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The effect of dietary exogenous digestive enzymes on ingestion, assimilation, growth and survival of gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae*

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Abstract

The success of microdiets commonly used in the cultivation of marine fish larvae is limited to serving as partial replacements for live food. This limited success is thought to be associated with a reduced digestive ability due to an incompletely developed digestive system. The enhanced growth obtained from live food has been partially attributed to the digestive enzyme activity of the food organism. The present study was designed to test the effect of an exogenous digestive enzyme incorporated into a microdiet on the growth of *Sparus aurata*.

Larval gilthead seabream, 20–32 days old, were fed ^{14}C labelled microdiets containing a commercial pancreatic enzyme at different concentrations (0, 0.1 and 0.05g / 100 g dry diet). Rates of ingestion and assimilation were measured and their relationship to dry weight was determined. Our results show that the success of the microdiet as a food for larval gilthead seabream was limited by the larva's low ingestion rate which only approached its maintenance requirement. In addition, the presence of digestive enzyme in the microdiet enhanced its assimilability by 30%. Larval growth over ten days was 0, 100 and 200% on microdiet free of added enzymes, one with added enzymes and a live food regime, respectively. It is our opinion that successful development of microdiets for *Sparus aurata* must be based on diets improved both in digestibility and attraction to the larvae. Further studies are now underway to determine the nutritional requirements of gilthead seabream larvae using the experimental method developed in the present study.

Introduction

Nutrition of fish larvae is one of the dominant factors influencing their survival in culture. Therefore, to ensure high survival, a continuous supply of a suitable, attractive diet is necessary. Food, alive or formulated, must provide the necessary nutrition for good growth and development and have physical and chemical properties which maximize larval encounter, ingestion and assimilation.

There are two major developmental trends in larval feeding: 1) use of live food organisms such as rotifers, *Brachionus plicatilis* (Gatesoupe and Robin 1982; Fukusho *et al.* 1985; Lubzens *et al.* 1989) copepods, *Calanoidea* (Kahan 1982) and brine shrimp, *Artemia salina* (Watanabe *et al.* 1980; Fluchter 1982; Walford and Lam 1987), 2) the use of formulated microdiets. From a nutritional point of view, live food is not always consistent or suitable. In addition, the supply

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Table 1. Microdiet composition

Component	% diet (DW)
Squid powder	40.6
Rotifers	46.8
Capelin oil	4
Lecithin	1
Vitamins	1
Vitamin C	1
Choline chloride	1
$CaHPO_4.2H_2O$	1
$CaCO_3$	0.6
Gelatin	3

of live food can be interrupted by a sudden collapse of the culture due to a number of reasons (Watanabe *et al.* 1979, 1980, 1983; Fukusho *et al.* 1980; Tandler 1985). Furthermore, the mass culture of live food requires considerable space and expense due to required energy, equipment and manpower. Microdiet in contrast, offers off-the-shelf availability, lower production costs and greater diet flexibility (Gatesoupe and Luquet 1981; Teshima *et al.* 1982). Unfortunately, in studies with larvae reared on artificial diets, the larvae have not matched the growth and survival performance of larvae fed live food organisms (Adron *et al.* 1974; Kanazawa *et al.* 1982, 1988; Teshima *et al.* 1982).

Poor performance with microdiets may result from an incompletely developed digestive tract in the early stages of larval growth, which cause low digestive enzyme activity in these fish (Dabrowski 1984; Lauff and Hofer 1984). It has been suggested that juvenile (Jancaric 1964) and larval fish (Dabrowski and Glogowski 1977) utilize the exogenous enzymes of the live food they consume as activators of zymogens in their gut to help complete the digestive process. The fact that these enzymes are not usually included in microdiets, could explain the greater success of live foods, if larvae were in fact utilizing exogenous enzymes.

The objective of the present study was to investigate the influence of exogenous digestive enzymes (porcine pancreatic extract) included in microdiets on rates of ingestion, assimilation and growth in 20–32 days old gilthead seabream (*Sparus aurata*) larvae. Three types of trials were run to meet these objectives: trials of 60 min to determine ingestion; trials up to 24h for assimilation and ten day trials to measure growth and survival.

