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Effect of nutrition on *Crassostrea gigas* larval development and the evolution of physiological indices: Part B: Effects of temporary food deprivation

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Abstract:

In the natural environment bivalve larvae are exposed to variable conditions and can therefore face periods of food shortage. To understand the resistance of early life stages of Pacific oyster (*Crassostrea gigas*), larvae were experimentally starved for 4 days at different periods of their development, and the resulting variations in their lipid reserves then analysed using image analysis and biochemical techniques. Faced with a temporary lack of food, the larvae halted their growth in size and weight and started to live off their lipid reserves; this effect was highly marked when the period of food deprivation was at the beginning of larval rearing, but was less marked when food deprivation was after day 14. The larvae conserved their developmental capacity despite these starvation treatments: when feeding was resumed, the larvae started to grow once more, rebuilt their reserves and showed a survival rate between 45 and 91% to the end of the experiment. Apart from the treatment where larvae were starved between days 6 and 10, high percentages became competent (56–85%) leading to a metamorphosis rate of 58–93%. Coloration of neutral lipids with Nile Red, followed by their quantification using image analysis to calculate lipid surface relative to total larval surface (OLI: overall lipid index) was particularly suited to studying the evolution of reserves through larval development. Results collected in this way supported those obtained in parallel using biochemical tests: triacylglycerols/sterols ratio (TAG/ST). The image analysis method offers the advantage of requiring less biological material to obtain a result; the correlation coefficient between OLI and TAG/ST ratio was 0.75.

Keywords: *Crassostrea gigas*; Larvae; Growth; Metamorphosis; Starvation; Lipids

1. Introduction

The technical knowledge underlying the controlled reproduction of bivalves is relatively recent, as it dates from the 1960s (Loosanoff and Davis, 1963; Walne, 1974), and despite the unquestionable progress that has been made in genitor conditioning, phytoplankton production and developments in rearing of larval and postlarval stages (Helm et al., 2004), hatchery production techniques are still not fully defined for most economically important species. *Crassostrea gigas* has become a species of vital importance for world shellfisheries, and accounts for 99 % of oyster production in France (FAO, 2008). Although the majority of cultivated oysters originate form natural recruitment (5.6 billion wild captured spat: 80 % of the supply), the demand from hatchery juveniles continues to increase (1.4 billion in 2008). This expansion has occurred due to the interannual variation and, therefore, unreliability of natural spat collection in the production basins. Producers have also shown a growing interest in new products for shellfish farming such as triploids, which offer reduced fertility, increased growth and potentially a greater resistance to summer mortality (Samain and McCombie, 2007).

Because the larval stages are presently thought to be the most critical stages in the life of bivalves and despite a high level of technical know-how in commercial hatcheries today, many biological questions on these stages remain unanswered. This means that, as yet, there are no suitable condition indices for bivalve larvae that allow quality and success of larval growth and/or metamorphosis to be predicted. Among environmental factors, food supply has been shown to have a predominating effect on larval development in many previous studies (His et al., 1989; Robert et al., 2004; Rico-Villa et al., 2006; Rico-Villa et al., 2009). Moreover, in the natural environment, the concentration and quality of phytoplankton resources fluctuate continually on both spatial and temporal scales. Larvae can therefore be faced with a shortage or even an absence of food during certain phases of their development. Little information is available today on the effects of starvation on marine invertebrate larvae, particularly by controlled experiments on bivalve molluscs. Invertebrate larvae have been reported to have a generally high tolerance of food deprivation, and survival rates are relatively high in individuals exposed to such stress (Qiu et al., 1997; Moran and Manahan, 2004; Böer et al., 2006). Thus, no effect of larval survival or nutritional capacity was detected after 33 d without food in the study by Moran and Manahan (2004), while His and Seaman (1992) found that larvae reached a ‘point of no return’ after 4 days stress at the beginning of development, and similar results were found in *Ostrea edulis* by Labarta et al. (1999b). A temporary absence of food resources during the larval phase has been seen to alter growth and survival in *Crepidula onyx* (Zhao et al., 2003), and even to affect growth of juveniles post metamorphosis in *C. fornicata* (Pechenik et al., 2002). Results on food deprivation in the same species are accordingly contradictory between studies. Moreover these previous studies reported only the effects of starvation on growth and survival.

However, some physiological indices have shown to be very informative. Thus, several quality indices have been described for marine organisms and most of these emphasize the importance of lipids, particularly triacylglycerols in the case of bivalve physiology (Waldock and Nascimento, 1979; Gallager et al., 1986; Whyte et al., 1987; Castell and Mann, 1994; Soudant, 1995, Pernet, 2003). The importance of these compounds in larvae is shown by their accumulation in the tissues under conditions with abundant trophic resources, and their catabolism in cases of trophic stress (Holland, 1978) or at critical points of development, such as embryogenesis and metamorphosis (Haws and DiMichele, 1993, Labarta et al., 1999a), Robert et al., 1999). To study the evolution and relevance of some physiological indices throughout the pelagic cycle in *C. gigas,* a recent study was carried out under different feeding conditions (Ben Kheder et al., 2010). The triacylglycerol/sterol (TAG/ST) ratio was shown to be closely related to the feeding level as did the second index, the overall lipid index (OLI), corresponding to the ratio of the area of lipids, revealed by Nile Red coloration of whole larvae, to the total larval surface. The third index tested was that of larval

condition (Organic Matter/Dry Weight), previously described as a good indicator of larval competence in the mussel *Mytilus edulis* (Lucas et al., 1986) and scallop *Patinopecten yessoensis* (Whyte et al., 1987), and a good indicator of metamorphosis in *C. gigas* (His and Maurer, 1988).
We decided to use the same physiological indices to look at the effects of a marked stress on larval development, and planned the present study to examine the effect of starvation stress during this critical phase. All of the previous studies dedicated to mollusc starvation concentrate on an early starvation period imposed following the end of embryogenesis; no information is therefore available on the effects of food deprivation occurring later in larval development. The objective of the present study was therefore to examine the effects of a temporary period of starvation of 4 days applied at different times during larval development on larval rearing performances and the expression of three physiological indices.

2. Materials and methods

2.1. Broodstock conditioning, larval development and competence

The techniques used in this study were previously described in Ben Kheder et al. (2010), so only a brief outline will be given here. The larvae were produced from genitors conditioned using tested methods: open circuit, 19 °C, feeding at 6 % of oyster dry weight with a mixture of microalgae (in equal quantity at an equivalent volume) *Isochrysis affinis galbana* (T. Iso = T, strain CCAP 927/14), *Chaetoceros gracilis* (Cg, strain Utex LB 2375) and *Skeletonema marinoï* (S, strain CCAP 1077/3). The larvae were reared at a density of 5 larvae ml^{-1}, in cylindro-conical tanks of 30 or 150 L according to the part of the trial. Batches were reared sequentially with total water replacement every 2-3 days. The seawater, filtered to 1 µm, was maintained at 24 ± 1 °C, ambient salinity (34 ppm). The nutritional input was made up, in equal volume parts of a mixture of T. Iso and *Chaetoceros calcitrans* forma *pumilum* (CCAP 1010/05 (Cp)), which was fed daily at a concentration of 50 cells $µl^{-1}$ during the first week and 100 cells subsequently. Light air bubbling (0.5 l min^{-1}), from the base of the cone was used throughout larval rearing to mix the algae and larvae in the water column and avoid accumulation of organic matter that could encourage the development of bacteria.
Larval mortality was assessed and survival rates calculated on days 6-7, 14 and at the end of larval rearing. Growth was studied every 4 days by image analysis of samples of 200-300 larvae fixed in formaldehyde (5 %) and photographed using Win Imager software under an inverted microscope to determine the surface area and length of each larva (de Pontual et al., 1998).
Towards the end of the pelagic phase of development the larvae considered competent, i.e. those with a foot and an eye spot, were counted. When their number exceeded 40 %, a selective sieving was made on a 225-µm screen. Competent larvae were put separately in 30-l tanks in which plastic discs for settlement of 15-cm diameter had been placed. Rearing conditions were similar to those maintained during larval development. Larvae were then fed on an equal volume mix of *Isochrysis affinis galbana* and *Chaetoceros calcitrans* forma *pumilum* fed daily at a concentration of 100 - 150 cells $µl^{-1}$. After 5 days, the percentage metamorphosis was calculated indirectly by the estimation of the number of free non-settled larvae (swimming and dead).

