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The effect of glycerol dissolved in the rearing water on the transition to exotrophy in gilthead sea bream *Sparus aurata* larvae

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Abstract

The effects of glycerol added to seawater at different concentrations were tested in monocultures of *Chlorella minutissima* and *Brachionus plicatilis* in mixed cultures of these two species and on starved or fed *Sparus aurata* larvae. Glycerol enhanced the growth of *Chlorella* and seemed to indirectly benefit the rotifers that feed on it. Its beneficial effect on gilthead sea bream prelarvae and larvae resulted in glycogen accumulation in the liver and absence of signs of cholestasis-like pathology. This effect varied according to the glycerol concentration in the water, to its addition timing and to light and feeding conditions. The gluconeogenic origin of the hepatic glycogen reserves is discussed, together with the mediating role of the food chain in glycerol larval assimilation.

Keywords: Glycerol; Glycogen; Liver; *Sparus aurata*; *Chlorella minutissima*; *Brachionus plicatilis*

1. Introduction

It is known that in fish larvae rearing, the transition from endotrophy to exotrophy is a critical phase of development characterised by high mortality, which seems to be mostly due to the inadequacy of first feeding to meet the nutrient requirements and/or

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to the digestive capacity of larvae. A number of studies have been carried out in order to determine a means to avoid such failure by testing a kind of feeding easy to assimilate by larvae. This procedure is expected to support at a nutritional level the transfer to exogenous feeding. Since organic matter dissolved in seawater is important for marine invertebrates (Le Gal, 1988), a number of studies have been focused on the evaluation of the role of various substances during the post-embryonic development in fish. Absorption and metabolism of dissolved amino acids and vitamins have been observed in European sea bass larvae (Pavillon and Vu T.T., 1981) and that of glucose in rainbow trout fry (Fauconneau et al., 1989). In addition, the beneficial effect of glucose, dissolved in the rearing water, on the general health condition of larvae has been shown in rainbow smelt (Bedard and Lalancette, 1989), in European sea bass and in gilthead sea bream (Diaz et al., 1994). This positive effect was suggested by the increased accumulation of glycogen in the hepatocytes and in the later appearance of the pathological signs characteristic of starvation. Nevertheless, their effects at a zootechnical level have not yet been investigated.

The importance of glycerol in metabolism, especially as a gluconeogenic precursor, led us to study its influence on the delicate transition from endotrophy to exotrophy in gilthead sea bream. The aims of our study were to evaluate the effect of glycerol on the liver of gilthead sea bream larvae and to see whether this compound could constitute a complement to the first feeding. The assimilation of glycerol was monitored in starved and fed larvae by histological and cytochemical analysis of the liver. When larvae were fed rotifers, we sought to know whether its effect was direct through absorption from the medium or indirect through the food chain. We have therefore also investigated the impact of its addition to the culture medium on the densities of *Chlorella* and rotifers reared under different experimental conditions.

2. Material and methods

2.1. Experiments on gilthead sea bream larvae

Gilthead sea bream (*Sparus aurata*) eggs were supplied by the Institute of Marine Biology of Crete (IMBC) and obtained from broodstock held in captivity for 5 years.