Materials and methods

Diet preparation

Microdiets of 150–400 μm particle size were prepared for these experiments. Composition of the basic microdiet is given in Table 1. Some of the microdiets were labelled using ^{14}C radioactive rotifers (*Brachionus plicatilis*), prepared after the methods of Sorokin (1966, 1968) and Tandler and Mason (1984). Rotifer labelling was accomplished by feeding [^{14}C]-labelled algae which had been prepared in the following way: microalgae (*Nannochloropsis* sp.) at a concentration of 120×10^6 cells/ml were sealed in a 4.5l glass carboy using a rubber stopper. Two ml of $NaH[^{14}C]O_3$ (Amersham, 2 mCi/ml) were injected into the carboy through the rubber stopper. All excess $[^{14}C]O_2$ was removed by a KOH trap. The carboy was illuminated by 6 fluorescent lamps (daylight 40W) which supplied light at an intensity of 1700 lux at the culture surface. The algae medium was maintained at 22°C ± 0.5°C, pH 6.5–6.9 and magnetically stirred to ensure a homogeneous exposure to light. After 27h of algae labelling the remaining $[^{14}C]O_2$ was bubbled out, and trapped in KOH. The efficiency of labelling was 85% with final activity of 1.393×10^{-2} dpm/algal cell.

Rotifers used in the microdiets were reared in mass cultures which were maintained at a concentration of 100–300 rotifers/ml, a salinity of 35–38 ppt and a temperature of 18–28°C. Cultures were fed daily with yeast (*Saccharomyces cerevisiae*, $1g/10^6$ rotifers) and algae *Nannochloropsis* sp.. Prior to labelling, the rotifers were washed in seawater, and filtered through 80 μm mesh. They were then stocked with 4.5l of labelled algae (50×10^9 cells), in a 20l carboy at a concentration of 4200/ml (18.9×10^6 rotifers). Oxygen was maintained in the carboy at 5.5 ppm by continuous aeration. After 4h of incubation, the specific activity of rotifers was 200 ± 20 dpm/rot. at a labelling efficiency of 75%. After incubation, rotifers were concentrated to a

wet paste on a 80 μm filter and heated at 80°C for 5 min to ensure no enzymatic activity. The rotifer paste was mixed with the other components of the diet (Table 1) after dry weight was calculated (Minkoff 1987). After mixing all the diet components together, gelatin dissolved in water was added and the wet diet spread on an aluminum pan to dry in a 45°C oven for 72h. The dry microdiet was ground using a mortar and pestle and sieved into three sizes, 150 μm, 150–250 μm, >250 μm.

Two labelled microdiets were prepared, one supplemented with 0.05% (dry weight of microdiet) commercial preparation of porcine pancreas (Pancreatin, Sigma) and the control microdiet without enzyme supplementation. These diets were used to determine food ingestion and assimilation. The specific activity of the two labelled diets were: 95 and 85 dpm/μg for the control microdiet and Pancreatin supplemented microdiet, respectively.

The proteolytic (Edelstein *et al.* 1968) and amylolytic activities (Berenfeld 1951) of Pancreatin were measured in the microdiet after incorporation[2] and they retained 80% of their activity. The control microdiet, showed no proteolytic or amylolytic activity.

For the 10 day growth and survival trial, three additional unlabelled microdiets based on the same composition (Table 1) were prepared having 0, 0.05 and 0.1% Pancreatin. A live food regime (Tandler *et al.* 1989) of rotifers and Artemia acted as the control in this trial.

Experimental tank system

Experiments were carried out in 600 ml polycarbonate beakers, continuously supplied (150 ± 50 ml/min) with filtered sea water at temperatures of 20.5, 23 or 24.5°C. Water exited through a 350 μm filter at the bottom of the beakers, to retain fish larvae. The temperature in the beakers corresponded to the temperature regime routinely used for *S. aurata* larval rearing (Tandler *et al.* 1989). The 30 beaker experimental system was kept under a fume hood and illuminated 15h/day with daylight 40W fluorescent lamps which provided a light intensity of 800 lux at the water surface.

The experimental beakers were stocked with 23 ± 5 larvae, at least 12–18h prior to the experiments, and the larvae deprived of food while acclimating to the system. Following acclimation, dead larvae were removed before experimental trials started. Larval survival up to 24h was 85%.

Larval fish

S. aurata larvae, 20 to 32 days old, used in these trials were reared in 600l conical tanks following the routine protocol of Tandler *et al.* (1989) prior to use. Larval feeding started after pigmentation (3 days after hatching) with n-3 HUFA enriched rotifers, (Koven *et al.* 1989) reared on baker's yeast and *Nannochloropsis* sp. At 15 days, larvae were offered newly hatched *Artemia* nauplii followed by n-3 HUFA enriched Artermia after day 18.