2.2. Experimental trials

Short periods of food deprivation were imposed at different times during larval life on a series of treatment batches. These periods of starvation started at 2, 6, 10, 14, and 18 days after fertilization and lasted 4 days each, after which the larvae were fed once more; treatments were named D2D6, D6D10, D10D14, D14D18 and D18D22, respectively. There were also

two types of control: the first was fed on the reference ration TCp throughout the larval rearing period, and the second was left unfed throughout the experiment. In total, seven treatments were applied in triplicate and this kind of experimental design was carried out twice, once in spring and once in summer using different larval batches. During the first experiment, the larvae were reared in triplicate, exclusively in 30-l tanks, and lipids were measured by OLI alone. In the second experiment, which was more comprehensive, the reared batches were studied using all three physiological indices: overall lipid index (OLI), structural biochemical index (TAG/ST) and larval condition index (OM/DW). The experiment was conducted in three 30-l and two 150-l cylindro-conical tanks. The 30-l tanks served as control references for monitoring larval development, because the larval density remained almost constant throughout the rearing period. The 150-l tanks were used for the biological samples (physiological indices) which required a greater number of larvae. The effect of tank volume (30-l *vs.* 150-l) had previously been tested and no significant effect on larval performances detected (Robert, unpublished data).
The effect of food deprivation on larval development and metamorphosis success was measured considering that the end point of larval rearing was when 40 % of larvae had eyespots.

2.3. Physiological indices

The three indices were previously described in detail in Ben Kheder et al., (2010) so we will provide only an outline here.

2.3.1. Overall Lipid Index

The lipid reserves of *C. gigas* larvae were stained according to the protocol of Castell and Mann (1994). After a 90 min contact time with Nile Red, the larvae were rinsed in filtered seawater and fixed in formaldehyde (95 %). Microscopic examination and photography was done within the following 4 h to avoid any decrease in fluorescence. The photographs were then treated by image analysis using Imaq Vision Builder version 6, with a batch treatment function. The images were converted, using a previously-established script, so that colours were represented by a grey-scale corresponding to the lipid surface revealed by Nile Red and the total surface of the larva, represented in pixels. From these two values, the ratio of the surface of the image occupied by lipids to the total surface of the larva was determined. This analysis was made on a sample of 50 individuals per tank.

2.3.2. Structural biochemical index (triacylglycerols / sterols: TAG/ST)

Every 4 days, depending on the larval stage, samples of larvae (n = 50 000 to 200 000) were collected on GF/C filter (previously burned at 450 °C) and put in 6-ml tubes of Folch's liquid (2:1 chloroform : methanol) in which they were stored at -20 °C until use. Neutral lipid classes were analysed by HPTLC (high performance thin layer chromatography) using a CAMAG system consisting of a sampler (TLC Sampler 4) and a reader (TLC Scanner 3). Results obtained after taking readings at 370 nm and quantifying the marks with the Wincats program were expressed in µg sterols and triglycerides per larva (Ben Kheder et al., 2010).

2.3.3. Larval condition index: relative organic matter content (OM/DW)

Every 4 days, depending on the larval stage, samples of n = 10 000 to 1 000 000 larvae, made in duplicate, were concentrated on a 40-µm sieve and rinsed with an aqueous isotonic solution of ammonium formiate (32 g l^{-1}). After transfer into hemolysis tubes, the samples were preserved at -20 °C until use. The larvae were then transferred into an aluminium cap that had previously been weighed and burnt at 450 °C. Dry and organic weights were taken

on a microbalance after treatment in a drying oven at 65 °C and muffle furnace at 450 °C, respectively.

2.4. Statistical analyses

Statistical analyses were made in Statview 5. Significant differences were detected between the means at the 5 % threshold using ANOVA and an *a postiori* multiple comparison test between the means (Scheffe's test), after transformation of percentage data by the function [arcsin (racine xi/100)].

3. Results

3.1. First Experiment

3.1.1. Larval Performances

The rearing of the control larvae under continuous food deprivation conditions was continued until day 21, with a high survival rate of 80 % (Table 1). It is interesting to note that 91 % of these larvae were still alive at the end of the second week and that this survival rate was similar to that of the continuously-fed control and sometimes higher than those of the treatments that only experienced temporary starvation (*e.g.,* D6D10 and D10D14). Treatments D6D10 and D10D14 led to a significant drop in survival following the food deprivation period, as only 58 – 64 % of larvae survived after 2 weeks of rearing, compared with 96 % in the continuously-fed control (Table 1, $p < 0.0001$). However, while re-feeding caused mortality to stabilize in D10D14, at a level similar to the control ($p = 0.3214$), survival of the individuals starved between days 6 and 10 (D6D10) continued to decrease significantly, even after re-feeding, to reach 45 % at the end of the experiment, compared with 76 % in the fed control ($p < 0.0001$). For the other treatments, survival to the end of rearing was between 76 and 85 % and was not significantly different from the control (Table 1, $p > 0.05$).

Apart from the larvae deprived of food throughout rearing and those deprived of food between days 6 and 10, neither of which achieved metamorphosis within the time provided, all the other treatments enabled larvae to become competent, although there was a time-lag of a few days, according to the treatment (Table 1). Competent larvae were observed at day 16 in the control and in the early (D2) and very late (D14) food deprivation treatments. It was delayed until day 21 in the other treatments.

Metamorphosis percentages were generally high (> 58 %, Table 1). The continuously-fed control reached 74 %, which was statistically similar to the percentage reached in the D10D14 batches (63 %). In contrast, the larvae deprived of food at the end of larval development (D14D18) showed a significantly lower metamorphosis percentage (58 %), while those deprived early in development (D2D6) suffered no detrimental effects to metamorphosis and even showed a significantly higher percentage (93 %) than the continuously-fed control.

As for larval development, a slowing or even a complete halt in growth was observed in larvae as soon as feeding stopped, whatever the period of rearing (Fig. 1). Larvae continued their development as soon as feeding was resumed. When food deprivation was experienced at the start of rearing (D2D6) compensatory growth occurred rapidly, but when the food deprivation period was imposed beyond the first week of rearing (D6D10), subsequent development was slower. When food deprivation was during the second week of rearing (D10D14), the larvae only achieved a final size equivalent to the control after 21 days (compared with 14 days for the fed control). When the deprivation period was late in larval development (D14D18), a slowing of growth was noted between days 14 and 16, but this

could not be attributed to lack of food as the same change was seen in the controls, just before metamorphosis (Fig. 1).

3.1.2. Overall lipid index

On day 2, endogenous lipids occupied 22 % of the larval surface (Fig 2). After day 2, the reserves were intensively consumed during the first week in all treatments, going down to 0 - 3 %, (including the controls) and were then reconstituted in all except larvae in continuous food deprivation (data not shown). In the fed control, the reserves were rapidly restored to their initial level by day 10 (20 %) and this level was maintained until day 14. At day 16, just before metamorphosis, this lipid ratio doubled, reaching 42 % of the larval surface (Fig. 2). When food deprivation had been imposed early in development (D2D6 and D6D10), the physiological response was immediate, leading to the consumption of the entirety of the reserves, but as soon as they were fed again, the larvae built their resources up again just as fast. Their lipid reserves continued to increase progressively to reach 34 % for D2D6 and 25 % for D6D10 at the end of the experiment (day 16). In contrast, later food deprivation (D10D14) caused different behaviour, as the absence of food only induced a partial consumption of lipid reserves: the lipid level dropped from 14 % on day 10 to 6.4 % at the end of food deprivation on day 14. This decrease was followed by a rapid increase in lipid reserves by day 16 (to 41 %: a level equivalent to the controls). The latest food derivation treatment, applied close to the end of the larval phase (D14D18), did not lead to a loss of lipid reserves but rather to a stabilisation of values around 21 %, which was about half the level observed in the fed control. However, as the larvae were already competent by day 16, measurements were not made on day 18 (Table 1). The larvae were put in the tanks with the plastic discs and the food deprivation was continued for the remaining 2 days of the treatment.