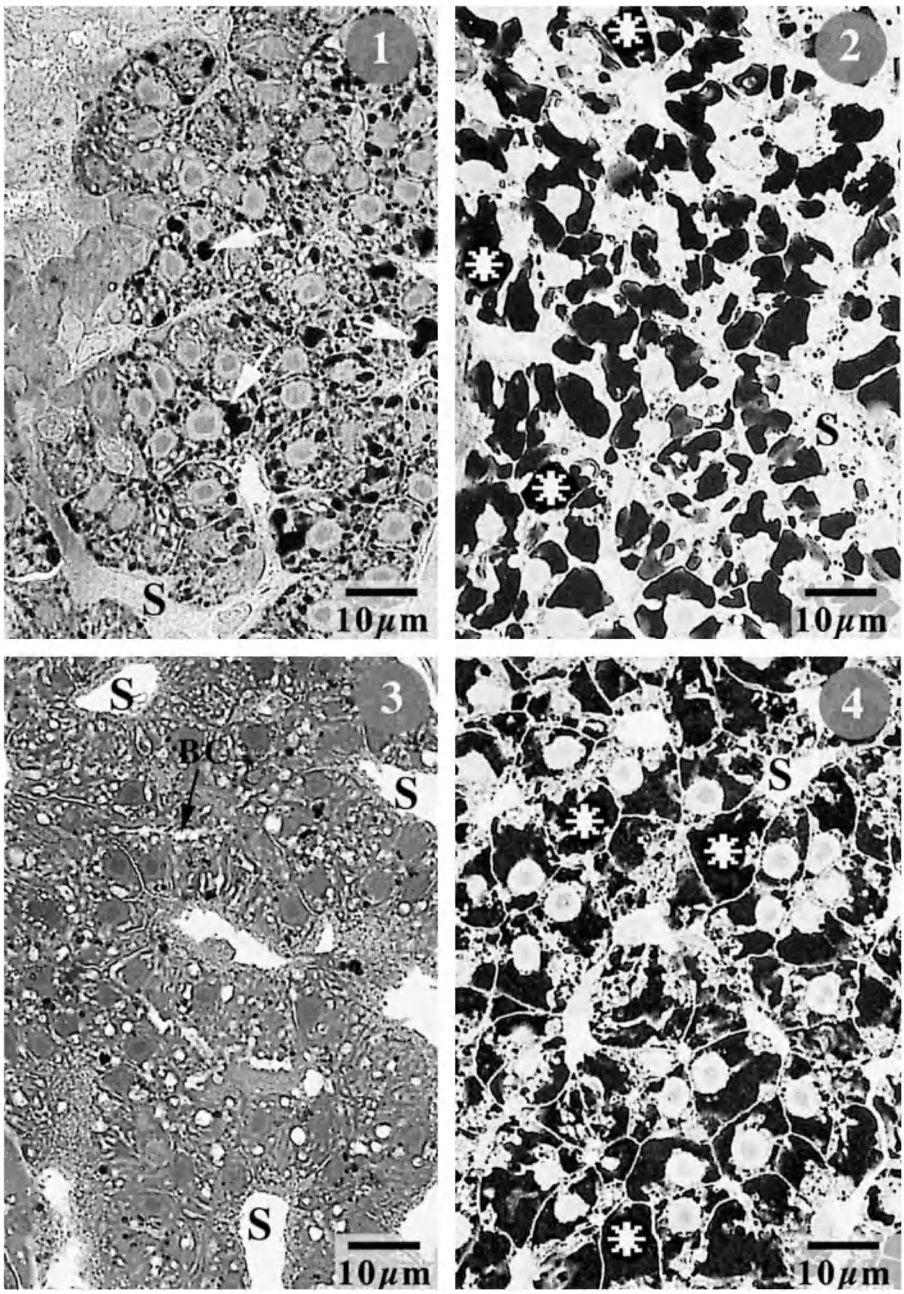
Fig. 1. Small amount (+) of glycogen (arrows) in the liver of a gilthead sea bream larva at mouth opening, reared in clear water without glycerol. (S) Sinusoid. (Argentodiamine hydroxide method.)

Fig. 2. Medium amount (+ + +) of glycogen (*) in the liver of a gilthead sea bream larva at mouth opening, reared in clear water with 1000 mg/l of glycerol added at hatching. (S) Sinusoid. (Argentodiamine hydroxide method.)

Fig. 3. Liver containing no glycogen in a 6-day-old starved larva reared in clear water, without glycerol addition and maintained in the dark. (BC) Bile canaliculus; S, sinusoid. (Argentodiamine hydroxide method.)

Fig. 4. Large amount (+ + + +) of glycogen (*) in the liver of a 6-day-old starved larva reared in clear water with 1000 mg/l of glycerol added at hatching and maintained in the dark. (S) Sinusoid. (Argentodiamine hydroxide method.)

