7		175 ± 29	117.4 ±	*** *

Values are given in percentage (mole basis) as means±sd of pooled samples of 10-20 larvae (n = 4). EAA and NEAA values are percentages of EAA+NEAA. Taurine (tau) and GABA are expressed as a percentage of all AA. FAA gives the total FAA content (nmol/individual). Significant differences between groups on the same day for each amino acid are shown as (t-test): * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Significant differences for the contents of each amino acid in each group at the different days are shown by different letters (ANOVA, followed by a Bonferroni t-test, $p < 0.05$).

However, the profile of the PAA was quite different from that of the FAA pool. After pooling the five sampling points from Table 1, the «average amino acid» in larval turbot protein could be calculated as $C_{4.61}H_{9.30}O_{2.58}N_{1.24}S_{0.02}$, with a molecular weight of 123.8. This average AA profile allowed to calculate that 1 gram of larval nitrogen is equivalent to 6.094 grams of protein, with 1 mg of protein containing 271.4 nmoles of phenylalanine.

Free amino acids

Within each of the two groups significant, but mostly small, differences among the different sampling days were found for all AA (Table 2). In general, the proportions of EAA decreased with development. The most striking differences were the very high proportions of tyrosine and phenylalanine at day 3 (22.6 and 14.2 % of all AA, respectively), compared to later samples (ranging from 3 to 4 %). Taurine was the most abundant FAA in all samples, and its contribution to the FAA pool increased with development (Table 2). Relative γ-amino-butyric acid (GABA) contents tended to decrease as the larvae grew.

Larvae fed natural zooplankton (group ZOO) or *Artemia* (group ART) had small differences in the profile of the FAA pool on day 16 post-hatch, but larger differences were noticed during later development, especially on days 23 and 26 (Table 2). On days 23 and 26 the proportion of methionine was significantly lower in the *Artemia*-fed group, and within this group the methionine proportion decreased between days 23 and 26 post-hatch. The relative content of taurine (% of all AA) was higher for larvae fed zooplankton than for those fed *Artemia* on day 20, and the opposite was found on day 26 (Table 2). However, when expressed in absolute (nmol.individual^{-1}) or mass specific (nmol.μg^{-1}DW) amounts, taurine contents were always lower in the *Artemia*-fed group.

On day 23 post-hatch some significant variations between the morning (Table 2) and afternoon (Table 1) in the AA profile of the FAA pool could be detected in both groups. The contributions of arginine, lysine and threonine to the FAA pool in the zooplankton-fed group increasing during the day, and phenylalanine and serine decreasing. Daily variations in the contribution of individual AA to the free pool were larger in the *Artemia*-fed group, with valine, isoleucine, lysine, threonine, tyrosine and proline increasing in the course of the day, while glycine and methionine decreased. There was an increase in the proportion of EAA in the free pool from the morning to the afternoon, and this was most noticeable in the *Artemia*-fed group. However, the methionine proportion decreased in the *Artemia*-fed group. During the course of day 11 small increases were found in the proportions of histidine, aspartate and glutamine, while the proportions of isoleucine, tyrosine and glycine decreased (Table 1).

The profile of the FAA pool in the food organisms was quite different in the various plankton types (results not shown). The plankton FAA profile was also considerably different

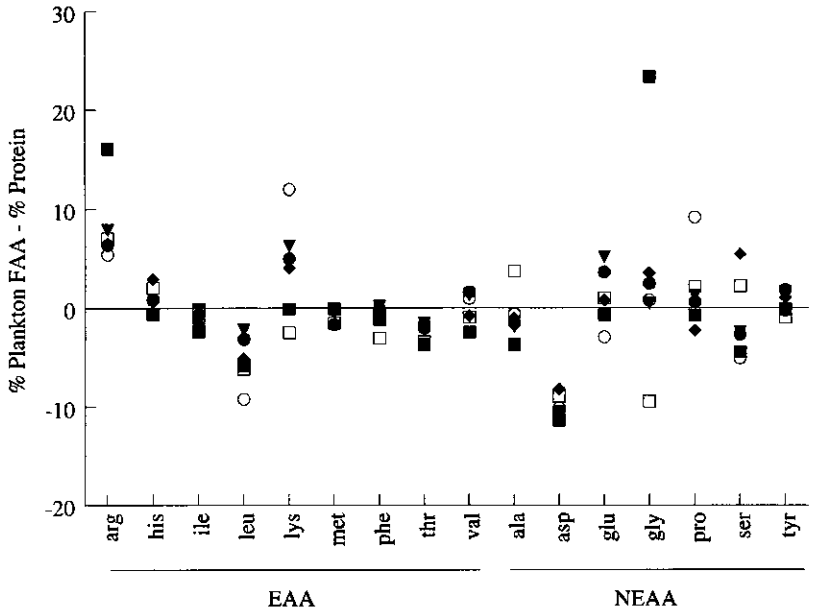

Figure 1. Difference between AA profiles of larval protein (Table 1) and of the FAA pool of the plankton organisms used as food (Table 5): Rotifers day 10 (□); Artemia day 25 (○); natural zooplankton day 9 (◆); natural zooplankton day 18 (▼); small natural zooplankton day 25 (●); big natural zooplankton day 25 (■).

from the AA profile of the larval protein (Figure 1). Highest deviations were observed for the larger zooplankton on day 25, followed by the enriched *Artemia* and the rotifers. All plankton types analysed had a FAA pool which was low in aspartate and leucine, and high in arginine and lysine.

Protein turnover

In a separate incubation with labelled phenylalanine it was shown that the specific activity of phenylalanine in both the free pool and protein increased linearly with time (Figure 2). During the incubations to determine the rates of protein synthesis, the free phenylalanine pool was almost doubled (Table 3). Both the fractional rates of protein synthesis (k_s, %.day^{-1}) and protein degradation (k_d, %.day^{-1}), as well as the fractional rate of protein growth (k_g, %.day^{-1}), were higher for the zooplankton-fed group than for the larvae fed *Artemia* on day 17 (Table 3). However, the efficiencies of retention of the protein synthesised (ERPS = $k_g / k_s .100$) were

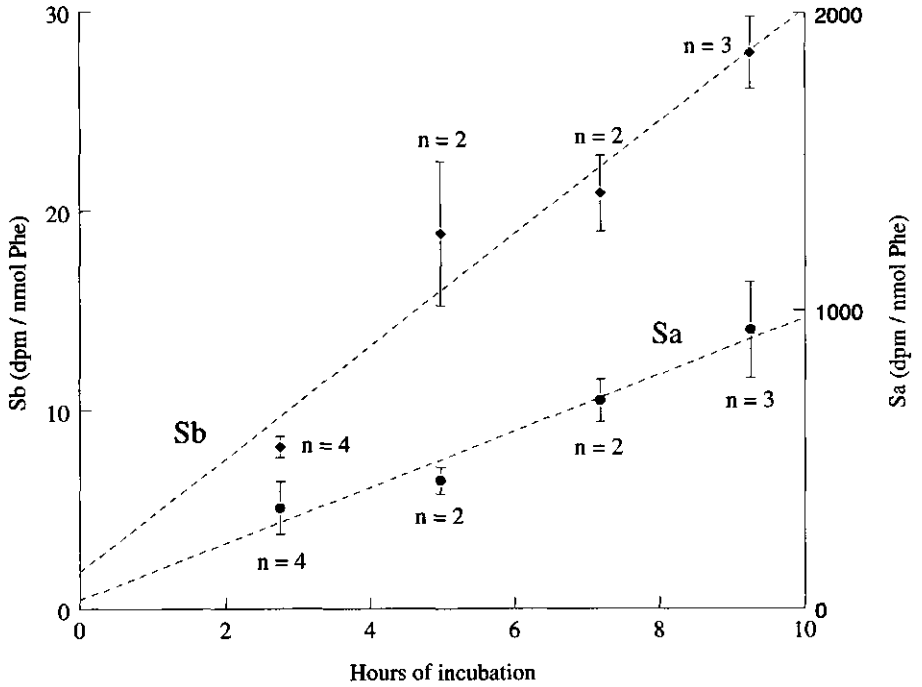

Figure 2. Time course of the specific activities of phenylalanine in the protein (Sb) and in the free pool (Sa) in the preliminary incubation. Values are means±sd of pooled samples of 30-40 larvae. Regression (dashed) lines are also given (Sb = 0.94 + 2.95. t, r^2 = 0.93, $p < 0.0001$; Sa = 55.0 + 92.1. t, r^2 = 0.87, $p < 0.0001$).

higher in the *Artemia*-fed group. The rates of protein synthesis, degradation and growth were higher on day 17 than on day 11, but the efficiency of retention of protein synthesised decreased (Table 3). On day 17 the RNA/protein ratio and the RNA efficiency were higher in the zooplankton-fed group. RNA contents were not measured on day 11.

Amino acid flux

Flux of AA in turbot larvae was estimated (Figure 3) based on the model of Millward and Rivers (1988) and Houlihan et al. (1995a). The values observed for the FAA pool (Table 2) and for the rates of protein synthesis, protein degradation and protein growth (Table 3) were used to calculate the respective flux components. The protein pool was calculated using the larval protein contents (Conceição et al. 1997b) and assuming that one gram of protein is equivalent to 8.1 mmol of FAA, according to the average AA calculated in the present study. Amino acid intake values referred to the amount of AA offered to the larva per day (Conceição et al. 1997b).

Table 3. Fractional rates of protein synthesis (k_s) growth (k_g) and degradation (k_d) together with the specific activities of phenylalanine in the free pool (Sa) and in protein (Sb) in turbot larvae. The efficiencies of retention of the synthesised protein (ERPS), RNA/Protein ratios, RNA efficiencies (K_{RNA}) are also given. The free phenylalanine pools (Free Phe) are given for larvae before (BI) and after (AI) the incubation.

Day Group		11 ZOO	17 ZOO	17 ART
Sa	(dpm/nmol)	1710.4±128.9	1006.8± 9.3	1519.2±166.5
Sb	(dpm/nmol)	47.1 ± 1.4	39.7 ± 5.3	62.0 ± 11.0
k_s	(%/day)	33.0 ± 2.8 b	69.7 ± 9.9 a	44.2 ± 7.4 b
k_g	(%/day)	31.0	41.6	32.6
k_d	(%/day)	2.0	28.1	11.6
ERPS	(%)	93.9 ± 8.0 a	59.7 ± 7.8 c	73.7 ± 11.6 b
RNA/Protein	(µg/mg)		48.8 ± 2.1 a	42.9 ± 0.8 b
K_{RNA}	(g/g/day)		14.3 ± 1.7 a	10.3 ± 1.8 b
Free Phe (BI)	(nmol)	1.1 ± 0.2	3.4 ± 0.4	1.8 ± 0.3
Free Phe (AI)	(nmol)	2.0 ± 0.1	6.6 ± 0.6	2.4 ± 0.5

Values are means±sd of pooled samples of 10 larvae (n=3) on day 11, and 3 larvae (n = 6) on day 17. Values with different letters in the same row are significantly different (ANOVA, followed by a Bonferroni t-test, $p < 0.05$).

Amino acid losses were calculated as intake minus net growth. It was estimated that turbot larvae on day 11 post-hatch deposited (as net growth) only 59% of their AA intake in body proteins, whereas on day 17 larvae fed on zooplankton deposited 93% of the AA intake (Figure 3). The AA losses could then be estimated to amount to 41% of intake at day 11 and 7% at day 17. The FAA pool seemed to be 6% of the protein pool on day 11, decreasing to 4% on day 17 (Figure 3). The flux model for the *Artemia*-fed larvae at day 17 (not shown) was very similar to the one for larvae fed zooplankton on the same day.

Discussion

Amino acid profile in protein

The present study suggests small changes in the AA profile of turbot larvae body proteins during development (Table 1). Furthermore, Finn et al. (1996) have found a PAA profile for turbot larvae at the end of the yolk sac-stage differing considerably different from what we observe on day 6 after hatching. The contributions of alanine and glycine to the PAA profile are higher in our study, while isoleucine, valine, methionine and serine are significantly lower. Compared with the PAA profiles in turbot eggs (Finn et al. 1996), the differences are even more

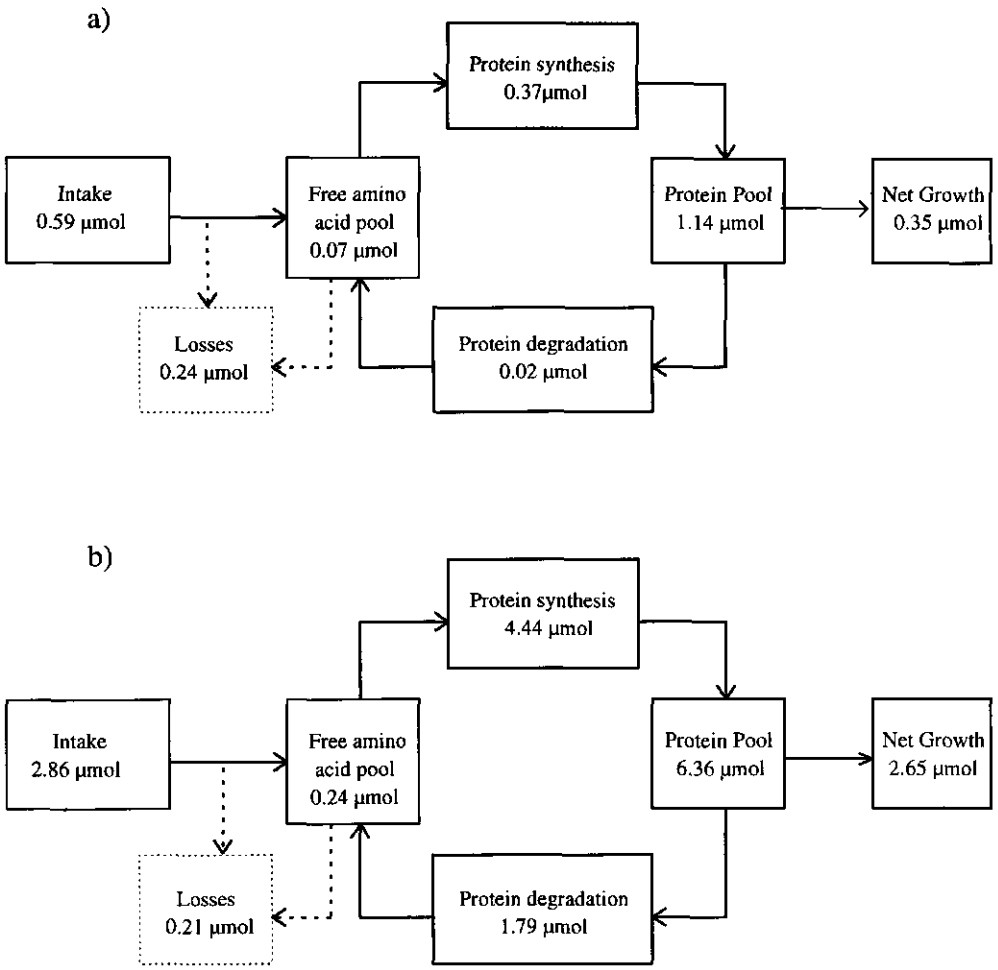

Figure 3. Amino acid flux for turbot larvae 11 (a) and 17 (b) days post-hatch fed with natural zooplankton, based on the model of Millward and Rivers (1988). The values observed for the FAA pool (Table 2) and for the rates of protein synthesis, protein degradation and protein growth (Table 3) were used. According to the average amino acid profile calculated in this study one gram of protein is equivalent to 8.1 mmol of free amino acids. Intake values refer to amount of amino acids offered to the larva per day (Conceição et al. 1997b). Amino acid losses are calculated as intake minus net growth.

pronounced. Considerable differences in the AA profile of eggs and whole body have also been reported for other fish species (Ketola 1982; Wilson and Poe 1985; Ng and Hung 1994). However, it is often accepted that AA profiles of the whole body change little between and within species (Wilson and Poe 1985; Wilson 1994; Ramseyer and Garling 1994). Nevertheless, significant differences in AA profiles during development have also been observed in dolphin fish (Ostrowski and Divakaran 1989), white sturgeon (Ng and Hung 1994) and African catfish (Conceição et al., unpublished data). This variation in AA profiles is probably a result of differences in the relative importance of individual proteins. Compared to other species, turbot larvae have proteins with considerable higher amounts of leucine and aspartate, and lower amounts of glutamate. Therefore, the requirement for leucine may be higher in turbot larvae than in other fish species.

Effects of free amino acids on growth and development

The losses of EAA through catabolism are probably reduced during development by a decrease in the fraction of EAA in the free pool. The affinity of the enzymes involved in protein synthesis for AA is higher than that of the enzymes involved in AA catabolism (Cowey and Walton 1989) and thus lower concentrations of EAA should allow for a lower oxidation of EAA. Interestingly, based on the AA flux diagrams (Figure 3) the losses of AA seem to have been much higher on day 11 than on day 17.

The contribution of tyrosine and phenylalanine to the FAA pool on day 3 post-hatch (22.6 and 14.2 % of all AA, respectively) is high when compared to later samples in this study (ranging from 3 to 4%). Finn et al. (1996) have also found the relative contents of tyrosine and phenylalanine to increase towards complete yolk-sac absorption in turbot larvae, reaching up to 16 and 7% of the total FAA pool, respectively. There seems to be a temporary rise in the proportions of these two aromatic AA in the FAA pool around first feeding. Such an increase in the relative contents of tyrosine and phenylalanine close to complete yolk absorption has also been observed in gilthead sea bream (Rønnestad et al. 1994) but not in Atlantic cod (Finn et al. 1995a). Phenylalanine is the precursor for the synthesis of tyrosine, and the latter AA makes up the carbon-skeleton of the thyroid hormones, melanin, dopamine and catecholamines (Cowey and Walton 1989; Bender 1985). It is tempting to suggest the rise in tyrosine (and phenylalanine) is related to the start of the activity of the thyroid gland. The first appearance of thyroid follicles in pelagic marine fish larvae appears just before complete yolk absorption (Tanaka et al. 1995). Marked increases in thyroid hormone levels around first feeding have been observed in two salmon species (Kobuke et al. 1987; Tagawa and Hirano 1987) and in striped bass (Brown et al. 1988).

High levels of taurine in the FAA pool, comparable to those observed in this study, have been observed in yolk-sac larvae of turbot (Finn et al. 1996), Atlantic cod (Finn et al. 1995a) and Atlantic halibut (Finn et al. 1995b). Such high taurine contents are also common in the FAA pool of tissues, but not plasma, of juvenile fish (e.g. Walton and Wilson 1986; Cowey and Walton, 1989; Lyndon et al. 1993; Carter et al. 1995) and mammals (Hayes and Sturman 1981). The function of these high levels of taurine is not well understood, although taurine is known to be the sole AA that conjugates with cholic acid to produce the bile salts in teleost fishes (van Waarde 1988). In addition, taurine seems to be an important osmolyte involved in cell volume regulation in fish (Fugelli and Zachariassen 1976; Vislie 1982) and takes part in a series of neuronal and membrane related functions (Hayes and Sturman 1981; Huxtable 1992). In animals, taurine cannot be broken down for energy (Huxtable 1992). Taurine is synthesised from methionine via cysteine. High levels of cysteine or methionine in the diet increase taurine production in the rat (Tanaka et al. 1993; Yamada et al. 1995). The same seems to be true for rainbow trout (Yokoyama and Nakazoe 1989). Based on data for skate, it has been suggested that fish are unable to synthesise taurine (Cowey and Walton 1989). If this is the case for turbot larvae, there may be a specific dietary requirement for taurine, as has also been found for cats (Hayes and Sturman 1981).

The present study suggests a correlation between taurine levels and growth rates of turbot larvae. The low growth rate observed in group ART at the end of the experiment (Conceição et al. 1997b) was accompanied by lower taurine contents than in group ZOO. Furthermore, turbot larvae growing at lower rates, due to food limitation, have also had lower taurine contents (Conceição et al., unpublished data). This indicates that an insufficient dietary supply of taurine, and/or its precursors methionine and cysteine may reduce growth in turbot larvae.

Between day 23 and day 26 post-hatching, *Artemia*-fed turbot larvae (group ART) had lower growth rates (16.6% body dry weight.day^{-1}) and higher lipid deposition (19.5% dry matter) than group ZOO (25.6% body dry weight.day^{-1} and 14.1% lipids in dry matter) (Conceição et al. 1997b). This may be related to a sub-optimal dietary quality of the enriched *Artemia*, which may be deficient in methionine for turbot larvae. The contribution of methionine to the FAA pool on days 23 and 26 was lower for the *Artemia*-fed larvae, and also decreased between days 23 and 26 within this group (Table 2). Furthermore, on day 23, while generally the *Artemia*-fed group had an increase in EAA during the day, methionine was the sole EAA which decreased significantly. The methionine content in *Artemia* protein (Seidel et al. 1980) is considerably lower than that observed in turbot larvae protein (Table 1).

Another AA which may limit growth in turbot larvae is leucine. The FAA pool in the different plankton organisms used in this study, and in *Artemia* in particular, is poor in leucine

when compared to the content of leucine in larval protein (Figure 1). Comparing with literature values (Seidel et al. 1980) the leucine level in *Artemia* protein is also considerably lower than that found in larval turbot protein (Table 1). The AA profile of the dietary plankton food seems rather imbalanced if the FAA profile of the plankton organisms is representative for the assimilated AA in the gut (Figure 1). However it is doubtful whether this is the case as the AA in protein are quantitatively much more important than FAA (Conceição et al. 1997b). In rotifers, unenriched *Artemia* and in various freshwater zooplankton organisms, large differences have been found between the AA profiles in protein and in the FAA pool (Dabrowski and Rusiecki 1983).

The profile of the FAA pool in turbot larvae is quite variable. The daily and the day to day variations (Tables 1 and 2) may be attributable to dietary influences. A good correlation between the individual AA levels of dietary EAA and plasma EAA has been found in juveniles and in adults of several fish species (Plakas et al. 1980; Wilson et al. 1985; Walton and Wilson 1986; Lyndon et al. 1993; Kaushik et al. 1994; Schuhmacher et al. 1995). No correlation has been found in these studies between dietary and plasma NEAA. On the contrary, liver and muscle levels of free EAA show a poor correlation with dietary EAA (Walton and Wilson 1986; Lyndon et al. 1993; Carter et al. 1995). However, the variations in the AA profiles from samples taken in the morning (Table 2) should be relatively diet independent, since food intake during this part of the day seems to be low in turbot larvae (Danielssen et al. 1990). Therefore, the variability in the profile of the FAA pool also seems to be affected by developmental events and physiological conditions.

Protein synthesis and turnover

The measurements of protein synthesis met the criteria for successful measurements with the flooding dose method (Garlick *et al.* 1980; Houlihan *et al.* 1988). There was a linear labelling of protein with time in larval turbot (Figure 2). Taking into account the fast increase in the specific activity of the free pool of phenylalanine and the high growth rate of the larvae, labelled phenylalanine was assumed to be homogeneously distributed among the different metabolic pools. The comparison of the protein synthesis rates with the rates of protein growth, and subsequent calculation of protein degradation rate, is somewhat uncertain due to the difference in time scale of the two measurements (Houlihan et al 1995a). In the present study, synthesis rates were measured over a period of 2-4 hours, while growth rates were measured over a period of 4-6 days.

Results on day 17 post-hatch (Table 3) support the general hypothesis that higher protein growth rates are associated not only with increased rates of protein synthesis but also with higher protein turnover (Waterlow et al. 1978; Houlihan et al. 1986,1988). However, unchanged

rates of protein turnover at different protein synthesis rates have been observed in larval nase (Houlihan et al. 1992) and has also been suggested for yolk-sac larvae of the African catfish (Conceição et al. 1997a).

Interestingly, the efficiencies of retention of the protein synthesised are reduced with increasing protein synthesis rates (Table 3). The efficiency on day 11 is surprisingly high compared to published values for fish larvae (Houlihan et al. 1992, 1995c; Conceição et al. 1997a). It is the first experimental support for the suggestion that fish larvae may reduce their levels of protein turnover in order to save energy for growth (Kiørboe et al. 1987; Kiørboe 1989; Wieser and Medgyesy 1990; Wieser 1994).

The RNA/protein ratios are comparable to values normally reported for fish larvae (about 45 μg RNA.mg^{-1}protein) which are much higher than values for adult fish (Houlihan et al. 1995b). However, the RNA efficiencies are high compared to published values, typically ranging from 3 to 6 g protein synthesised.g^{-1}RNA.day^{-1} (Houlihan et al. 1995b). Comparable RNA efficiencies have only been found in 10 mg tilapia (Houlihan et al. 1993b), in yolk-sac larvae of the African catfish (Conceição et al. 1997a) and in mammals (Reeds and Davies 1992). High protein synthesis rates in larval turbot are obtained through very high RNA efficiencies, associated with high RNA concentrations.

Amino acid flux and growth

The AA flux diagram for day 17 (Figure 3b) suggests a very high protein conversion efficiency (growth / intake) compared to the 63% found in larval herring (Houlihan et al. 1995b), and values between 20 and 48% found in juvenile and in adult fish (Bowen 1987; Houlihan et al. 1995a). Protein conversion efficiencies of larval fish are not available in the literature, probably due to the difficulties in measuring food (and protein) intake. The estimates of protein conversion efficiencies in the present study should be treated with caution, as AA intake is estimated based only on the food offered to the larvae. The estimates of AA losses are also dependent on the uncertainties of AA intake. Amino acid losses include both AA oxidation and AA which are eaten but not assimilated in the gut. Conway et al. (1993) found that the AA assimilation efficiency of different zooplankton organisms is close to 100% in turbot larvae with an age of 21 to 27 days post-hatch. However, in the present study the larvae are younger, and therefore the digestive system may be less developed. Striped bass larvae have been found to have protein assimilation efficiencies increasing from 30% in young stages to 60% around metamorphosis (Rust 1995).

