Towards an inert diet for first-feeding gilthead seabream *Sparus aurata* L. larvae

M. YÚFERA[1], C. FERNÁNDEZ-DÍAZ[1], E. PASCUAL[1], M.C. SARASQUETE[1], F.J. MOYANO[2], M. DÍAZ[2], F.J. ALARCÓN[2], M. GARCÍA-GALLEGO[3] & G. PARRA[1]

[1] *Instituto de Ciencias Marinas de Andalucía (CSIC), Puerto Real, Cádiz, Spain;* [2] *Department of Biología Aplicada, Universidad de Almería, Almería, Spain;* [3] *Department of Biología Animal, Vegetal y Ecología, Universidad de Granada, Granada, Spain*

Abstract

The development of an inert food to replace live prey during the early stages of marine fish larvae requires research in different fields and therefore a precise work strategy. Our research on this subject has been carried out in successive steps using the gilthead seabream *Sparus aurata*. The first step was the design of a food particle that would be well accepted and ingested by free-swimming marine larval fish during the first developmental stages. We chose microencapsulation by polymerization of the dietary protein as the most appropriate method for making the particles; different types of microcapsules were made using a basic diet containing only the major dietary components. In the second step, our aim was to keep the larvae alive in a routine rearing system in 300-L tanks, using exclusively this kind of food, long enough to detect any changes in growth, survival, or anatomical and histological status of the larvae, in order to verify whether the technological changes were positive. The third step focused on diet formulation and searching for clues to inefficient assimilation and growth. The use of '*in vitro*' digestibility techniques allowed us to detect the inhibitory effect of some diet ingredients on larval proteases and to determine more suitable sources of protein. We now have a microcapsule able to efficiently support growth and development of *S. aurata larvae*, at least during the first 2 weeks of life, although the larvae still need to feed on rotifers during the first 2–4 days of exogenous feeding. This microcapsule will make it possible to make advances in determining the specific nutritional requirements of larval fish.

KEY WORDS: digestive system, larval fish, microencapsulated diets, proteases, *Sparus aurata*

Received 29 September 1998, accepted 16 February 1999

Correspondence: M. Yúfera, Instituto de Ciencias Marinas de Andalucía (CSIC), Apartado Oficial, 11510 Puerto Real, Cádiz, Spain. E-mail: manuel.yufera@icman.csic.es

Introduction

The replacement of live prey in the larval rearing of marine fish has been a challenge since production attained industrial scale (Adron *et al*. 1974; Barnabé 1976; Gatesoupe & Luquet 1977; Girin & Person-Le Ruyet 1977). Different types of inert feeds have been proposed, designed and tested, from dry or frozen plankton organisms (Kentouri 1980; Gatesoupe & Luquet 1981) to formulated food microparticles made using different methodologies and degrees of complexity (Kanazawa *et al*. 1989; Jones *et al*. 1993). Some of the main problems to be solved are acceptability and stability of inert particles — aspects directly related to water quality. Consequently, considerable effort has been focused on particle characteristics such as size and structure, as well as on an appropriate food dosage and supply method. In addition, an adequate chemical composition satisfying energetic and nutritional requirements has been a permanent aim while designing prepared microdiets. The problems become more difficult when first-feeding larvae are concerned. The digestive tract is not completely functional and the larvae are very weak, requiring strict rearing conditions that make experimentation difficult. An additional point to consider is the variability in feeding physiology and behaviour of larvae belonging to different species. In addition to the well-known size and developmental differences found with freshwater fish larvae in general, marine fish larvae can also show remarkable ontogenic differences among families.

All these constrains have contributed to a lack of success in feeding inert diets in larvae. Thus, despite hopeful early results by Adron *et al*. (1974) and Appelbaum (1985)

working with flatfish larvae, relatively good growth of marine fish larvae fed exclusively on inert diets has only recently been reported.

In this paper, we describe the strategy followed in researching the replacement of live prey in the first larval stages of the gilthead seabream *Sparus aurata*, a very important species in the aquaculture industry of Mediterranean countries and whose larvae are very sensitive at early stages. Therefore, in this report we summarize our progress in developing an inert diet for marine fish larvae, including our latest results.

Materials and methods

Research strategy

The most common approach observed in the literature for studying the replacement of live prey has been to advance progressively the time of weaning to prepared diets by partial replacement in cofeeding experiments (live + prepared food). The inconvenience of such an approach is that, when live, the prey mask the real contribution of prepared feeds, as well as the physiological response of larvae to such food — aspects that are crucial for advancement in this area.

Our research focused on larvae at first feeding, in the early stages of development. The research programme has been carried out in successive steps, trying to isolate problems associated with the particles themselves, rearing water quality and growth and survival of larvae. The goals were: to design a food particle, to keep the larvae alive using this kind of food and to advance knowledge of larval nutrition.

The first step was the development and manufacture of a food particle able to simulate the physical and structural characteristics of live prey. The basic aim at this stage was to make a particle that would be well accepted and ingested by free-swimming marine larval fish during the first developmental stages. We chose microencapsulation by polymerization of the dietary protein as the most appropriate method for making the particles. We made and tested different types of microcapsules using a basic diet containing only the major dietary components.

In a second step, our aim was to keep the larvae alive in a routine rearing system in 300-L tanks, using exclusively this type of food, long enough to detect any changes in the growth, survival, or anatomical and histological status of larvae, to determine if the technological changes were positive. Working on both physical and chemical characteristics of the particle and on larval-rearing technology, we obtained a microencapsulated diet prototype that keeps larvae alive for weeks. In addition, we developed a methodology for detecting poor growth at these early stages (Fernández-Díaz & Yúfera 1997).