3.2. Second Experiment

3.2.1. Larval performances

Apart from the continuously-starved control larvae, which were strongly affected (45 %), the survival levels at the end of the experiment were high (76 - 86 %). It is particularly notable that at the end of the second week of rearing, 92 % of the larvae in the unfed batch were still alive (Table 2). In the same way as in the previous experiment, competence was delayed according to treatment. When the food deprivation period was at the beginning of development, (D2D6), the acquisition of competence was not observed until day 18 (35 %) but by day 22 a level had been reached that was similar to the control at day 18 (85 % and 76 %, respectively); these two treatments received the same cumulative amount of food during the 16 days considered. The case of treatment D6D10 was similar, although a lower percentage competence was observed for a similar feeding period (37 %; Table 3). Later food deprivation treatments (D10D14 and D14D18) caused a greater time-lag in development, so that at day 22 only 14 and 21 % competence was recorded in treatments D10D14 and D14D18, respectively. At day 25, however, after having been fed for 19 cumulative days, their percentage competence was high, ranging between 67 and 72 % (Table 3). No impact of food deprivation was shown on metamorphosis, which was around 65 % and not significantly different from the fed control (79 %, Table 3). Only food deprivation at the end of larval rearing (D14D18) resulted in lower metamorphosis, which nevertheless attained 56 % (Table 3).

As in the first experiment, the temporary food deprivation periods were accompanied by a sudden halt in growth, which resumed when the larvae were fed again but without any compensatory effect (Fig. 3). The control larvae that were fed throughout had the highest growth (12.9 µm d^{-1}), while those that experienced periods of food deprivation had

significantly lower growth (9.5-10.5 µm d^{-1}; $p < 0.0001$). Growth was negligible in the unfed control (0.5 µm d^{-1}).

3.2.2. Overall lipid index

On day 2, lipids occupied only 6 % of the larval surface (Fig. 4) in the second experiment. This level remained stable until day 6 in the batches that were fed (Fig. 5A), then sharply increased to reach 21 % on day 10 (Figs. 4 and 5C). Later, slight fluctuations were seen, followed by a decrease in values (13 %) at day 18, just before metamorphosis (Fig. 4. and Figs. 5E, 5G). In the individuals subjected to an early period of food deprivation (D2D6), there were no lipids on the larval surface at day 6, at the end of the food deprivation period itself (Fig. 4 and Fig. 5B). As soon as these larvae were re-fed, however, they started to build up lipid reserves quickly (19 % was reached on day 10) and these levels then stabilised (Fig. 4), in contrast to the larvae under continuous food deprivation conditions, which never had a recordable lipid level. The same pattern was observed in the D6D10 batch, with the total consumption of lipid reserves during food deprivation (Fig. 5D), followed by a high recovery (31 % on day 16) then a decreasing trend just before metamorphosis (Fig. 4). When the temporary food deprivation periods were later (D10D14 and D14D18) reserve lipids were only partially consumed (Fig. 4 and Figs. 5F and 5H), therefore giving values of 5 and 9 %, respectively, at the end of the treatment periods. In both cases, after 4 days of re-feeding, the lipid contents reached about 20 % and decreased just before metamorphosis, on day 25 (11 - 13 %, Fig. 4).

3.2.3. Structural biochemical index (TAG/ST)

The continuously-fed control larvae only showed a very slight increase in TAG over the first week, with values increasing from 0.9 to 1.2 ng $larva^{-1}$ between day 2 and day 6. Values then increased more steeply during the second week (35 ng $larva^{-1}$ on day 14, Table 4). A pre-metamorphic larva of 18 days contained 125 ng TAG on average, meaning that 8 ng $larva^{-1}$ were accumulated per day. As with the colorimetric study, the biochemical analysis showed that all reserves of neutral lipids (TAG) were totally consumed when food deprivation was imposed early (D2D6 and D6D10) whereas, low levels were still detected following later food deprivation treatments (D10D14 and D14D18: 2 and 9 ng $larva^{-1}$, respectively, Table 4). TAG was rapidly restored following re-feeding in all treatments (Table 4).

Sterols also increased slowly during the first week (≈ 0.07 ng $larva^{-1}$ d^{-1}). The levels in continuously-fed larvae increased regularly to reach 15 ng $larva^{-1}$ at the end of larval rearing on day 18 (Table 5), corresponding to an accumulation of 1 ng $larva^{-1}$ d^{-1}. The increase in sterols followed the increase in dry weight and there was a strong correlation between these two variables ($R^2 = 0.89$, Fig. 6). Food deprivation led to a lesser impact on these structural lipids than on the reserve lipids.

While triacylglycerols were almost wholly consumed (93 - 100 %), sterols diminished little (30 and 12 % decreases in treatments D10D14 and D14D18, respectively) during food deprivation. In the early food deprivation treatments (D2D6 and D6-D10), sterol catabolism was virtually inexistent (Table 5).

The TAG/ST ratio evolved over time in a specific way according to the nutritional conditions. In the control larvae fed throughout rearing, this index was low at the start of rearing (3) and decreased slightly by day 6 (2). An optimum was seen on day 10 (10), and values subsequently remained between 5 and 9 (Fig. 7). Periods of food deprivation always led to a decrease in the ratio, but this increased rapidly on re-feeding as reserves were rebuilt (Fig. 7). A linear regression was found between the TAG/ST ratio and the overall lipid index analysed above with OLI = 1.5 (TAG/ST) + 4.8 and $R^2 = 0.75$.

Similar regression was also found between TAG content per larvae (%) and OLI with OLI = 3.6 TAG + 6.6 and $R^2 = 0.72$.

3.2.4. Larval condition index (OM/DW)

Dry weight increased progressively over the first week in the fed control (Table 6). It then doubled approximately every 4 days to attain 4800 ng larva^{-1}, just before metamorphosis (day 18). The resulting overall rate of dry weight increase was about 292 ng d^{-1} larva^{-1}. Food deprivation led to a halt in weight increase, which was resumed on re-feeding. During the period without food, the dry weight increase rate was between 11 and 13 ng d^{-1} larva^{-1}, except in D14D18 where a loss in weight was recorded.

Organic matter increased rapidly in the fed control over the first week, reaching a mean of 200 ng larva^{-1} on day 6, equivalent to a 5-fold increase (Table 7). The rate of organic growth then slowed between days 10 and 14 (375 and 436 ng, respectively) and doubled just before metamorphosis (849 ng larve^{-1}, Table 7). Apart from the earliest food deprivation treatment (D2D6), diminution by half (D6D10 and D14D18) or stabilisation was observed in the index values at the end of the food deprivation period (Table 7).

The larval condition index (OM/DW) went from 29 % on day 2 to 39 % on day 6 in the larvae fed continuously (Fig. 8). It stabilised from day 6 until day 10, and then decreased until the end of larval rearing (18 % on day 18, Fig. 8). A similar evolution was noted in the larvae exposed to food deprivation early (D2D6) except that values were lower at the end of larval rearing (12-13 %, Fig. 8). The same pattern was seen once more in treatment D10D14, except that the food deprivation period led to a stabilisation of the index rather than a fall (27-31 %, Fig. 8). Inversely, food deprivation at the end of the first week (D6D10) or just before metamorphosis (D14D18) caused values of this index to fall by half (14 and 8 %, respectively, Fig. 8).