Experimental trials

In order to determine microdiet ingestion, each experimental group of larvae was offered 3.5 mg of diet every 15 min. A preliminary trial had determined this to be the *ad libitum* ration for 20–32 day old larvae. The interval between feedings was sufficient for most food particles from the previous feeding to be flushed out. After 60 min of dry food ingestion, the larvae were killed using 1 ml of 15% quinaldine (Sigma) in methanol. The larvae were individually siphoned out, washed with Distilled water, dried and counted into scintillation vials. One ml of tissue solubilizer (Soluen 100, Packard) was added to each tube and the tubes were incubated at 50°C for 120 min. After incubation, 5 ml of scintillation fluid (Hionic flour, Packard) was added and radioactivity in the sample was then counted, using scintillation counter (Packard 4530). The counts were corrected for quench and dpm converted into the amount of dry food ingested by the specific activity of the labeled microdiets.

[2]Amylose breakdown – release of $4.5*10^{-4}$ μmole soluble Carbohydrates/min/mg microdiet (40°C); Casein breakdown – release of 0.1 μmole FAA/min/mg microdiet (40°C, pH 7.5).

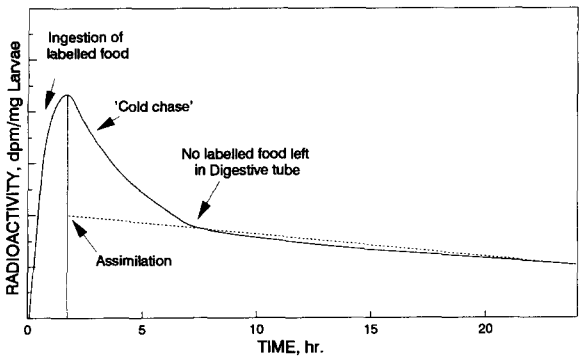

Fig. 1. Change in radioactivity of *Sparus aurata* larvae during short feeding trials.

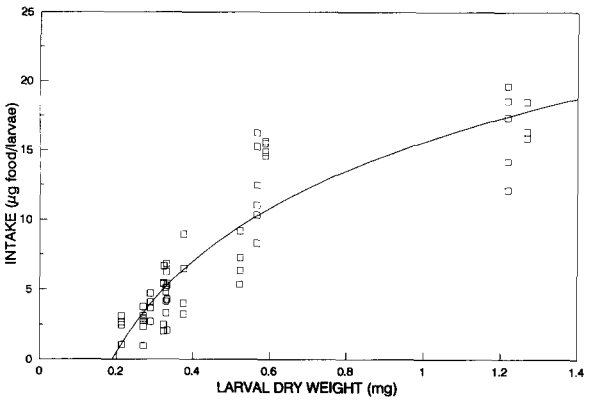

Fig. 2. Ingestion rate of microdiet by *Sparus aurata* larvae of different weights.

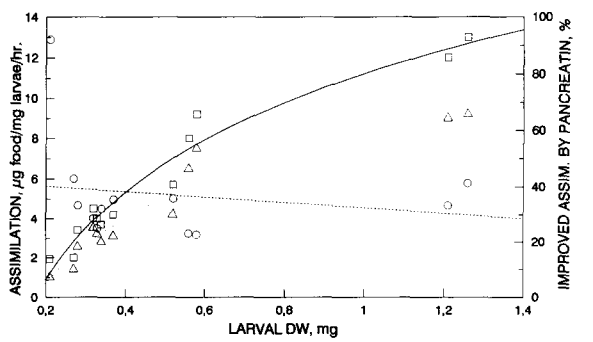

Fig. 3. Assimilation rates of dry microdiet in *Sparus aurata* Larvae. –□– microdiet with Pancreatin, ... Δ ... microdiet without Pancreatin, --○-- % improvement in assimilation by Pancreatin addition.

From the ingestion rate experiments, it was determined that larvae fill up their digestive tube within 60 min, therefore, larvae in the assimilation experiments were fed labelled microdiet for only 60 min. After feeding, the labelled food was washed out of the experimental containers by flushing until the outflow water reached background activity levels of 40–60 dpm/ml. Following flushing, unlabelled microdiet was fed at the same rate of 3.5 mg/15 min to help evacuate the digestive tube of labelled diet. After 1, 3, 6 and 24h of 'cold chase' the larvae were killed and sampled to measure radioactivity using the procedure mentioned previously. The assimilation rate of dry food was determined by extrapolation using the data of decrease in radioactivity with time: 1, 3, 6 and 24h after termination of feeding on labelled food (Fig. 1).

Growth and survival of larvae fed microdiets for a ten day period were determined using 23 ± 5, 20 day old larvae in each experimental beaker. The three unlabelled microdiets and live food control mentioned in the diet preparation section were each replicated 6 times in this trial. After 10 days of growth larvae were counted to determine survival and sampled for dry weight.