The AA flux diagram for day 11 (Figure 3a) is comparable to the one published for larval herring (Houlihan et al. 1995b), except in what concerns the size of the free pool. On day 11 the size of the FAA pool is 6% of the protein pool (4% on day 17) compared to 29 % in

herring larvae. In this respect our results are closer to the values around 2.3% found in juvenile rainbow trout (Carter et al. 1995; Houlihan et al. 1995a,b). This means that on a daily basis the dietary AA supply may be up to 10 times the larval FAA pool. In addition, protein synthesis will daily remove the equivalent of 5 (day 11) or 20 (day 17) times the size of the FAA pool. Similarly, protein degradation will return to the FAA pool less than one (day 11) or 10 (day 17) times its size. So, the FAA pool in growing turbot larvae is extremely dynamic, being very sensitive to the arrival of dietary AA, as well as to the AA profiles of proteins that are being synthesised and/or degraded at a given moment. The high variation in the profiles of the FAA pool supports this idea.

The present study suggests that there are some important ontogenic changes in AA metabolism in larval turbot. In an initial phase (day 11 post-hatch), high growth rates seem to involve a reduction in the turnover of proteins, while AA losses through oxidation appear to be high. This high AA oxidation may be related to the high larval FAA concentration when compared with older larvae (see above). Lower protein turnover may save energy for growth (or other processes) as protein turnover is responsible for a large fraction of the energy budget (Hawkins et al. 1989; Conceição et al. 1997a). Furthermore, a reduced protein turnover rate is also likely to diminish losses by oxidation of the AA involved in turnover (Carter et al. 1995). However, it has been proposed that reducing protein turnover may have costs for the larvae in terms of viability and survival (Kiørboe et al. 1987). High protein turnover will allow for more metabolic plasticity, enabling a fast response of the organism to environmental/disease stress, through the synthesis of specific enzymes and other proteins.

At a later stage (day 17 post-hatch), a much higher protein turnover is observed, but the oxidative losses of AA seem to be much smaller. During development, turbot larvae may acquire the capacity for (down) regulation of the catabolism of AA. Dabrowski (1986,1989) proposed a decreasing role of AA catabolism for energy production during fish development. Furthermore, the activities of enzymes involved in AA catabolism are reduced with increasing size of larval African catfish (Segner and Verreth 1995). This suggests that an eventual increase in AA oxidation associated with higher protein turnover rates can be minimised. In such conditions, investment of energy in protein turnover may be a beneficial strategy, that increases viability. Older larvae may also attempt to optimise the AA resources by reducing both the size and the proportion of EAA in the FAA pool.

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Chapter 6

Amino acid profiles and amino acid utilisation in larval African catfish (*Clarias gariepinus*): effects of ontogeny and temperature

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Amino acid profiles and amino acid utilisation in larval African catfish (*Clarias gariepinus*): effects of ontogeny and temperature

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Abstract

The qualitative amino acid (AA) requirements of larval African catfish *Clarias gariepinus* were investigated. In yolk-sac larvae, changes in larval AA profiles were measured at different temperatures. In larvae fed *Artemia* nauplii or an experimental dry diet AA profiles were measured in animals reared at 28°C. The AA profile of *C. gariepinus* larvae changed during ontogeny, especially before the start of exogenous feeding. The AA profiles of the food items (yolk, *Artemia* and the dry diet) differed considerably from the larval ones. No selective absorption of yolk AA could be detected in yolk-sac larvae. Depletion rates of individual AA varied. This may mainly be due to differences between larval and yolk AA profiles, but also to changes in the larval AA profile during ontogeny. There is little regulation of catabolism of individual AA in yolk-sac and starved larvae, and no sparing of essential AA. Until more detailed information is available, larval AA profiles can be used as an index of the qualitative larval AA requirements. Higher temperatures lead to increased absorption and depletion rates of AA, and also to a higher retention efficiency of yolk nutrients. However, changes in temperature did not induce preferential absorption or depletion of individual AA, and caused only small variations in the AA profile.

Introduction

Fish growth and feed conversion efficiencies can be optimised through manipulation of the amino acid (AA) profile of the dietary protein. AA imbalances in the diet will cause an increased AA oxidation and will lead to a decrease in growth rate (Tacon and Cowey 1985; Fauconneau et al. 1992). The amounts and balance of the essential AA (EAA) are of particular importance as the fish depend on their dietary supply (Tacon and Cowey 1985; Wilson 1994).

Since free AA pools are small and their size kept within narrow limits (Houlihan et al. 1995), the absorbed dietary AA are either used for the synthesis of proteins or used otherwise. AA which are not polymerised into proteins can be used for energy production (catabolised), transaminated into another AA, used in gluconeogenesis and lipogenesis, or used in the synthesis of other N-containing molecules (e.g., purines, pyrimidines, hormones). The dietary AA profile that will allow for a maximum protein growth will depend on the efficiency of absorption of each AA, on the AA profile of proteins being synthesised, as well as on the use of

individual AA for energy or other purposes. This optimal AA profile may be different between species, and also within species, depending on the environmental conditions (e.g., temperature) and the physiological state of the animal.

The EAA profile of fish carcass or muscle is considered to be a good index of the EAA requirements of larval fish (Watanabe and Kiron 1994) and of juvenile and adult fish (Tacon and Cowey 1985; Wilson 1994; Mambrini and Kaushik 1995). Little is known about the specific AA requirements of larval fish and whether these change during ontogeny. Fiogbé and Kestemont (1995) found that goldfish, *Carassius auratus,* larvae had much higher EAA requirements (g AA.g^{-1}protein) than juvenile and adult fish. Fish larvae are also reported to have a higher protein requirement than older fish (Dabrowski 1986).

The present paper studies the utilisation of the individual AA for larval growth, in relation to their availability in the diet and to the larval (protein) AA profile. It intends to reveal information on the qualitative AA requirements of fish larvae, in particular those of the African catfish *Clarias gariepinus* (Burchell). Changes in larval AA profiles were studied from hatching until the post-larval stage in *C. gariepinus.* Larval and selected dietary AA profiles were compared in order to detect possible AA imbalances. Selective absorption of individual AA from the yolk-sac and preferential depletion of individual AA in developing eggs, yolk-sac larvae and starved larvae were investigated at different temperatures. Eggs, yolk-sac larvae and starved larvae were used as a model in this study as they can be considered a semi-closed system, were macro-nutrients come exclusively from the yolk-sac, and there is no faecal excretion. AA absorption (and intake) can be estimated through the decrease in yolk AA contents in time. Similarly, the decrease in AA contents in whole eggs/larvae, in this paper termed AA depletion, is the net result of AA catabolism, transamination, and the use of AA for synthesis of other molecules.

Material and methods

Fish

Larvae of the African catfish, *C. gariepinus*, were obtained by artificial fertilisation (Hogendoorn and Vismans 1980) of eggs from a broodstock held under conditions described by Richter et al. (1995). Three independent batches of eggs were used, one for an experiment with yolk-sac larvae and larvae starved after complete yolk absorption, and the other two for experiments with exogenously fed larvae. Each batch was produced by mixing the eggs of four females (individual wet weight ranging from 0.76 to 1.08 kg) with the sperm from two males (0.76 to 1.25 kg).

Experiment with yolk-sac larvae

Approximately 35000 eggs were incubated in each of three 120 l aquaria receiving water from a recirculation system at 25.0±0.3(daily fluctuation)°C. The aquaria had a flow rate of 1 to 1.5 $l.min^{-1}$ and the water was aerated continuously. One aquarium was kept at 25.0±0.3°C, a second thermostated at 28.0±0.1°C, and a third at 31.0±0.1°C. Upon hatching, yolk-sac larvae were transported by the water flow to a second set of 3 aquaria (120 l) kept at the same temperatures as above. Larvae were kept in these aquaria, without feeding. After complete yolk-sac absorption the experiment continued until about 50% mortality by starvation.

To synchronise sampling for ontogenetic development in the three temperature groups, samples were taken at fertilisation (0 time), and at about 32, 55, 86, 150, 192, 249, 320 and 397 physiological day degrees (PD°). PD° (Huisman 1974) are the amount of day degrees corrected for the curvilinear relationship between temperature and the rate of physiological processes, using the q correction factors of Winberg (1956):

$$PD° = (\text{Temperature} \cdot \text{hours post-fertilisation}) / (24 \cdot q)$$

The q values for 25, 28 and 31°C are 0.659, 0.520 and 0.389, respectively.

At each sampling point, samples of 50-100 individuals were taken in triplicate for dry matter contents (DW) and in four replicates for total AA analysis. During the yolk-sac stage, samples were taken both for whole larvae and for larval bodies (whole larvae minus yolk-sac). Due to a fungus infection during egg incubation in the 25°C group, a smaller number of larvae were available for the latter samples. Therefore, the sampling schedule was reduced in this group and no samples were taken at 150, 249 and 397 PD°. Samples were stored at -20°C until analysed. For dry matter determinations, samples were dried at 70°C overnight, kept at 103°C for 5h, and weighed in a micro-balance (Mettler AE160, accuracy of ±10μg).

Experiments with fed larvae

Eggs were incubated in a recirculation system at 28.0±0.2°C. Yolk-sac larvae hatched around 22 hours after fertilisation, and were reared at 28.0±0.2°C in 16 l aquaria until complete yolk absorption. The aquaria had a continuous water flow (0.2 to 0.5 $l.min^{-1}$) from a recirculation system, and gentle aeration. When yolk absorption was almost completed (48h after hatching) groups of 1200 larvae were transferred to each of nine 16 l aquaria. Larvae were reared in a recirculation system, with 5 to 10% of the total water volume being renewed every day and with UV filtration. The aquaria had a flow rate of 1 to 1.5 $l.min^{-1}$, and were kept at 28.0±0.3°C with continuous aeration in the water. For 10 days, the larvae were fed equal portions 5 times a day,

Table 1. Composition ($g.Kg^{-1}$) of the micro-bound dry diet used in experiment II.

Danish fish meal [1]	350	Vitamin C [2]	10
Protibel [1,3]	489	Methionine premix [1]	10
Cod liver oil [1]	30	Glycine [4]	1
Soya oil	30	Vitamin and	
Gelatine	30	mineral premix [1]	50

[1] Supplied by PROVIMI BV (Rotterdam, The Netherlands); [2] Rovimix Stay C (Roche Nederland BV, Mijdrecht, The Netherlands); [3] Brewer's yeast; [4] Crystalline (Sigma-Aldrich NV, Bornem, Belgium)

from 9.00 to 23.00h (interval of 3.5h). Daily ration was calculated to be near satiation according to Verreth and den Bieman (1987). In experiment I larvae were fed *Artemia* nauplii. *Artemia* cysts (strain Great Salt Lake, Sanders Co.) were hatched once a day in 35 $g.l^{-1}$ salt water at 28°C for 24 hours. Newly hatched nauplii were stored in salt water at 4°C until fed to the larvae. In experiment II larvae were fed with a micro-bound dry diet (Table 1). Until day 3 larvae were fed particles with a diameter of 160 - 315 µm, and after that 315 - 630 µm particles.

A sample of 10 to 50 larvae was taken from each of three aquaria for wet weight and dry matter determination at 0, 2, 4, 6, 8 and 10 days of exogenous feeding, before the first daily meal. Samples were stored at -20°C until analysed. Dry matter contents were determined as above. At days 3 and 7 of exogenous feeding three aquaria were fed only the first meal of the day, and larvae sampled for AA analysis just before the meal (time 0) and 24h after the meal. Samples of 30 (day 3) or 15 (day 7) larvae were taken in four replicates and stored at -20°C until analysed.

Amino acid analysis

For AA analysis, an acid and an oxidative hydrolysis were performed separately, both in duplicate. AA contents were derived with nynhidrin after passage of the samples through a LC-ion column (Biotronik LC 5001). Norleucine was used as internal standard. The estimated contents of threonine, serine, isoleucine and valine were corrected for incomplete recovery after hydrolysis by multiplying by: 1.05, 1.10, 1.08 and 1.07, respectively (Slump 1969).

Data handling and statistics

Tyrosine and cysteine are non-essential AA (NEAA) but can only be synthesised from the EAA phenylalanine and methionine, respectively (Bender 1985). In this study these two AA were therefore included in the EAA.

In yolk-sac and starved larvae, absorption and depletion of each AA was estimated by regression on time (hours) of AA contents (ng.individual^{-1}) in yolk and whole individuals, respectively. Yolk AA were estimated by the difference of whole and dissected bodies of yolk-sac larvae. To enable comparison of absorption and depletion rates between abundant and less abundant AA in the yolk, AA contents were expressed as a percentage of the AA content at the start (i.e., unfertilised egg for absorption for regressions referring to yolk-sac larvae, and complete yolk for starved larvae). Regression lines were calculated for each AA at the three different temperatures. Significant regression lines were compared by analysis of covariance (ANCOVA) using the following model:

$$AA_{i,t,T} = \mu + TYPE + b_1 .TIME + b_2 .TEMP + TYPE .TIME + TYPE .TEMP + e$$

where $AA_{i,t,T}$ is the relative content in AA_i at a given time (TIME, t) of development and temperature (TEMP, T), and TYPE is a class variable referring to each AA. A significant effect of the TYPE .TIME interaction together with a non-significant effect of TYPE was interpreted as an indication of significant differences between the slopes of depletion (or absorption) of the individual AA. When significant differences were detected, regression slopes were compared by a test for the equality of slopes of multiple regression lines (Sokal and Rohlf 1995). For pair-wise comparison of regression slopes a t-test for independent samples with different variances (Snedecor and Cochran 1989) was used.

AA profiles (pAA_i, g per 100g) were calculated by dividing the individual AA content by the total AA content at each sampling point. Differences in AA profiles were studied by analysis of variance and pairwise t-test taking each AA separately. When significant differences were detected by ANOVA a Bonferroni t-test was performed to analyse differences between class levels. Differences in the AA profile during development (STAGE, the sampling points in PD°) in eggs and unfed larvae at different temperatures (TEMP) were tested using the following model:

$$pAA_i = \mu + STAGE + TEMP + STAGE .TEMP + e$$

A similar model was used to test the effects of day of exogenous feeding (day 3 or day 7) and of feeding state (9 or 24 hours after a meal) on AA profile. One-way ANOVA was used to study differences in the AA profile of yolk and the two diets, and also the effect of time of development on the AA profile from hatching until day 7 of exogenous feeding in larvae growing at 28°C.

Data were analysed using the procedures REG, GLM and TTEST of the SAS Statistical Software package (SAS Inc. 1995). Differences were considered significant when $P<0.05$.

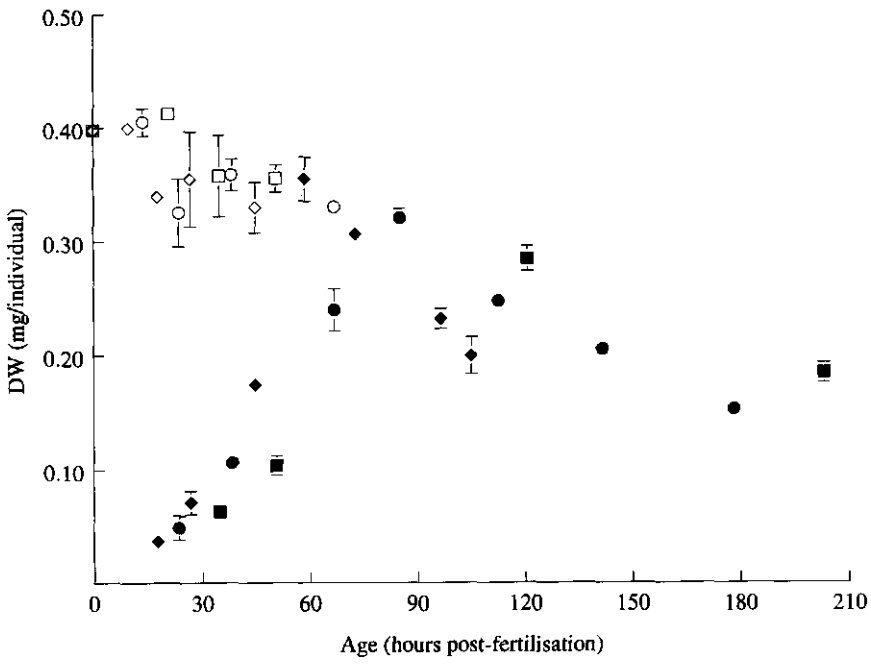


Fig. 1. Whole egg/larvae (empty symbols) and larval body (full symbols) dry matter contents (DW) in developing eggs, yolk-sac larvae and starved larvae of *C. gariepinus* reared at 25°C (squares), 28°C (circles) and 31°C (diamonds). Error bars are standard deviations of three pooled samples of 50-100 individuals.

Results

Growth

Yolk-sac larvae (larval bodies) grew faster at higher temperatures (Figure 1 and Table 2). The relative growth rates were 99, 237 and 484 %DW.day^{-1} between hatching and complete yolk-sac absorption, at 25, 28 and 31°C, respectively. Dry matter contents in eggs and unfed larvae decreased with time of development (Figure 1), as energy was spent for the metabolic processes. The decrease in larval DW seemed to proceed at different rates before and after complete yolk absorption, therefore two separate linear regressions were calculated. The slopes (and intercepts) of those two regressions (Table 2) were found to be significantly different from each other at the three temperatures. The rate at which DW decreases after complete yolk absorption was higher with increasing temperatures, and the thermal effect was more pronounced at 31 than at 28°C. However, in the egg and yolk-sac stage the slopes of DW were very similar, with no apparent effect of temperature.

Table 2. Linear regression of dry matter and amino acid contents (µg / individual) on time in *Clarias gariepinus* eggs, yolk-sac and starved larvae. Q10 values are also given.

Temp (°C)	Intercept (µg)	Slope (µg/h)	r2	n	P	Q10	Temp Range[1]
Larval bodies dry weight until CYA							
25	-27.5 ± 0.3	2.60 ± 0.00	1.00	3	0.0020	5.97	25 - 31
28	-60.4 ± 5.3	4.48 ± 0.09	1.00	4	0.0004	6.18	25 - 28
31	-121.3 ± 54.0	7.58 ± 1.34	0.94	4	0.0298	5.77	28 - 31
Egg and whole larvae dry weight before CYA							
25	413.4 ± 13.7	-1.04 ± 0.21	0.93	4	0.0376	0.99	25 - 31
28	405.6 ± 7.8	-1.04 ± 0.15	0.94	5	0.0062	1.02	25 - 28
31	396.7 ± 14.3	-1.03 ± 0.40	0.69	5	0.0829	0.97	28 - 31
Larval dry weight after CYA							
25	433.4	-1.22		2		5.24	25 - 31
28	459.2 ± 26.1	-1.75 ± 0.19	0.98	4	0.0122	3.35	25 - 28
31	548.3 ± 6.3	-3.29 ± 0.07	1.00	4	0.0005	8.20	28 - 31
Egg and whole larvae AA content							
25	308.6 ± 9.3	-0.94 ± 0.09	0.96	6	0.0005	2.23	25 - 31
28	299.2 ± 8.9	-1.11 ± 0.10	0.95	9	0.0001	1.75	25 - 28
31	311.8 ± 7.7	-1.52 ± 0.12	0.96	9	0.0001	2.85	28 - 31
Yolk AA content							
25	332.9 ± 118.5	-2.43 ± 1.35	0.76	3	0.3223	3.99	25 - 31
28	315.3 ± 17.7	-3.69 ± 0.30	0.99	4	0.0066	4.02	25 - 28
31	354.9 ± 49.7	-5.58 ± 1.23	0.91	4	0.0455	3.96	28 - 31

Values are means ± sd. [1] Temperature ranges used to calculate Q10 values, based on the regression slopes. CYA stands for complete yolk-sac absorption.

After 10 days, larvae fed *Artemia* nauplii were about twice as big as larvae fed the dry diet (Table 3). Growth during the first two days of exogenous feeding in the *Artemia* fed larvae was very low due to an insufficient amount of food presented to the larvae (very low *Artemia* hatching rates). Between day 2 and day 6, larvae fed *Artemia* had considerably higher growth rates than the ones fed the dry diet. After day 6 growth rates were similar.

Amino acid profiles

The diets used in this study had quite different AA profiles (Table 4). The main differences in yolk (egg), *Artemia* nauplii and the dry diet, were observed for cysteine, glycine, serine and proline. The yolk AA composition was more similar to the dry diet than to the *Artemia* nauplii.

The AA profile of yolk-sac larvae and starved larvae of *C. gariepinus* changed considerably during development (Table 5). The only AA which did not change significantly were threonine, cysteine and serine. The AA whose participation in the profile changed most

Table 3. Growth of Clarias gariepinus larvae fed Artemia or dry diet.

	Wet Weight (mg)		Dry Weight (mg)		Relative Growth Rate (%/day)	
Day	Artemia	Dry Diet	Artemia	Dry Diet	Artemia	Dry Diet
0	2.96 ± 0.25	2.16 ± 0.00 **	0.53 ± 0.04	0.36 ± 0.01 **		
2	3.92 ± 0.16	4.20 ± 0.61	0.59 ± 0.04	0.62 ± 0.04	5.9 ± 6.1	31.0 ± 5.8 **
4	17.43 ± 7.53	7.00 ± 0.51	1.59 ± 0.12	0.98 ± 0.09 **	63.7 ± 4.8	25.9 ± 2.2 ***
6	29.91 ± 4.39	12.90 ± 0.79 **	4.47 ± 0.58	1.87 ± 0.16 **	67.6 ± 5.2	38.1 ± 1.0 ***
8	48.12 ± 1.35	26.53 ± 3.25 ***	7.71 ± 0.31	3.64 ± 0.63 ***	31.8 ± 6.9	39.3 ± 13.4
10	98.78 ± 10.2	45.32 ± 3.01 **	17.13 ± 1.88	7.17 ± 0.24 ***	48.9 ± 7.5	41.5 ± 13.8

Values are means±sd of three pooled samples of 10-50 larvae. Significant differences between the two diets are shown as : * ($P<0.05$), ** ($P<0.01$). Relative Growth Rate = (e^g-1) . 100, where $g = (\ln DW_{t_2} - \ln DW_{t_1}) / (t_2 - t_1)$.

were histidine, glycine and methionine, which changed up to 41, 32 and 25% above their lower levels, respectively. In general, the magnitude of the changes in AA profile with development increased at higher temperatures. An effect of temperature on the AA profile was also detected by ANOVA; arginine, lysine, valine, alanine and proline had lower contributions to the AA profile at 25°C than in the two other temperatures, while histidine and glycine were lower at 28 and 31°C, respectively. However, these changes were quite small, never exceeding 9% of the lower level of the AA in question.

The AA profiles of yolk-sac larvae and *Artemia* fed larvae growing at 28°C (Table 6) showed a similar trend to what was observed in yolk-sac larvae and starved larvae (Table 5). The contribution (%) of lysine, aspartate, glycine and proline to the AA profile increased during development, while histidine and isoleucine decreased. The most noticeable changes were the ones of histidine and glycine, with 41 and 31% above their lower levels, respectively.

In larvae fed both *Artemia* and dry diet, the AA profile changed little between day 3 and day 7 of exogenous feeding, or when larvae fed *Artemia* were starved for 24 hours (Table 7). The biggest changes were observed for methionine, glycine, histidine and proline, and those were always less than 10% above the lower level of the AA in question. On day 3 of exogenous feeding, no significant differences were found between the AA profiles of larvae fed *Artemia* and dry diet. On day 7, proline and histidine, respectively, had a higher and a lower contribution to the AA profiles of *Artemia* fed larvae when compared to larvae fed the dry diet.

The AA profile of yolk-sac larvae bodies and starved larvae was considerably different from the AA profile of the yolk (egg). When compared to yolk-sac larvae, yolk was poor in glycine (difference in contribution to the AA profiles of 60 to 100% of the profile in yolk) and

Table 4. AA profile (g / 100g AA) of *Clarias gariepinus* eggs, *Artemia* nauplii and the micro-bound dry diet.

AA	Egg	Artemia nauplii	Dry diet
lys	7.54 ± 0.21 b	8.51 ± 0.21 a	7.70 ± 0.05 b
leu	9.81 ± 0.16 a	7.67 ± 0.14 b	7.99 ± 0.14 b
arg	6.54 ± 0.24 b	7.33 ± 0.18 a	6.06 ± 0.28 c
val	6.47 ± 0.05 a	5.96 ± 0.10 b	5.68 ± 0.11 c
ile	6.35 ± 0.10 a	5.46 ± 0.20 b	5.17 ± 0.11 b
thr	5.20 ± 0.08	5.12 ± 0.27	4.83 ± 0.14
phe	3.88 ± 0.03	4.18 ± 0.55	4.23 ± 0.04
tyr	3.28 ± 0.05	2.72 ± 1.80	3.13 ± 0.18
his	2.91 ± 0.07 a	2.36 ± 0.05 b	2.83 ± 0.06 a
met	2.80 ± 0.04	2.33 ± 0.28	2.34
cys	0.95 ± 0.01 b	1.74 ± 0.27 a	0.81
glu	12.73 ± 0.30 b	13.20 ± 0.48 b	15.38 ± 0.20 a
asp	8.89 ± 0.16 b	9.30 ± 0.30 b	9.87 ± 0.14 a
gly	3.23 ± 0.05 c	5.13 ± 0.15 b	7.24 ± 0.14 a
ala	7.63 ± 0.16 a	5.53 ± 0.40 c	6.92 ± 0.04 b
ser	7.29 ± 0.11 a	6.37 ± 0.42 b	5.05 ± 0.13 c
pro	4.74 ± 0.26 b	6.83 ± 0.60 a	4.75 ± 0.37 b
EAA	55.72	53.39	50.77
NEAA	44.51	46.36	49.21

Values are means ± sd (n = 2 for met, cys and tyr; n = 4 for other AA). Different letters in the same row stand for significant differences.

cysteine, glutamate and phenylalanine (difference around 20%). Depending on the stage of development, 11.4 to 21.7% of the AA intake was unavoidably lost due to the imbalance between the dietary and the larval AA profile. This imbalance was estimated by summing the amounts of each AA in the diet above the amount that can be incorporated into protein. The AA amounts that can be incorporated into protein were calculated based on the larval AA profile and the first-limiting AA. The first limiting AA changed during development of yolk-sac larvae, being phenylalanine initially, then lysine, and then cysteine close to complete yolk-absorption.