The third step focused on diet formulation, searching for clues to efficient assimilation and growth. The use of *in vitro* digestibility techniques allowed us to detect any inhibitory effect of some diet ingredients on larval proteases, as well as to determine more suitable sources of protein.

Elaboration of food particles

Protein-walled microencapsulated diets for aquatic organisms were first developed for crustacean and bivalve suspension-feeders (Jones *et al*. 1975; Langdon *et al*. 1985) and then tested on fish (Kanazawa *et al*. 1982; Appelbaum 1985; Walford *et al*. 1991). Microencapsulation is a process that allows the variation of several factors (food ingredients, reagents and procedure) that influence the characteristics of the final product. Different types of microcapsules were successively made and tested by changing both manufacturing procedure and diet composition. In the present report, we refer only to those prototypes which represented significant progress in our work. These prototypes are named Prototypes 0, 1 and 2, respectively.

Microcapsules were manufactured by interfacial polymerization of the dietary protein. The process was described in detail in Yúfera *et al*. (1996) and Fernández-Díaz & Yúfera (1997). The process is basically as follows. The dry diet (complete formulation of different prototypes in Table 1) was dispersed in a basic pH, buffered aqueous solution (100 g L^{-1}). Two parts of this solution were emulsified in five parts of a soy lecithin and cyclohexane solution (20 g L^{-1}), with the aid of a homogenizer for 8 min at room temperature (20°C approximately). The stirring speed was constant during the process, and ranged from 600 to 1200 r.p.m depending on the particle diameter required. The cross-linking agent, dissolved in diethyl ether, was then added to the emulsion, stirring continuously, and the reaction was continued for 8 min. The amount of cross-linking agent 1,3,5-benzenetricarboxylic acid chloride (trimesoyl chloride) represents one-third (w/w) of the total crude protein in the dry diet. The microcapsules formed were allowed to settle, and the cyclohexane-lecithin solution was decanted. After washing with cyclohexane, the microcapsules were dispersed in a gelatine solution (150 g L^{-1}), stirring at the same fixed speed for 3 min. Two-thirds of the water, at ≈ 38°C, were added while stirring. The capsules were then repeatedly washed with fresh water and finally with a buffered saline solution (pH 8) in order to remove undesirable materials. The microcapsules were freeze-dried.

Table 1 Diet formulations used in manufacturing the microcapsules (g kg^{-1} of dry diet)

Ingredients	PT0 and PT1A	PT1B	PT2
Albumin[1]	500	430	
Casein[2]			500
Dextrin	105	80	60
Fish lipids & vitamins emulsion			120[3]
Fish oil	112.5	70	
Fish protein hydrolysate[4]	170	350	120
Mineral complex	50		
Octopus meal			100
Premix C[5]	20	20	
Soy lecithin	12.5		30
Vitamin complex		50[6]	50[7]

[1] ICN 901633 (Costa Mesa, CA, USA).
[2] ICN 901293
[3] Kurios (France) lipids emulsion: fatty acids (67%), W3 HUFA (35.87%), DHA + EPA (32.2%). Vitamins (IU mL^{-1} emulsion): vitamin A 100, vitamin D 50, vitamin C 250; Minerals and pigments; Emulsifiers(5%).
[4] Norse fish powder, Norway.
[5] Dibaq (Segoria, Spain) (80 mg vitamin C/g^{-1} premix).
[6] Abbot Laboratories, Dayamineral.
[7] Kurios polyvitamin fish complex (mg mL^{-1} complex): vitamin B_1 3.75; vitamin B_2 1.5; vitamin B_6 1.25; vitamin B_5 11.25; vitamin B_3 11.25; methionine 1; choline 10; vitamin B_{12} 0.01; vitamin C 5; vitamin E 7.5; vitamin K_3 0.75; and vitamin A 10 000 IU mL^{-1}, vitamin D_3 7500 IU mL^{-1}.

The main difference in the manufacturing process of the different prototypes is that, to isolate Prototype 0 microcapsules, the final phase of preparation was performed in alcohol (30 mL L^{-1}), whereas Prototypes 1 and 2 microcapsules are made in a gelatin solution (150 g L^{-1}) as a dispersant, as explained above. The nitrogen content of the manufactured microcapsules was determined with an Elemental Analyser model 1106 (Carlo Erba Science, Milan, Italy) using subsamples weighing about 1 mg. Cyclohexanone 2,4-dinitrophenyl-hydrazone was used as standard. Energy content was determined with a semimicro bomb calorimeter Parr 1421 (Moline, IL, USA) using samples weighing about 20 mg. Three subsamples per replicate were used in all analyses.

Larval rearing techniques

Any change in particle design was tested on larvae. The experimental protocols and rearing system were established for obtaining answers rather than to maximize growth and survival. Therefore, two types of experiment were performed:

1 Experiments for detecting acceptability, ingestion and digestibility of food particles, required only a small water volume (2–15 L) and a short experimental time (1–5 h). These experiments were carried out under permanent illumination using a stocking density of 20–40 larvae L^{-1}, at 19.5°C and 33 g L^{-1} salinity. Tests were performed on first-feeding larvae and on larvae of several ages reared on rotifers (Fernández-Díaz *et al.* 1994; Fernández-Díaz & Yúfera 1995; Yúfera *et al.* 1995).

2 Experiments for detecting larval growth required a large water volume and a duration of several days. In this case, water quality was a concern in connection with food supply (Yúfera *et al.* 1996, 1999; Fernández-Díaz & Yúfera 1997).