4. Discussion

Invertebrate larvae have been reported to have a generally high tolerance of food deprivation, Thus larvae of Antarctic species such as echinoderms can survive several months on their endogenous resources alone (Fraser and Manahan, 1994). Similarly, the arctic pteropod *Clione limacina* can survive 100 to 260 days without food, depending on its initial reserves. Larvae of temperate and tropical regions can only withstand the absence of food for a much shorter period, amounting to days or weeks (Fraser and Manahan, 1994). Larvae of *Ostrea edulis*, reared for 12 days without any food input had a survival rate of 73 % (Labarta et al., 1999b). In *C. gigas*, the survival rate of larvae that had been starved for their first 14 days was not found to be significantly different from that of larvae that had been fed continuously (Moran and Manahan, 2004). In the present work, after 2 or even 3 weeks of continuous food deprivation (1st and 2nd experiments, respectively), the survival rates also were similar to the continuously-fed controls. Survival was only significantly affected after 25 days without food, but another study on the same species found that almost all larvae died after only 8 days without food (His and Seaman, 1992), and suggested that food input is necessary from the first days following fertilisation. This conclusion contradicts recent findings on the Pacific oyster in which it was clearly shown that at temperatures ≤ 27°C and a stable phytoplankton concentration of 40 cells µl^{-1}, the ingestion of microalgae by oyster larvae remains low until day 5 (Rico-Villa et al., 2008, 2009). Considering this finding, the mortalities reported by His and Seaman (1992) cannot have occurred as a direct result of lack of food; the hypothesis of vibriosis is quite possible as vibrios are known to be particularly virulent towards mollusc larvae (Elston, 1995). Our results show that food deprivation has repercussions for larval growth, with almost no size increase when the treatment is prolonged and a transitory halt in growth when the treatment is temporary. It should be noted that when food deprivation is early (D2D6), dry matter and organic matter increase, nevertheless at a relatively minor scale compared with the continuously-fed control. As the water was systematically filtered to 1 µm in our experiments, it is probable that this increase in size, nevertheless at a relative lesser scale, was due to dissolved materials, as already shown in other invertebrate larvae (Jaeckle and Manahan, 1992). As previously

demonstrated elsewhere (Ben Kheder et al., 2010), the OM/DW ratio is unsuitable for representing the differences in performance observed and appears to be a poor indicator of quality in *C. gigas* larvae. These results disagree with those of Lucas et al. (1986) and Whyte et al. (1987) who consider this ratio to be a good indicator of larval competence for metamorphosis in the mussel *Mytilus edulis* and the Yesso scallop *Patinopecten yessoensis* but without providing evidence to support this assertion. These previous studies do, however, agree with results reported by Robert et al. (1999), demonstrating a weak correlation between this ratio and the percentage metamorphosis in *P. maximus.*

Our results also show that *C. gigas* larvae have a certain tolerance to limited food deprivation and rapid physiological recuperation capacity. The first food deprivation period, D2D6, did not have a strong influence on the biology of the studied individuals. In the first experiment, the larvae in this treatment had a similar growth rate to the control, despite having a shorter cumulative period of feeding (12 *vs* 14 days), and subsequently had a better percentage metamorphosis. Conversely, in the second experiment, the growth rate of the larvae in the earliest feed deprivation treatment was below that of the control, although this difference had no repercussions for the percentage metamorphosis achieved. These differences in larval growth could be explained by difference in the initial levels of lipid reserves between the two experiments. In fact, at day 2, while lipids covered 22 % of the larval surface in the first experiment, they only covered 7 % in the larvae of the second experiment. Differences of this type cannot, however, explain differential mortalities such as those previously reported in *O. edulis* (Holland and Spencer, 1973), *Mercenaria mercenaria* and *C. virginica* (Gallager and Mann, 1986). Although food deprivation at the start of larval development (D2D6) induces low subsequent consequences because larvae of this stage are still to a large extent dependent on their vitellinous reserves (Rico-Villa et al., 2009), food deprivation at the end of the first week (D6D10) appears to be more critical. In the first experiment, the D6D10 treatment led to relatively low survival, poor growth recovery and an incapacity for metamorphosis. The second experiment had high survival and larval competence despite the low initial lipid content. It is interesting to see that the normally-fed controls of the first experiment had higher growth than those of the second experiment (16 *vs* 13 µm d^{-1}), while other indicators of larval development (survival, competence and metamorphosis) were similar between the two experiments. Difference in larval resistance between the two experiments to the imposed nutritional stress at D6-D10 could be explained by a higher energetic consumption in the first experiment, during the first week, due to faster growth. In this condition initial reserves, however higher in experiment 1, were actively catabolized and accordingly larvae were more fragile. In *C. gigas*, this period of larval development corresponds to a transition between the mixotrophic phase, during which the larvae draw most of their energy from their endogenous reserves and, to a lesser extent, from exogenous planktonic particles, and the exotrophic phase, where microalgae added to the rearing vessel come to play a vital role in larval development, notably in metamorphosis success. Whatever index is used, (OLI or TAG/ST) results show that, following food deprivation, neutral lipids were totally consumed by day 6; this demonstrates that *C. gigas* uses all its endogenous reserves in the first week at a rearing temperature of 25 °C. As a result, larval use of the exogenous food supply is limited during the early days of rearing, in line with results of Labarta et al. (1999b) on *O. edulis*, but is necessary, probably for stimulating the digestive system. The transition to strict exotrophy is a period when larvae are particularly sensitive to energy deficits. While in *O. edulis*, a point of no return is reached when endogenous reserves are exhausted (Labarta et al., 1999b), the temporary food deprivation period of 4 days applied in this study led to intense catabolism of reserves in C. gigas larvae, which did not affect their ability to reconstitute their reserves once they were re-fed. Additionally, in continuously-fed larvae, the low level of neutral lipids present at day 6 (3 - 6 %, 1.2 ng TAG) confirms that the endogenous reserves had been consumed and that the larvae had started to consume microalgae, although they were not yet able to accumulate lipid reserves. Ingestion of microalgae has been reported from D-stage, only a few hours after the formation of the prodissoconch I (Lucas and Rangel, 1983; Robert, 1998). This incapacity for lipid storage during the first week of larval life could be due either to a vital need to use up the

endogenous lipid reserves before accumulating new exogenous reserves, or to a deficient enzymatic system capable of lipolysis but not yet able to perform liposynthesis, as shown by larvae of Solea senegalensis (Fehri-Bedoui et al., 2000). To decide between these hypotheses, the quantities of specific enzymes would need to be measured in the early larval stages of C. gigas. Our work demonstrates that the lipid reserves start to become evident from the tenth day (19 – 21 % and 28 ng TAG); it is these reserves, linked to high microalgae consumption, that prevent the food deprivation treatments after the 10th day (D10D14 and D14D18) from causing complete exhaustion of the lipid reserves. This lipid accumulation appears to occur earlier in the scallop P. maximus, as it starts at day 6, 4 days after the first feeding (Delaunay et al., 1992). It is slightly later in M. edulis, with the end of vitellus use at day 8, followed by a strong increase in the lipid content corresponding to reserves build- up (Lucas et al., 1986). It is also interesting to note that metamorphosis is not linked to a particular level of lipid reserves: batches D2D6 and D6D10 contained more TAG and neutral lipid reserves than the control at day 10 but were not capable of metamorphosis. Similar observations were made on M. edulis, where the addition of lipid microspheres at the end of larval rearing did not improve the rate of metamorphosis (Pernet et al., 2004). Other criteria, like minimum size, are probably necessary for individuals to become competent to metamorphose. The minimum size necessary for metamorphosis is estimated at 250-290 µm in M. edulis (Coon et al., 1990) and 300-320 µm in C. gigas (Helm et al., 2004).

C. gigas larvae show a great plasticity when faced with temporary food deprivation, which is a strong stress but a short one, and have a large capacity for recovery and ability to return to their initial physiological state as soon as good nutritional conditions are re-established. To cope with a trophic stress such as this, the larvae showed adaptive strategies based on a reduction of their metabolism (a vital strategy for energy economy); this was revealed by the slowing or stopping of increases in weight and size and a total or partial consumption of their lipid reserves to maintain their basal metabolism. Additionally, we noted that unfed veligers stopped swimming and confined themselves to the bottom of their tanks or beakers, presumably to minimise energy use.