The AA profiles of fed larvae also differed considerably from the ones of the yolk, *Artemia* nauplii and dry diet. *Artemia* nauplii had an AA profile poor in histidine, leucine, glutamate, glycine, tyrosine and aspartate, while the dry diet was poor in lysine, arginine, histidine, phenylalanine and cysteine. The first limiting AA was tyrosine for the *Artemia*-fed larvae and cysteine for the larvae fed dry diet. The unavoidable AA loss was 27.0 and 39.4% of the AA intake, for larvae fed *Artemia* nauplii and dry diet, respectively.

Table 5. AA profiles of yolk-sac and starved larvae of *Clarias gariepinus* reared at 31°C, together with the results of the two-way ANOVA with developmental stage and temperature as main effects.

	Yolk-sac larvae	Starved larvae						Temperature		
	18 HPF	27 HPF	45 HPF	59 HPF	73 HPF	97 HPF	105 HPF	25	28	31
lys	8.13 ± 0.31 c	8.17 ± 0.11 bc	8.34 ± 0.16 ab	8.36 ± 0.14 abc	8.62 ± 0.14 a	8.56 ± 0.05 ab	8.04 ± 0.21 bc	b	a	a
leu	8.84 ± 0.16 a	8.75 ± 0.16 ab	8.58 ± 0.11 bc	8.60 ± 0.05 cd	8.15 ± 0.08 de	8.13 ± 0.12 e	8.21 ± 0.40 e			
arg	6.37 ± 0.18 b	6.38 ± 0.25 ab	6.38 ± 0.25 ab	6.44 ± 0.21 ab	6.68 ± 0.04 a	6.72 ± 0.09 a	6.28 ± 0.17 a	b	a	a
val	5.94 ± 0.20 a	5.83 ± 0.17 ab	5.89 ± 0.12 ab	5.94 ± 0.09 abc	5.79 ± 0.07 bc	5.76 ± 0.26 cd	5.09 ± 0.26 d	b	ab	a
ile	5.24 ± 0.17 a	5.11 ± 0.12 ab	5.09 ± 0.23 bc	5.24 ± 0.12 bcd	4.93 ± 0.14 cd	4.89 ± 0.08 d	4.45 ± 0.26 e			
thr	4.80 ± 0.44	4.87 ± 0.05	4.90 ± 0.11	5.00 ± 0.16	4.81 ± 0.03	4.76 ± 0.04	4.60 ± 0.07			
phe	4.77 ± 0.24 a	4.79 ± 0.16 a	4.50 ± 0.11 b	4.20 ± 0.08 b	4.25 ± 0.04 b	4.29 ± 0.08 b	4.06 ± 0.07 b			
tyr	3.71 ± 0.04 ab	3.93 ± 0.00 a	3.62 ± 0.12 bc	3.33 ± 0.15 bc	3.57 ± 0.12 bc	3.66 ± 0.03 c	3.43 ± 0.08 c			
his	3.41 ± 0.21 a	3.53 ± 0.13 a	3.37 ± 0.15 b	3.23 ± 0.24 c	3.03 ± 0.10 cd	2.81 ± 0.07 de	2.55 ± 0.06 e	a	b	a
met	2.67 ± 0.26 b	2.66 ± 0.10 ab	2.76 ± 0.01 ab	3.15 ± 0.25 a	2.63 ± 0.01 b	2.53 ± 0.02 b	2.82 ± 0.01 b	b	b	a
cys	1.15 ± 0.04	1.88 ± 1.01	1.08 ± 0.00	1.13 ± 0.09	1.10 ± 0.10	1.17 ± 0.01	0.99 ± 0.04			
glu	14.28 ± 0.76 c	14.20 ± 0.12 bc	14.75 ± 0.25 ab	14.62 ± 0.10 a	15.07 ± 0.22 a	15.07 ± 0.30 a	15.18 ± 0.92 ab			
asp	9.52 ± 0.43 d	9.60 ± 0.12 cd	9.92 ± 0.06 abc	9.88 ± 0.16 ab	10.26 ± 0.19 a	10.18 ± 0.14 a	9.58 ± 0.20 bcd			
gly	5.30 ± 0.29 c	5.25 ± 0.15 c	5.38 ± 0.06 c	5.06 ± 0.06 b	5.89 ± 0.13 b	6.19 ± 0.08 a	6.13 ± 0.07 a	a	a	b
ala	5.64 ± 0.45 ab	5.44 ± 0.31 b	5.76 ± 0.14 ab	6.13 ± 0.17 a	5.66 ± 0.02 ab	5.73 ± 0.06 ab	5.49 ± 0.19 ab	b	a	ab
ser	5.76 ± 0.24	5.54 ± 0.13	5.57 ± 0.23	5.89 ± 0.24	5.43 ± 0.23	5.35 ± 0.17	5.43 ± 0.33			
pro	3.82 ± 0.16 c	3.89 ± 0.11 bc	4.02 ± 0.13 bc	4.06 ± 0.20 b	4.14 ± 0.29 bc	4.14 ± 0.49 b	7.61 ± 0.12 a	b	ab	a
EAA	55.04	55.90	54.50	54.62	53.54	53.27	50.53			
NEAA	44.31	43.92	45.42	45.64	46.45	46.67	49.42			

Values are means ± sd (n=2 for met, cys and tyr; n=4 for other AA); HPF stands for hours post-fertilisation; complete yolk absorption occurred close to 59 hours post-fertilisation; Different letters for the same AA (row) at the various sampling points or temperatures stand for significant differences according to a Bonferroni t-test. These differences refer to the two-way ANOVA model , and not to the 31°C group alone.

Table 6. AA profiles of yolk-sac and *Artemia*-fed larvae of African catfish reared at 28°C.

	Yolk-sac larvae				Artemia fed larvae	
	24 HPF	38 HPF	67 HPF	85 HPF	Day 3	Day 7
lys	8.03 ± 0.12 c	8.54 ± 0.11 b	8.47 ± 0.16 bc	8.39 ± 0.24 bc	8.56 ± 0.08 ab	8.72 ± 0.16 a
leu	8.96 ± 0.37 a	8.76 ± 0.08 ab	8.48 ± 0.05 ab	8.36 ± 0.17 b	8.25 ± 0.14 ab	8.20 ± 0.13 ab
arg	6.44 ± 0.16 ab	6.64 ± 0.28 ab	6.53 ± 0.12 ab	6.28 ± 0.13 b	6.61 ± 0.07 a	6.54 ± 0.18 a
val	6.12 ± 0.23 a	5.80 ± 0.05 ab	5.75 ± 0.05 ab	5.71 ± 0.19 ab	5.74 ± 0.38 ab	5.46 ± 0.26 b
ile	5.39 ± 0.26 a	5.06 ± 0.12 ab	5.01 ± 0.06 ab	4.85 ± 0.12 b	4.75 ± 0.31 b	4.87 ± 0.43 b
thr	4.99 ± 0.11	4.84 ± 0.04	4.84 ± 0.05	4.66 ± 0.37	4.98 ± 0.22	4.74 ± 0.32
phe	4.65 ± 0.32	4.72 ± 0.06	4.30 ± 0.03	4.34 ± 0.12	4.32 ± 0.04	4.36 ± 0.10
tyr	3.67 ± 0.10 a	3.83 a	3.64 ± 0.00 a	3.63 ± 0.04 a	3.87 ± 1.60 a	3.57 ± 0.50 b
his	3.46 ± 0.11 a	3.43 ± 0.17 a	3.13 ± 0.06 b	2.97 ± 0.06 bc	2.73 ± 0.03 d	2.64 ± 0.06 cd
met	2.55 ± 0.10	2.63 ± 0.02	2.71 ± 0.02	2.76 ± 0.09	2.54 ± 0.04	2.66 ± 0.00
cys	1.16 ± 0.01	1.13 ± 0.05	1.17 ± 0.00	1.21 ± 0.11	1.31 ± 0.01	1.34 ± 0.21
glu	13.80 ± 0.27 c	14.70 ± 0.18 b	15.10 ± 0.13 ab	15.55 ± 0.16 a	14.45 ± 0.00 b	14.56 ± 0.17 b
asp	9.43 ± 0.11 c	9.66 ± 0.11 bc	9.92 ± 0.10 ab	10.11 ± 0.18 a	9.95 ± 0.21 a	9.97 ± 0.28 a
gly	5.24 ± 0.20 b	5.29 ± 0.17 b	5.52 ± 0.20 b	6.18 ± 0.22 a	5.94 ± 0.03 a	6.21 ± 0.12 a
ala	5.93 ± 0.53 ab	5.32 ± 0.16 b	5.58 ± 0.13 ab	5.64 ± 0.09 ab	5.62 ± 0.20 ab	5.88 ± 0.15 a
ser	5.90 ± 0.08	5.36 ± 0.09	5.45 ± 0.08	5.03 ± 0.72	5.29 ± 0.12	4.95 ± 0.52
pro	4.02 ± 0.15 c	3.96 ± 0.01 c	4.22 ± 0.02 bc	4.44 ± 0.23 b	4.26 ± 0.33 b	4.72 ± 0.10 a
EAA	55.42	55.39	54.04	53.18	53.66	53.10
NEAA	44.32	44.29	45.78	46.95	45.50	46.29

Values are means ± sd (n=2 for met, cys and tyr; n=4 for other AA); HPF stands for hours post-fertilisation; complete yolk absorption occurred close to 85 hours post-fertilisation; Different letters for the same AA (row) stand for significant differences according to a Bonferroni t-test.

Amino acid utilisation

Through analysis of covariance it was determined that AA absorption was significantly affected by time of development and temperature, but the effect of AA type and the interactions of AA type with time and temperature were not significant. These results indicate that there is a temperature effect on AA absorption, but within each temperature the individual AA are absorbed at similar relative rates (%.hour^{-1}). Absorption rate increased with rising temperatures (Table 2).

For almost all AA, depletion rates (regression slopes) before and after complete yolk absorption were significantly different at the three temperatures. Therefore, regression slopes of the individual AA were compared separately in these two developmental periods. Glycine increased with time until complete yolk absorption. It was thereby quite different from the other AA. Therefore, glycine was excluded from the ANCOVA analysis and the comparisons of regression slopes. Cysteine (three temperatures) and methionine (31°C) were also excluded in these tests in the period before complete yolk absorption as the regressions on time were not significant. Similarly, cysteine (25°C) and proline (31°C) were excluded from the statistical analysis in the period after complete yolk absorption. At 31°C, proline had a clear increase between 320 and 397 PD° (end of the starvation period). There was a significant effect of developmental time, temperature and the interaction TYPE . TIME on AA depletion, both before and after complete yolk absorption. In turn, the AA type and the interaction TYPE .TEMP had no effect on AA depletion. These results indicate that there is a selective depletion (%.hour^{-1}) of AA, which is not affected by temperature. When regression slopes (see Figure 2) were compared, cysteine, tyrosine, phenylalanine, lysine, glutamate and aspartate had lower relative depletion rates before complete yolk absorption than valine, leucine, isoleucine, proline, alanine and serine. Differences between slopes of individual AA were less pronounced in starved larvae. However, no significant differences were detected between the regression slopes within each temperature in both developmental periods. In general, depletion rates increased with rising temperatures, and were higher in starved larvae than in eggs and yolk-sac larvae. The slopes (and intercepts) of the regressions of the summed amounts of EAA and NEAA on time were not significantly different, neither before nor after complete yolk absorption. The decrease in the total amount of AA with time at each temperature could be well described by a single regression line from the egg stage until starvation (Table 2), as the slopes (and intercepts) of the two developmental periods were not significantly different. Apparently, the increase in total AA depletion rate was higher between 28 and 31°C than between 25 and 28°C.

Comparison of the retention efficiencies of the individual AA at complete yolk absorption (Figure 3) suggests that some AA were retained more efficiently by yolk-sac larvae were associated with higher retention efficiencies. NEAA were retained more efficiently than

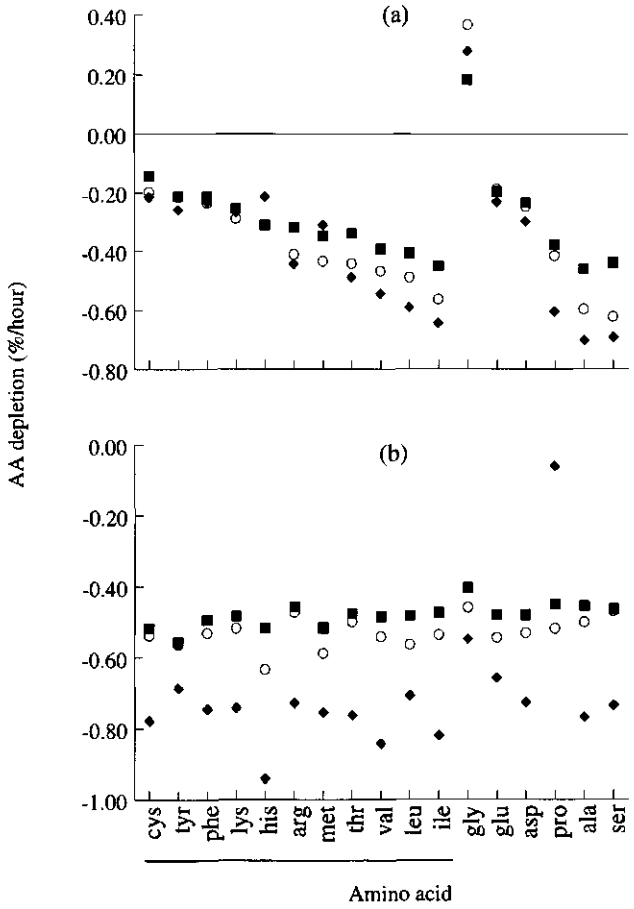


Fig. 2. Amino acid (AA) depletion rates (%.hour^{-1}) in developing eggs and yolk-sac larvae (a) and starved larvae (b) of *C. gariepinus* reared at 25°C (diamonds), 28°C (circles) and 31°C (squares). Depletion rates were calculated as the slopes of the linear regression of relative AA amounts (amount at sampling time / amount at the regression starting point * 100; starting point was unfertilised egg for regressions until complete absorption, and complete yolk absorption for starved larvae regressions) on time.

than others, in a pattern comparable to that of the regression slopes above. Higher temperatures EAA, but that is due to the net increase in the amount of glycine. If this AA is excluded the retention efficiencies between EAA and NEAA were very similar. Interestingly, the average retention efficiency of the sum of all AA (62.4, 67.3 and 74.2%, at 25, 28 and 31°C, respectively) was considerably lower than the one of dry matter contents (71.9, 80.7 and

89.3%). The regression slopes of the contents of all AA summed were also lower than the ones of dry matter contents (Table 2). The differences were only significant for starved larvae.

Discussion

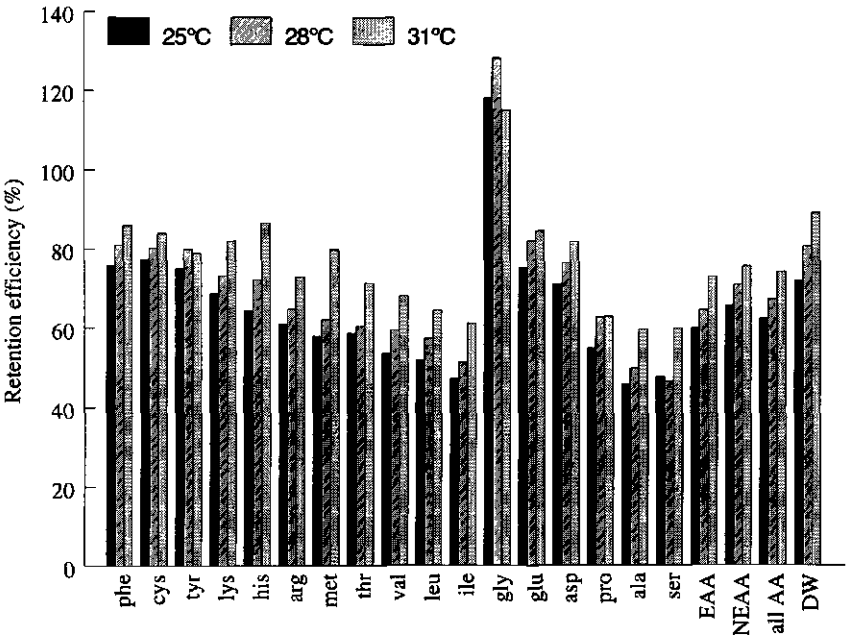
Ontogenic changes in AA profiles

The AA profile of *C. gariepinus* larvae changed with ontogenic stage, especially in the period prior to exogenous feeding (Tables 5 and 6). This probably reflects a change in the proportions of the various proteins being synthesised during larval growth and/or the synthesis of different proteins. Such changes are probably associated with the allometric growth of the larvae, as different organs and tissues develop at varying rates and at different times (Oikawa and Itazawa 1984; Osse and van den Boogaart 1995) and the various organs have different AA profiles. Furthermore, the AA profile of *C. gariepinus* changes further towards the juvenile stage. Juvenile catfish (Hoffman et al. 1995) have considerably higher proportions of proline, alanine and glycine in their AA profile than larvae, the opposite being true for aspartate, leucine, isoleucine and valine. Juvenile and adult fish whole body AA profiles are commonly referred to vary little both between and within species (Wilson and Cowey 1985; Wilson and Poe 1985; Wilson 1994; Ramseyer and Garling 1994). However, these authors based their conclusions mostly on studies of cold-water species. Fish growing at higher temperatures may have more variable AA profiles: some differences in the AA profiles of juvenile and adult fish have been found in dolphin fish, *Coryphaena hippurus* (Ostrowski and Divakaran 1989) and in white sturgeon, *Acipenser transmontanus* (Ng and Hung 1994). Different strains of *C. gariepinus* also had variable AA profiles (Hoffman et al. 1995). Further, the variation between larval and post-larval AA profiles is, besides in *C. gariepinus*, also apparent in Atlantic salmon, *Salmo salar* (Wilson and Cowey 1985; Srivastava et al. 1995), and in turbot, *Scophthalmus maximus* (Finn et al. 1996; Conceição et al. 1997b). Small changes in the AA profile may have important implications in terms of AA requirements. For instance, a 0.5% decrease in the contribution of methionine to the AA profile can result in an increase of 21% of the methionine requirement.

Feeding catfish larvae with *Artemia* nauplii or with a dry diet, or fasting the larvae up to 24 hours, had little effect on the larval AA profile. The changes in AA profiles in larvae fed the two diets on day 7 was probably more a result of the different larval weights (see Table 2) than a dietary effect. Development of catfish larvae correlates better with size than age (Verreth 1994).

Dietary AA profiles

Egg AA profiles have been proposed as an index for requirements of EAA in young fish (Ketola 1982; Dendrinos and Thorpe 1987; Shcherbina et al. 1988). However, considerable differences



larvae of *C. gariepinus*. The same has been shown for larval turbot (Finn et al. 1996; Conceição

Fig. 3. Retention efficiency (amount at complete yolk absorption / amount at fertilisation .100) of individual amino acids (AA) , summed essential AA (EAA), summed non-essential AA (NEAA), sum of all AA and dry matter contents (DW) in yolk-sac larvae of *C. gariepinus* reared at three temperatures.

in the AA profile of eggs and of juvenile and adult whole body have been reported for different fish species (e.g., Ketola 1982; Wilson and Poe 1985; Ng and Hung 1994). Egg (yolk) AA profiles are also considerably different from those of yolk-sac larvae and exogenous feeding et al. 1997b). This is remarkable, because the AA profile of yolk proteins is often assumed to match closely the AA requirements in early life stages, as a result of a high selective pressure for maximum growth. Nevertheless, also for larval fish the AA profile of the egg is rather imbalanced, and is probably a bad indicator of AA requirements. The fact that yolk proteins have a sub-optimal AA profile may be related to the considerable changes in AA profile during the yolk-sac stage, including changes in the first limiting AA (Tables 5 and 6). Another possible explanation is that the larvae will use some AA for energy production anyway (an obligatory AA loss), and therefore there is no need for further optimisation of the AA profile.

The two diets used to feed larvae also had an imbalanced AA profile. Tyrosine and cysteine were probably limiting growth in *Artemia*-fed larvae and larvae fed dry diet, respectively. The dry diet seemed to be more imbalanced, leading to higher unavoidable AA losses. In fact, larvae fed the dry diet had lower growth rates than *Artemia* fed larvae until day 6 of exogenous feeding. This may also have been correlated with the poor development of the digestive system on the first days of feeding, as the stomach only becomes functional in *C. gariepinus* larvae at a size of around 20 mg wet weight (Verreth et al. 1992).

Yolk AA absorption

Our results support that AA absorption from the yolk occurs in bulk, by endocytosis of yolk mass (Heming and Buddington 1988). Indeed, the absorption rates of the different AA were similar after correction for the relative abundance of each AA in the yolk. A selective uptake of free AA from the yolk was however observed in Atlantic halibut, *Hippoglossus hippoglossus* (Rønnestad et al. 1993). It has been suggested that free AA may be absorbed by mechanisms other than bulk endocytosis (Rønnestad and Fyhn 1993). Such selective mechanisms of free AA absorption may also exist in *C. gariepinus*, but they are not quantitatively important due to the low contents (less than 5% of the total AA, Polat et al., unpublished) of free AA in the egg. Even in fish larvae with higher free AA content (halibut and other marine species) the differences in absorption rates of the individual AA may be reduced when the absorption of AA from yolk protein is taken into account.

Amino acid utilisation

In the present study we considered AA depletion to be an index of AA use for energy production. However, depletion rates may also reflect the use of the individual AA for transamination to other AA, or for synthesis of glucose, lipids or other N-containing molecules. Furthermore, when AA are synthesised (NEAA) the depletion rate of an individual AA will be equal to the AA use minus the AA synthesis. Therefore, depletion rates of NEAA should be analysed with care. In fact, the present study showed that yolk-sac larvae of *C. gariepinus* are capable of synthesising glycine (Figure 3), probably at the expense of serine (its direct precursor, Bender 1985), which is in excess in the yolk compared to the larval AA profile. The summed amount of serine and glycine absorbed from the yolk is sufficient to allow for the increase in larval body contents of these two AA. However, this study provides no evidence for whether other NEAA can be synthesised before exogenous feeding. High activity of transaminases has been found from the onset of exogenous feeding in *C. gariepinus* (Segner and Verreth 1995).

The observed changes in AA depletion rates can be, at least partly, explained by differences in the larval and yolk AA profiles, and also by changes in AA profile during ontogeny. The apparently high depletion rates of the branched chain AA (leu, ile, val) (Figure 2) are associated with a decrease in their contribution to the larval AA profile (Table 5), and also with the excess of these AA in the yolk when compared to the larval AA profile. In turn, the low yolk contents of phenylalanine and tyrosine when compared to larval AA profile are associated with relatively low depletion rates for these AA, even though their contribution to the larval AA profile decreases with development. Lysine and cysteine also had low depletion rates, reflecting low contents of those AA in the yolk. Furthermore, the differences in depletion rates of the different AA (Figure 2) were much lower in starved larvae, when no dietary influence existed, than in yolk-sac larvae. Overall, changes in depletion rates were more related to AA imbalances in the yolk, than to ontogenic changes in AA profile. Changes in depletion rates can also be explained by modulation of the activity of the specific enzymes involved in AA metabolism. Different enzymes are involved in transamination and catabolism of AA (Cowey and Walton 1989; Jürss and Bastrop 1995) allowing for the differential use of individual AA in these processes. An efficient use of the available AA resources is assured by the higher affinity of the enzymes involved in protein synthesis than those involved in AA catabolism (Cowey and Walton 1989). In the rat, alanine, glutamate, glutamine and aspartate are preferentially used for energy production, while the other NEAA and the EAA are spared for synthetic purposes, in particular when the amount of dietary protein is limiting or when there are AA imbalances (Tanaka et al. 1995). However, in fish the AA catabolising pathways are less adaptable to dietary influences than in mammals (Walton 1985). NEAA were oxidised at faster rates than EAA in juveniles of turbot (Cowey and Sargent 1979) and rainbow trout, *Oncorhynchus mykiss* (Kim et al. 1992). However, while trout had the ability to conserve EAA better than NEAA when fed protein deficient diets (Kim et al. 1992), dietary protein level had no effect on AA catabolism rates in common carp, *Cyprinus carpio* (Nagai and Ikeda 1972, 1973) and turbot (Cowey and Sargent 1979). In the present study, no significant differences between the depletion rates of the summed amounts of EAA and NEAA were found. Furthermore, the low retention efficiency of AA in comparison with the retention efficiency of dry matter (Figure 3), suggests that AA are an important energy source during the yolk-sac stage. This agrees with previous observations on *C. gariepinus* yolk-sac larvae (Polat et al. 1995; Verreth et al. 1995). The high utilisation of AA for energy in fish larvae has been attributed to a reduced catabolic adaptability, related to their strictly carnivorous nature (Dabrowski 1986). Thus, it is unlikely that *C. gariepinus* larvae have the ability to spare individual AA by other means than (eventually) synthesising proteins with different AA profiles.