The effect of Pancreatin addition to a microdiet on *S. aurata* larval ingestion rate and assimilation rate was analyzed by covariance analysis (ANCOVA, SAS; Sokal and Rohlf 1969). All variances were tested for normality and homogeneity (Hartley's F-max test; Sokal and Rohlf 1969). Data were log transformed when the variances were significantly different ($p < 0.05$). The critical level of significance for testing hypothesis was $p < 0.05$. In the growth and survival experiment, ANOVA and a multiple range test (Duncan) was used to determine which of the treatments significantly differed from each other (Sokal and Rohlf 1969).

Results

Ingestion rate of 20–32 day old larvae rose from 3 to 17 µg/h as larvae grew from 0.2 to 1.2 mg (Fig. 2). Since no significant difference ($p > 0.05$) was found in ingestion rate of diets with or without Pancreatin the data were pooled. The following function describes the relationship between larval dry weight and ingestion rate:

Table 2. Growth and survival of *Sparus aurata* larvae fed for a 10 day period

Treatment	Initial weight (mg)	Final weight DW, (mg)	Survival (%)
Live food	0.165[c]	0.479[a]	24.4[a]
Micro-no panc.	0.165[c]	0.233[c]	13.7[a]
Micro-0.05% panc.	0.165[c]	0.345[b]	16.5[a]
Micro-0.1% panc.	0.167[c]	0.346[b]	17.5[a]

Values in a given column having the same superscripts are not significantly different ($p<0.05$).

$$Y = 15.64 + 9.37\ln(dw) \quad (R^2=0.806, n=58)$$

where Y is ingestion rate (μg dry food/h/mg larvae). dw is average larval dry weight (mg).

Assimilation rate was about 30% higher ($p < 0.05$) in larvae fed the microdiet with Pancreatin as compared to the control diet (Fig. 3). The assimilation rate in larvae increased logarithmically with an increase in the larval dry weight. The two relationships can be best represented by the following equations:

$$A_{panc} = 11.22 + 6.401\ln(dw) \quad (R^2=0.956, n=12)$$
$$A_{no\,panc} = 8.43 + 4.791\ln(dw) \quad (R^2=0.928, n=13)$$

where A_{panc} is the assimilation rate (μg dry food/h/mg larvae) of dry food containing 0.05% (dw) Pancreatin and $A_{no\ panc}$ the assimilation rate without Pancreatin; dw = larval average dry weight (mg).

The influence of Pancreatin on ^{14}C retained in the body was significant ($p<0.05$) 3, 6 and 24h after the labelled meal and independent of larval weight.

Twenty day old *S. aurata* larvae fed 10 days on the Pancreatin diets had 48% (Table 2) dry weight gain ($p<0.05$) as compared the larvae fed on microdiet without pancreatin addition. Larvae fed 0.1 or 0.05% Pancreatin in the diet for 10 days did not differ significantly ($p>0.05$) from each other in growth. In contrast, larvae fed the live food control in these trials had 100% dry weight gain ($p<0.05$), as compared to the larvae fed the non pancreatin microdiet. Survival of larvae in all treatments was not significantly different ($p>0.05$).

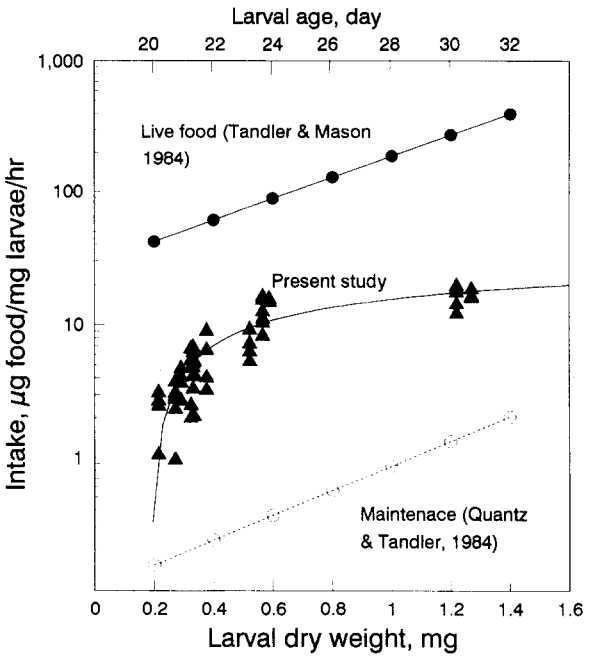

Fig. 4. Rate of ingestion of dry and live food in *Sparus aurata* Larvae. -●- live food ingestion from Tandler and Mason (1984); -▼- microdiet from the present study, --○- maintenance ingestion from Quantz and Tandler (1984).