The colorimetric approach is a simple technique, which is rapid to set up and requires little biological material (Ben Kheder et al., submitted). Nile Red is a fluorescent dye specific for lipids (Greenspan and Fowler, 1985), with different coloration according to lipid type: neutral lipids are coloured yellow and phospholipids emit weak red-orange fluorescence (Greenspan et al., 1985; Brown et al., 1992). The results provided by this technique corroborate the data obtained by the biochemical technique, as a good correlation is shown between these two indices (OLI and TAG/ST). The results reported here and those from our previous study (Ben Kheder et al., 2010) demonstrate that this method can be used to understand the evolution of lipid reserves over time. The correlation between OLI and larval rearing performances was not entirely satisfactory, however, as low or null indices were not always associated with poor results in terms of growth, survival or metamorphosis. This is probably linked to the large capacity for recuperation shown by *C. gigas* larvae and because lipids are most likely not the only limiting factor for *C. gigas* larval development.

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Figures

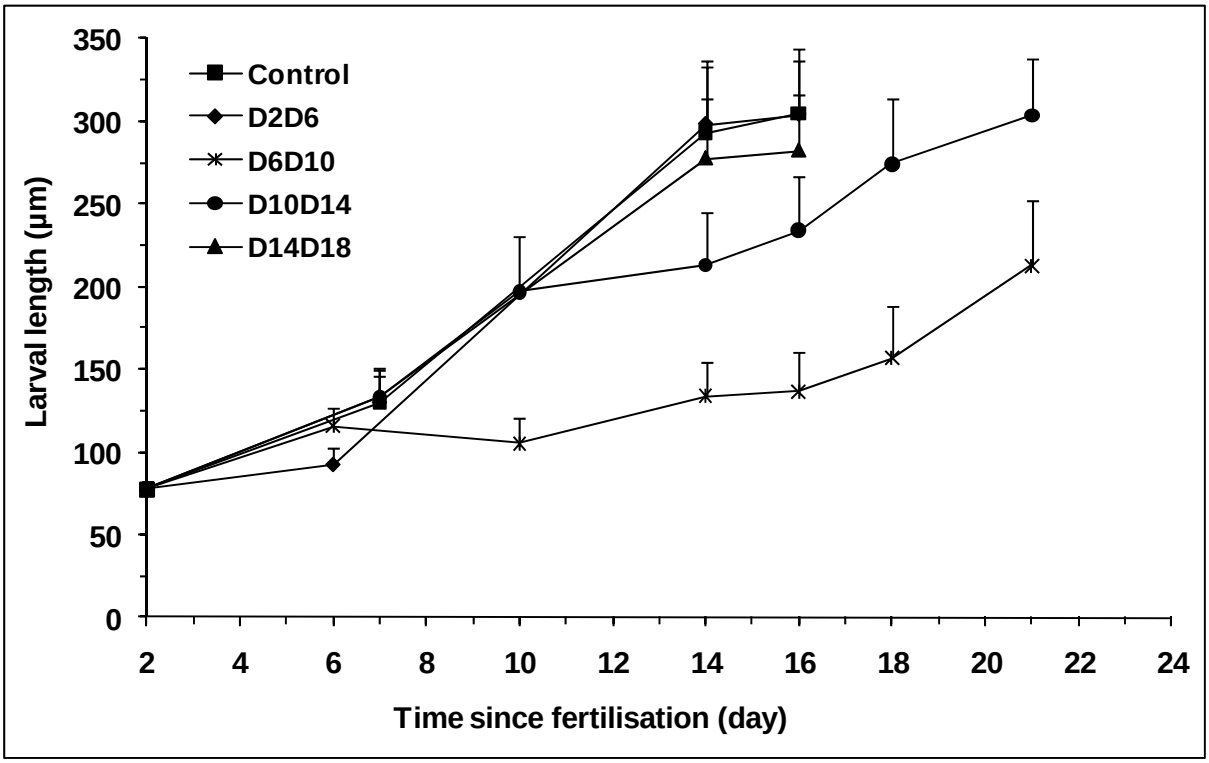

Fig. 1

Fig. 1. Larval growth (mean ± SD; n ≥ 100) of *C. gigas* larvae normally fed (control) or exposed to 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (first experiment).

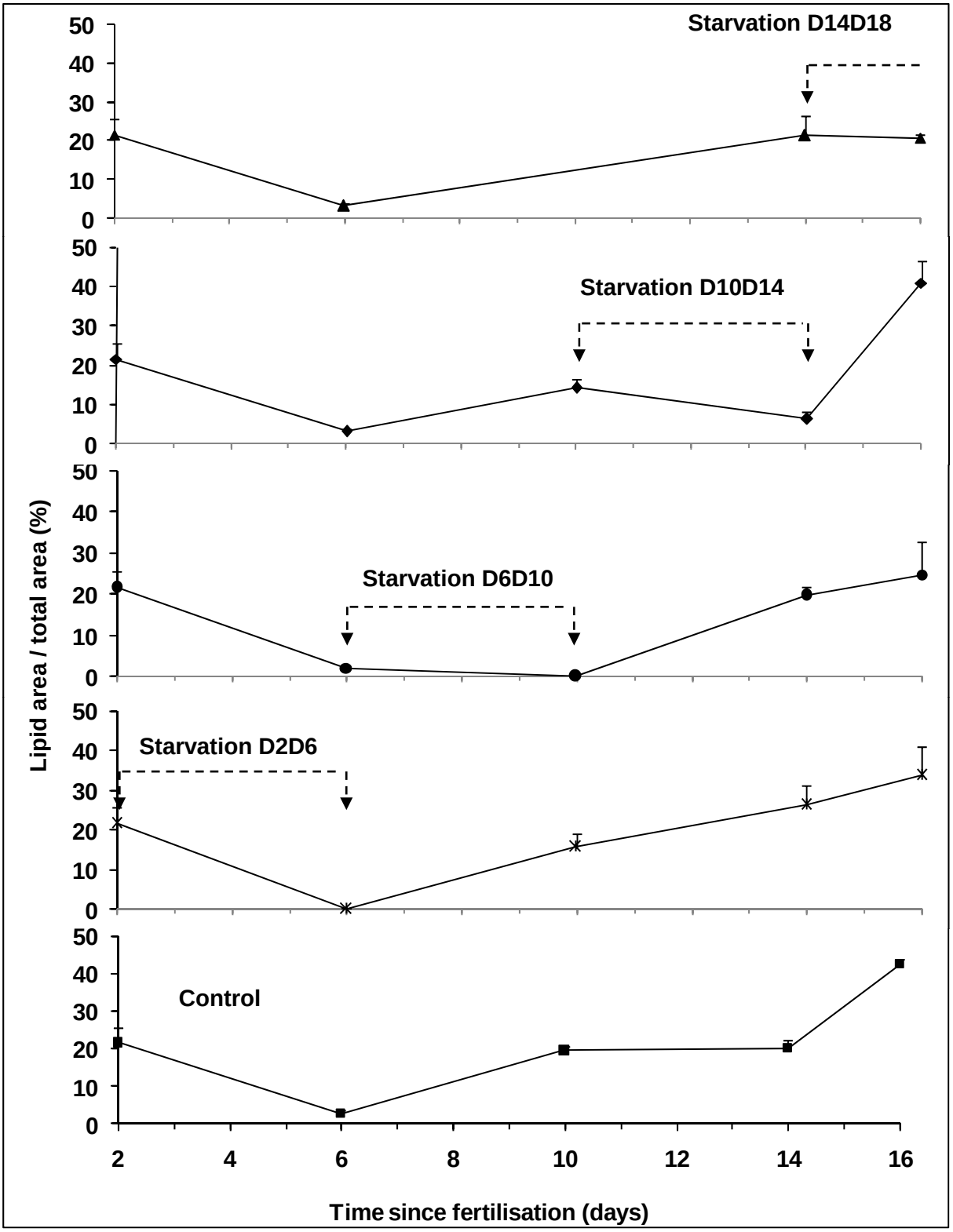

Fig. 2.