The large-volume larval rearing experiments were carried out in 300-L tanks (19.5°C and 33 g L^{-1} salinity). The initial stocking density was 35–60 larvae L^{-1}. Constant illumination and slight aeration were provided. The first 24 h after hatching were considered as day 0. Larvae were reared under different conditions:

1 Larvae fed on rotifers from first feeding (day 4) to the end of the experiment (day 15) (control);

2 Larvae fed on microcapsules from first-feeding to the end of the experiment (day 15 after hatching);

3 Larvae fed on rotifers from day 4 after hatching to day 7, and on microcapsules from day 8 to day 15.

The experiments ended on day 15 and were carried out in triplicate using larvae from separate batches of eggs. Included in the present report is a new experiment in which the microcapsule supply started on day 6 and ended on day 22, and that was performed at 20 and 18°C, respectively.

Rotifers *Brachionus rotundiformis* (Bs strain) and *Brachionus plicatilis* (S1 strain) were used in the experiment (mean dry weight 0.20 and 0.45 μg per individual, respectively; Yúfera *et al.* 1997). Rotifer concentration was adjusted daily to 10 individuals mL^{-1}. The microcapsules were distributed in six feedings per day (0.2 g each) by an automatic feeder. Microalgae (*Nannochloropsis gaditana*) cells (0.5×10^6–1×10^6 cells mL^{-1}) were added to all the rearing tanks with rotifers.

Larvae were reared in a semi-open system, with no water exchange during the first 7 days, and from day 8 with 35–75% of the sea-water volume replaced daily. In the tests with microcapsules from first-feeding, the water exchange started on day 5. In the test with microcapsules from day 8 (or day 6), the rotifers were removed from the rearing tanks during the first 24 h after the food shift. The new experiments with Prototype 2 microcapsules were also carried out in tanks equipped with a recirculating water system through a biological filter (Yúfera *et al.* 1999). Water flow started on day 6 and the flow rate increased progressively from 8 L h^{-1} on day 6 to ≈ 30 L h^{-1} on day 15.

Studies on physiology and nutrition

It is obvious that a better understanding of the feeding physiology and nutrition of larvae during the early moments of trophic life is a necessary step for advancement in the replacement of live prey. In *S. aurata*, as in other marine fish in general, studies on larval biology are progressing continuously and, at present, the pattern of development at the tissue level is relatively well-known (Yúfera *et al*. 1996).

The digestive system is not fully functional during the larval stage, compared with that of juveniles. This has been given as the reason for the insufficiency of inert foods. More specifically, poor enzymatic activity in marine larval fish has been suggested as the main reason for a strict dependence on live prey that would supply exogenous enzymes. To examine this hypothesis, we analysed the ontogeny of the digestive enzymes, as well as enzymatic capacity in relation to food offered (Moyano *et al*. 1996; Díaz *et al*. 1997).

An efficient diet formulation requires adequate ingredient selection and suitable preparation that preserves the bioavailability of the nutrients. In addition, appropriate testing of the suitability of a given formulation is only possible when larvae are growing on such a diet. As a substitute, in order to progress more quickly in diet development, we performed a series of *in vitro* protein digestibility analyses using an enzymatic extract prepared from 8-day-old larvae. These experiments assessed the effect of the protein source used in microcapsules on total digestive protease activity of larvae, as well as the potential inhibitory effect of some specific protein sources on larval proteases (Alarcón *et al*. 1999).

Diagnostic methods

The first step was to observe the larvae under the microscope to examine whether the different prototypes were ingested and disintegrated within the gut. The normal method for observing the effect of a given diet or culture condition is the analysis of growth and survival. Larval dry weight was determined by drying samples of 20–30 individuals per replicate at 90°C to constant weight. Survival at the end of the experiments was estimated as a percentage from first feeding or from the first day of microcapsule supply. However, growth and survival were ineffective when examining the effect of a given diet when no or limited growth occurs. Histological techniques were therefore used to analyse health status at the tissue level (Sarasquete *et al*. 1993, 1995), concentrating mainly on aspects related to digestion, such as the structure of liver, pancreas and gut epithelium. A thin intestinal epithelium lacking supranuclear protein inclusions in the hindgut or infranuclear lipids droplets and intracellular small lipid particles in the midgut are symptoms associated with poor assimilation (Yúfera *et al*. 1993; Sarasquete *et al*. 1995).

Some anatomical observations can also be made directly in fresh larvae under the microscope. The liver index (liver width × 1000/total length) and miotome index (miotome width × 1000/total length), determined by image analysis, give a quick diagnosis of the effect of a diet or a rearing system. Low liver and/or low miotome index in relation to the control larva are indicative of poor growth potential.

These diagnosis methods and experimental protocols (based on 2-week experiments with larvae) allowed us to test continuous changes in the prototypes (diet and encapsulation procedure) and in the rearing system.

Calculations and statistics

At least three replicates per treatment were used to elaborate the growth curves, except for the experiments up to day 22, in which only two replicates per treatment were performed. Growth rate (G) was calculated as the slope of the exponential regression of dry weight vs. larval age. Growth rate and survival data were expressed as mean ± SD. Data of control groups were grouped as there were no significant differences among the different experiments. Differences in growth rate and survival between treatments were compared by one-way ANOVA followed by Neuman–Keul's multiple-range test. Data given as percentages were arcsine-transformed prior to analysis. A minimum significant level $P < 0.05$ was considered in all tests.