Discussion

In the present study we demonstrated a positive effect of exogenous digestive enzymes on the assimilation rate of 20–32 day old *S. aurata* larvae. This supports the hypothesis that reduced diet digestibility by larval fish is associated with a lack of exogenous enzymes in the food (Lauff and Hofer 1982; Dabrowski 1984). Studies on morphology and the enzymatic capacity of the digestive tract of larval fish suggest that it is not fully developed (Baragi and Lovell 1986; Cousin *et al.* 1987; Segner *et al.* 1989). Additional studies indicate that digestive capacity increase with larval age as a result of increased enzymatic activity associated with the more developed digestive tracts of older larvae (Buckley and Dillman 1982; Govoni *et al.* 1986). Based on our results, we propose that enzyme activity in young seabream larvae was not sufficient for digestion of the microdiets. The mode of action of Pancreatin in our microdiet could be associated with both the supplementation of digestive enzymes as well as zymogen activation

(Jancaric 1964; Maugle *et al.* 1983).

The present study provides the first published data on feeding rates of marine fish larvae fed on a microdiet, using radioactive labelling. These data facilitate distinguishing between the true dietary effects of a microdiet and the amount actually ingested by the larvae. A comparison between feeding rates on the microdiet in this study and live rotifers (Fig. 4, Tandler and Mason 1984) shows a higher intake for larvae offered the live food; for example, 0.46 mg (dw) larvae ate 54.7 µg rotifer (dw)/mg larvae/h (Tandler and Mason 1984) as compared to 3–7 µg dry food/mg larvae/h in 0.52–0.55 mg larvae in the present study. Similarly, 28 day old *Leiostomus xanthurus* larvae, 0.7 mg in weight, ate 20–28 µg rotifer (dw)/mg larvae/h (Govoni *et al.* 1986) as compared to 14–17 µg dry food/mg larvae/h in the 0.58–1.2 mg (dw) larvae of this study. Also, Weinhart and Rosch (1991) concluded that "low amount of dry diet eaten by coregonid larvae is one of the main reasons for the lower growth rate of larvae fed on dry diet compared to larvae fed on living zooplankton/*Artemia* nauplii". Therefore, we suggest that in *S. aurata* larvae, both, the rate of ingestion and assimilation of the microdiet were low.

Quantz and Tandler (1984) calculated the carbon intake for maintenance in *S. aurata* larvae and proposed the following equation to describe this relationship:

$$M = 5.87e^{0.215age}$$

where M is the maintenance requirement in mg carbon/larvae/h and age in days after hatching. This equation can be converted to rotifers (dw) required for seabream maintenance since 1 µg rotifer contains 0.36 µg C (Scott and Baynes 1978). Based on calculations using this conversion the maintenance requirement of *S. aurata* larvae is close to the rate of dry food ingestion observed in the present study. 25 day old larvae require about 1 µg dry weight rotifers/h for maintenance while microdiet ingestion of 5–10 µg dry food/hr was found in the present study. Laurence (1977) suggested that fish larvae cannot survive long at maintenance feeding rates as larval growth is very high and without a constant supply of energy, larvae will die.

S. aurata larval growth in the present study was positively affected by the inclusion of Pancreatin in microdiets. The final weight of larvae fed on supplemented microdiets (0.05 or 0.1% Pancreatin) was 100% higher than the initial dry weight, while the final weight of larvae fed on microdiet without pancreatin addition was only 40% higher than the initial weight. However, the final weight of larvae fed microdiet with pancreatin in the same trial was 70% of larvae fed live food. These results on a marine species agree with studies on the influence of enzyme (trypsin) supplemented diets in a larval freshwater fish, *Cyprinus carpio* (Dabrowski and Glogowski 1977; Dabrowska *et al.* 1979).

The observed lack of effect of Pancreatin level in the microdiet (0.05 or 0.1%) on larval growth could be explained by Jancaric's hypothesis (1964) that exogenous digestive enzymes activate larval zymogens. Furthermore, Dabrowski (1984) proposed that the activating time of fish zymogens is shorter, and the activity of their digestive enzymes much higher, than the mammalian ones. As Pancreatin, in the present study is of a mammalian origin and the amount of zymogens in the digestive tract is limited, the lower level of Pancreatin supplementation could be sufficient for maximum activation in the larvae.

In conclusion, in the present study, we propose that lack of efficient use of microdiets by 20–32 day *S. aurata* larvae was due to low food intake and digestibility. The addition of digestive enzymes to the microdiet increased the diet's assimilation by the larvae. Improvement in the rate of microdiet ingestion by *S. aurata* larvae and the use of fish digestive enzymes are the goal of our future studies.

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