Incubation, hatching (day 0), prelarval phase (until mouth opening, i.e. days 3–4) and larval rearing (until day 8 or day 14 according to the experimental protocol) were carried out in 35-l cylindroconical tanks with an initial density of 100 individuals/l. Larvae



were kept in total obscurity until mouth opening (prelarval stage), and either under 24 h lighting (700–1000 lx at the surface of the tanks) or under total obscurity afterwards, according to the experimental protocol. The rearing medium (39‰ salinity, 18 ± 0.5°C temperature) was either 'clear water', i.e. pure filtered seawater, or 'pseudo-green water', containing a culture of *Chlorella minutissima*, whose density was maintained constant (0.5×10^6 cells/ml) by daily algae additions (Divanach et al., 1999). Glycerol was added to part of the rearing tanks according to the experimental protocol.

The following three experiments were performed.

The first experiment was aimed at evaluating the influence of the light conditions and of the timing of glycerol addition in the rearing tanks on the hepatic glycogen content of starved larvae. This procedure allowed also the setting of the parameters of the experimental protocol of the second trial. The rearing was performed following three procedures: in pure clear water and in clear water in which 1000 mg/l glycerol were added either at hatching or at mouth opening. In all cases, half of the tanks were kept in the dark for the entire experiment while the other half was kept under a 24-h photoperiod from mouth opening onwards. Samples for liver analysis were collected in all the tanks at mouth opening (MO) on day 6 and on day 8.

The second experiment was aimed at evaluating the influence of glycerol concentration in the rearing water and of its administration mode on the hepatic glycogen content of starved larvae. The results of this trial allowed also the setting of the concentration and the administration mode of the third trial. The rearing was performed following three procedures: in pure clear water, in clear water in which 10, 100 or 1000 mg/l of glycerol were added once at hatching (flash mode), and in clear water in which 100 mg/l of glycerol were added daily starting at hatching (continuous mode). Larvae were kept in total obscurity during the whole experiment. Samples for liver analysis were collected in each tank at mouth opening (MO) on day 6 and on day 8.

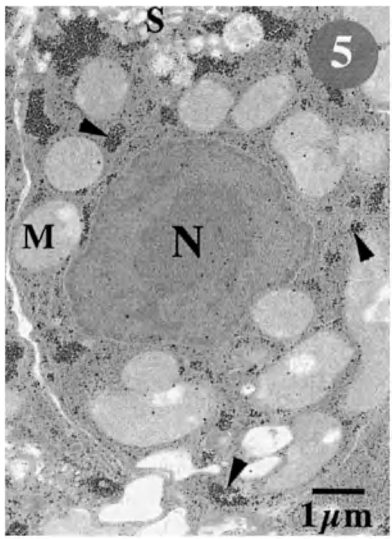
Fig. 5. Hepatocytes of gilthead sea bream larvae at mouth opening reared in pseudo-green water. Larvae reared without dissolved glycerol accumulated little hepatic glycogen (+) (arrowheads), which was partially concentrated in small patches. (M) Mitochondrion; (N) nucleus; (S) sinusoid. (Thiosemicarbazide silver proteinate method.)

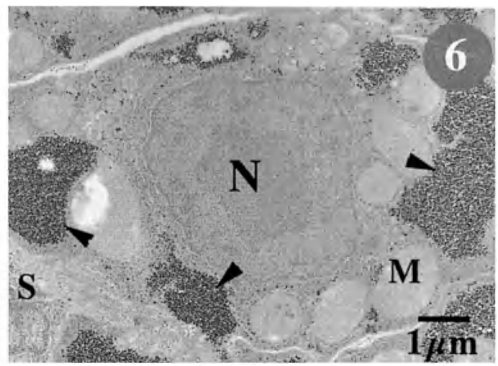
Fig. 6. Hepatocytes of gilthead sea bream larvae at mouth opening reared in pseudo-green water. Larvae reared with dissolved glycerol from hatching accumulated medium amount (+++) of glycogen (arrowheads) stored in broad patches. (M) Mitochondrion; (N) nucleus; (S) sinusoid. (Thiosemicarbazide silver proteinate method.)