Results

Microcapsule types and characteristics

The three main types of microcapsules illustrate our progress in developing inert food particles. Prototype 0 (PT0) was the first well-formed, stable capsule obtained and was elaborated using a basic diet. At this stage, the ingredients and their relative proportions (Table 1) were chosen to obtain effective microencapsulation and a particle that would be useful for starting experimentation with larvae. Prototype 1 (PT1) was similar in diet composition to PT0 but the manufacturing process was changed to achieve a soft particle. (There were two versions of this prototype, PT1A and PT1B, with only small differences in the proportion of ingredients (Table 1), which illustrates that

Table 2 Characteristics of the different microcapsule prototypes (PT). Crude protein (N × 6.25) is given as g kg^{-1} of total dry matter

	PT0	PT1A	PT1B	PT2
Manufactured using	Alcohol	Gelatin	Gelatin	Gelatin
Texture	Rigid	Soft	Soft	Soft
Shape	Spherical	Irregular	Spherical	Spherical
Main protein source	Albumin	Albumin	Albumin	Casein
Ingested	Yes	Yes	Yes	Yes
Digested	No	Yes	Yes	Yes
Assimilated	No	+	+	+ + +
Crude protein (g kg^{-1})	465	571	546	619
Energy content (J mg^{-1})	18.2	21.7	21.7	23.9

+, Poor assimilation; +++, good assimilation.

there were transition types other than the three main types described in this paper.) Prototype 2 (PT2) was made using with the same procedure as PT1, but changing the diet composition, mainly the protein source.

The principal characteristics of the different types are shown in Table 2. All prototypes showed a good floatability and were well ingested by the seabream larvae from the first feeding (Fernández-Díaz *et al.* 1994; Yúfera *et al.* 1995). All these microcapsules kept their shape and structure after rehydration for 24 h, with little detectable loss of matter (from 0 to 5% of the total dry weight). Studies on larvae feeding behaviour indicated clearly from our first stable prototype that availability in the water column and acceptability by larvae were not constrained by dietary particles manufactured using this technology.

Larval growth

Figures 1 and 2 show the progress in larval growth and survival with the different prototypes. Larvae fed on PT0 showed no growth or survival and were not included in the graph. Although larvae ingested the particles well, they were unable to disintegrate them within the gut (Fernández-Díaz & Yúfera 1995). They died within 2 days of the onset of feeding, showing strong erosion of the intestine epithelium and degeneration of the liver and pancreas (Yúfera *et al.* 1996). PT1, a soft diet particle manufactured using gelatin, was well disintegrated in the gut. Larvae fed PT1 from the first feeding survived several days (at least up to day 15), although little growth was observed ($G = 0.001 \pm 0.002$ day^{-1}; Fig. 2). Nevertheless, some growth was detected after the theoretical point of irreversible starvation. Growth was better when PT1 was offered from day 8 ($G = 0.053 \pm 0.015$ day^{-1}). Prototype 2, with a different diet formulation, including casein and octopus meal instead of albumin, when offered from day 8, allowed a reasonable growth and survival similar to larvae fed on rotifers ($P > 0.05$; Fig. 2), when microcapsule-fed larvae were reared with a water recirculation system (Yúfera *et al.* 1999). When PT2 was offered from first feeding, larvae showed poor growth ($G = 0.052 \pm 0.011$ day^{-1}) but were able to grow up to 40 μm dry weight without any live prey.

Figure 1 includes recent growth curves obtained when the shift to microcapsules was made on day 6 larvae reared to day 22. At 20°C, seabream larvae showed excellent growth

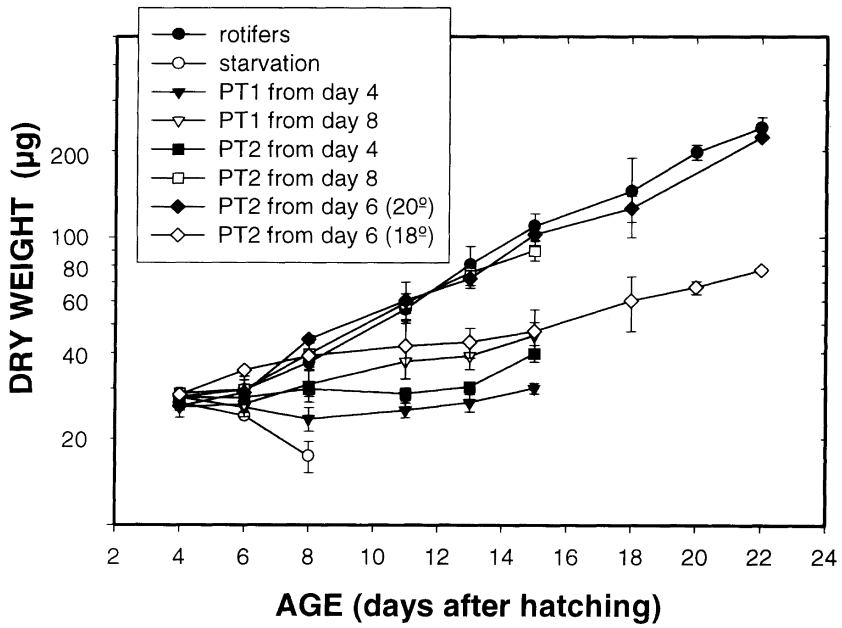

Figure 1 Growth curves of *Sparus aurata* larvae fed on the different microcapsule prototypes and reared under different conditions. Larvae fed on Prototype 2 (PT2) were reared in recirculating water system.