Effect of temperature

In our study, the larval AA profile changed slightly with the different temperatures (Table 5). This suggests that different temperatures induce the synthesis of different proteins, or more likely, a change in the proportions of the various proteins being synthesised during larval growth. Besides a quantitative effect on larval growth, temperature also affects the timing of differentiation of different organs and tissues (Johnston 1993; Gibson and Johnston 1995; Rombough 1995). Temperature may also lead to alternative patterns of muscle differentiation, with consequences for growth later in development (Johnston 1993). It remains to be established whether these (small) temperature related changes in the AA profile have any significance in terms of the AA requirements.

Retention efficiency of both AA and dry matter yolk contents increased with temperature in the range of 25-31°C (Figure 3). This agrees with our previous observations (Conceição et al. 1993), and disagrees with a maximum yolk retention efficiency at 25°C (Kamler et al. 1994). This increase in retention efficiency with temperature is explained by higher Q10 found for growth (6.0) than for the catabolism-related processes. Before complete yolk absorption, there was no thermal effect on dry matter depletion (Q10 = 1.0) and it ranged between 1.75 and 2.85 for AA depletion (Table 2). In earlier studies of yolk-sac larvae of *C. gariepinus* the Q10 for oxygen consumption, was estimated to be 2.0 for temperatures between 25.0 and 29.5°C (Verreth et al., unpublished data). Kamler et al. (1994) found a much higher Q10 for oxygen consumption (4.1), and a much lower Q10 for growth (1.9), for temperatures ranging from 25 to 28°C. These discrepancies are difficult to explain, and may be due to differences in spontaneous activity due to the rearing systems used or to different genotypes. In the present study, different Q10 values were estimated for both AA depletion and dry matter depletion after complete yolk absorption in the ranges 25-28°C and 28-31°C. These differences indicate a non-linear temperature effect in the two processes. Depletion of both AA and dry matter accelerates with increasing temperatures. This implies that while retention efficiency increases with temperature, the gain in retention efficiency will be decreasing.

Growth optimisation

Dietary AA imbalances increase the oxidation of AA (Tacon and Cowey 1985; Fauconneau et al. 1992). The close matching in this study between the differences in yolk and larval AA profiles and the variation in depletion rates of the individual AA supports this hypothesis. Considering the high potential for growth and the high food intake (%DW.day^{-1}) of fish larvae (Houde 1989; Wieser and Medgyesy 1990; Conceição et al. 1997a), such dietary AA imbalances may have a much larger impact on growth depression, reduction of feed conversion efficiencies and increased nitrogen waste production, in larvae than in older fish.

As larvae can only store AA in the form of proteins, imbalances between dietary and larval AA profiles will tend to bring a unavoidable AA loss. Small differences between dietary and larval AA profile may lead to high unavoidable AA losses. The contribution of tyrosine to the AA profile of *Artemia* nauplii was 1% lower than to that of *C. gariepinus* larvae, tyrosine being the first limiting AA. This difference suggests a unavoidable AA loss of 27% of the total AA intake. Increased unavoidable AA losses will be reflected in higher AA requirements. However, several factors may reduce (or amplify) the impact of the dietary imbalances on AA losses. There is an obligatory AA loss, independent of the AA profile of the diet, as some AA will always be used for energy production even when there is a perfect matching between dietary and larval AA profiles. If the AA losses induced by dietary imbalance are below this obligatory AA loss, larval growth cannot be improved by manipulation of the AA profile of the diet. Furthermore, possible dietary AA imbalances may be compensated by the endogenous release of free AA through protein turnover. In fish larvae the daily turnover rate of proteins is correlated with the growth rate and can be more than 50% (Houlihan et al. 1993). In addition, the absorption of individual AA in the gut depends on different transport systems (Jürss and Bastrop 1995) which may proceed at different rates (Dabrowski 1983). Therefore, variation in the rates of absorption of individual AA may attenuate or aggravate AA imbalances. Finally, the visceral organs, e.g., liver and gut, may play a buffering role in AA imbalances. After a meal, the dietary AA are essentially deposited into these organs with high metabolic activity. Muscle has a much lower metabolic activity and has a continuous supply of AA from the visceral protein turnover. Furthermore, the AA profiles of muscle and viscera are different (e.g., Ostrowski and Divakaran 1989; Ng and Hung 1994).

Besides increasing AA oxidation, AA imbalances can also affect conversion efficiencies by increasing metabolic rate. Poor dietary AA balance has been shown to increase the rates of protein synthesis and turnover (Langar et al. 1993). As protein synthesis is highly energy demanding (Houlihan 1991) this will probably result in higher metabolic rates. In fact, infused diets with an imbalanced EAA composition have been shown to lead to a higher oxygen consumption than balanced diets (Kaczanowski and Beamish 1996).

Whole fish AA profiles change considerably during larval development, and are different from adult profiles. Whether these changes are significant in terms of AA requirements remains to be determined. However, it may be advisable to consider the above AA profile variations when formulating larval diets. As long as more detailed information is not available, larval AA profiles are the best index we have to estimate the qualitative AA requirements of fish larvae. The AA profile of *Artemia* nauplii may be sub-optimal for larvae of *C. gariepinus*, but also for turbot larvae (Conceição et al. 1997b), and possibly other species.

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Chapter 7

An explanatory model for dynamic simulation of growth in fish larvae: application to the African catfish (*Clarias gariepinus*) and turbot (*Scophthalmus maximus*)

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An explanatory model for dynamic simulation of growth in fish larvae: application to the African catfish (*Clarias gariepinus*) and turbot (*Scophthalmus maximus*)

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Abstract

The present study describes an explanatory model that simulates growth and body composition of fish larvae. Its objective is to improve the understanding of the growth process in larval fish, and by doing so, to enable the optimisation of the feeding strategies in larviculture. The model is driven by nutrient intake, with the absorbed dietary nutrients being used for energy production or for biosynthetic processes, and it is based on the stoichiometry of intermediary metabolism. The model was parameterised using literature data and validated for the African catfish (*Clarias gariepinus*) and turbot (*Scophthalmus maximus*). According to the model, high unavoidable losses of amino acids due to imbalances between the dietary and the larval amino acid profiles lead to an increase in lipid deposition in periods of high food intake. In catfish this occurs with the contribution of gluconeogenesis. Model simulations indicate that an increase in dietary protein stimulates growth, and leads to a reduction in lipid deposition. Model simulations also show that an increase in dietary lipid leads to an increase in body lipid content and a small protein-sparing effect.

Introduction

Growth is a complex phenomenon, which is affected by several processes, many of them interacting with one another. Firstly, food is ingested and nutrients are subsequently digested and absorbed; some will be used for energy production, others transformed into other types of molecules. The retained nutrients are deposited as proteins and other macro-molecules in the body tissues. The relative importance of these processes can change due to several factors, e.g., feeding regime, diet physical properties, diet composition, developmental stage of the animal, and water temperature. Therefore, studying only a few of these processes may lead to erroneous conclusions. The ideal would be to study them all simultaneously, but this is difficult to achieve with classical experimental designs.

Modelling can be a powerful tool in growth studies. An explanatory, or mechanistic, model predicts growth based on the underlying biochemical processes. Such a model can be used to predict growth under conditions different from those it was calibrated for. Such a model can also be used to reveal gaps in knowledge, and suggest directions for further research. In

animal nutrition literature, the principles for the development of mechanistic models and their possible uses were reviewed by Gill et al. (1989) and Baldwin and Sainz (1995). An explanatory model for growth of juvenile African catfish (*Clarias gariepinus*) has been developed by Machiels and Henken (1986, 1987) and further elaborated by Machiels and van Dam (1987) and Machiels (1987). Recently this model was slightly modified and validated for rainbow trout, *Oncorhynchus mykiss*, and tilapia, *Oreochromis niloticus* (van Dam and Pauly 1995; van Dam and Penning de Vries 1995). A similar model has also been developed for yolk-sac larvae of the African catfish (Conceição et al. 1993). The present study is a further elaboration of the latter model and it simulates growth and body composition of fed fish larvae. In the present study the model is calibrated and validated for the African catfish (*Clarias gariepinus* Burchell) and the turbot (*Scophthalmus maximus* L.), using literature data. The relative importance of the different underlying processes and model parameters for larval growth is analysed and discussed.

Model Description

General

The model described in the present paper is a further elaboration of the model of Conceição et al. (1993), which in turn was developed after the model of Machiels and co-workers (Machiels and Henken 1986, 1987; Machiels and van Dam 1987; Machiels 1987). The main difference between those two models and the present model is the calculation of the energy partitioning, a crucial step in the simulation of growth. In the previous models it was calculated based on the ratio of absorbed fatty acids to absorbed amino acids (AA). In the present model, a more explanatory approach was used, as energy resource partitioning is driven by the dietary protein quality and quantity. In addition, the cost of protein deposition depends on the growth rate in the current model, whereas this cost was fixed in the two previous models. This change is of particular importance for fish larvae, due to their highly variable growth rates.

The model is driven by nutrient intake. It simulates growth on a dry matter basis together with protein and lipid content. The stoichiometry of intermediary metabolism is used to calculate the transfer of matter and the costs of growth, thus the oxygen consumption, carbon dioxide production and ammonia excretion can also be estimated. The model considers three pools, comprising the whole larval body protein, lipid and ash (Figure 1). Absorbed dietary nutrients are used either for energy production or for biosynthetic processes. All absorbed carbohydrates are assumed to be converted to fatty acids.

The equations used in the model are given in Appendix 1. The variables used in the model are listed in Appendix 2.

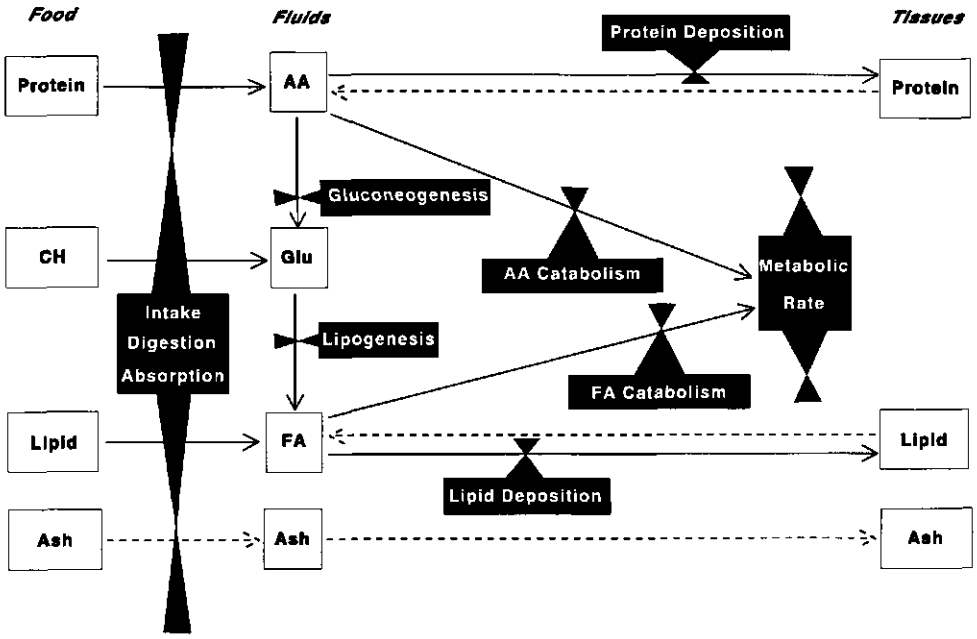

Figure 1. Relational diagram representing the quantities and fluxes of nutrients considered in the dynamic model to simulate growth of fish larvae. White boxes stand for amounts of nutrients (state variables), but only the ones referring to the tissues column are explicit in the model. Black polygons stand for processes responsible for the flux of nutrients (rate variables). Nutrient fluxes are represented by arrows. Dashed arrows stand for fluxes which are not explicit, but implicit in the model. Abbreviations: AA - amino acids; FA - fatty acids; CH - carbohydrates; GLU - Glucose.

Food intake and nutrient absorption

In fish larvae, estimation of food intake and food digestibility is difficult due to the small size of the animals. Such estimates will tend to be highly variable, depending on prey type and density, larval size, and environmental conditions such as temperature and light. Daily food intake (FOODIN) is a model parameter entered as the amount of food eaten per unit of larval dry weight (%DW day^{-1}). Protein and lipid digestibilities (mg nutrient absorbed per mg of nutrient intake, in %) are assumed to be identical (PRODIG), but can change with larval size. Carbohydrate digestibility (CHDIG) is a separate parameter.

Energy expenditure

Total energy expenditure at each moment is calculated as the sum of the maintenance metabolism and the energy needed for protein and lipid deposition, minus the energy produced by gluconeogenesis.

Maintenance metabolism (H_m, nmol ATP.g^{-1}DW.h^{-1}) is defined as an allometric relation of larval dry weight (LADW):

$$H_m = \alpha * LADW^{\beta}$$

where α and β are model parameters, the rate constant and the weight exponent, respectively, for maintenance metabolism. If the model is to be used at different temperatures, a Q_{10} factor can be added to this equation.

The cost of lipid deposition (COSTLD, mmol ATP.g^{-1} lipid deposited) is assumed to be constant. The cost of protein deposition is assumed to change with growth rate. The cost of growth (dry matter deposition) decreases with increasing growth rates (Wieser 1994) probably because of the cost of protein deposition (Conceição et al. 1997a). The cost of protein deposition (COSTPD, mmol ATP.g^{-1}protein.deposited) is represented in the model by an exponential decrease to the minimum theoretical cost of protein deposition (50 mmol ATP.g^{-1}protein deposited, Conceição et al. 1997a):

$$COSTPD = 50 + a * e^{-b * RGR}$$

where RGR is the relative growth rate (%DW.day^{-1}) and a and b are constants.

Energy resource partitioning

The amounts of AA and fatty acids that are used for energy production are mainly determined by the quality and quantity of dietary protein. First a unavoidable AA loss is calculated based on the difference between the AA profiles of the diet and of the larval body. A variable AA loss is also assumed in such a way that it increases with the protein feeding level. Fatty acid oxidation is calculated based on the difference between the total energy expenditure and energy provided by AA oxidation. Obligatory fatty acid and AA losses are also considered in the model, since some AA and fatty acids will always be used for energy, independent of the dietary composition.

Not all the AA losses will result in AA catabolism. Gluconeogenesis may divert part of the AA loss into lipid formation. The model defines an obligatory component for gluconeogenesis, depending on the amount of absorbed AA, but also a variable component, which plays a role when the unavoidable losses of AA are very high.

Table 1. Model parameters.

Abreviation	Description	Unit	Type[2]
FOODIN_j[1]	Daily food intake	%DW/day	E
AAGLU	Absorbed amino acids used for gluconeogenesis	% absorbed amino acids	S
AAVLOSS	Rate constant for amino acid variable loss	mg	S
PRODIET_i[1]	Protein content in diet	% diet weight	E
LIPDIET_i[1]	Lipid content in diet	% diet weight	E
CHDIET_i[1]	Carbohydrate content in diet	% diet weight	E
PRODIG_i[1]	Protein and lipid digestibility	% intake	S
CHDIG	Carbohydrate digestibility	% intake	S
AAILOSS	Amino acid loss due to imbalance	% absorbed amino acids	E
AAOLOSS	Amino acid obligatory loss	% absorbed amino acids	S
FAOLOSS	Fatty acid obligatory loss	% absorbed fatty acids	S
α	Rate constant for maintenance metabolism	nmol ATP/mg DW^{β}/h	S
β	Weight exponent for maintenance metabolism	-	S
COSTLD	Cost of lipid deposition	mol ATP/g lipid	S
COSTPDa	Parameter for the cost of protein deposition	-	S
COSTPDb	Parameter for the cost of protein deposition	-	S
ASHPERC	Percentage of ash in dry matter	%	S

[1] Parameter values may change with development. [2] Parameters were considered as species-specific (S) or simulation run-dependent (E).

Growth

The model calculates larval growth (in dry matter) as the sum of protein, lipid and ash deposition. Protein and lipid deposition are estimated by subtracting the amounts of AA and fatty acids used for energy production from the amounts absorbed, and taking into account the amount of AA converted into fatty acids via gluconeogenesis. Ash deposition is calculated by assuming a constant ash content in the dry weight (ASHPERC).

Parameterisation

A list of model parameters is given in Table 1. Parameters were either determined from literature (Table 2) or calibrated with the model using experimental data (Table 3), for both African catfish and turbot. These parameters can be divided into two groups: species-specific and simulation run-dependent.

The dietary protein, lipid and carbohydrate contents are obviously dependent on the simulation run for which they are to be applied. The values of the respective parameters were obtained together with the experimental data sets used to calibrate and validate the model.

Table 2. Initial values of the state variables, and model parameters derived from literature (see text for explanation and references), for the different simulation runs used to calibrate and validate the model for the African catfish (*Clarias gariepinus*) and turbot (*Scophthalmus maximus*).

Species		catfish			turbot		
Simulation run		I	2	3	I	II	III
LADWi	(mg)	0.3485	0.2866	0.3695	0.0378	0.0246	0.8274
LAPROi	(mg)	0.2659	0.1991	0.2539	0.0252	0.0172	0.5828
LALIPi	(mg)	0.0453	0.0491	0.0650	0.0097	0.0076	0.1232
PRODIET	(%)	59.1	59.1	60.0	49.5	49.5	46.9
FATDIET	(%)	16.4	16.4	17.4	9.2	9.2	6.5
CHDIET	(%)	12.3	12.3	11.3	31.3	31.3	36.5
PRODIG_a	(%)	50.0	50.0	40.0	60.0	60.0	60.0
PRODIG_b	(%)	85.0	85.0	75.0	70.0	70.0	70.0
PRODIG_c	(%)	90.0	90.0	90.0	82.2	82.2	82.2
CHDIG	(%)	50.0	50.0	50.0	30.0	30.0	30.0
AAILOSS	(%)	27.0	27.0	39.4	28.4	28.4	24.3
AAOLOSS	(%)	10.0	10.0	10.0	10.0	10.0	10.0
FAOLOSS	(%)	10.0	10.0	10.0	10.0	10.0	10.0
α	(nmol ATP/mg DW^{β}/h)	1586	1586	1586	1140	1140	1140
β	-	0.789	0.789	0.789	1.000	1.000	1.000
COSTLD	(mol ATP/g lipid)	14.0	14.0	14.0	14.0	14.0	14.0
COSTPDa	-	80.977	80.977	80.977	763.316	763.316	763.316
COSTPDb	-	-0.0544	-0.0544	-0.0544	-0.5171	-0.5171	-0.5171
ASHPERC		11.0	11.0	11.0	16.0	16.0	16.0

The suffix _i for a dietary nutrient or digestibility parameter means that it was changed during the course of simulation. Protein/lipid digestibility was considered to change on days 1 and 5 of exogenous feeding for the African catfish, and on days 9 and 16 post-hatching for turbot. See Table 1 for explanation of parameters.

The AA loss due to dietary AA imbalances is simulation run-dependent, since it is calculated based on the difference between dietary and larval AA profiles. To calculate this unavoidable AA loss, the amount of each AA which could be polymerised into protein AA was first estimated. The latter estimation was based on the amount of the first-limiting AA and the larval AA profile. Finally, the amounts of each AA in excess in the diet were summed. AA profiles for the African catfish and for the diets (*Artemia* nauplii and dry diet) were taken from Conceição et al. (1997b). The AA profiles for larval turbot, enriched *Artemia* nauplii and natural zooplankton were taken from Conceição et al. (1997d), Seidel et al. (1980) and Watanabe et al. (1983), respectively. The first-limiting AA for *Artemia*-fed catfish was tyrosine, while it was

cysteine for dry diet-fed catfish, leucine for natural zooplankton-fed turbot, and threonine for enriched *Artemia*-fed turbot. AA and fatty acid obligatory losses were fixed as 10% of the respective absorbed amounts.

Little information is available on food digestibility in the two species considered. Therefore the used values were based on assumptions, and considered as species-specific. For the African catfish, high protein/lipid digestibility coefficients were assumed since high gross food conversion efficiencies were found in larvae of this species (Conceição et al. 1997a). For turbot larvae before day 16 post-hatching, the assumed protein/lipid digestibility coefficients were based on literature data for pleuronectiforms and other marine fish larvae (Govoni et al. 1986; Houde 1989; Rust 1995; Day et al. 1996) and on the work of Conway et al. (1993) for older larvae. The carbohydrate digestibility coefficients for both species were assumed to be considerably lower than the ones for protein and lipid, based on the (little) data available in literature (Hemre et al. 1989; NRC 1993).

The parameters for maintenance metabolism are species-specific. Values for the African catfish were taken from Conceição et al. (1997a) and for turbot from Conceição et al. (1997c). The cost of lipid deposition was calculated based on the stoichiometry of lipid synthesis (see Machiels and Henken 1986) and assumed to be equal for the two species. The parameters for the growth rate dependent cost of protein deposition were assumed to be species-specific. The values for the African catfish were calculated based on data given in Conceição et al. (1997a). For turbot the parameter values were calculated based on data for larvae of eight different species (see General Discussion).

The percentage of ash in larval dry weight, a species-specific parameter, was taken from Verreth et al. (unpublished) for the African catfish, and from Conceição et al. (1997c) for turbot.

The parameters referring to nutrient fluxes, consumption of oxygen, and production of ATP, carbon dioxide and ammonia, which are driven by AA catabolism, fatty acid catabolism, gluconeogenesis, lipogenesis, lipid deposition and protein deposition, are based on the stoichiometry of the intermediary metabolism (Machiels and Henken 1986; Conceição et al. 1993). Their values are assumed to be universal for fish species, and are given directly in the model equations (see Appendix 1).

The parameters referring to the daily food intake (FOODIN_a..j), the proportion of absorbed AA used for gluconeogenesis (AAGLU), and the variable AA loss (AAVLOSS), were calibrated for with the model. They were adjusted to obtain a good fit between the simulated and the experimental data. Food intake is simulation run-dependent, while the two other parameters were considered species-specific. Food intake was estimated daily (10 days) for the African catfish, while it was assumed to be constant for turbot for periods of 2 - 3 days, so that a maximum of 10 parameters per simulation run could be calibrated. All parameters were

Table 3. Values of the parameters calibrated using the model, for the different simulation runs used to calibrate and validate the model for the African catfish (*Clarias gariepinus*) and turbot (*Scophthalmus maximus*).

Species		catfish			turbot		
Simulation run		1	2	3	I	II	III
FOODIN_0	(%DW/day)	52.9	41.2	40.8	47.2	74.7	
FOODIN_1	(%DW/day)	112.1	144.1	107.5	65.0	77.8	
FOODIN_2	(%DW/day)	85.1	99.7	80.1	47.5	63.3	
FOODIN_3	(%DW/day)	114.8	66.7	84.6	62.0	92.0	
FOODIN_4	(%DW/day)	75.3	132.3	71.6	65.0	69.5	
FOODIN_5	(%DW/day)	72.1	20.0	70.6	41.9	99.1	
FOODIN_6	(%DW/day)	20.0	53.4	62.7	52.5	61.7	50.0
FOODIN_7	(%DW/day)	36.5	87.0	89.0	92.4	103.3	94.0
FOODIN_8	(%DW/day)	75.4	64.4	74.9	48.7	59.0	84.8
FOODIN_9	(%DW/day)	49.4	51.4	67.5	80.8	57.6	51.0
AAGLU	(%)	25.0	**25.0**	**25.0**	1.053	**1.053**	**1.053**
AAVLOSS	(mg)	0.022	**0.022**	**0.022**	^ 0043	**0.0043**	**0.0043**

Values in bold were not calibrated in the respective simulation runs. Food intake was calibrated daily for the African catfish (suffix _i , refers to days of exogenous feeding) and in intervals of 2 or 3 days for turbot (the interval considered started at days 3, 5, 7, 9, 11, 13, 15, 17, 20 and 23 post-hatching). See Table 1 for explanation of parameters.

calibrated simultaneously using the programme STEM 2.1 (Remedy Systems Modelling, Enschede, The Netherlands). Calibration aimed at minimising the differences between observed values and the values of the three state variables: dry weight, protein content and lipid content. This programme uses the simplex minimisation method from Nelder and Mead (1965). The Euler integration method was used, with a fixed time step of 1 hour. The model output was not sensitive to smaller time steps.

As no data on food intake were available for model validation, also those parameters were calibrated for.

To check the goodness of fit between experimental and simulated values, during both model calibration and validation, the deviation of the simulated values from the experimental data (DEV) were calculated as a percentage of the experimental values:

$$DEV = ABS\ (simulated - experimental) / experimental * 100$$

where ABS stands for absolute value. The maximum deviation (DEV_{max}) and the average of all deviations (DEV_{mean}) for a given simulation run were used as goodness of fit criteria.