Fig. 2. Overall lipid index evolution (mean ± SD; n =3) in *C. gigas* larvae normally fed (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (first experiment). Arrows and dashed lines indicate food deprivation periods.

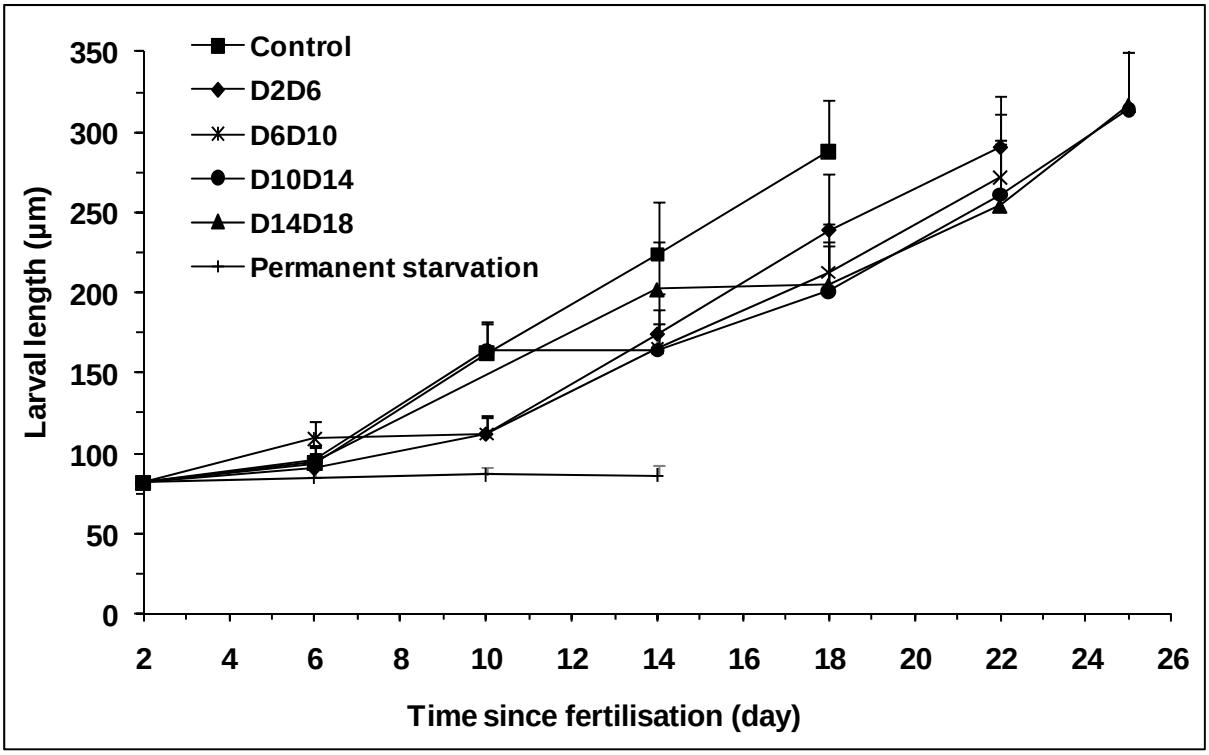

Fig. 3

Fig. 3. Larval growth (mean ± SD; n ≥ 100) of *C. gigas* larvae normally fed (control), unfed (permanent starvation) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment).

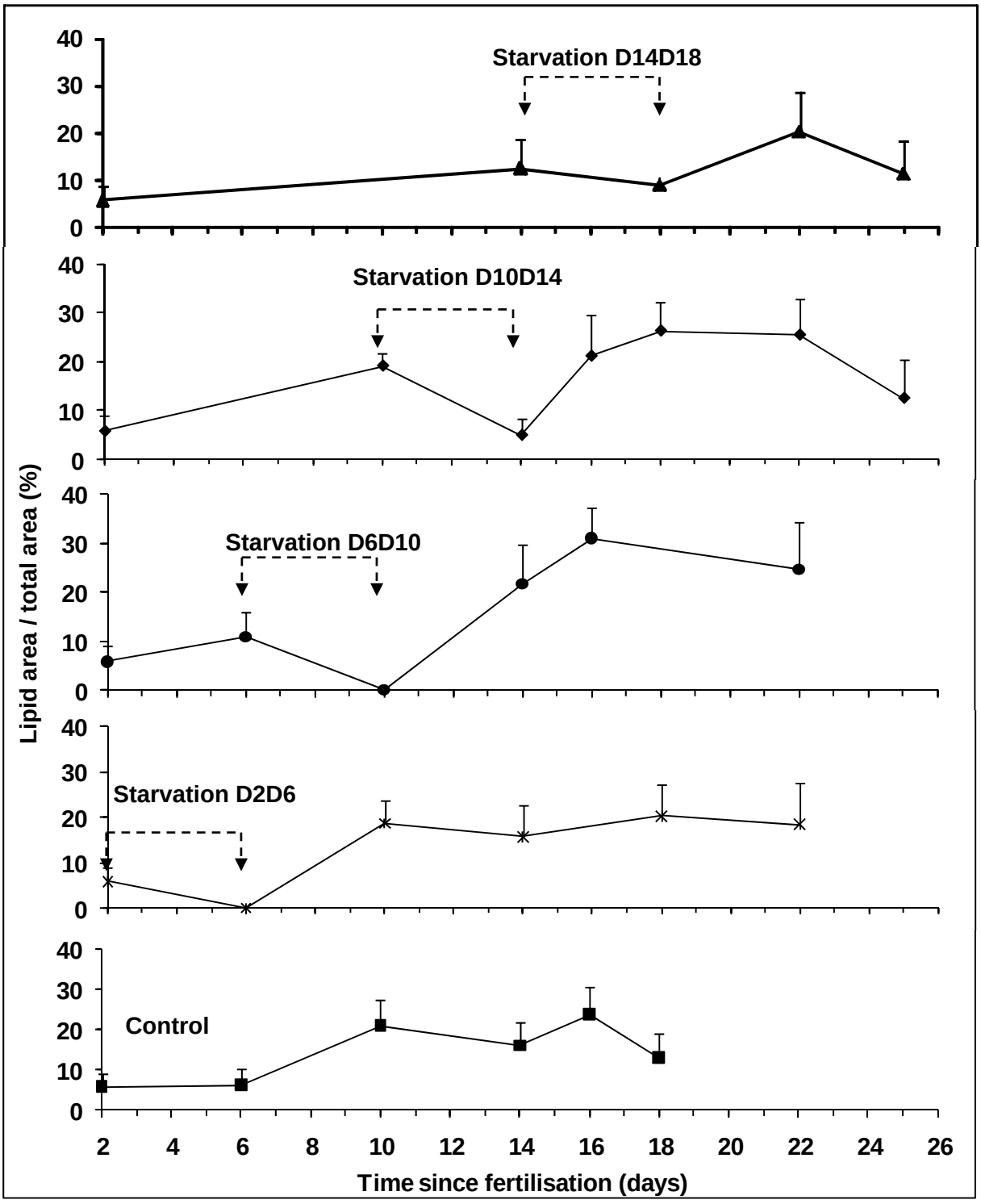

Fig. 4

Fig. 4. Overall lipid index evolution (mean ± SD; n =3) in *C. gigas* larvae normally fed (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Arrows and dashed lines indicate food deprivation periods.