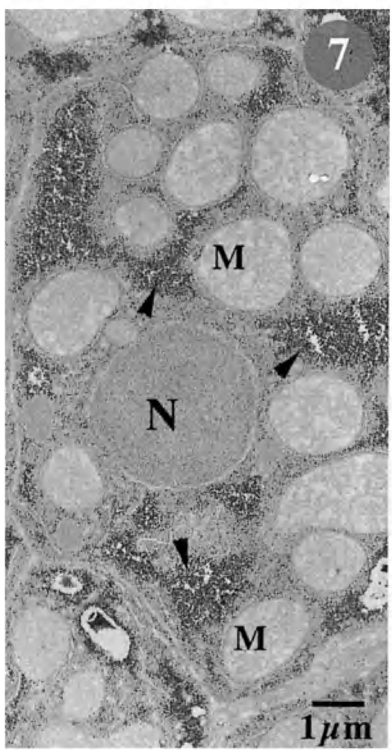
Fig. 7. Hepatocytes of 8-day-old gilthead sea bream larvae reared in pseudo-green water and fed with rotifers. Larvae reared without dissolved glycerol accumulated medium amount of hepatic glycogen (+++) (arrowheads). (M) Mitochondrion; (N) nucleus. (Thiosemicarbazide silver proteinate method.)

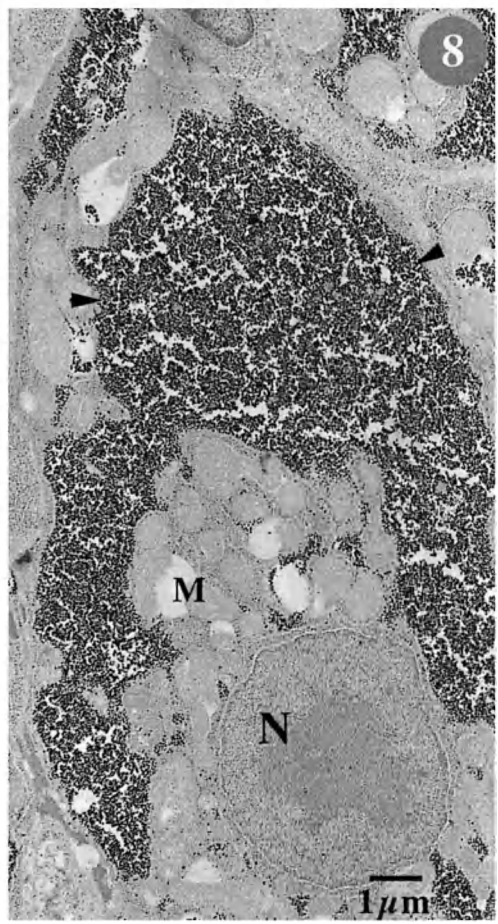
Fig. 8. Hepatocytes of 8-day-old gilthead sea bream larvae reared in pseudo-green water and fed with rotifers. In fed larvae reared with dissolved glycerol, these reserves were very large (++++++), giving the cytoplasm a compartmentalised appearance. (N.B.: Increasing the glycogen storage in the hepatocytes resulted in the increasing of the cell size.) (M) Mitochondrion; (N) nucleus. (Thiosemicarbazide silver proteinate method.)

The third experiment was aimed at evaluating the influence of glycerol, utilised as complementary dissolved feeding, on the hepatic glycogen content of rotifer-fed larvae. Rearing conditions were standard of intensive hatcheries. The experiment was performed









following two procedures — in pseudo-green water alone and in pseudo-green water in which 100 mg/l glycerol were added daily starting at hatching (continuous mode). Prelarvae were kept in the dark and larvae under a 24-h photoperiod. Larvae were fed three times per day on rotifers (*Brachionus plicatilis*) starting at mouth opening. Prey density was maintained at 7 individuals/ml. Samples for liver analysis were collected in each tank at mouth opening (MO) on day 8 and on day 14.

Histology analysis was performed on larvae anaesthetised in cold water and subjected to double fixation in toto in 2.5% glutaradehyde (1 h), then in 1% OsO_4 (1 h), at 350 mosM (pH 7.2). They were then dehydrated in a graded series of alcohol and embedded in Epon 812. Semi-thin (0.5 μm) and ultra-thin (90–150 nm) sections were prepared with LKB ultramicrotome for light and electron microscopy analysis, respectively. Glycogen was enhanced on ultra-thin sections by the thiosemicarbazide silver proteinate staining method (Thiéry, 1967), and on semi-thin sections by the argentodiamine hydroxide staining method (Singh, 1964). The quantity of glycogen was assessed by the comparison of histological sections from three levels of the liver, and the following annotations were attributed: (−) no glycogen (Fig. 3); (+) small amount of glycogen (Figs. 1 and 5); (+ +) moderate amount; (+ + +) medium amount (Figs. 2, 6 and 7); (+ + + +) large amount (Fig. 4); (+ + + + +) very large amount (Fig. 8).