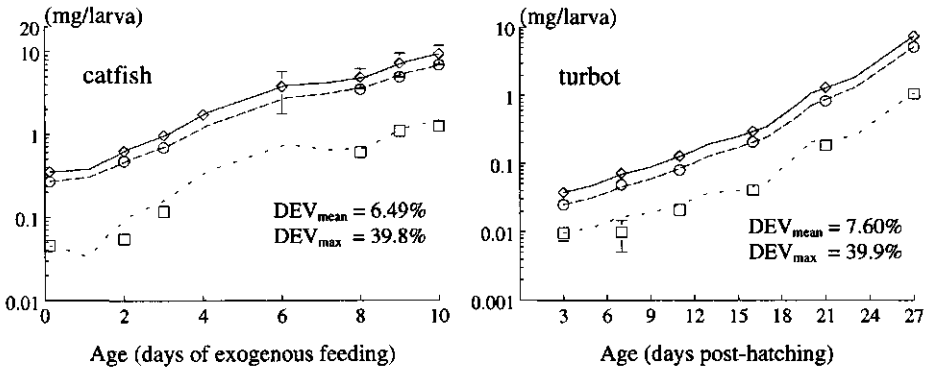

Figure 2. Model calibration. Comparison of model simulated (lines) and experimental (markers) values for larval dry weight (—, ◇), larval protein (---, ○) and larval lipid (···,□) for *Clarias gariepinus* fed *Artemia* nauplii during 10 days (left) and *Scophthalmus maximus* fed natural zooplankton during 24 days (right) (simulation runs 1 and I, respectively). See tables 2 and 3 for parameter values, and text for sources of experimental data.

Experimental data

For both African catfish and turbot, one data set was used to calibrate the unknown parameters, and two additional independent data sets were used to validate the model. The data set used to calibrate the model for African catfish (simulation run 1) originated from the main experiment of Conceição et al. (1997a) where larvae were fed *Artemia* nauplii for 10 days at 28°C. The two data sets used to validate the model for this species (Conceição et al., unpublished) were obtained under identical experimental conditions. In one experiment larvae were also fed *Artemia* nauplii (simulation run 2), while in another a dry diet (see formulation in Conceição et al. 1997b) was used (simulation run 3).

The turbot data sets originated from three experimental groups reared at 18°C under identical conditions, as described by Conceição et al. (1997c). The model was calibrated with data (simulation run I) from a group fed natural zooplankton from day 3 to day 27 post-hatching (Conceição et al., unpublished data). Model validation was done by using data from a group fed natural zooplankton from day 3 to day 26 post-hatching (simulation run II), and data from a group fed a mixture of natural zooplankton and enriched *Artemia* nauplii from day 16 to day 26 post-hatching (simulation run III), both shown in Conceição et al. (1997c,d).

All data sets included data on dry matter, crude protein, and total lipid contents. The calibration data sets (simulation runs 1 and I) also included data on oxygen consumption.

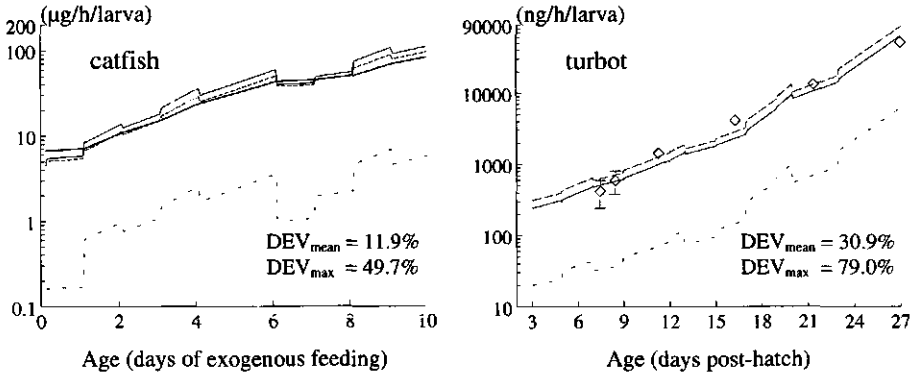

Figure 3. Simulated oxygen consumption (—), carbon dioxide production (---) and ammonia production (···) for *Clarias gariepinus* fed *Artemia* nauplii during 10 days (left) and *Scophthalmus maximus* fed natural zooplankton during 24 days (right), after model calibration (simulation runs 1 and I, respectively). Oxygen consumption values for catfish (thick line) after the allometric relation by Conceição et al. (1997a), and experimental data (Conceição et al., unpublished) for turbot (✧), are also given.

Results

Calibration

The unknown parameters were successfully calibrated for with the model for both the African catfish and turbot (Figure 2). Deviations (DEV) between simulated values and experimental data were always less than 4 and 9% of the experimental value for dry matter and protein contents, respectively, in both species. Deviations for lipid contents were higher, with both species having a maximum of around 40%, and an average value of 18% (Figure 2). The deviation between simulated and experimental oxygen consumption was 11.9% in average for *C. gariepinus* and 30.9% for *S. Maximus* (Figure 3). The experimental oxygen consumption data were not used during model calibration. Therefore, the deviations of this variable are an external control of the model definition and calibration.

Values for the calibrated parameters are given in Table 3. The model suggests that gluconeogenesis is considerably more important in the African catfish than in turbot. Changes in losses of AA related to increases in protein food intake were also more significant in the African catfish.

As food intake was calibrated simultaneously with two parameters involved in energy resource partitioning, it is important to know the interaction between these two parameters and the food intake parameters. In turbot, changes of 25% for the value of the fraction of AA used for gluconeogenesis had little effect on the estimation of food intake (Table 4). In contrast, the

Table 4. Dependence of the model calibrated estimates for daily food intake on the two parameters involved in energy resource partitioning that were calibrated simultaneously, in simulation runs I (*Clarias gariepinus*) and I (*Scophthalmus maximus*)[1].

		catfish			turbot		
		Maximum	Minimum	Average	Maximum	Minimum	Average
		(difference to the original calibration, in % of the original calibration)					
AAGLU	-25%	81.9	-45.0	4.3	5.1	-4.2	0.3
	+25%	93.4	-73.0	1.5	3.6	-5.7	-0.1
AAVLOSS	-25%	1.7	-3.5	-0.3	8.5	-8.5	-0.2
	+25%	10.7	-8.7	0.3	18.6	-8.9	1.0

[1] Daily food intake was re-calibrated after changing 25% in the calibrated value (Table 2) of one of the other parameters. Values are differences between the original (Table 3) and the re-calibrated food intake, expressed as percentage of the original value. Values given are the maximum, minimum and average values over the 10 time intervals considered (see Table 3). See Table 1 for explanation of parameters.

calibration of daily food intake appeared to be sensitive to changes in the fraction of AA used in gluconeogenesis for the African catfish. However, this interaction seems to have mainly affected the daily variation in food intake, since for the overall simulation run the average daily food intake values changed little (Table 4). In turn, 25% changes in the rate constant for AA loss dependent on the protein food intake lead to little changes in the food intake estimates for both species.

Model validation

The model was validated with two independent experimental data sets for each species. However, due to the absence of reliable experimental data on food intake, these parameters were also calibrated in those model runs (see Table 3). Simulated values were in good agreement with the experimental data for African catfish fed either *Artemia* nauplii or a dry diet (Figure 4). Deviations were lowest for dry matter and highest for lipid contents, but they never reached more than 13% of the experimental value. The agreement between the simulated and the experimental values was worse (average deviation of 16.9% from the experimental value) for larval turbot (Figure 5) fed natural zooplankton than for larvae fed a mixture of enriched *Artemia* nauplii and natural zooplankton (average deviation of 6.8%).

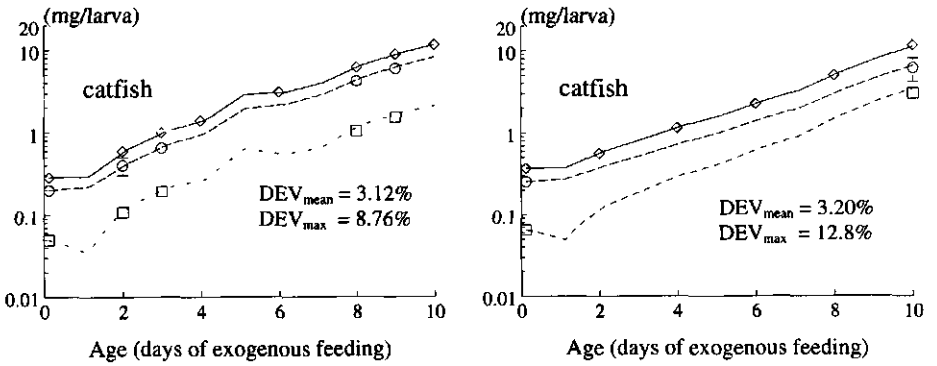

Figure 4. Validation of the model simulations (lines) with experimental values (markers) for larval dry weight (—, ◇), larval protein (---, ○) and larval lipid (···,□) for *Clarias gariepinus* during two simulation runs were larvae were fed *Artemia* nauplii (simulation run 2, left) or a dry diet (simulation run 3, right) during 10 days. See tables 2 and 3 for parameter values, and text for sources of experimental data. Food intake values were calibrated with the model.

Sensitivity analysis

The sensitivity of the model to its different parameters was analysed by changing each one separately by 25%, and comparing the final larval dry matter, protein and lipid contents with a reference simulation (Table 5). The model is highly sensitive to changes in food intake, protein/lipid digestibility and dietary protein for both African catfish and turbot. The model indicates that an increase in dietary protein will lead to an increase in the final dry matter and protein contents in both the African catfish and turbot. However, for the African catfish the increase in dry matter will be small compared to what happens in turbot, probably because of a strong concomitant decrease in lipid content. Simulations for turbot were highly sensitive to the dietary carbohydrate. Carbohydrate digestibility also affected the growth of larval turbot to a greater extent than that of larval catfish. Changes in the fraction of absorbed AA that is used for gluconeogenesis have a small effect on catfish growth, whereas in turbot it was negligible. Changes in the dietary lipid content affected mostly the larval lipid content. The model output was not very sensitive to the rate constant for AA loss dependent on the protein food intake, nor to the cost of lipid deposition. Model output was moderately sensitive to the rate constant for the cost of maintenance, the cost of protein synthesis, and the AA losses due to dietary AA imbalance.

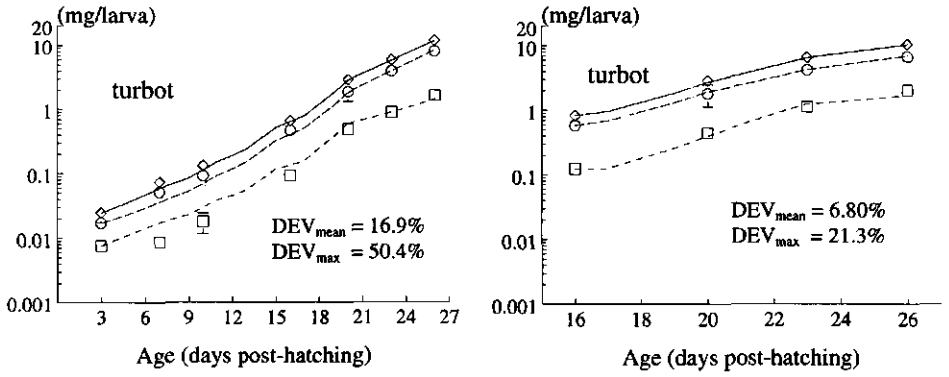

Figure 5. Validation of the model simulations (lines) with experimental values (markers) for larval dry weight (—,◇), larval protein (---, ○) and larval lipid (···,□) for *Scophthalmus maximus* with two simulation runs where larvae were fed natural zooplankton during 23 days (simulation run II, left) or enriched *Artemia* nauplii and natural zooplankton during 10 days (simulation run III, right). See tables 2 and 3 for parameter values, and text for sources of experimental data. Food intake values were calibrated with the model.

Model output

In addition to simulating larval growth and body composition under different feeding regimes (Figs. 2, 4 and 5) and larval respiration and excretion (Figure 3), the model also allows for the study of all the other variables and parameters considered. Figure 6 shows the simulated partitioning of the total energy expenditure in the costs of maintenance, protein deposition and lipid deposition, for catfish and turbot. The cost of maintenance takes the bigger share of energy expenditure, and seems to be more important in turbot than in African catfish. The cost of lipid deposition seems to be of little significance in the overall energy budget in both species.

Increases in the percentage of unavoidable AA losses due to AA imbalance in the diet were simulated while the other model parameters were kept constant (Figure 7). This resulted in a decrease in both dry weight and protein content, and an increase in the relative lipid content.

Discussion

Design, parameterisation and validation

The model simulates whole body changes in dry matter, protein and lipid contents. To improve the present model a representation of more compartments would be required. This would entail at least the representation of a "visceral" compartment and a "carcass" compartment. In such a multi-compartmental model, nutrient fluxes could be described by chemical relationships (Gill et al. 1989), which facilitate the biological interpretation of the model parameters. However,

Table 5. Sensitivity of the model to 25% changes in each parameter[1]. The larval contents (mg/larva) in dry matter, protein and total lipids after 10 days of exogenous feeding (*Clarias gariepinus*) or 26 days post-hatching (*Scophthalmus maximus*), are used as criteria.

		catfish			turbot		
		Dry Weight	Protein	Lipids	Dry Weight	Protein	Lipids
		(difference to a reference simulation, in %)					
FOODIN	-25%	-70.98	-67.86	-83.39	-84.65	-83.87	-88.94
	+25%	234.91	216.11	309.04	520.05	500.52	631.44
AAGLU	-25%	9.19	6.73	18.84	0.32	0.24	0.78
	+25%	-8.48	-6.32	-16.95	-0.35	-0.27	-0.81
AAVLOSS	-25%	0.45	0.76	-0.79	4.62	4.72	3.99
	+25%	-0.45	-0.77	0.79	-5.34	-5.44	-4.73
PRODIET	-25%	-13.12	-31.93	60.26	-59.47	-63.71	-34.83
	+25%	15.11	38.34	-75.55	140.14	157.27	40.76
LIPDIET	-25%	-9.86	-2.08	-40.26	-11.80	-8.71	-29.61
	+25%	10.82	1.77	46.19	13.38	9.54	35.54
CHDIET	-25%	16.34	16.33	16.45	118.12	119.76	108.31
	+25%	-14.07	-14.06	-14.15	-54.96	-55.44	-52.05
PRODIG	-25%	-58.23	-59.20	-54.61	-82.30	-81.88	-84.44
	+25%	70.99	71.93	67.54	401.41	394.34	441.14
CHDIG	-25%	-4.03	-3.02	-7.98	-13.30	-11.55	-23.38
	+25%	4.20	3.12	8.39	15.32	13.08	28.22
AAILOSS	-25%	4.01	12.51	-29.17	12.99	22.80	-43.74
	+25%	-5.05	-12.93	25.71	-21.56	-28.02	15.80
AAOLOSS	-25%	0.00	0.00	0.00	0.00	0.00	0.00
	+25%	0.00	0.00	0.00	0.00	0.00	0.00
FAOLOSS	-25%	0.00	0.00	0.00	0.74	0.71	0.91
	+25%	0.00	0.00	0.00	-1.01	-0.97	-1.29
α	-25%	21.67	16.34	42.57	27.90	21.40	65.36
	+25%	-18.66	-14.47	-35.11	-26.81	-22.46	-51.93
β	-25%	2.57	-1.56	18.69	-50.23	-52.25	-38.41
	+25%	-7.84	-1.73	-31.71	-7.71	5.32	-82.99
COSTLD	-25%	0.41	0.32	0.79	0.34	0.31	0.49
	+25%	-0.41	-0.31	-0.78	-0.35	-0.33	-0.52
COSTPD[2]	-25%	11.24	8.02	23.82	9.40	7.24	21.85
	+25%	-10.32	-7.51	-21.31	-10.48	-8.54	-21.64

[1] Values are differences to a reference simulation (simulation runs 2 and II, Table 2) and expressed as percentages.

[2] The overall cost of protein deposition (see equation in text). See Table 1 for explanation of parameters.

representing more compartments in the model would require information about nutrient utilisation and growth in fish larvae which is not available at present.

During model parameterisation, several parameters were simultaneously calibrated for. This adds some uncertainty to the estimated values, as different combinations of parameter values can lead to similar model outputs. However, in the present study three variables were used simultaneously as criteria of goodness of fit, and thus the possibility of bias is reduced. Furthermore, we analysed the interaction between the calibrated estimates for daily food intake and the two parameters involved in energy resource partitioning (Table 4). Only for the African catfish was there some interaction between the calibrated values for food intake and the fraction of absorbed AA used for gluconeogenesis. Nevertheless, the set of calibrated values is the one that provides the best goodness of fit between simulated and experimental data, when the simplex method is used for calibration.

The presented validation of the model is a partial one, as in the validation runs the parameters for food intake were also calibrated for. An indisputable validation of the model will only be possible when experimental data for food intake are available. Nevertheless, the present validation shows that the mechanism proposed for energy resource partitioning is consistent with experimental results. Furthermore, by simultaneously using the larval dry matter, protein and lipid contents to calibrate for food intake, the possibility of bias was reduced. The reasonable agreement between simulated and experimental larval oxygen consumption during the calibration runs (Figure 3) also supports the model validity.

Food intake and digestibility

Food intake was calibrated for with the model, while digestibility parameters were set according to the information available in literature. Digestibility of proteins and lipids was considered to increase with larval age as the digestive system becomes more complex. The digestive tract is fully developed at a size of about 2.5 mg dry weight in the African catfish (Verreth et al. 1992), which corresponds to 4-5 days of exogenous feeding in larvae fed *Artemia* nauplii at a level close to maximum food intake. In turbot, the digestive tract develops as the larvae grow, but a functional stomach appears only at the end of metamorphosis (Segner et al. 1995). However, the digestive efficiency of fish larvae may also change considerably with food intake (Govoni et al. 1986; Houde 1989; Day et al. 1996). This effect was not considered in the present model. A possible bias in the digestibility coefficients used in the model will be reflected in the calibrated estimates of food intake. Obviously, to further improve the present model, a better knowledge of its "input" processes, i.e., food intake and digestibility, and the factors regulating them is required.

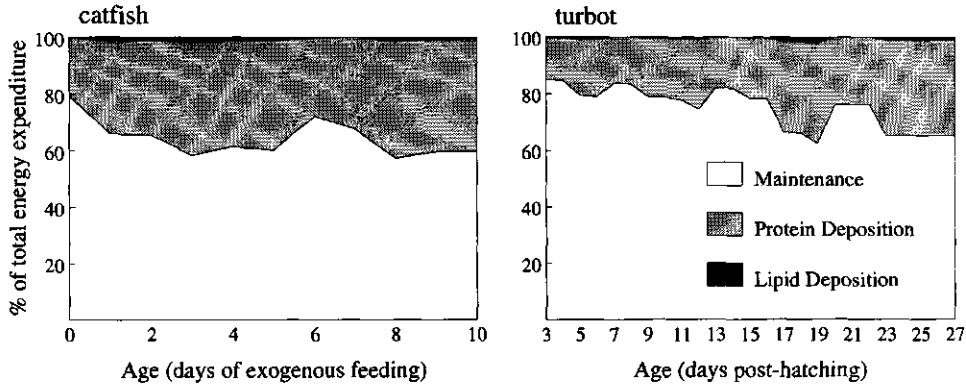

Figure 6. Model prediction of the partitioning of total energy expenditure between the costs of maintenance and costs of protein and lipid deposition for *Clarias gariepinus* fed *Artemia* nauplii during 10 days (left) and *Scophthalmus maximus* fed natural zooplankton during 24 days (right). Simulation runs were performed with the initial values and parameters for simulation runs 1 and I (Table 2), respectively.

As expected, the model is very sensitive to changes in food intake and digestibility (Table 5). The higher sensitivity for carbohydrate intake and digestibility in turbot larvae can be related to the lower digestibility of carbohydrate assumed for turbot.

Energy resource partitioning

The mechanism chosen to simulate energy resource partitioning proved to be adequate for both the African catfish and turbot in the analysed range of dietary conditions. As a comparison, the model was run by using the ratio of absorbed fatty acids to absorbed AA to calculate energy resource partitioning, as given in Machiels and Henken (1986) and Conceição et al. (1993) (results not shown). Also then could the model be successfully calibrated for both species. However, in that situation model simulations deviated considerably more from experimental data when trying to validate the model for catfish fed the dry diet (simulation run 3) and turbot fed enriched *Artemia* nauplii in combination with natural zooplankton (simulation run III). This means that the model was less accurate in predicting growth and body composition when larvae received other diets than the ones used to calibrate the model. Therefore, the present algorithm used to calculate the amounts of AA and fatty acids that are used for energy production seems preferable. In addition, calculating the energy resource partitioning based on the dietary protein quality (AA profile) and quantity is a more explanatory approach.

The model suggests that African catfish relies on a high gluconeogenic activity to support high growth rates. In contrast, gluconeogenesis seems to be of little importance in turbot

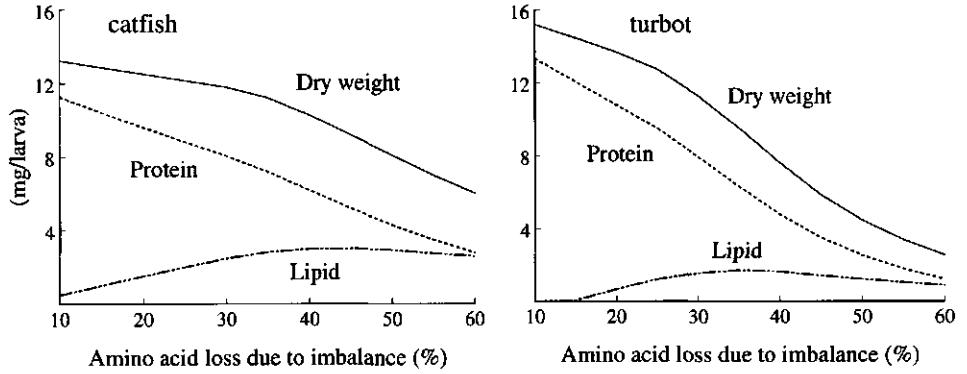

Figure 7. Simulated changes in dry matter, protein and lipid contents with increasing percentage of amino acid unavoidable losses due to dietary amino acid imbalance, in larvae of *Clarias gariepinus* (left) after 10 days of feeding and in larvae of *Scophthalmus maximus* (right) after 23 days of feeding. Simulation runs were performed with the initial values and parameters for simulation runs 2 and II (Table 2), respectively, and amino acid losses increased from 10 to 60% of amino acid absorption.

larvae. Gluconeogenic capacity seems to be present in catfish larvae from first feeding, although enzymatic activity is relatively low until the stomach is developed (Segner and Verreth 1995). In the model simulations, the high gluconeogenic activity in catfish allows for part of the AA lost due to dietary AA imbalance to be stored temporarily as lipids when larvae are feeding at high rates.

Costs of maintenance and growth

The model output shows that the cost of maintenance constitutes 60 to 85% of the total energy expenditure in both catfish and turbot, and the cost of protein deposition 15 to 40% (Figure 6). The cost of lipid deposition is of very little significance, contributing with a maximum of 2% to the total energy expenditure. The relative importance of the costs of maintenance and protein deposition changed with growth rate. Higher growth rates were associated with a higher relative importance of the cost of protein deposition, and a lower contribution of the cost of maintenance, to the total energy expenditure

In the model, the definition of maintenance metabolism is still highly descriptive. It would be preferable to replace it by a more explanatory approach, i.e., splitting maintenance into its main components. The main components of maintenance metabolism are probably the cost of protein turnover and the cost of ionic transport (Reeds et al. 1985; Houlihan et al. 1995) together with the cost of voluntary activity. At this moment there is insufficient information available to enable such an improvement of the model.

The model indicates that turbot larvae use more of their energy expenditure for maintenance costs than the African catfish (Figure 6). This may be due to the lower relative growth rates of turbot (Conceição et al. 1997a,c) and/or to the higher activity of this species during the larval period. Larvae of the African catfish are meal feeders, with long resting periods on the bottom of the tanks between meals, while turbot larvae feed continuously.

Growth and body composition

Differences between dietary and larval AA profiles suggest a high unavoidable loss of AA for both the African catfish and turbot. This was estimated to be around 25% for larvae fed live organisms and up to 39% in catfish larvae fed with a dry diet. According to the model output, these high unavoidable losses lead to an increase in lipid deposition. During periods of high food intake in the catfish this occurred with the contribution of gluconeogenesis. Furthermore, the higher those AA losses are, the higher will lipid deposition be (Figure 7). Although more lipid reserves are beneficial in periods of low food availability, very high lipid contents may be detrimental to fish larvae; they may reduce the swimming performance by increasing the "inert" mass and/or by increasing buoyancy, reducing the larval fitness both as prey and predator. Little is known about food intake regulation in fish larvae, but it is tempting to suggest that larval lipid contents above a certain level will lead to a reduction in food intake. A lipostatic control of feed intake in fish with high lipid contents was proposed for juvenile African catfish (Machiels and Henken 1987). This could mean that feeding fish larvae close to satiation may lead to an oscillatory pattern of growth. Larvae will feed at maximum rates until they reach a critical lipid content, and then they will reduce food intake to avoid further fattening. Growth, and in particular lipid deposition, will then be depressed, but growth rates will still be relatively high (15% body weigh day^{-1} or more). Therefore, the larvae will grow lean and soon there will be space for further lipid deposition, so that the larvae can increase food intake again. This hypothesis is in line with the large variations in growth rate observed in larvae of catfish (Conceição et al. 1997a) turbot (Conceição et al. 1997c) and other species (Houde 1989; Wieser and Medgyesy 1990a) when they are fed close to satiation. Furthermore, the model output predicts a large variation in food intake (Table 3) and growth rates (results not shown) during the course of development. Such a feeding strategy also makes sense for larvae growing under natural conditions. As abundance of food for longer periods probably is rare in nature, larvae may have the strategy of eating as much as possible in periods of food abundance until their lipid stores reach a limit.