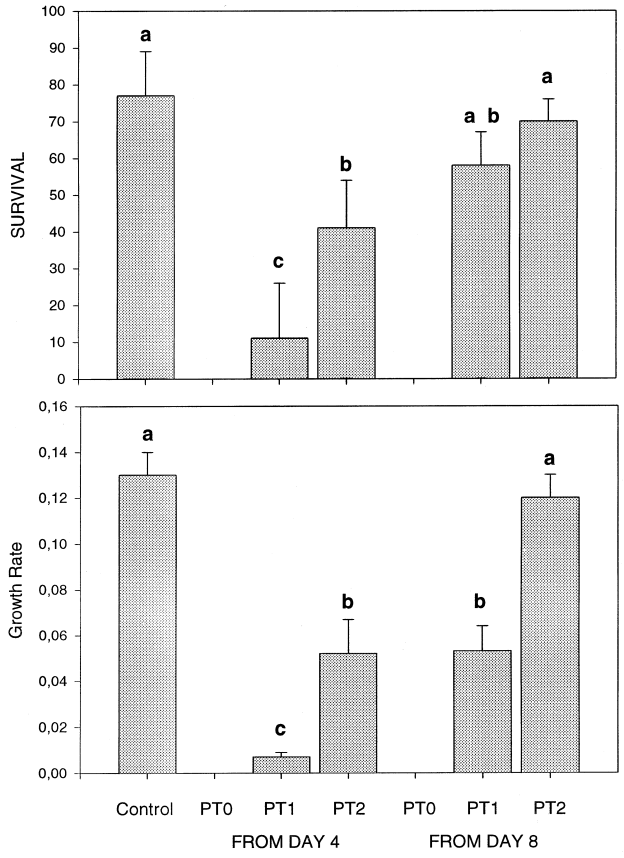

Figure 2 Survival and growth rates of *Sparus aurata* larvae fed on the different microcapsule prototypes and reared under different conditions. Means with the same letter are not significantly different ($P > 0.05$).

($G = 0.127$ day^{-1}) with only two days of feeding on rotifers. However, at 18°C, the larvae showed poor growth over the first 2 weeks, although they appeared to recover afterwards. This different response may be related to slower development of enzymes at 18°C.

Figure 3 shows the excellent status of the digestive epithelium at the end of the experiment in larvae fed PT2, indicating the good potential to continue growing. Even larvae that ingested PT2 from first feeding (Fig. 3a,b) showed excellent tissue quality, with protein inclusion vacuoles in the hindgut, well-developed intestine folds and a well-organized liver with the presence of zymogens in the pancreas. Thus, despite the lower growth rate observed in larvae fed exclusively on PT2 from the onset of feeding, the digestive tract appeared fully functional and without pathologies. It is interesting to note that when larvae were fed on PT1 from first feeding to day 15, the epithelium appeared thinner and some cell desquamation was observed, indicating limited digestive capacity and potential for growth (Yúfera *et al.* 1996). Similarly, excellent quality and functionality of the digestive tissue was observed in larvae fed on PT2 from day 6 to day 22 (Fig. 3c) and from day 8 (Fig. 3d).

Enzymatic activity and nutrition

In terms of enzymes, seabream larvae are reasonably well equipped. Protease activity was detected from the first feeding (day 4). A complete set of bands corresponding to the different proteases existing in 30-day-old larvae was detected at day 6. Protease activity increases progressively with larval age but no more protease types appeared during the first month (Moyano *et al.* 1996; Díaz *et al.* 1997). Likewise, larvae showed increasing amylase and phosphatase activity. It appears that seabream have the capacity to digest inert feeds during the larvae stage.

The selection of ingredients would appear to be the primary factor involved in the success of an inert microdiet. Nevertheless, adequate composition can be studied properly only when larvae are able to live and grow. Therefore, only when we obtained reasonable survival and detectable growth did we start the nutritional studies. Thus, PT0 included a basic composition of macronutrients (protein, lipids and carbohydrates). The main protein source was albumin but the rigidity of the structure did not allow us to make any nutritional observation. When fed on PT1, which also included albumin as main protein source, the larvae living to the end of the experiment grew poorly. Histological analysis revealed symptoms of poor assimilation which could be due to limited enzyme activity or to inappropriate diet ingredients.

Enzymatic problems associated with ingredients were detected through *in vitro* experiments (Alarcón *et al.* 1999). These experiments revealed that both ovalbumin and capsules made with a high proportion of this ingredient, induced strong enzymatic inhibition in early seabream larvae (Fig. 4). Other sources of protein, such as casein and octopus meal, as well as the other compounds used in manufacturing microcapsules, did not induce noticeable inhibition. The suitability of these ingredients was confirmed in *in vivo* experiments with larvae (see growth curves with PT2 in Fig. 2).

Discussion

The main problem with the replacement of live prey in diet of marine fish larvae has been the large number of factors and the disciplines involved. Other problems derive from the scarcity of fish larvae for research (these are usually grown to the juvenile stage at any cost) and classical weaning experiments