2.2. Experiment on Chlorella and rotifers

This experiment was aimed at evaluating the influence of glycerol on the first levels of the food chain in gilthead sea bream rearing. Nine polyethylene bottles containing 1.5 l seawater (39‰ salinity, 18 ± 0.5°C temperature) were inoculated either with *Chlorella* at a concentration of 10×10^5 cells/ml, or with rotifers (*B. plicatilis*) at a concentration of 7 individuals/ml, or with a mixture of both (co-culture). Photoperiod was assessed under a 24-h lighting (700–1000 lx at the surface of the tanks). The suspension of the medium in the tanks was provided by an air diffuser, which created a vertical convection current from the tank bottom. A third of the tanks of each rearing condition was used as a control, while 100 and 1000 mg/l of glycerol was added to the rest of the tanks. The experiment was conducted in duplicate. *Chlorella* and rotifers densities were recorded after 5, 24, 48 and 72 h from the beginning of the experiment. The effects of the glycerol addition and of the co-culture on the concentrations of *Chlorella* and rotifers were tested using ANOVA and the a posteriori Games–Howell test.

3. Results

3.1. The influence of glycerol on the hepatic glycogen accumulation in gilthead sea bream prelarvae and larvae

The early post-embryonic development of gilthead sea bream consists of two periods. The first one, the prelarval or endotrophic period, starts at hatching (day 0) and ends at

mouth opening (days 3–4). It is characterised by yolk reserve utilisation, organ differentiation in the digestive tract and accumulation of glycogen in the liver. The second period, or larval period, starts at mouth opening with an endo-exotrophic phase where larvae depend both on their endogenous reserves and on external food. Larvae enter their exotrophic phase towards day 15 when exogenous food becomes the only energy source. Glycogen reserves vary drastically after mouth opening according to the larval nutritional status (Guyot et al., 1995).

3.1.1. Influence of light conditions and of the timing of glycerol addition on the hepatic glycogen accumulation in fasting larvae reared in ''clear water''

At mouth opening, prelarvae reared in pure clear water showed little hepatic glycogen (Fig. 1) partially concentrated in small dense patches (Table 1). At the same stage, prelarvae reared with glycerol stored much more glycogen in their hepatocytes (Fig. 2), in which it was accumulated in small and large dense patches. Larvae in all experimental conditions rapidly exhausted these reserves after mouth opening (Fig. 3), with the exception of those reared with glycerol in the dark from hatching onwards (Fig. 4). In the latter larvae, the stock was important at day 6 and decreased slightly afterwards. When glycerol was added at mouth opening, larvae reared in the dark reconstituted moderate amount of glycogen at day 8.

3.1.2. Influence of glycerol concentration in the rearing water and of its administration mode on the hepatic glycogen accumulation in fasting larvae reared in clear water

At the end of the endotrophic period (MO), prelarvae reared with or without glycerol in the rearing water showed a moderate hepatic glycogen content (Table 2). This storage was then totally exhausted during the days following mouth opening. Two exceptions should be pointed out. Firstly, larvae reared with 1000 mg/l of glycerol had high glycogen reserves at mouth opening, which were not fully exhausted at day 8. Secondly, larvae reared with glycerol given in continuous mode reabsorbed part of their hepatic glycogen from mouth opening to day 6, whereas they partially reconstituted it afterwards.

Table 1
Variations of the hepatic glycogen content in starved gilthead sea bream larvae according to the timing of glycerol addition and to the light condition. (MO: mouth opening.)

Glycerol concentration and addition timing	Light condition	Hepatic glycogen content		
		MO	Day 6	Day 8
0 mg/l (control)	Light	+	−	−
	Dark	+	−	−
1000 mg/l at mouth opening	Light	+	−	−
	Dark	+	−	+ +
1000 mg/l at hatching	Light	+ + +	−	−
	Dark	+ + +	+ + + +	+ + +

Table 2
Variations of the hepatic glycogen content in starved gilthead sea bream larvae according to the glycerol administration procedure and its concentration in the rearing water. (MO: mouth opening.)