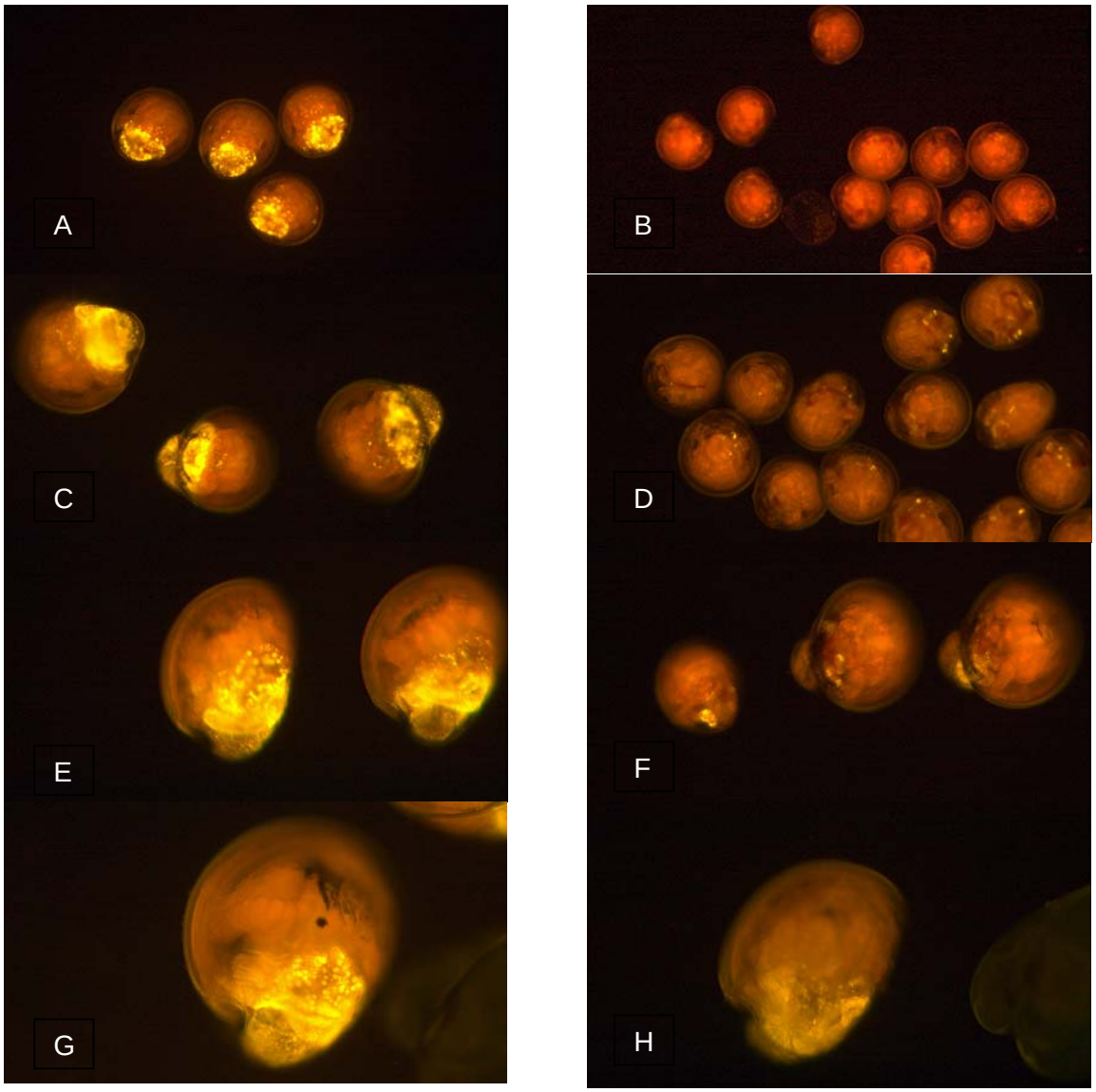

Fig. 5.

Fig. 5. Metabolism of lipid reserves in *C. gigas* larvae normally fed (control) having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). On the left, the yellow areas correspond to Nile Red-stained lipid reserves after fluorescent light exposition on continuously-fed larvae aged 6 d (94 ± 12 µm: **A**), 10 d (162 ± 20 µm: **C**), 14 d (224 ± 33 µm: **E**), 18 d (288 ± 33 µm: **G**).
On the right, the small or absent areas of lipid reserves correspond to larvae exposed to 4 consecutive days of food deprivation at 4 different periods of larval life (second experiment). Larvae were aged 6 d (treatment D2D6: **B**), 10 d (treatment D6D10: **D**), 14 d (treatment D10D14: **F**) and 18 d (treatment D14D18: **H**).

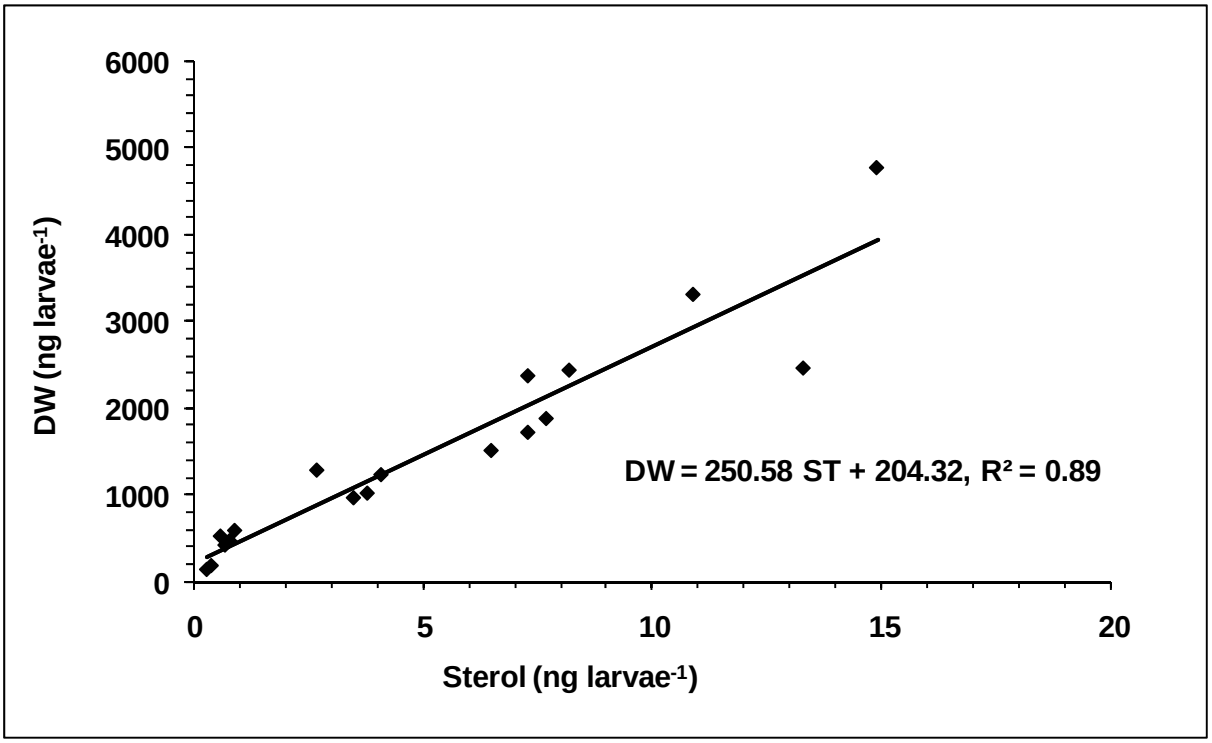

Fig. 6.

Fig. 6. Correlation between sterol content and dry weight in *C. gigas* larvae.