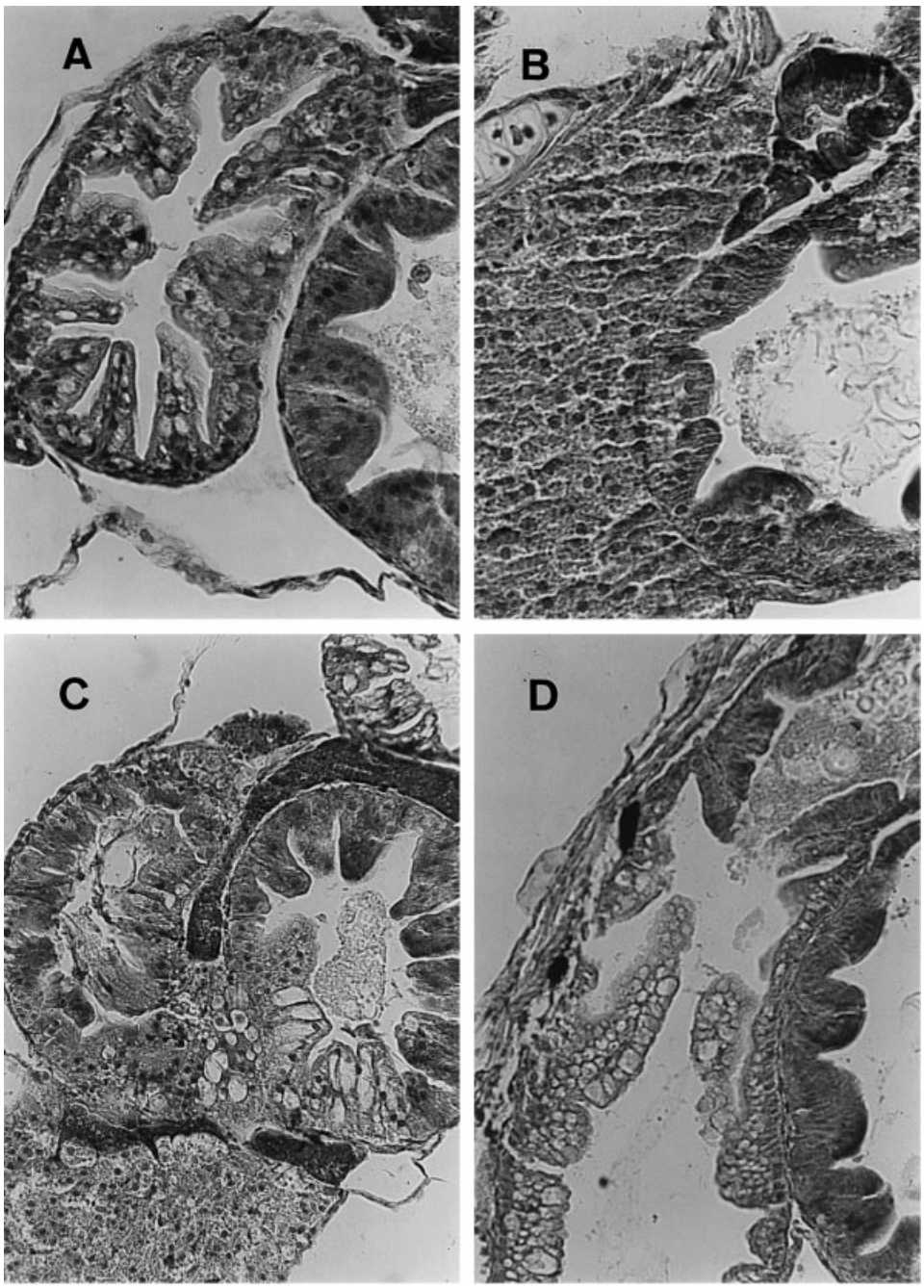

Figure 3 Photomicrographs showing the digestive tract of *Sparus aurata* fed on Prototype 2 from different ages. (a) Midgut and hindgut of 15-day-old larvae fed on microcapsules from first feeding, showing thick and well-developed intestine epithelium and the protein inclusion vacuoles. (b) Liver, pancreas with zymogen granules of 15-day-old larvae fed on microcapsules from first feeding. (c) Midgut, hindgut, liver and pancreas of 22-day-old larvae fed on microcapsules from day 6 after hatching. (d) Hindgut and midgut of 18-day-old larvae fed on microcapsules from day 8 after hatching.

that tend to extrapolate directly the experience in juveniles to larvae. This means few long experiments in a year.

The strategy used in this research programme, based on the isolation of separate factors and short-term experimental protocols, has allowed us to advance considerably. The continuous change and testing of diet formulations, the adjustment of food dosage and rearing technology yielded excellent results. At present, we have a microcapsule able to

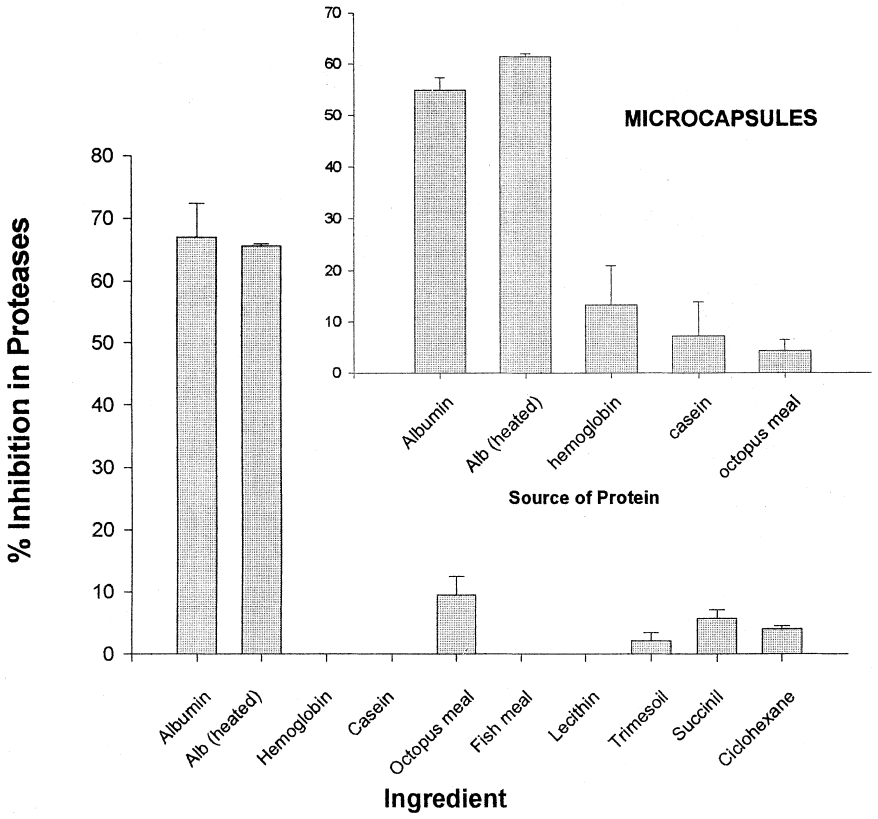

Figure 4 Inhibition of protease activity in 8-day-old larvae induced by microcapsules made with different protein sources and by the different ingredients used in their manufacture.