Glycerol concentration and glycerol administration mode	Hepatic glycogen content		
	MO	Day 6	Day 8
0 mg/l (control)	+ +	−	−
10 mg/l once at hatching	+ +	−	−
100 mg/l once at hatching	+ +	+	−
1000 mg/l once at hatching	+ + +	+ +	+
100 mg/l daily starting at hatching	+ +	+	+ +

3.1.3. Influence of glycerol added as complementary dissolved feeding, on the hepatic glycogen accumulation in rotifers-fed larvae reared in pseudo-green water

At the end of the endotrophic period (MO), glycogen reserves were much lower in larvae reared in pure pseudo-green water (Fig. 5) than in larvae reared with glycerol dissolved in the medium (Fig. 6; Table 3). In the first case, this storage increased to medium values at day 8 (Fig. 7) and decreased slightly at day 14, while in the latter, it was large to very large at day 8 (Fig. 8) and even at day 14. Glycogen particles were accumulated in broad patches that pushed most of the organelles around the nucleus, giving the cytoplasm a compartmentalised appearance. Besides, the liver of larvae reared in a medium containing glycerol did not show, at mouth opening, on day 8 and day 14, any cholestasis-like alteration. This pathological feature, typical of starving larvae (Diaz et al., 1998), was observed in most control larvae.

3.2. The influence of glycerol on the first levels of the food chain in gilthead sea bream larvae rearing

The cell density of *Chlorella* reared as a pure culture, without addition of glycerol, was constant throughout the experimental period, except for a peak ($P < 0.0001$) observed 24 h after the beginning of the experiment (Fig. 9). When glycerol was added to the rearing medium, density showed significant change ($P < 0.0001$): it increased from 24 h, reached maximum value at 48 h, and levelled off to the end of the experimental period.

The density of rotifers reared as monoculture without addition of glycerol changed through the experimental period ($P < 0.0001$): it was constant during the first 24 h, then decreased steadily to the end of the experiment. When glycerol was added, this downwards trend was also observed until 48 h, but from this time density levelled off.

Table 3
Influence of glycerol on the hepatic glycogen content in rotifer-fed gilthead sea bream larvae reared in pseudo-green water. (MO: mouth opening.)

Glycerol concentration	Hepatic glycogen content		
	MO	Day 8	Day 14
0 mg/l (control)	+	+ + +	+ +
100 mg/l daily starting at hatching	+ + +	+ + + + +	+ + + +