The model further suggests that an increase in dietary protein content will have a positive effect on growth, and lead to a reduction in lipid deposition (Table 5). This could mean that the live food based feeding regimes currently used in larviculture are sub-optimal in terms

of the protein level in the diet. The model also predicts that an increase in dietary lipid content will lead mainly to an increase in larval lipid content, although a small protein-sparing effect is also suggested. This protein sparing-action would be more significant for turbot than for catfish. Increased lipid deposition and protein-sparing with higher dietary lipid have been demonstrated earlier for juvenile catfish (Henken et al. 1986) and juvenile turbot (Bromley 1980; Andersen and Alsted 1993).

Understanding of the growth process in fish larvae is hindered by difficulties in estimating the importance of the different factors affecting food intake and digestibility. The role of gluconeogenesis in diverting unavoidable AA losses into synthesis of lipids also deserves further study. It is also important to determine to which extent the imbalances in dietary protein can affect food utilisation and growth.

Due to its mechanistic nature, the present model can be of use for other fish species provided that the model parameters can be estimated. Hence it can be a useful tool for developing adequate feeding strategies for new cultivated species.

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Appendix 1. Model equations

Note: *All transactions made on a hourly basis. Model parameters are shown in italics.*
Description of parameters is given in Table 1, and of the model variables in Appendix 2.
Differential equations (value is added to the value of the previous hour) are shown in **bold**.

*** FOOD INTAKE ***

FOODINT = FOODIN / 100 * LADW / 24
FI_PRO = PRODIET / 100 * FOODINT * 10^6
FI_LIP = LIPDIET / 100 * FOODINT * 10^6

FI_CH = CHDIET / 100 * FOODINT * 10^6

*** ABSORPTION OF NUTRIENTS ***

ABS_PRO = PRODIG / 100 * FI_PRO * (1 / 0.85)
ABS_LIP = PRODIG / 100 * FI_LIP
ABS_CH = CHDIG / 100 * FI_CH

*** METABOLIC RATE ***
$H_m = \forall * LADW^3$
MET_RATE = H_m + PRDATP + LIDATP - GLUNATP

*** ENERGY RESOURCE PARTITIONING ***
* Obligatory losses *
AAIMBLOSS = AAILOSS / 100 * ABS_PRO

AAOBLLOSS = AAOLOSS/ 100 * ABS_PRO
if AAIMBLOSS < AAOBLLOSS then
AAUNVLOSS = AAOBLLOSS
else
AAUNVLOSS = AAIMBLOSS
FAOBLLOSS = FAOLOSS / 100 * ABS_LIP
ATPFAOBL = FAOBLLOSS / 1.96
AAOBLGLUN = AAGLU / 100 * ABS_PRO
EXCAAGLUN = 0
* Amino Acid catabolism *
AAVARLOSS = AAVLOSS * (ABS_PRO / LAPRO)
AACAT = AAUNVLOSS+AAVARLOSS - AAOBLGLUN
ATPAA = AACAT * 0.33
if MET_RATE < (ATPAA + ATPFAOB) then
EXCATPAA = ATPAA - MET_RATE + ATPFAOBL
AACAT = (AACAT - EXCATPAA) / 0.33
EXCAAGLUN = EXCATPAA / 0.33
ATPAA = AACAT * 0.33
AACATCO2 = AACAT * 1.696
AACATO2 = AACAT * 1.336
AACATNH3 = AACAT * 0.17

* Fatty Acid catabolism *
ATPFA = MET_RATE - ATPAA
FACAT = ATPFA * 1.96
FACATCO2 = FACAT * 2.8
FACATO2 = FACAT * 2.9

*** GLUCONEOGENESIS ***

AAGLUN = AAOBLGLU + EXCAAGLU
GLUGLUN = AAGLUN* 0.506
GLUNO2 = GLUGLUN * 1.581
GLUNCO2 = GLUGLUN * 1.905
GLUNNH3 = GLUGLUN * 0.340
GLUNATP = GLUGLUN * 0.236

*** LIPOGENESIS ***

GLULIP = (GLUGLU + ABS_CH) * 0.29
LIPGO2 = GLULIP * 0.76
LIPGCO2 = GLULIP * 2.17

*** PROTEIN DEPOSITION ***

AAPRO = ABS_PRO - AACAT - AAGLUN
PRODEP = 0.85 * AAPRO
LAPRO = PRODEP / 10^6
COSTPD = (50 + COSTPDa . $e^{\frac{COSTPDb * RGR}{}}$) / 10^3
PRDATP = PRODEP * COSTPS

*** LIPID DEPOSITION ***

FALIP = ABS_LIP - FACAT
FADATP = FALIP * COSTLD/1000
LALIP = (GLULIP + FALIP) / 10^6

*** EXCHANGES WITH ENVIRONMENT ***

O2 = GLUNO2 + LIPGO2 + FACATO2 + AACATO2

CO2 =GLUNCO2+LIPGCO2+FACATCO2 + AACATCO2
NH3 = GLUNNH3 + AACATNH3

*** GROWTH ***

ASHGAIN = (PRODEP + GLULIP + FALIP) * (1/(1-ASHPERC/100)-1)
DWGAIN = (ASHGAIN+PRODEP+GLULIP+FALIP) /10^6
LADW = DWGAIN

Appendix 2. Model state variables (**bold**) and rate variables.

Abbreviation	Definition	Unit
LADW	**Larval dry weight**	**mg**
LAPRO	**Larval protein content**	**mg**
LALIP	**Larval lipid content**	**mg**
FOODINT	Food intake	mg/h
FI_PRO	Protein intake	ng/h
FI_LIP	Lipid intake	ng/h
FI_CH	Carbohydrate intake	ng/h
ABS_PRO	Absorbed protein	ng/h
ABS_LIP	Absorbed lipid	ng/h
ABS_CH	Absorbed carbohydrate	ng/h
H_m	Maintenance metabolism	nmol ATP/h
MET_RATE	Total energy expenditure	nmol ATP/h
AAIMBLOSS	Amino acid loss due to imbalanced dietary amino acid profile	ng/h
AAOBLLOSS	Amino acid obligatory loss	ng/h
AAUNVLOSS	Amino acid unavoidable loss	ng/h
FAOBLLOSS	Fatty acid obligatory loss	ng/h
ATPFAOBL	ATP produced from fatty acid obligatory loss	nmol ATP/h
AAOBLGLUN	Amino acids used in obligatory gluconeogenesis	ng/h
EXCAAGLUN	Amino acids used in gluconeogenesis due to amino acid excess	ng/h
AAVARLOSS	Amino acids variable loss	ng/h
AACAT	Amino acids used for energy production (catabolism)	ng/h
ATPAA	ATP produced from amino acid catabolism	nmol ATP/h
EXCATPAA	ATP value of excess amino acid	nmol ATP/h
AACATCO2	Carbon dioxide produced due to amino acid catabolism	ng/h
AACATO2	Oxygen consumed due to amino acid catabolism	ng/h
AACATNH3	Ammonia produced due to amino acid catabolism	ng/h
ATPFA	ATP produced from fatty acid catabolism	nmol ATP/h
FACAT	Fatty acid used for energy production (catabolism)	ng/h
FACATCO2	Carbon dioxide produced due to fatty acid catabolism	ng/h
FACATO2	Oxygen consumed due to fatty acid catabolism	ng/h
AAGLUN	Amino acid used for gluconeogenesis	ng/h
GLUGLUN	Glucose produced from gluconeogenesis	ng/h
GLUNO2	Oxygen consumed due to gluconeogenesis	ng/h
GLUNCO2	Carbon dioxide produced due to gluconeogenesis	ng/h
GLUNNH3	Ammonia produced due to gluconeogenesis	ng/h
GLUNATP	ATP produced by gluconeogenesis	nmol ATP/h
GLULIP	Lipid synthesised from glucose	ng/h
LIPGO2	Oxygen consumed due to lipogenesis	ng/h
LIPGCO2	Carbon dioxide produced due to lipogenesis	ng/h
AAPRO	Amino acids polymerised into protein	ng/h
PRODEP	Protein deposited	ng/h
COSTPD	Cost of protein deposited	mol ATP/g
PRDATP	ATP needed for protein deposition	nmol ATP/h
FALIP	Fatty acids deposited in lipids	ng/h
FADATP	ATP needed for lipid deposition	nmol ATP/h
O2	Total oxygen consumption	ng/h
CO2	Total carbon dioxide production	ng/h
NH3	Total ammonia production	ng/h
ASHGAIN	Increase in ash mass	ng/h
DWGAIN	Increase in dry weight	ng/h
RGR	Relative growth rate	%/day

General Discussion

The ultimate objective of this thesis has been to develop an explanatory model that can simulate growth during the early life stages of fish. It should contribute to the understanding of growth and growth metabolism in larval fish, and therefore contribute to elucidate their nutritional requirements and develop appropriate feeding strategies. Protein metabolism and the energetics of growth received particular attention during the experimental phase of the thesis, as these processes are determinant for growth and its modelling.

This general discussion will focus on the prospects for using and improving the present model, and debates some of the main factors determining fish larval growth.

The growth model

Modelling growth and metabolism is always a compromise between an accurate presentation of the many processes involved and the information available to define their equations and determine the respective parameters. The simulation model developed for larval growth that is presented in Chapter 7, simulates whole body changes in dry matter, protein and lipid contents. Absorbed nutrients are considered to be used either directly for energy production or deposited in body tissues. However, in the body the organs and tissues have specific roles in the various metabolic processes. Upon absorption, nutrients are extensively processed by the intestine and liver before being transported to the musculature and the other peripheral tissues. Therefore, to develop a model that describes and predicts growth in fish larvae more accurately and mechanistically than the present one, more compartments should be included. It is suggested that a "visceral" and a "carcass" compartment should be distinguished. Such an increase in the number of model compartments would also allow for the use of chemical kinetic relationships to describe the flux of nutrients as proposed by Gill et al. (1989). The parameters in this type of equations have a clearer biological interpretation than the ones in the first-order equations used in Chapter 7. Simulation models for growth of whole animals using several body compartments and chemical kinetic relationships already exist for sheep (Gill et al. 1984), mice (Bernier 1986), steers (France et al. 1987) and pre-ruminant calves (Gerrits 1996). Little information is available on nutrient utilisation and growth in fish larvae, and the study of several compartments in a larva is limited by the small size of the organism. Therefore, a simpler representation of the nutrient utilisation and growth, considering the whole larva as one compartment is the only feasible option at present. Nevertheless, as knowledge about larval growth and metabolism increases, it may be worthwhile to increase the complexity of the model.

The predicting power of the model would also certainly gain if the effect of environmental factors such as temperature and oxygen availability would be included in the model. The effect of temperature was included in the models of Chapter 1 and of Machiels and

co-workers (Machiels and Henken 1986, 1987; Machiels and van Dam 1987; Machiels 1987) by introducing a Q_{10} factor in the maintenance metabolism equation. This can also be easily done in the present model. However, this may be a simplistic approach for fish larvae as temperature may also affect other processes, as well as the interrelationship between these processes (Chapter 6; Kamler 1992).

In the model of van Dam and Pauly (1995) the limiting effect of oxygen availability on feed intake was simulated. This would be difficult to do for fish larvae, as the respiration of these small animals is initially cutaneous and changes to branchial during development, and respiratory capacity is therefore hard to estimate.

The present model can be classified as deterministic, as it considers only average pools and average fluxes of nutrients. In such a model the size-distribution and the biological variation are not accounted for. This limits the application of the model to larviculture. Therefore, it would be interesting to transform the model from a deterministic to a stochastic one. Techniques to produce stochastic models are available (e.g., Nicolaï 1994; Kinder and Wiechert 1995; Hinckley et al. 1996). However, to apply them more information would be needed on the variability of the processes controlling the fluxes of nutrients, and therefore on the variability of the model parameters.

Food intake is introduced in the model as one or more input parameters. However, when more information is available on the mechanisms regulating feed intake, it would be possible to introduce only the fed ration and the feeding scheme as input parameters. Furthermore, the development of a food intake module for this model could also be a tool to study the regulation of food intake.

In its present state the model (Chapter 7) can be used to make predictions of growth, body composition, oxygen consumption and ammonia excretion, when larvae of the African catfish, *Clarias gariepinus*, or turbot, *Scophthalmus maximus*, are fed diets of different compositions. However, before the model is used in practice, a ultimate validation of the model should be done when reliable data on food intake are available (see Chapter 7). Due to its mechanistic nature, the present model could also be used for other fish species, providing that the model parameters can be estimated. The model may even help to define provisional feeding strategies for new cultivated species.

Two crucial aspects of the model are how the costs of growth and maintenance are defined, and how the energy resource partitioning is simulated. During development of the model it was apparent that to define energy resource partitioning the impact of the quality and quantity of the dietary protein on larval growth should be understood. In this respect, the capacity of fish larvae to regulate AA catabolism should also be evaluated. The following

sections discuss the protein metabolism and the energetics of growth in fish larvae, and to which extent these differ from older fish.

How can fish larvae grow so fast?

An intriguing question is how fish larvae manage to accommodate the cost of high growth rates with the cost of maintenance. Fish larvae have a limited capacity for oxygen uptake, and they probably cannot simultaneously accommodate the cost of growth at high growth rates with the cost of maximum activity (Wieser et al. 1988; Wieser 1991). In comparison to larger fish, larvae require specific energetic solutions to accommodate these costs. It has been suggested that high growth rates are possible because larval fish may have a reduced protein turnover in comparison to juvenile and adult fish (Kiørboe et al. 1987; Kiørboe 1989; Wieser and Medgyesy 1990a,b; Wieser 1994). An alternative strategy would be to reduce the cost of protein synthesis (Kiørboe 1989).

Protein synthesis retention efficiency (protein growth rate . 100 / rate of protein synthesis) is a good measure for comparing turnover rates of animals with widely different protein growth rates (Houlihan 1991). A high retention of the synthesised protein stands for a low protein turnover, with 100% corresponding to a protein turnover rate of zero. In fish larvae there is a general tendency for an increase in protein synthesis retention efficiency with increasing growth rates (see Fig. 1). However, at higher growth rates there may be a tendency for a reduction in the rate of increase in retention efficiency. This means that although protein turnover is reduced at high larval growth rates, this reduction has a limit. On the first days of feeding, turbot larvae deviate from this pattern, due to their very low protein turnover (Chapter 5). In fry of sea bass, *Dicentrarchus labrax*, a dietary amino acid (AA) imbalance lead to small reductions in growth rate, associated with a sharp decrease in the efficiency of retention of the synthesised protein (Langar et al. 1993; Fig. 1). Tilapia, *Oreochromis mossambicus*, fry and juveniles have a rather constant protein synthesis retention efficiency with increasing growth rates (Houlihan et al. 1993; Fig. 1).

The cost of protein synthesis was close to the theoretical biochemical minimum in African catfish yolk-sac larvae (Chapter 2) and in juvenile tilapia (Houlihan et al. 1993). However, a much higher cost of protein synthesis was reported for both larval nase, *Chondrostoma nasus* (Houlihan et al. 1992) and larval herring, *Clupea harengus* (Houlihan et al. 1995c). This apparent discrepancy may be explained by a change in the cost of protein synthesis with growth rate (see General Introduction), as nase and herring had lower growth rates than catfish and tilapia. The decreased cost of protein synthesis at higher growth rates may also explain the lack of correlation between oxygen consumption and growth rates in yolk-sac larvae of rainbow trout, *Oncorhynchus mykiss* (Rombough 1994) and in larval herring

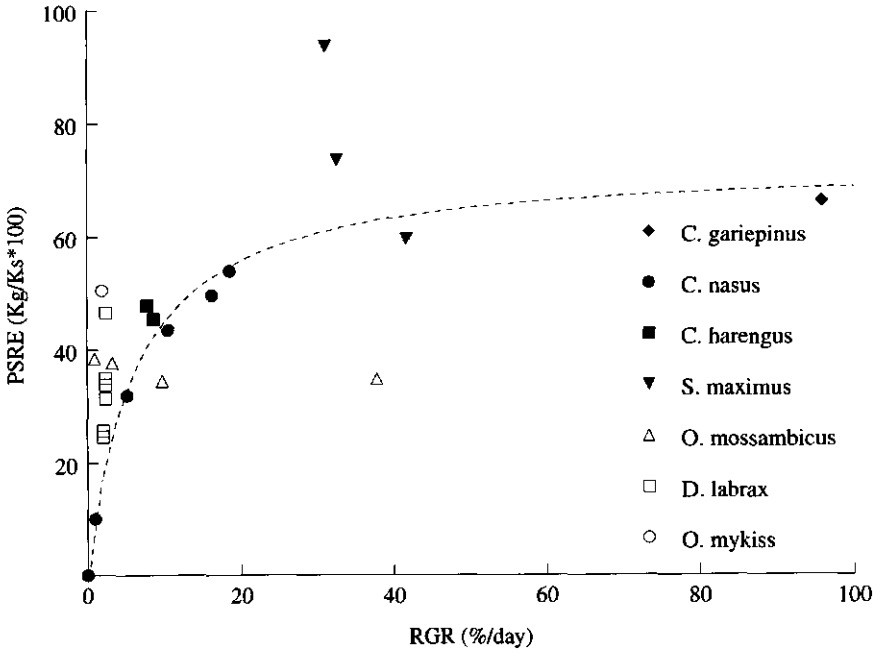

Fig. 1. Relation between relative growth rate (RGR) and protein synthesis retention efficiency (PSRE) in fish larvae (full symbols) and fry (open symbols). Data Sources: *Clarias gariepinus* (Chapter 3); *Chondrostoma nasus* (Houlihan et al. 1992); *Clupea harengus* (Houlihan et al. 1995c); *Scophthalmus maximus* (Chapter 5); *Oreochromis mossambicus* (Houlihan et al. 1993); *Dicentrarchus labrax* (Langar et al. 1993); *Oncorhynchus mykiss* (Houlihan et al. 1995b). RGR = $(e^g - 1)*100$ and $g = (\ln DW_2 - \ln DW_1) / (t_2 - t_1)$.

(Houlihan et al. 1995c), as well as the independence between oxygen consumption and growth rate at high growth rates in larval whitefish, *Coregonus wartmanni*, and roach, *Rutilus rutilus* (Wieser and Medgyesy 1990a,b).

Despite their high growth rate, fish larvae allocate a considerable amount of the total energy expenditure to maintenance costs. Fed larvae of the African catfish (Chapters 3 and 7) allocate 60 to 75%, and larval turbot (Chapter 4 and 7) 70 to 85 %, of the total energy expenditure to maintenance. Even the extremely fast growing yolk-sac larvae of the African catfish allocate 30 to 45% of the total energy expenditure to maintenance (Chapter 3). Furthermore, the energy expenditure due to protein turnover seems to be only 27% of the cost of maintenance in yolk-sac larvae of the African catfish (Chapter 2). Therefore, the hypothesis that larval fish would drastically reduce their maintenance functions, namely protein turnover, to

create metabolic space for high growth rates (Kiørboe et al. 1987; Kiørboe 1989; Wieser and Medgyesy 1990a,b; Wieser 1994) does not hold.

The main factor enabling fast growth of larval fish is their high rates of protein synthesis at the minimum theoretical cost. In general, fast growing fish larvae are only slightly more efficient in retaining synthesised protein than slow growing larvae and older fish. However, for larval turbot close to first feeding a strong suppression of protein turnover was observed (Chapter 5). It would be interesting to verify if a suppression of protein turnover around first feeding also occurs in larvae of other species. This could have an explanation in the regulation of AA metabolism (see section on AA catabolism), rather than in the need to create metabolic space for growth.

Growth and cost of growth

Based on data for seven species of aquatic metazoans, Wieser (1994) suggested that the cost of growth decreases asymptotically as growth rate increases: the cost of growth was estimated to be 66 mmol ATP g^{-1}dry weight deposited for animals growing faster than 16.8% body dry weight day^{-1}. In the African catfish the cost of growth also decreases with increasing growth rates, towards a value close to the minimum biochemical cost of protein synthesis (see Chapter 3). The relationship between growth rate and cost of growth is plotted in Figure 2, based on available data from literature on fish larvae. It seems to be a general case that in fish larvae the cost of growth is variable, decreasing towards the minimum biochemical cost of protein synthesis (50 mmol ATP.g^{-1}protein synthesised, Reeds et al. 1985) at high growth rates. This was also the case for turbot larvae growing between 15.4 and 22.8% body dry weight day^{-1} (Chapter 4). Furthermore, it has been shown that the cost of protein synthesis, measured by using the cycloheximide inhibition method, is close to the biochemical minimum in yolk-sac larvae of the African catfish (Chapter 2) and in fast growing juvenile tilapia (Houlihan et al. 1993). However, the pattern of change in the energetic cost per unit of growth with increasing growth rates is quite variable between species and also, at least in the roach, between temperatures (Fig. 2). Both the rate of decrease in the cost of growth and the intercept (cost of growth when growth is close to zero) seem to be variable. Furthermore, Figure 2 suggests a lower cost of growth for larvae reared at lower temperatures, at comparable growth rates. It is possible that the concurrent high rates of protein synthesis and not the high growth rates are the direct cause of the reduced cost of growth. The cost of protein synthesis decreased with augmenting rates of protein synthesis in larval nase (Houlihan et al. 1992), in trout hepatocytes (Pannevis and Houlihan 1992) and in different cell lines (Smith and Houlihan 1995). This may be due to a fixed component of this cost, independent of the rate of protein synthesis (Pannevis and Houlihan 1992). Houlihan et al. (1995a) suggested that this fixed energy expenditure is

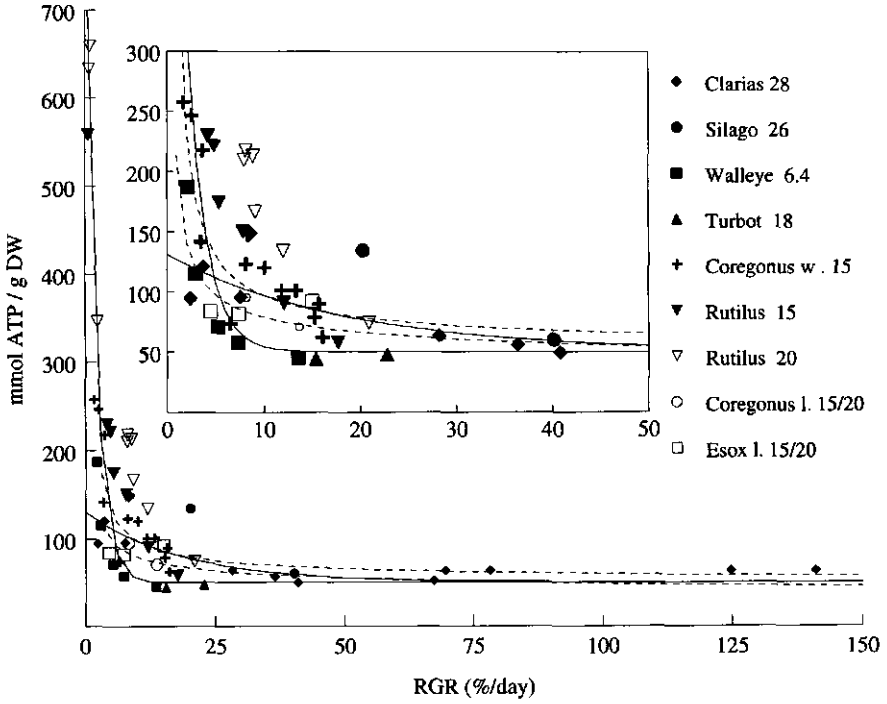

Fig. 2. Relation between relative growth rate (RGR) and the cost of growth (COG) in fish larvae. The different species and the rearing temperatures are given in the legend. The regression lines are $COG = 50 + 80.977 \cdot e^{0.0544 \cdot RGR}$ ($r^2 = 0.69$ n = 12) for *Clarias gariepinus* (dashed line) and $COG = 50 + 763/316 \cdot e^{-0.5171 \cdot RGR}$ ($r^2 = 0.77$ n = 55) for all data pooled together (full line). Data Sources: *Clarias gariepinus* (Chapter 3); *Sillago japonica* (Oozeki and Hirano 1994); *Theragra chalcogramma* (Yamashita and Bailey 1989); *Scophthalmus maximus* (Chapter 4); *Coregonus wartmanni* and *Rutilus rutilus* (Wieser and Medgyesy 1990a,b); *Coregonus lavaretus* and *Esox lucius* (Wieser et al. 1992). The cost of growth was calculated as described in Chapter 3.

related to a constant activation of tRNA and a constant production of rRNA. At low growth rates this fixed component of the cost of protein synthesis will have a large contribution to the cost of growth in comparison to the cost of peptide-bond formation. Therefore, the wide variation found between species in the pattern of decrease of the cost of growth with increasing growth rates may be explained by differences in the ratio between growth rate and the rate of protein synthesis. It would be interesting to ascertain whether this ratio growth rate / rate of protein synthesis changes with temperature.

The present study confirms the hypothesis that the cost of growth is largely influenced by the costs of protein metabolism. The biochemical cost of protein synthesis sets the lower limit for the cost of growth, which is reached at high growth rates (Chapters 2, 3 and 4).

However, the cost of protein growth can be expected to be higher than the cost of protein synthesis. Higher protein growth rates are usually associated with increased rates of protein synthesis and also with higher protein turnover rates, both in fish (Houlihan et al. 1986; Houlihan et al. 1988) and in mammals (Waterlow et al. 1978). Consequently, the rate of protein turnover may be higher in a growing organism than in an organism that is not growing. This means that in growing fish the cost of growth includes the energy needed for protein turnover in addition to the cost of protein turnover at maintenance. However, in yolk-sac larvae of the African catfish (Chapter 2) the estimates for the cost of protein synthesis (measured by cycloheximide inhibition) were identical to the cost of protein growth (measured by regression of mass-specific oxygen consumption on growth rate). This indicates that costs associated with protein growth other than the synthesis costs are insignificant. This in turn implies that the rate of protein turnover is fixed, and independent of the rate of protein synthesis. Unchanged rates of protein turnover at different protein synthesis rates have been observed earlier in larval nase (Houlihan et al. 1992). Therefore, in fast growing fish larvae the cost of protein growth is probably almost entirely attributable to the cost of protein synthesis because protein turnover remains fixed, although at a high rate.