efficiently support growth and development of *S. aurata* larvae, at least during the first 3 weeks of life, although the larvae still need to feed on rotifers during the first 2–4 days of exogenous feeding. In fact, even larvae fed exclusively on microcapsules (PT2) from the opening of the mouth show a well-developed and functional digestive system at day 15. However, prolonged rearing experiments up to juvenile stages are required in order to explore possible nutritional problems.

This microcapsule (PT2) will make it possible to advance knowledge of the specific nutritional requirements of fish larvae. In addition, microencapsulation is a technique that allows many possibilities to such studies (Langdon 1989; Villamar & Langdon 1993; Ozkizilcik & Chu 1996). Although a complete replacement diet with similar growth and survival from first feeding to juvenile stage on live prey has not yet been achieved, a basic conclusion can be emphasized: marine fish larvae can grow exclusively on prepared food from the early stages of development. This might seem obvious now, but such a possibility was not supported just a few years ago.

In addition to satisfying dietary requirements, success in rearing larvae on inert diets also depends on an adequate food supply, while at the same time avoiding an excess. A system for maintaining water quality needs to be compatible with the extreme sensitivity of the larvae of some species. Closed recirculation water systems applied to rearing fish larvae (Holt 1993) have resulted in significant improvements in larval growth when they are fed on microcapsules.

Special attention was paid to the relative composition of macronutrients and to supposed enzymatic incapacity. Our results with *S. aurata* and recent studies on other species indicated that marine fish larvae do not have as limited an enzymatic capacity as assumed and that they are able to digest prepared feeds (Cahu & Zambonino-Infante 1995; Moyano *et al.* 1996). Nevertheless, the process of opening the mouth and the functionality of the digestive tract is not instantaneous and could involve the contribution of external factors.

The appearance of protease bands corresponding to different proteases were observed from day 4 (first-feeding), and were complete by day 6. Results of replacement experiments on day 6 indicated that this age is the limit with our current PT2. Therefore, the possibility of dependence on exogenous enzymes at first feeding is still open. The role of plankton enzymes at the start of feeding remains unclear. Our results indicate that the contribution of rotifer proteases in the larval gut is not significant (Díaz *et al.* 1997). However, rotifers showed high acid protease activity and are suscep-

tible to autolysis (Kleinow 1993; Wethmar & Kleinow 1993). This process may be neutralized when such proteases are in contact with the alkaline content of the larval gut.

Therefore, understanding of the processes occurring at the onset of feeding with inert food has to be addressed to further advances in formulation. To optimize the source of ingredients and relative proportions of protein and lipids, the use of '*in vitro*' techniques followed by validation with live larvae experiments is needed. Special attention should also be given to the presence of free amino acids and protein hydrolysates in ingested particles. Finally, further research is needed on the colonization of the larval gut by bacterial flora and its role in the digestion processes.

Acknowledgements

This work was supported by the Comisión Interministerial de Ciencia y Tecnología, Spain (CICYT, MAR97–0924-C02–01 and MAR97–0924-C02–02). We are indebted to Cupimar SA (San Fernando, Cádiz, Spain) for supplying part of the biological material used in this research programme. Special thanks are also due to J.M. Espigares and J.A. Miquel for technical assistance.

References

Adron, J.W., Blair, A. & Cowey, C.B. (1974) Rearing of plaice (*Pleuronectes platessa*) larvae to metamorphosis using artificial diet. *Fish. Bull*., **72,** 353–357.

Alarcón, F.J., Moyano, F.J.M., Díaz, M., Fernández-Díaz, C. & Yúfera, M. (1999) Optimization of the protein fraction of microcapsules utilized in feeding of marine fish larvae using 'in vitro' digestibility techniques. *Aquacult. Int*., **5,** 107–113.

Appelbaum, S. (1985) Rearing of the Dover sole, *Solea solea* (L), through its larval stages using artificial diets. *Aquaculture*, **49,** 209–221.

Barnabé, G. (1976) Elevage larvaire du loup (*Dicentrarchus labrax* (L.); Pisces, Serranidae) à l'aide d'aliment sec composé. *Aquaculture*, **9,** 237–252.

Cahu, C. & Zambonino-Infante, J.L. (1995) Maturation of the pancreatic and intestinal digestive functions in sea bass (*Dicentrarchus labrax*): effect of weaning with different protein sources. *Fish. Physiol. Biochem*., **14,** 431–437.

Díaz, M., Moyano, F.J., García-Carreño, F.L., Alarcón, F.J. & Sarasquete, M.C. (1997) Substrate–SDS-PAGE determination of protease activity through larval development in sea bream. *Aquacult. Int*., **5,** 461–471.

Fernández-Díaz, C. & Yúfera, M. (1995) Capacity of gilthead seabream, *Sparus aurata* L., larvae to break down dietary microcapsules. *Aquaculture*, **134,** 269–278.

Fernández-Díaz, C. & Yúfera, M. (1997) Detecting growth in gilthead seabream, *Sparus aurata* L., larvae fed microcapsules. *Aquaculture*, **153,** 93–102.