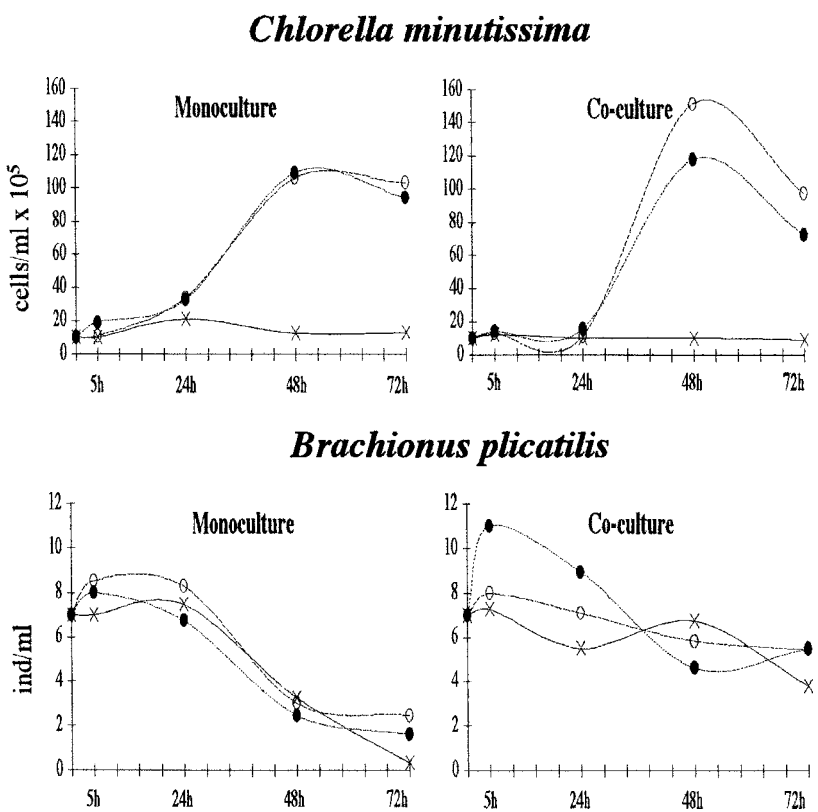


Fig. 9. Influence of glycerol addition and of the co-culture on the densities of *Chlorella* and rotifers. (Glycerol concentration: (-X-) 0 mg/l; (-○-) 100 mg/l; and (-●-) 1000 mg/l.)

The density of *Chlorella* reared as mixed culture without glycerol remained constant through the experimental period ($P = 0.5353$). When 100 or 1000 mg/l was added, bloom type growth was observed from 24 to 48 h ($P < 0.0001$). Throughout the experiment, there was no significant difference in *Chlorella* density between 100 and 1000 mg/l of glycerol cultures.

In mixed culture, the density of rotifer cultured without glycerol displayed a weak downwards trend between the beginning and the end of the experiment ($P = 0.0252$). This trend was also observed in 1000 mg/l glycerol culture ($P = 0.0002$), while there was no significant difference (0.1825) in 100 mg/l glycerol culture through the experiment.

4. Discussion

Glycogen accumulation in the liver is a constant process in the development of vertebrates, and it coincides with the hepatocyte differentiation period (Houssaint et al.,

1970; Sandstrom and Westman, 1971; Devos and Hers, 1973; Bashan et al., 1979; Daimon et al., 1982). In fish, it has been demonstrated in rainbow trout (Byczkowska-Smyk, 1967; Vernier and Sire, 1976), whitefish (Appelbaum et al., 1986), European sea bass (Diaz and Connes, 1991), pike-perch (Mani-Ponset et al., 1994) and gilthead sea bream (Guyot et al., 1995). These observations are confirmed by our results. Indeed, during the endotrophic period control prelarvae reared with no glycerol stored glycogen in their hepatocytes, and these reserves varied afterwards according to the larval nutritional status. Prelarvae and larvae maintained in the presence of glycerol in the rearing water showed similar or larger glycogen storage than the controls. This difference suggests that glycerol could be absorbed and metabolised by both prelarvae and larvae. This implies that before mouth opening glycerol crossed the integument whereas it was absorbed by the intestinal mucosa afterwards.

Glycerol is probably involved in hepatic glycogen synthesis via the gluconeogenesis pathway. Indeed, it is known in fish, that during the endotrophic period yolk is the only protein and lipid source available and is released as lipoprotein particles to the organism. Therefore, hepatic glycogen synthesis by prelarvae can only be explained by the use of the yolk. When yolk is exhausted, starved larvae utilize the glycogen previously accumulated. It is also known that in vertebrates, hepatic glycogen synthesis depends mostly on a gluconeogenetic mechanism (Katz and McGarry, 1984), which enables the organism to remove from the circulation gluconeogenic precursors such as lactate, amino acids and glycerol (Radziuk, 1991). Glycerol, as well as serine, also seems to be an important gluconeogenic precursor in some fish species (Moon et al., 1985). Thus, Lech (1970) showed, in vitro and in vivo, that trout liver contains glycerolkinase, which is involved in glycerol phosphorylation and in its conversion into CO_2, glycogen and lipid. Moreover, in lamprey, labelled glycerol was incorporated in muscle and liver glycogen (Savina and Wojtczak, 1977). Finally, the addition of glycerol to a trout hepatocyte culture medium significantly increased the synthesis of glucose, which was used as an indicator of the gluconeogenic capacity (Segner et al., 1994). In our experiments on starved specimens, glycerol dissolved in the rearing water was the only exogenous energy source available to prelarvae and larvae. This strongly suggests its utilisation in the liver for glycogen synthesis via the gluconeogenic pathway.