High growth rates in fish larvae are associated with a low cost of growth. Chapter 3 suggests that the transition from a larval to a juvenile pattern of fish growth and metabolism is a gradual process. If growth rates reach levels that allow for a low cost of growth, food conversion efficiencies will increase, allowing for higher growth. Thus, higher growth may bring an amplification effect on growth itself. Once fish are reared under optimal oxygen levels, feeding regime, diet quality and water temperature, the larval pattern of high growth rates / low cost of growth / high food conversion efficiencies might be extended for a longer time span in the larval / juvenile transition period. Conversely, sub-optimal conditions will probably lead to increasingly restrained growth rates and food conversion efficiencies.

Control of amino acid catabolism in fish larvae

An animal can control its AA catabolism by modulating either the activity of the enzymes involved in catabolism or the rates of protein synthesis and turnover. A coarse control of AA utilisation exists through the affinity (K_m) of the enzymes involved in the AA metabolism (Christiansen and Klungsøyr 1987), as enzymes involved in AA catabolism usually have low K_m values. On the other hand, aminoacyl-tRNA synthetases have high K_m values. Hence, when in a given cell the AA charge is low, protein synthesis will be "preferred" (Cowey and Walton 1989).

In comparison to mammals, juvenile and adult fish have a lower adaptability of the AA catabolism (Walton 1985; Cowey and Walton 1989). This means that fish will be more

sensitive to diets poor in protein and to diets with an imbalanced AA profile. Fish larvae have even less control of their AA catabolism, leading to higher catabolic losses of AA (Dabrowski 1986). However, Dabrowski (1986,1989) proposed a decreasing role of AA catabolism for energy production during fish development, associated with an increasing capacity of reducing the catabolism of AA. In fact, in larvae of the African catfish the activities of enzymes involved in AA catabolism decreased with increasing size (Segner and Verreth 1995). In yolk-sac larvae of the same species, AA are a major energy source (Chapter 6; Polat et al. 1995; Verreth et al. 1995) and there is little control of AA catabolism (Chapter 6). Apparently, catfish yolk-sac larvae have no ability to spare EAA, in contrast to juvenile and adult fish (Cowey and Sargent 1979; Kim et al. 1992). However, larval turbot may develop the capacity of sparing EAA during ontogeny (Chapter 5).

A reduced protein turnover is likely to reduce the losses of the AA involved in protein turnover (Carter et al. 1995). However, reducing protein turnover may have costs for the larvae in terms of viability and survival (Kiørboe et al. 1987). High protein turnover allows for higher metabolic plasticity, enabling a faster response of the organism in case of environmental/disease stress, through the synthesis of specific enzymes and other proteins. Therefore, reducing protein turnover in early stages may be a compromise between the risks of mortality in case of stress and the benefits of a faster growth in terms of survival. In the natural environment, larger larvae have higher chances of survival, as they are more competitive in prey capture, and have a higher chance of escaping predators (Blaxter 1988). Through evolution, there may have been a selective pressure to increase protein turnover as the larva develops, and increases its control of AA catabolism. So far, not much evidence has been found for this hypothesis. Only in larval turbot was protein turnover reduced in early stages of development (Chapter 5). In contrast to this hypothesis, the few other available studies suggest that larval protein turnover is comparable to older fish (Houlihan et al. 1995a,b; Chapter 2). It would be interesting to further investigate the relationship between efficiency of AA retention and protein turnover at different larval stages. The extent to which fish larvae manage to compensate for imbalances in dietary AA profile through selective catabolism of individual AA also deserves more attention.

According to the simulation model developed in the present study (Chapter 7), gluconeogenesis may play an important role in the African catfish larvae since considerable amounts of absorbed AA can be saved from catabolism and diverted into lipid deposition. This would be the case in periods of high food intake when high unavoidable AA losses would occur. However, in the African catfish the activity of the rate-limiting enzyme for gluconeogenesis (FDPase) had a relatively low value from first feeding (0.3 mg dry weight) until a size of about 2.5 mg dry weight, when it began to increase (Segner and Verreth 1995). This increase was related to the onset of anaerobic muscle metabolism, and the concomitant

regeneration of lactate to glucose. It remains to be determined if the relatively low initial levels of FDPase activity are sufficient to explain the predicted shunt of AA into lipid synthesis.

Dietary protein and growth

Comparison between the dietary and larval AA profiles indicates dietary imbalances in the AA profiles of the diets normally employed in larviculture operations. According to the results of the present study (Chapters 5, 6 and 7), these imbalances may lead to rather high (20 to 40%) losses of the AA in the diet. Considering the high potential for growth and the high food intake of fish larvae (Chapter 3; Houde 1989; Wieser and Medgyesy 1990b), such dietary AA imbalances may have a much larger negative impact on growth, food conversion efficiencies and nitrogen waste production than in older fish. Furthermore, several factors may reduce the impact of the dietary imbalances on AA losses. These include an obligatory AA loss independent of the dietary AA profile, the level of protein turnover, the absorption rate of the individual AA in the gut, and a possible buffering role of the visceral organs (see Chapter 6). The significance of each of these factors is difficult to determine.

It would be interesting to check whether different dietary AA profiles affect growth qualitatively, i.e., lead to different development patterns. According to Kamler (1992), the composition of larval diets would modify the rate of formation of the digestive organs, and influence the relationship between body size and organ size.

Whole fish AA profiles change during larval development (Chapters 5 and 6), and are different from adult profiles. Changes are more important in the yolk-sac stage. Such changes are probably associated with the allometric growth of the larvae, as different organs and tissues develop at varying rates and at different times in ontogeny (Oikawa and Itazawa 1984; Osse and van den Boogaart 1995). Whether these changes are significant in terms of AA requirements remains to be determined. However, it may be advisable to consider the mentioned AA profile variations when formulating larval diets. As long as more detailed information is not available, larval AA profiles are the best index for estimating the qualitative AA requirements of fish larvae.

The AA profile of *Artemia* nauplii may be sub-optimal to fish larvae. The present study indicates that this is probably the case for turbot larvae (Chapter 5), and larvae of the African catfish (Chapter 6). Based on this hypothesis, one could consider to enrich the *Artemia* nauplii with certain AA. In this respect, threonine, leucine and methionine/cysteine, could be tested for turbot larvae (Chapter 5), and phenylalanine/tyrosine, leucine and histidine for the African catfish (Chapter 6). However, in practice this may be difficult. The AA profile of *Artemia* is probably genetically controlled (Seidel et al. 1980), and it is unknown whether *Artemia* nauplii are able to synthesise proteins with different AA profiles when the dietary AA supply changes.

Nevertheless, the profile of the FAA pool can probably be manipulated by incubating *Artemia* in AA-enriched water. However, this may be of little significance due to the small size of the *Artemia* FAA pool. Dissolving crystalline AA in the water were larvae are reared, or supplying micro-particles or micro-algae rich in the target AA to the larvae, could be valuable alternatives.

Results from model simulations (Chapter 7) suggested that an increase in dietary protein content would stimulate growth and reduce lipid deposition. This can be an indication of sub-optimal dietary protein levels in the live foods currently used in larviculture. In turn, the model predicts that an increase in the dietary lipid content would lead mainly to an increase in larval lipid content, although a small protein-sparing effect is also suggested. This protein sparing-action would be more significant for turbot than for catfish. An increase in dietary lipid has been shown to lead to both protein-sparing and increased lipid deposition in juvenile catfish (Henken et al. 1986) and in juvenile turbot (Bromley 1980; Andersen and Alsted 1993).

A window to the future...

The present study shows that a better knowledge of the different aspects of AA metabolism and its regulating factors, can contribute to growth optimisation in larviculture. In this respect, definition of the ideal AA profiles, and the extent of their variation in ontogeny is paramount. Studies with isotope-labelled AA combined with modelling techniques is a promising direction to pursue. The role of gluconeogenesis in diverting unavoidable AA losses to synthesis of lipids also deserves further study. The practical application of the information obtained in growth studies with fish larvae, is still hindered by the lack of knowledge, and the difficulties in estimating the importance of the different factors affecting food intake and digestibility. The further development of an explanatory simulation model that can accurately predict growth in early life stages of fish for different species, fed various diets, in a range of rearing conditions, is a challenge for the future. Such a model is especially promising for estimating the dietary requirements when new species are brought into cultivation.

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Summary

Resumo

Samenvatting

For many fish species a further increase in production volume is hampered by an irregular and sometimes insufficient fingerling supply. This is usually the case when new species are brought into cultivation. Dietary nutrient imbalances are often responsible for low survival and low growth rates in fish larvae. If survival is dependent on micro-nutrients like polyunsaturated fatty acids and vitamins, growth is essentially protein deposition. Therefore, optimising growth is closely linked to understanding protein and amino acid (AA) metabolism in order to supply dietary protein of appropriate quality in the right quantity.

Although fish larvae grow very fast, little is known about how they accomplish it, and to which extent their metabolism and nutritional requirements differ from larger fish. Modelling can be a powerful tool to promote understanding and optimisation of growth in fish larvae. In a model, sound hypotheses are integrated with information from different sources, so that growth can be simulated under different conditions. An explanatory model predicts growth based on the underlying biochemical processes. Therefore, in addition to its predicting power, such a model may promote understanding of the growth process and may reveal gaps in knowledge, thereby suggesting directions for further research.

The ultimate objective of this study was to develop an explanatory model that can simulate growth during the early life stages of fish. It should contribute to the understanding of growth and growth metabolism in larval fish, and therefore contribute to elucidate their nutritional requirements and to develop appropriate feeding strategies. Protein metabolism and the energetics of growth received particular attention during the experimental phase of the present study, as these processes are believed to be determinant for growth and its modelling.

In **Chapter 1**, the framework of the model was developed using a relatively simple system, the yolk-sac larvae of the African catfish, *Clarias gariepinus*. It considered the larvae as a two compartment system: the yolk-sac and the larval body. This system exchanges oxygen, carbon dioxide, water, ammonia and heat with the environment. Yolk is absorbed through the syncytium, digested, and the resulting nutrients are released into the blood circulation. Circulating AA and fatty acids are considered to be used firstly for the production of energy, and what is left will be used for growth. From the moment that absorbed nutrients do not satisfy the energy needs, embryo tissue starts to be catabolised. From the comparisons between simulations and experimental data, the simulations were fairly accurate until complete yolk absorption, losing accuracy thereafter. The improvement of the model accuracy probably involves a better definition of the energetics of growth and of protein metabolism. Simulation outputs suggest that fat is the main energy substrate during yolk absorption, with protein becoming progressively more important, and being predominant during starvation. Ideal culture conditions for African catfish yolk-sac larvae seem to include: the highest temperature that combines with

acceptable mortality rates; selection of broodstock towards obtaining the largest egg size, insofar as viability is not affected; the start of feeding as close as possible to 144 physiological day-degrees.

Chapters 2 to 6 supplied experimental data to parameterise and support the design and the assumptions, so that the model could be further developed to simulate growth of fed fish larvae. **Chapter 2** deals with the question of how fish larvae manage to accommodate the cost of high growth rates with the cost of maintenance within their limited energy budget. In fish larvae the cost of rapid growth may be accommodated by a decrease in the rate of protein turnover or by a reduction in the cost of protein synthesis. Protein growth, synthesis and degradation were measured in yolk-sac larvae of the African catfish and the costs of protein synthesis and protein growth were estimated. Growth rates were over 100% protein weight.day^{-1}. Protein synthesis retention efficiency (retained protein per unit of synthesis) was estimated to be 66.6%, a value comparable to that of larger fish. The larvae used 43% of their oxygen consumption for protein synthesis. Nevertheless, the cost of protein synthesis was close to the theoretical minima. Therefore, the high growth rates of African catfish yolk-sac larvae are possible because the cost of protein synthesis is minimised. This low cost was associated with high rates of protein synthesis (138% protein weight.day^{-1}), and elevated RNA concentrations (102 μg RNA.mg^{-1}protein), which together lead to very high RNA efficiencies (13.5 g protein synthesised.g^{-1}RNA.day^{-1}).

In **Chapter 3** the cost of growth was estimated at different stages of development in the African catfish, and related to food intake and growth rate. Measurements were performed on larvae reared at 28°C, and the results were compared to those of juveniles (recalculated from literature). As the African catfish grows (0.07 mg to 38 g body DW), the cost of growth increases from 64 to 149 mmol ATP.g^{-1}DW deposited. A higher cost of growth is associated with reduced growth rates (from 141 to 2.4% body DW.day^{-1}), and is also reflected in lower gross food conversion efficiencies (93 to 24%). Decreasing growth rates are a result of reductions in relative rates of food intake (152 to 6.2% body DW.day^{-1}) and are accompanied by a reduction in oxygen consumption (1.0 to 0.033 μmol O_2.mg^{-1}DW.h^{-1}). Fish size was allometrically related to growth rate, food intake and oxygen consumption. The absence of any distinct breakpoint in these relations suggests a gradual transition from the larval to the juvenile pattern of fish growth and metabolism. Providing that optimal conditions are met (e.g., temperature, oxygen availability and feeding) the pattern of fast growth at low cost might be extended for a longer time span during the transition period from the larval to the juvenile stage, resulting in improved growth and food conversion efficiency.

The cost of growth for larval turbot, *Scophthalmus maximus*, was estimated in **Chapter 4**, and its biochemical composition was studied in relation to development and feeding regime.

In general, body composition in terms of protein, total lipids and ash contents was stable throughout the larval period. The contribution of glycogen to body composition increased slightly, and the contribution of free AA was small but variable. Larvae fed enriched *Artemia* grew less and had a higher lipid content than larvae feeding on natural zooplankton. *Artemia* may cause sub-optimal larval growth due to its high lipid content, when using standard enrichments. High growth rates in turbot larvae are associated with the deposition of new tissues at a cost close to the theoretical minimum cost of protein synthesis.

The cost of growth is variable in fish larvae, decreasing asymptotically towards the minimum biochemical cost of protein synthesis as growth rate increases. It is possible that the changes in the rates of protein synthesis associated with the changes in growth rates are the direct cause of the variability in the cost of growth. In fact, the cost of growth in fast growing African catfish yolk-sac larvae and turbot larvae is almost entirely attributable to the cost of protein synthesis. It has also been demonstrated in literature that the cost of protein synthesis decreases with increasing rates of protein synthesis.

In **Chapter 5** growth and AA utilisation of turbot larvae were related to the composition of the free AA pool, the rate of protein turnover, and the flux of AA. High contents on free tyrosine and phenylalanine were found around first feeding, what may be related to the development of thyroid follicles. Free taurine was present in high levels throughout the experimental period. Larval growth rates were positively correlated with taurine levels suggesting a dietary dependency for taurine and/or sulphur AA. Reduced growth rates in *Artemia*-fed larvae were associated with lower levels of free methionine, indicating that this diet is deficient in methionine for turbot larvae. Leucine may also be limiting turbot growth as the different diet organisms had lower levels of this AA in the free pool than that found in the larval protein. The AA profile of protein was stable during the larval period although some small, but significant, differences were found. The AA profile of the free pool in turbot larvae is highly variable, and considerably different from the larval protein AA profile. The FAA pool is extremely dynamic, being very sensitive to the arrival of the dietary AA, as well as to the AA profiles of proteins that are being synthesised and/or degraded at a given moment. In an initial phase, high growth rates in turbot larvae involve a reduction in the turnover of proteins, while AA losses through oxidation appear to be high. At a later stage a much higher protein turnover was observed, together with smaller AA oxidation losses. This difference may be related to different strategies for optimising the utilisation of dietary AA as the larvae develops and gains further control of AA catabolism. The contribution of essential AA to the free AA pool decreased with development, which may be a mechanism for sparing essential AA.

In **Chapter 6** the qualitative AA requirements of larval African catfish were investigated. Changes in larval AA profiles were studied in yolk-sac larvae at different

temperatures, and in larvae fed *Artemia* nauplii or an experimental dry diet at 28°C. The larval AA profile changed during ontogeny, especially before the start of exogenous feeding. The AA profiles of the food items (yolk, *Artemia* and the dry diet) differed considerably from the larval ones. No selective absorption of yolk AA could be detected in yolk-sac larvae. Depletion rates of individual AA varied, which could be largely explained by differences between larval and yolk AA profiles, and also by changes in larval AA profile during ontogeny. There is little regulation of catabolism of individual AA in yolk-sac and starved larvae, and no sparing of essential AA. Until more detailed information is available, larval AA profiles can be used as an index of the qualitative larval AA requirements. Higher temperatures lead to increased absorption and depletion rates of AA, and also to a higher utilisation efficiency of yolk nutrients. However, changes in temperature did not result in preferential absorption or depletion of individual AA, and caused only small variations in the AA profile.

In **chapter 7** the simulation model for growth and body composition of fed larvae was described and validated for African catfish and turbot. A sensitivity analysis was also performed in order to pin-point the key parameters governing larval growth. The model is driven by nutrient intake, with the absorbed dietary nutrients being used either for energy production or for biosynthetic processes, and it is based on the stoichiometry of the intermediary metabolism. According to model simulations, high unavoidable losses of AA due to imbalances between the dietary and the larval AA profiles lead to an increase in lipid deposition in periods of high food intake. In catfish this occurs with the contribution of gluconeogenesis. Results of simulations also indicate that an increase in dietary protein stimulates growth, and leads to a reduction in lipid deposition. Furthermore, an increase in dietary lipid leads to an increase in body lipid content and a small protein-sparing effect.

The present study suggests that *Artemia* nauplii may be a sub-optimal feed for fish larvae. Their AA profile is imbalanced for both the African catfish and the turbot. Furthermore, *Artemia* probably has an excess of lipids and too little protein in its body composition. It may therefore be advisable to review the enrichment procedures for *Artemia* nauplii currently used in larviculture.

A better knowledge of the different aspects of AA metabolism and its regulating factors, can contribute to growth optimisation in larviculture. In this respect, definition of the ideal dietary AA profiles, and the extent of their variation in ontogeny is paramount. Studies with isotopic AA associated with modelling techniques is a promising direction to pursue. The role of gluconeogenesis in diverting unavoidable AA losses to synthesis of lipids also deserves further study. The application of the information obtained in growth studies on fish larvae for practical conditions is still hampered by the difficulties in estimating the importance of the different factors affecting food intake and digestibility. The further development of an

explanatory simulation model that can accurately predict growth in early life stages of fish for different species, fed various diets and in a range of rearing conditions, is a challenge for the future. Such a model is especially promising in the estimation of the dietary requirements when new species are brought into cultivation.

O aumento da produção de muitas espécies de peixes é limitado pela irregular, e por vezes insuficiente, produção de larvas e juvenis. Este é normalmente o caso quando novas espécies são adoptadas para o cultivo. As baixas taxas de sobrevivência e crescimento, são frequentemente devidas a desiquílibrios nutricionais da dieta. Se as taxas de sobrevivência estão provavelmente relacionadas com as necessidades em micro-nutrientes, como ácidos gordos poli-insaturados e vitaminas, o crescimento é essencialmente deposição de proteína. Assim, a optimização do crescimento está intimamente relacionada com a compreensão do metabolismo de proteínas e amino ácidos (AA), com vista a incluir na dieta proteínas de qualidade apropriada, e na quantidade adequada.

Se bem que as larvas de peixes apresentam um rápido crescimento, pouco se sabe sobre como isso é alcançado, e até que ponto o seu metabolismo e requesitos nutritivos são diferentes dos peixes juvenis e adultos. A elaboração de modelos de simulação pode ser uma ferramenta poderosa na promoção de entendimento e na optimização do crescimento em larvas de peixe. Num modelo, hipóteses fundamentadas são integradas com informação proveniente de diferentes estudos, com vista a produzir simulações de crescimento em diversas condições. Um modelo mecanístico prevê o crescimento com base nos processos bioquímicos subjacentes. Para além do seu uso para previsões de crescimento, um modelo com estas características pode promover o entendimento do processo de crescimento, e revelar pontos obscuros, sugerindo desta forma direções a seguir para investigação futura.

O objectivo principal deste estudo foi desenvolver um modelo mecanístico que possa simular o crescimento durante a fase larvar dos peixes. Este estudo deverá contribuir para o entendimento do processo de crescimento, e respectivo metabolismo, em larvas de peixes, com a intenção de elucidar sobre as suas necessidades alimentares e desenvolver estratégias de alimentação adequadas. Durante a fase experimental deste estudo, o metabolismo proteico e a energética do crescimento foram alvo de particular atenção, uma vez que estes processos são tidos como determinantes para o crescimento e a elaboração de modelos de simulação do crescimento.

No **Capítulo 1** foram definidas as bases fundamentais do modelo, usando um modelo biológico relativamente simples, as larvas de saco vitelino do peixe gato Africano, *Clarias gariepinus*. Este modelo considera a larva como um sistema com dois compartimentos: o saco

vitelino, e o corpo larvar. Este sistema permuta oxigénio, dióxido de carbono, água, amónia, e calor com o meio ambiente. O vitelo é absorvido pelo sincício, digerido, e os nutrientes resultantes são libertados para a circulação sanguínea. Considerou-se que os AA e ácidos gordos circulatantes são usados primordialmente para satisfazer as necessidades energéticas, sendo o restante usado para o crescimento. A partir do momento em que a absorção de nutrientes do vitelo não é suficiente para as necessidades energéticas, considerou-se que tecidos larvares começam a ser catabolizados. Quando comparados com dados experimentais, os resultados de simulações com o modelo foram razoavelmente precisos desde a eclosão até à completa absorção do saco vitelino, perdendo precisão depois disso. A melhoria da precisão do modelo parece depender de uma melhor defínição no modelo da energética do crescimento e do metabolismo de proteínas. Os resultados das simulações sugerem que os lípidos são o principal substrato energético durante a absorção do saco vitelino, com as proteínas ganhando progressivamente importância, sendo o principal substrato quando as larvas entram em jejum. Condições ideais de cultivo para larvas de saco-vitelino do peixe gato Africano parecem incluir: temperatura mais alta que combine com taxas de sobrevivência aceitáveis; seleção de reprodutores com vista à obtenção do ovos de tamanho máximo, desde que as taxas de sobrevivência não sejam afectadas; começar a alimentação das larvas tão perto quanto possível dos 144 graus-dia fisiológicos.

Os capítulos 2 a 6 forneceram resultados experimentais para parameterizar e apoiar a estrutura e os pressupostos do modelo, de forma a que este pudesse ser desenvolvido para simular o crescimento de larvas de peixes alimentadas. O **Capítulo 2** trata da questão de como as larvas de peixes conseguem conciliar os custos energéticos resultantes das suas altas taxas de crescimento com as necessidades energéticas para o metabolismo basal, umas vez que a sua capacidade respiratória é limitada. Os custos energéticos das altas taxas de crescimento das larvas de peixes podem ser conciliados com as outras necessidades metabólicas, por uma diminuição da taxa de renovação de proteínas, ou por uma redução do custo de síntese proteica. As taxas de crescimento, síntese e degradação proteica foram medidas em larvas de saco vitelino do peixe gato Africano, e os custos de síntese e crescimento proteico foram estimados. As taxas de crescimento ultrapassaram 100% do peso em proteína por dia. A eficiência de retenção da proteina sintetizada (crescimento proteico por unidade de proteína sintetizada) foi estimada em 66.6%, um valor comparável com o

normalmente registrado em peixes juvenis e adultos. Estimou-se também que as larvas usaram 43% do oxigénio consumido na síntese de proteínas. No entanto, o custo de síntese proteica foi próximo do custo teórico mínimo (50 mmol ATP por grama de proteína sintetizada). Portanto, as altas taxas de crescimento das larvas de saco vitelino do peixe gato Africano são tornadas possíveis por uma minimização do custo de síntese proteica. Este baixo custo da síntese proteica esteve associado às altas taxas de síntese proteica verificadas (138% do peso em proteína por dia), bem como às elevadas concentrações de RNA (102 μg RNA.mg^{-1}proteína), o que em conjunto levou a altas eficiências de expressão do RNA (13.5 g proteína sintetizada.g^{-1}RNA.dia^{-1}).

No **Capítulo 3** o custo energético do crescimento foi estimado, e relacionado com as taxas de crescimento e de consumo de alimento, em diferentes estádios de desenvolvimento de larvas do peixe gato Africano cultivadas a 28°C. Estes resultados foram comparados com dados re-calculados para juvenis da mesma espécie obtidos na bibliografia. À medida que o peixe gato cresce (de 0.07 mg até 38 g peso seco), o custo de crescimento aumenta de 64 até 149 mmol ATP.g^{-1}peso seco depositado. Este aumento no custo de crescimento é associado com uma redução na taxa de crescimento (de 141 até 2.4% peso seco.dia^{-1}), e também se reflecte em eficiências de conversão do alimento mais baixas (de 93 até 24%). A diminuição da taxa de crescimento resulta da redução da taxa relativa de consumo de alimento (de 152 até 6.2% peso seco.dia^{-1}) e é acompanhada por uma redução na taxa de consumo de oxigénio (de 1.0 até 0.033 μmol O_2.mg^{-1}peso seco.hora^{-1}). O decréscimo nas taxas de crescimento, consumo de alimento e consumo de oxigénio pode ser descrito por relações alométricas com o peso seco das larvas e juvenis. A ausência de descontinuidades aparentes nestas relações alométricas sugere uma transição gradual do padrão de crescimento e metabolismo da fase larvar para a fase juvenil. Desde que se garantam condições óptimas de cultivo, tais como de temperatura, oxigénio dissolvido e alimentação, o padrão larvar de altas taxas de crescimento a baixos custos poderá eventualmente ser mantido por um maior período de tempo na transição da fase larvar para a juvenil, garantindo assim maiores taxas de crescimento e de eficiência de utilização do alimento.