Fernández-Díaz, C., Pascual, E. & Yúfera, M. (1994) Feeding behaviour and prey size selection of gilthead seabream, *Sparus aurata*, larvae fed on inert and live food. *Mar. Biol*., **118,** 323–328.

Gatesoupe, F.J. & Luquet, P. (1977) Recherche d'une alimentation artificielle adapté è l'élevage des stades larvaires des poisons. I. Comparaison de quelques techniques destinées à améliorer la stabilité à l'eau des aliments. *Actes Colloq. CNEXO*., **4,** 13–20.

Gatesoupe, F.J. & Luquet, P. (1981) Weaning of the sole (*Solea solea*) before metamorphosis. *Aquaculture*, **26,** 359–368.

Girin, M. & Person-Le Ruyet, J. (1977) L'élevage larvaire de poissons marins: chaines alimentaires et aliments composés. *Bull. Franc. Piscic*., **264,** 88–101.

Holt, G.J. (1993) Feeding larval red drum on microparticulate diets in a closed recirculating water system. *J. World Aquacult. Soc*., **24,** 225–230.

Jones, D.A., Kamarundin, M.S. & Le Vay, L. (1993) The potential for replacement of live feeds in larval culture. *J. World Aquacult. Soc*., **24,** 199–210.

Jones, D.D., Moller, T.H., Campbell, R.J., Munford, J.G. & Gabbott, P.A. (1975) Studies on the design and acceptability of microencapsulated diet for marine particle feeders. I. Crustacea. In: *Proc. 10th Eur. Mar. Biol. Symp*., (Persoone, G. & Jaspers, E. eds.) Vol. 1, pp. 229–239. Universa Press, Wetteren, Belgium.

Kanazawa, A., Teshima, S.-I., Inamori, S., Sumida, S. & Iwashita, T. (1982) Rearing of larval red sea bream and ayu with artificial diets. *Mem. Fac. Fish. Kagoshima Univ*., **31,** 185–192.

Kanazawa, A., Koshio, S. & Teshima, S.-I. (1989) Growth and survival of larval red sea bream *Pagrus major* and Japanese flounder *Paralichthys olivaceus* fed microbound diets. *J. World Aquacult. Soc*., **20,** 31–37.

Kentouri, M. (1980) Elevage de larves de loup (*Dicentrarchus labrax* L.) à l'aide d'organismes du zooplankton congelé: résultats préliminaires. *Aquaculture*, **21,** 171–180.

Kleinow, W. (1993) Biochemical studies on *Brachionus plicatilis*: hydrolytic enzymes, integument proteins and composition of trophi. *Hydrobiologia*, **255/256,** 1–12.

Langdon, C.J. (1989) Preparation and evaluation of protein microcapsules for a marine suspension-feeder, the Pacific oyster *Crassostrea gigas*. *Mar. Biol*., **102,** 217–224.

Langdon, C.J., Levine, D.M. & Jones, D.A. (1985) Microparticulate feeds for marine suspension-feeders. *J. Microencaps*., **2,** 1–11.

Moyano, F.J., Díaz, M., Alarcón, F.J. & Sarasquete, M.C. (1996) Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). *Fish Physiol. Biochem*., **15,** 121–130.

Ozkizilcik, S. & Chu, F.L.E. (1996) Preparation and characterization of a complex microencapsulated diet for striped bass *Morone saxatilis* larvae. *J. Microencaps*., **13,** 331–343.

Sarasquete, M.C., Polo, A. & González de Canales, M.L. (1993) A histochemical and immunohistochemical study of digestive enzymes and hormones during the larval development of *Sparus aurata* L. *Histochem. J*., **24,** 337–344.

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

Villamar, D.F. & Langdon, C.J. (1993) Delivery of dietary components to larval shrimp (*Penaeus vannamei*) by means of complex microcapsules. *Mar. Biol*., **115,** 635–642.

Walford, J., Lim, T.M. & Lam, T.J. (1991) Replacing live foods with microencapsulated diets in the rearing of seabass *Lates calcarifer* larvae: do the larvae ingest and digest protein-membrane microcapsules? *Aquaculture*, **92,** 225–235.

Wethmar, C. & Kleinow, W. (1993) Characterization of proteolytic activities stimulated by SDS or urea and two-dimensional gel

electrophoresis of proteins from *Brachionus plicatilis* (Rotifera). *Comp. Biochem. Physiol.*, **106B,** 349–358.

Yúfera, M., Pascual, E., Polo, A. & Sarasquete, M.C. (1993) Effect of starvation on the feeding ability of gilthead seabream (*Sparus aurata* L.) larvae at first-feeding. *J. Exp. Mar. Biol. Ecol.*, **169,** 252–272.

Yúfera, M., Fernández-Díaz, C. & Pascual, E. (1995) Feeding rates of gilthead seabream, *Sparus aurata* L., larvae on microcapsules. *Aquaculture*, **134,** 257–268.

Yúfera, M., Sarasquete, M.C. & Fernández-Díaz, C. (1996) Testing protein-walled microcapsules for the rearing of first-feeding gilthead sea bream (*Sparus aurata* L.). *Larvae Mar. Freshw. Res.*, **47,** 211–216.

Yúfera, M., Parra, G. & Pascual, E. (1997) Energy content of rotifers (*Brachionus plicatilis* and *Brachionus rotundiformis*) in relation to temperature. *Hydrobiologia*, **358,** 83–87.

Yúfera, M., Pascual, E. & Fernández-Díaz, C. (1999) A highly efficient microencapsulated food for rearing early larvae of marine fish. *Aquaculture*, **117,** 249–256.