Our experiments performed over a range of glycerol concentrations showed that the level of this alcohol in the rearing water must reach a certain threshold to have an effect on hepatic glycogen accumulation. Larvae reared in pure seawater and in seawater containing 10–100 mg glycerol/l showed similar trends, i.e. no glycogen accumulation. In contrast, 1000 mg glycerol/l added once at hatching and 100 mg glycerol/l added daily starting at hatching resulted in high hepatic glycogen storage.

In the presence of dissolved glycerol, starved larvae kept in the dark did not exhaust the hepatic glycogen stored during the endotrophic period, whereas it was completely resorbed under a 24-h photoperiod. This difference is probably related to the increased larval activities, swimming, hunting, etc., occurring under light conditions and to the higher energy source therefore required. The fact that dissolved glycerol alone did not meet the energy needs of larvae reared under continuous light is reminiscent of the results obtained in starved sea bass and gilthead sea bream larvae when glucose was dissolved in the rearing water (Diaz et al., 1994). Larvae did not survive to the

endo-exotrophic period and feeding was therefore necessary from a certain stage onwards.

Rotifer-fed larvae reared in pseudo-green water showed a much higher hepatic glycogen content when glycerol was added to the rearing medium. These reserves were sometimes very large. This can be explained by the fact that glycerol may be directly metabolised after crossing either the integument or the intestinal mucosa. If this is the case, it would certainly follow the way described above for the endotrophic period, and thus enters the gluconeogenic pathway as a precursor. However, its indirect action on feeding larvae via the first level of the trophic chain (*Chlorella* and rotifers) may also occur. The two hypotheses do not exclude each other.

It has been demonstrated that certain strains of *Chlorella* can grow in the dark and in the light utilising organic carbon sources such as glucose and acetate (Turner, 1979; Oh-Hama and Miyachi, 1988), although to the best of our knowledge, data on *C. minutissima* are not available. Besides, glycerol dissolved in the culture medium was taken up by *Poteriochromonas malhamensis* showing mixotrophic growth (Lewitus and Caron, 1991). Our results on *C. minutissima* show that, when glycerol is available to the alga, the growth is significantly higher than in the glycerol-free medium, confirming the potential nutritional role if this compound.

Although glycerol did not prevent the density of a population of rotifers reared in pure seawater from decreasing 24 h after its addition, it may affect their quality and behaviour. Indeed, glycerol drastically enhanced egg production of rotifers (our unpublished data), and this suggests that it was metabolised and could possibly modify their body composition and their food value. We also noticed (unpublished results) that glycerol increased rotifer mobility in the rearing tanks, making them more visible and therefore more available to the larvae. Rotifer growth was constant throughout the experiment when reared together with *Chlorella*, both in the glycerol-containing and in the glycerol-free media. Moreover, it was found that essential fatty acids and PUFA content in rotifers were improved when fed on microalgae (Nichols et al., 1989; Frolov and Pankov, 1992; Carie et al., 1993) or on frozen algae (Lubzens et al., 1995). Nevertheless, the higher accumulation of fat and starch occurring in unicellular green algae when cultured with organic carbon sources (Oh-Hama and Miyachi, 1988; Lid et al., 1992) may constitute a higher energy source for the rotifers grazing on them. The stimulation of *Chlorella* growth thus provides rotifers with a constantly abundant and high nutritional value food. This quantitative and qualitative improvement of the food chain in the medium containing glycerol affected gilthead sea bream larvae at a hepatic level. Larvae showed high glycogen content, while the downwards trend of these reserves, characteristic of gilthead sea bream at first feeding (Guyot et al., 1995), was not observed. They can therefore benefit from a higher energy storage. Besides, larvae reared in the presence of glycerol did not show any sign of cholestasis-like pathology, which is typical of periods of food deprivation or food unsuitability (Diaz et al., 1998) and characterises gilthead sea bream during the first days following mouth opening. Furthermore, the replenishment of glycogen reserves observed in starved specimens reared in the medium containing glycerol, suggests the potential nutritional role of this substrate during the delicate transfer from endotrophy to exotrophy. Therefore, it seems that the presence of glycerol in the rearing water has a positive effect on gilthead sea

bream larvae. In conclusion, the utilisation of glycerol as a complementary dissolved feed in first feeding gilthead sea bream larvae may constitute an important step in the rearing of this species, although the experimental protocol of a pilot scale rearing needs to be defined.

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