O custo energético de crescimento das larvas de pregado, *Scophthalmus maximus*, foi estimado no **Capítulo 4,** e a sua composição bioquímica foi estudada em diferentes fases de desenvolvimento e diferentes regimes alimentares. Em geral, a composição bioquímica em

termos de proteína, lípidos totais e cinzas, manteve-se estável durante a fase larvar. Por seu lado, a contribuição de glicogénio para a composição corporal aumentou ligeiramente durante a fase larvar, enquanto que o conteúdo larvar de AA livres foi pequeno mas variável. Larvas alimentadas com *Artemia* enriquecida cresceram menos e apresentaram um conteúdo de lípidos superior ao das larvas alimentadas com zooplâncton selvagem. Estes resultados indicam que o uso de *Artemia* com os correntes protocolos de enriquecimento poderá causar taxas de crescimento sub-óptimas devido ao seu elevado conteúdo em lípidos. As altas taxas de crescimento observadas em larvas de pregado estão associadas à deposição de novos tecidos a um custo próximo da custo teórico mínimo de síntese proteica.

O custo de crescimento é variável em larvas de peixes, diminuindo assintoticamente para o custo teórico mínimo de síntese proteica, à medida que a taxa de crescimento aumenta. É possível que as variações na taxa de síntese proteica, associadas às variações na taxa de crescimento, sejam a causa directa para as mudanças no custo de crescimento. Na verdade, o custo de crescimento nas larvas de saco vitelino de peixe gato Africano e em larvas de pregado, com altas taxas de crescimento, foi devido quase por completo ao custo de síntese proteica. Está também demonstrado que o custo de síntese proteica diminui com o aumento da taxa de síntese proteica.

No **capítulo 5** o crescimento e a eficiência de utilização de AA em larvas de pregado foram relacionados com a composição do pool de AA livres, a taxa de renovação proteica, e os fluxos de AA. Foram detectados altos níveis de tirosina e fenilalanina no pool de AA livres por altura da primeira alimentação, que poderão estar relacionados com o desenvolvimento de folículos da tiroide. Por seu lado, altos conteúdos de taurina estiveram presentes durante toda a fase larvar. Foi encontrada uma correlação positiva entre a taxa de crescimento larvar e os conteúdos de taurina, o que sugere uma dependência para o fornecimento de taurina ou AA sulfurados na dieta. De facto, as reduzidas taxas de crescimento em larvas alimentadas com *Artemia* coincidiram com baixos níveis de metionina no *pool* de AA livres, o que indica que nauplii de *Artemia* enriquecidos são uma dieta deficiente em metionina para larvas de pregado. O crescimento das larvas de pregado também poderá ser limitado por uma deficiência de leucina na *Artemia* bem como no zooplâncton selvagem, uma vez que os níveis deste AA no pool de AA livres daqueles organismos foi consideravelmente mais baixo que o encontrado no perfil da proteína do pregado. Este perfil de AA foi relativamente estável

durante a fase larvae, se bem que algumas pequenas, mas significantes, diferenças tenham sido detectadas. O perfil de AA do pool de AA livres das larvas de pregado é altamente variável, e consideravelmente diferente do perfil de AA da proteína. O pool de AA livres é extremamente dinâmico, e muito sensível à chegada dos AA provenientes da dieta, bem como dos perfis de AA das proteínas que estão a ser sintetizadas e/ou degradadas num dado momento. Numa fase inicial, as altas taxas de crescimento em larvas de pregado estão associadas a uma redução da taxa de turnover proteico, enquanto que as perdas de AA por oxidação parecem ser altas. Num estadio posterior, taxas de turnover proteico muito mais altas foram observadas, enquanto que as perdas de AA por oxidação parecem ser muito menores. Estas diferenças podem estar relacionadas com diferentes estratégias para maximizar a eficiência de utilização dos AA provenientes da dieta, a medida que a larva vai aumentando a sua capacidade de regulação do catabolismo de AA. A importância dos AA essenciais no pool de AA livres diminuiu com o desenvolvimento larvar, o que pode ser um mecanismo para a poupança de AA essenciais.

No **Capítulo 6** os requesitos qualitativos de AA para as larvas do peixe gato Africano foram estudadas. Variações no perfil de AA foram analisadas em larvas de saco vitelino a diferentes temperaturas, e em larvas alimentadas com nauplii de *Artemia* ou uma dieta artifícial a 28°C. O perfil de AA larvar variou consideravelmente durante o desenvolvimento larvar, e em particular antes do início da alimentação. Os perfis de AA dos alimentos usados (vitelo, *Artemia* e dieta artifícial) eram consideravelmente diferentes uns dos outros. Não foi detectada qualquer absorção selectiva dos diferentes AA em larvas de saco vitelino. As taxas de depleção de cada AA variaram consideravelmente, o que pode ser atribuído em grande parte a diferenças entre os perfis de AA do peixe gato e do vitelo, mas também às variações no perfil de AA do peixe gato durante o desenvolvimento larvar. Parece haver pouca regulação do catabolismo de AA em larvas de saco vitelino, e não há poupança de AA essenciais. Os perfis de AA das larvas podem ser usados como referência para as necessidades qualitativas de AA, até que informação mais detalhada esteja disponível. As taxas de absorção e depleção de AA, bem como as eficiências de utilização dos nutrientes do vitelo, aumentaram com o aumento da temperatura. No entanto, as mudanças de temperatura não provocaram a absorção ou depleção preferêncial dos diferentes AA, e causaram apenas pequenas variaçtes no perfil de AA.

No **Capítulo 7** o modelo de simulação para o crescimento e composição corporal de larvas alimentadas foi descrito e validado para o peixe gato Africano e para o pregado. Uma análise de sensibilidade foi também efectuada por forma a identificar os parâmetros chave que determinam o crescimento larvar. O modelo é essencialmente dependente do consumo de alimento, com os nutrientes absorvidos a serem usados para a produção de energia, ou para os processos de biossíntese, e é baseado na estequiometria do metabolismo intermediário. De acordo com o modelo, em períodos de alto consumo de alimento, altas perdas inevitáveis de AA devidas ao desiquilíbrio entre os perfis de AA na dieta e na larva, que para as dietas consideradas neste estudo foram entre 25 e 40% da quantidade de AA absorvidos, provocam um aumento na deposição de lípidos. No peixe gato Africano isto parece ocorrer com a participação da gluconeogénese. Resultados de simulações com o modelo indicam que um aumento do conteúdo proteico da dieta estimula o crescimento e reduz a deposição de lípidos. Simulaçtes com o modelo também sugerem que um aumento no conteúdo de lípidos na dieta, provoca um aumento na conteúdo larvar de lípidos, bem como um pequeno efeito de poupança de proteína.

Este estudo sugere que os nauplii de *Artemia* podem ser uma dieta sub-óptima para as larvas de peixes. Tanto para o peixe gato Africano como para o pregado, o perfil de AA da *Artemia* é desiquilíbrado. A composição química dos nauplii de *Artemia* também tem um excesso de lípidos, e deficiência em proteína. Como tal, é recomendável rever os protocolos de enriquecimento para os nauplii de Artemia usados currentemente no cultivo de larvas.

Com vista a optimizar as taxas de crescimento no cultivo de larvas de peixes, um melhor conhecimento dos diferentes aspectos do metabolismo de AA, bem como os factores que os regulam, parece essencial. Nomeadamente, a identificação dos perfis de AA ideais nas dietas, e até que ponto estes variam durante a ontogénese. Estudos com AA marcados com isotópos, combinados com a elaboração de modelos matemáticos para o fluxo de AA, parecem ser promissores neste respeito. O possível papel da gluconeogénese em desviar perdas inevitáveis de AA para a síntese de lípidos deveria ser também investigado. A aplicação práctica do conhecimento obtido nos estudos de crescimento de larvas de peixes é ainda limitado pelas dificuldades em estimar a importância dos diferentes factores que determinam o consumo de alimento e a sua digestibilidade. A continuação do desenvolvimento de um modelo mecanístico que possa prevêr com precisão o crescimento em

larvas de peixes de diferentes espécies, alimentadas com as mais variadas dietas, numa variedade de condiçtes de cultivo, é um desafio para o futuro. Um modelo com estas características será especialmente útil quando se quiserem determinar as necessidades nutritivas de novas espécies trazidas para o cultivo.

Voor veel vissoorten geldt dat de toename in produktievolume wordt bemoeilijkt door een onregelmatige - en soms een onvoldoende - aanvoer van pootvis. Dit is vooral het geval wanneer nieuwe soorten in kweek worden gebracht . Een niet gebalanceerde voeding is vaak de oorzaak van een geringe overleving en lage groeisnelheid bij vislarven. Overleving is waarschijnlijk in hoge mate afhankelijk van micronutrienten zoals poly-onverzadigde vetzuren en vitaminen terwijl groei hoogstwaarschijnlijk bepaald wordt door de hoeveelheid en kwaliteit van eiwitten. Een goed inzicht in het eiwit- en aminozuurmetabolisme is vereist om tot een kwalitatief en kwantitatief geschikt voeder te komen waarmee de groei kan geoptimaliseerd worden.

Alhoewel vislarven zeer snel groeien is nauwelijks bekend hoe ze dat doen en in welke mate hun metabolisme en voedingsbehoeften verschillen van volwassen vissen. Modellen kunnen een probaat middel zijn om de groei en de optimalisatie ervan beter te begrijpen. In een model worden betrouwbare hypothesen aangevuld met relevante informatie uit verschillende bronnen; op die manier worden groei-simulaties verkregen onder verschillende omstandigheden. Een verklarend model voorspelt groei op basis van de onderliggende biochemische processen. Naast zijn voorspellende waarde kan zo een model inzicht verschaffen in het groeiproces en kan het mede daardoor richtingbepalend zijn voor verder onderzoek.

De voornaamste doelstelling van deze studie was een verklarend model te ontwikkelen voor de groei gedurende de eerste levensfasen van vis. Dit model zou een bijdrage moeten leveren aan een beter inzicht in de groei en het groeimetabolisme van larvale vis. Op die manier kunnen hun voedingsbehoeften en geschikte voeder regimes voor de larvale teelt uitgewerkt worden. In de experimentele fase van dit onderzoek werd vooral aandacht besteed aan het eiwitmetabolisme en de energetische aspecten van groei omdat vermoed werd dat deze processen bepalend zijn voor de groei en voor de ontwikkeling van een goed groeimodel.

In **hoofdstuk 1** werd het kader van het model ontwikkeld door gebruik te maken van een relatief eenvoudig systeem: de dooierzak larve van de Afrikaanse meerval (*Clarias gariepinus*). Hierbij wordt de larve als een systeem bestaand uit twee compartimenten gezien: de dooierzak en het larvelichaam. Dit systeem wisselt zuurstof, koolstofdioxide, water , ammoniak en warmte uit met de omgeving. De dooier wordt geabsorbeerd in het syncytium, verteerd en de daaruit resulterende voedingsstoffen komen in de bloedcirculatie terecht. Verondersteld wordt dat de circulerende aminozuren en vetzuren in de eerste plaats gebruikt worden voor de ademhaling; wat rest wordt gebruikt voor de groei. Vanaf het ogenblik dat de geabsorbeerde voedingsstoffen niet kunnen voldoen aan de energiebehoeften, wordt embryoweefsel afgebroken. Uit vergelijkingen tussen de simulatiemodellen en de experimentele

gegevens blijkt dat de simulatiemodellen zeer betrouwbaar zijn tot de fase dat de dooier volledig is geabsorbeerd. Daarna neemt de betrouwbaarheid af.

Om de nauwkeurigheid van het model te verbeteren moeten de energetische aspecten van groei en het eiwitmetabolisme beter gedefinieerd worden. Uit resultaten van simulaties kunnen we opmaken dat gedurende de dooierabsoptie vet de voornaamste energiebron is, terwijl eiwit geleidelijk in belang toeneemt en duidelijk de dominante energiebron is in hongerende larven. De ideale kweekcondities voor de Afrikaanse meerval lijken dan ook te zijn: een zo hoog mogelijke temperatuur waarbij de mortaliteit aanvaardbaar blijft; een teeltdierselectie gebaseerd op het verkrijgen van zo groot mogelijke eieren (voor zover dit de vitaliteit van de larven niet aantast); beginnen met voederen zo kort mogelijk na 144 fysiologische daggraden.

Hoofdstukken 2 tot 6 behandelen experimenteel onderzoek waarvan de resultaten gebruikt werden om de voorveronderstellingen in het model te toetsen en het model te parametriseren. In **hoofdstuk 2** werd onderzocht hoe vislarven erin slagen om zowel de kosten van groei als van onderhoud binnen de voor hen beperkte energetische ruimte te besteden. De vraag was of vislarven de kosten van hun hoge groei mogelijk maken door de eiwit turnover te verminderen of door de kosten van de eiwitsynthese te verlagen. Daarom werden eiwit groei, synthese en degradatie in dooierzakbroed van Afrikaanse meerval gemeten en de kosten die met de synthese en aanzet van eiwit gepaard gaan berekend. Groeisnelheden bereikten waarden boven de 100% van het eiwitgewicht per dag. De hoeveelheid aangezette eiwit per eenheid van synthese werd geschat op 66,6%, een waarde die ook bij grotere vissen wordt gevonden. De larven in dit onderzoek gebruikten 43% van hun zuurstofbehoeften voor eiwitsynthese. Desondanks lagen de kosten van de eiwitsynthese dicht bij het theoretisch minimum. Met andere woorden, de hoge groeisnelheden die in dooierzak larven van Afrikaanse meerval worden gemeten lijken tot stand te komen door kosten van de eiwitsynthese te verminderen. Deze lage kosten gingen gepaard met zeer hoge snelheden van eiwitsynthese (138% van het eiwitgewicht per dag), en verhoogde RNA gehaltes (102 µg RNA/mg eiwit), die tesamen resulteerden in zeer hoge RNA efficienties (13,5 g gesynthetiseerd eiwit/g RNA/dag).

In **hoofdstuk 3** werden voor verschillende ontwikkelingsstadia van Afrikaanse meerval de kosten van groei gerelateerd aan de voedselopname en groeisnelheid. Metingen werden uitgevoerd aan larven die bij 28 °C werden opgekweekt, en de resultaten vergeleken met (op basis van literatuur) berekende waarden bij juveniele dieren. In het gewichtstraject van 0.07 mg tot 38 g stijgen de kosten van groei bij de Afrikaanse meerval van 64 tot 149 mmol ATP/g aangezette droge stof. Hogere kosten gaan gepaard met lagere groeisnelheden (van 141 tot 2.4% van het lichaamsgewicht per dag) en dit wordt ook weerspiegeld in lagere efficienties van voederconversie (van 93 naar 24%). Afnemende groeisnelheden zijn het gevolg van afnemende relatieve voederopname snelheden (van 152 naar 6.2% van het lichaamsgewicht per dag) en

gaan gepaard met een vermindering in de zuurstofconsumptie (1,0 tot 0.033 µmol O_2/mg ds/h). Groeisnelheid, voedselopname en zuurstofconsumptie waren allometrisch gerelateerd aan het lichaamsgewicht. Aangezien geen uitgesproken knikpunt in deze relatie kon gevonden worden, is het aannemenlijk dat de larven geleidelijk overschakelen van een larvaal naar een juveniel patroon van groei en metabolisme. Onder optimale omstandigheden van temperatuur, zuurstofbeschikbaarheid en voeding kan het larvale patroon van hoge groeisnelheid tegen een lage kosten misschien over een langere tijdspanne uitgepreid worden en dus leiden tot een verbeterde groei en efficientie van de voederconversie.

Hoofdstuk 4 is gewijd aan de kosten van groei bij tarbotlarven, *Scophthalmus maximus*, en aan hun lichaamssamenstelling in relatie tot ontwikkeling en voedselregime. In het algemeen bleek de lichaamssamenstelling (uitgedrukt in termen van eiwit, vet en as) vrij stabiel te zijn gedurende de larvale periode. Het aandeel aan glycogeen in de lichaamssamenstelling nam licht toe en dat van vrije aminozuren was klein maar zeer variabel. Larven die met aangerijkte *Artemia* gevoederd werden, groeiden slechter en waren vetter dan larven gevoederd met natuurlijk zooplankton. Het is mogelijk dat *Artemia* een suboptimale groei veroorzaakt door het hogere vetgehalte als gevolg van de standaard aanrijkingsprocedures. Hoge groeisnelheden kunnen in tarbotlarven geassocieerd worden met de aanzet van nieuw weefsel tegen kosten die dichtbij de theoretische minimale kosten van eiwitsynthese liggen.

In vislarven blijken de kosten van groei asymptotisch af te nemen naar de biochemisch minimale kosten van eiwitsynthese met toenemende groeisnelheid. Mogelijk zijn de met groei toe- of afname gepaard gaande veranderingen in de eiwitsynthese direct verantwoordelijk voor deze veranderingen in de kosten van groei. Inderdaad, zowel in dooierzak larven van Afrikaanse meerval als in tarbotlarven zijn de kosten van groei nagenoeg geheel toe te schrijven aan de kosten van eiwitsynthese.

In **Hoofdstuk 5** werd de groei en aminozuurbenutting van tarbotlarven gerelateerd aan de samenstelling van de pool van vrije aminozuren, de eiwit turnover en de flux van aminozuren (AZ). Het aandeel essentiele AZ in de vrije AZ pool nam af in de loop van de ontwikkeling van de dieren. Hoge gehaltes aan vrij tyrosine en phenylalanine werden gevonden op dag 3, terwijl de gehaltes aan vrij taurine hoog bleven gedurende de hele proefperiode. De larvale groeisnelheid was positief gecorreleerd aan het taurine gehalte, wat zou kunnen wijzen op een voer-afhankelijke aanvoer van taurine en/of zwavelhoudende AZ. De lagere groeisnelheden van larven die gevoederd werden met *Artemia* werden gerelateerd aan de lagere concentraties van methionine. Dit zou er op kunnen duiden dat dit voedselorganisme voor tarbotlarven te weinig methionine bevat. Ook leucine zou limiterend voor tarbotlarven kunnen zijn, aangezien de diverse geteste voedselorganismen minder leucine bevatten dan de concentratie gevonden in het larvale eiwit. Het AZ profiel in het larvale eiwit bleef relatief

stabiel gedurende de larvale periode alhoewel soms kleine significante verschillen werden gevonden. In tarbot larven is de samenstelling van de vrije AZ pool sterk wisselend en erg verschillend van deze in het larvale eiwit. De vrije AZ pool is buitengewoon dynamisch. Haar samenstelling is sterk gevoelig voor de samenstelling van de AZ die aangevoerd worden uit het dieet of uit interne eiwitafbraak en voor de vraag naar AZ voor synthese processen. In een beginfase gaan de hoge groeisnelheden in tarbotlarven gepaard met een reductie van de eiwit turnover terwijl de verliezen aan AZ door oxidatie hoog lijken. In een latere fase werd een veel hogere eiwit turnover gevonden en waren de oxidatieverliezen van AZ veel lager. Deze verandering kan mogelijks verklaard worden door verschillende strategieën die door de larven gehanteerd worden om de benutting van AZ te optimaliseren naarmate ze zich verder ontwikkelen en meer controle krijgen over de catabolische processen.

In **Hoofdstuk 6** werden de kwalitatieve AZ behoeften van larvale meerval onderzocht. De veranderingen in het AZ profiel van dooierzak larven werd bestudeerd bij verschillende temperaturen. Bij etende larven werden deze veranderingen onderzocht in larven die gevoederd werden met *Artemia* of met een droogvoeder, telkens bij 28 °C. Het AZ profiel veranderde gedurende de ontwikkeling en in het bijzonder vlak voor het begin van exogene voedering. De AZ samenstelling van het voedsel (dooier, *Artemia*, droogvoeder) was erg verschillend van deze in de larven. In dooierzak broed kon geen selectieve absorptie van specifieke AZ uit de dooier vastgesteld worden. De snelheid waarmee individuele AZ concentraties in het organisme afnamen was sterk wisselend. Dit werd verklaard door de grote verschillen tussen de AZ samenstelling van dooier en larve, en door de veranderingen die in het larvale AZ profiel tijdens de ontogenetische ontwikkeling optraden. In dooierzak larven en in hongerende larven is er nauwelijks controle over de catabolisatie van individuele AZ, en van selectief sparen van essentiële AZ is geen sprake. Zolang meer gedetailleerde informatie niet beschikbaar is, kan de larvale AZ samenstelling best gebruikt worden als een index voor de kwalitatieve AZ behoeften. Hogere temperaturen resulteerden in een hogere absorptie maar ook afnamesnelheid van de AZ en tot een betere benuttingsefficiëntie van dooiernutrienten. Veranderingen in de temperatuur leidden niet tot een meer selectieve absorptie of afname van individuele AZ en resulteerden slechts in kleine verschillen in de AZ samenstelling.

Hoofdstuk 7 behandelt een simulatiemodel voor de groei en lichaamssamenstelling van etende larven van afrikaanse meerval en tarbot. Het model werd beschreven en gevalideerd en ook aan een gevoeligheidsanalyse onderworpen om de belangrijkste parameters die groei in larvale vis beïnvloeden te identificeren. Het model wordt aangestuurd door de opgenomen nutrienten. Na absorptie kunnen nutrienten ofwel voor energieproductie ofwel voor biosynthetische processen gebruikt worden. De berekeningen die het model maakt zijn gebaseerd op de stoicheometrische vergelijkingen van de biochemische reacties in het

intermediair metabolisme. Volgens het model resulteren de verschillen tussen de AZ samenstelling van het voer enerzijds en de larve anderzijds tot onafwendbare AZverliezen. Deze verliezen leiden op hun beurt tot een hogere vetaanzet in periodes van hoge voedselconsumptie. In meerval verloopt dit proces via de gluconeogenese. Model simulaties geven aan dat hogere concentraties aan voedereiwit aanleiding geven tot een betere groei en lagere vetaanzet. Op dezelfde manier wordt aangegeven dat verhoogde vetgehaltes in het voedsel resulteren in hogere gehaltes aan lichaamsvet en een licht eiwit-sparend effect.

Uit dit onderzoek kan afgeleid worden dat *Artemia* nauplii mogelijks een suboptimaal voeder is voor vislarven. Zowel voor larven van Afrikaanse meerval als van tarbot is de AZ samenstelling van de nauplii niet gebalanceerd. Bovendien lijkt de lichaamssamenstelling van *Artemia* teveel vet en te weinig eiwit te bevatten. Het lijkt daarom aangewezen om de gebruikelijke aanrijkingsprocedures voor *Artemia* te herzien.

Een beter inzicht in de diverse aspecten van het AZ metabolisme kan bijdragen tot een verbeterde larvale groei in de larvale teelt. In dit verband is het van groot belang om de ideale AZprofielen en hun veranderingen tijdens de ontogenetische ontwikkeling nauwkeurig vast te stellen. Een combinatie van modelonderzoek met experimenteel onderzoek naar de AZstofwisseling mbv isotopen lijkt daarbij een veelbelovende weg. De rol van gluconeogenese om onafwendbare AZverliezen om te zetten in vetaanzet verdient eveneens nadere aandacht. De praktische toepassing van kennis die verkregen wordt uit groei studies bij larvale vis wordt nog steeds bemoeilijkt omdat niet duidelijk is wat het relatieve belang is van de diverse factoren die de opname en verteerbaarheid van het voedel beïnvloeden. Het is een uitdaging voor toekomstig onderzoek om een verklarend simulatiemodel (verder) te ontwikkelen dat in staat is om de groei tijdens de larvale stadia van vis precies te voorspellen, en dit voor verschillende vissoorten, gekweekt onder verschillende omstandigheden en gevoederd met verschillende voeders. Zo een model zou zeer geschikt zijn om de voedselbehoeften van vislarven in te schatten, in het bijzonder wanneer nieuwe soorten in kweek worden gebracht.

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Curriculum Vitae

Luis Eugénio Castanheira da Conceição, was born on the 23rd of April 1966, in Lisboa, Portugal, as son of Maria Helena Lopes Castanheira de Carvalho e Silva da Conceição and Alberto Eugénio da Conceição. He followed his high school studies (Sciences, Aquatic Production) at the Escola Secundária D. João de Castro (Lisboa), and subsequently studied Aquatic Environment Sciences, at the Institute of Biomedical Sciences "Abel Salazar", University of Porto. After his graduation in 1989, he was a Junior Consultant at the Aquaculture Consultancy Office of the Institute of Biomedical Sciences "Abel Salazar", working mostly with trout farming. In 1990 he enrolled in the MSc Course in Aquaculture at the Wageningen Agricultural University, and graduated in 1992 with *cum laude*. He started his PhD studies in October 1992 at the Wageningen Agricultural University. In 1993 he did a research period at the Department of Zoology of the University of Aberdeen, under supervision of Prof. D.F. Houlihan. In 1995, he conducted 4 months of research in Norway at the Zoological Laboratorium (University of Bergen) and the Austevoll Aquaculture Research Station (Institute of Marine Research), under the guidance of Prof. H.J. Fyhn and Dr. T. van der Meeren. Since February 1997, he has held a post-doctoral position at SINTEF Applied Chemistry, Center of Aquaculture (Trondheim, Norway).