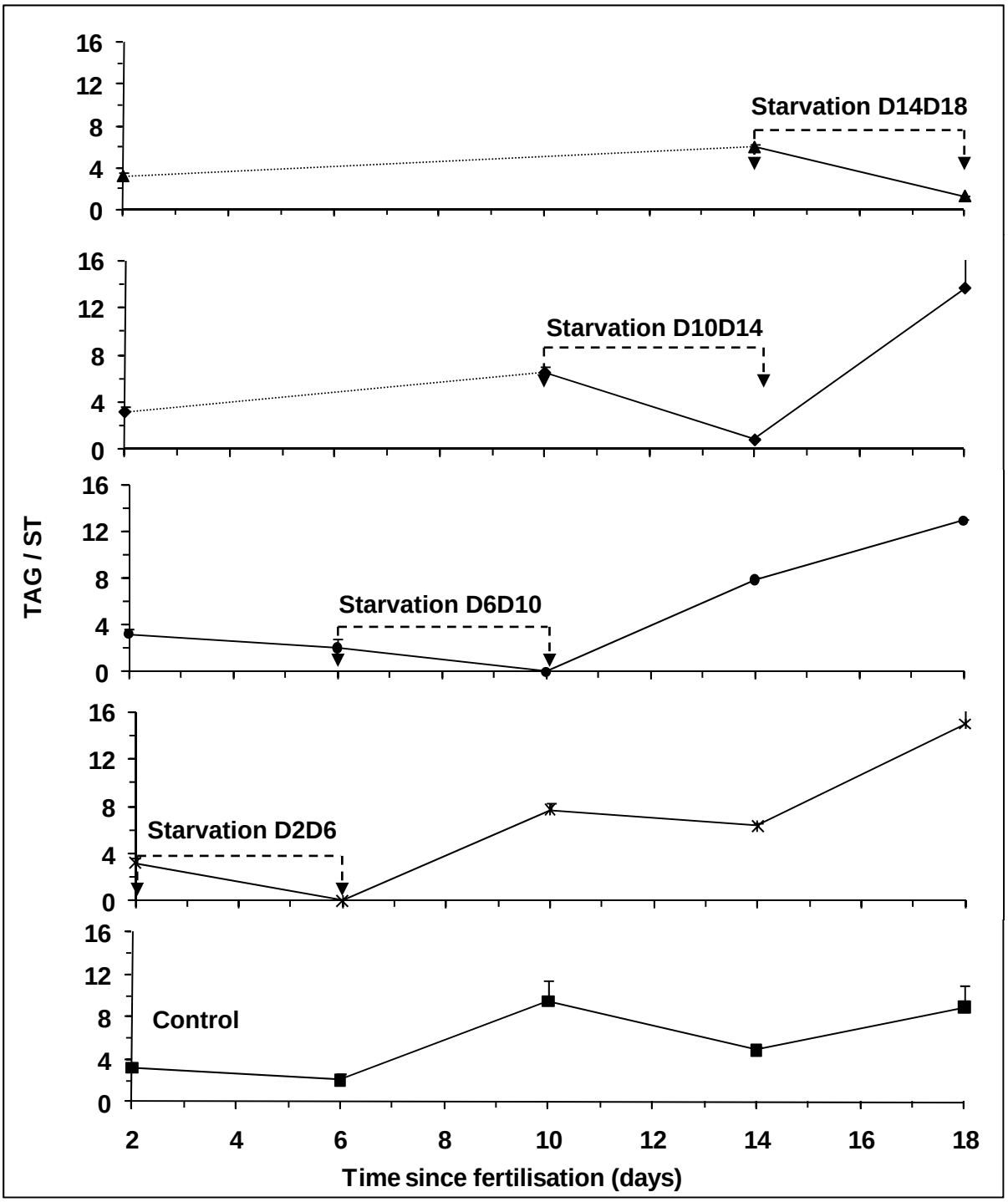

Fig. 7.

Fig. 7. Triacylglycerols / sterols (TAG/ST) content evolution (mean ± SD; n = 2) in *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Arrows and dashed lines indicate starvation periods.

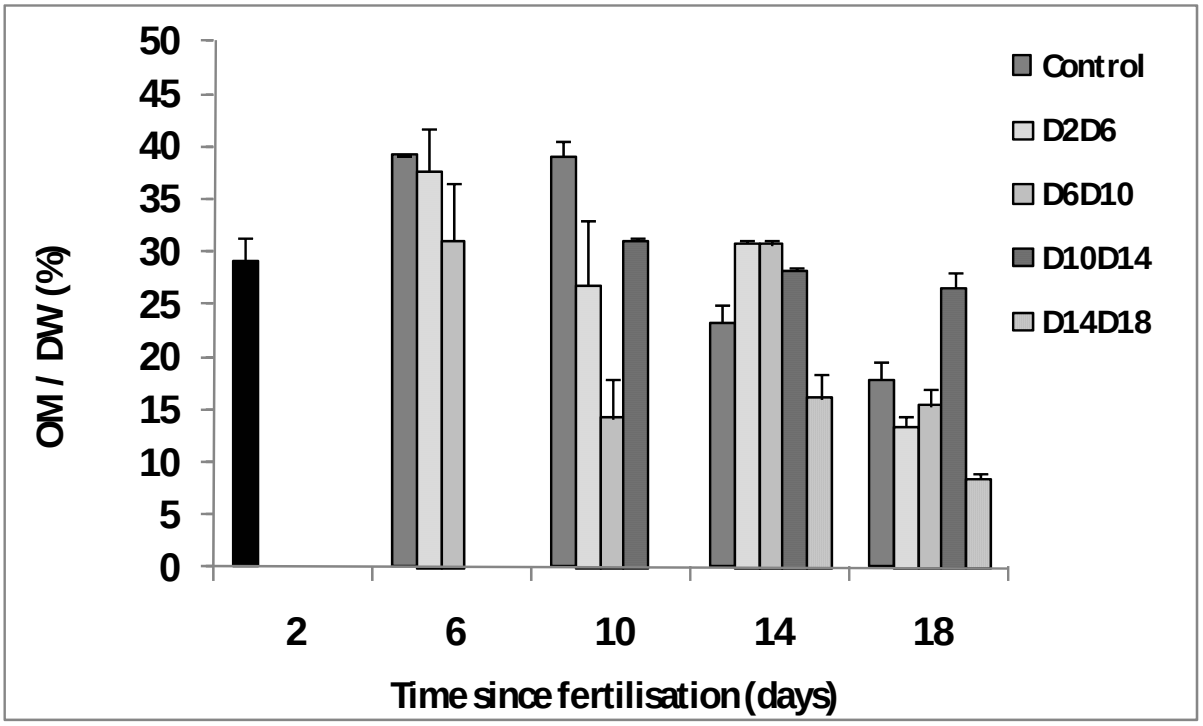

Fig. 8.

Fig. 8. Organic matter / Dry weight (OM/DW) (mean ± SD; n = 2) evolution in *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment).

Tables

Table 1.
Survival, competence and metamorphosis (mean ± SD; n = 3) of *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (first experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.

Table 2.
Survival (mean ± SD; n = 3) of *C. gigas* larvae continuously fed (control), unfed (starved larvae) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.

Table 3.
Larval period length (days), competence and metamorphosis (mean ± SD; n = 3; %) of *C. gigas* larvae continuously fed (control), unfed (starved larvae) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.

Table 4.
Triacylglycerol content (mean ± SD; n = 2; ng $larva^{-1}$) evolution in *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.
nd: not determined

Table 5.
Sterol content (mean ± SD; n = 2; ng $larva^{-1}$) evolution in *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.
nd: not determined

Table 6.
Dry weight (mean ± SD; n = 2; ng $larva^{-1}$) evolution in *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.
nd: not determined

Table 7.
Dry organic weight (mean ± SD; n = 2; ng $larva^{-1}$) evolution in *C. gigas* larvae fed continuously (control) or having had 4 consecutive days of food deprivation at 4 different periods of larval life according to treatment (second experiment). Values within the same column with a common superscript letter are not significantly different at $p > 0.05$.

nd: not determined

Table 1.

Treatments	Larval life (days)	Larval survival (%)			Larval competence (%)	Metamorphosis (%)
		D7	D14	End of experiment		
Control (TCp)	**16**	**97.3 (3.6)^{ab}**	**95.8 (6.1)^{ac}**	**75.5 (12.9)^{ac}**	**80.5 (10.9)^{a}**	**74.0 (8.1)^{bd}**
Starved larvae	**21**	**98.0 (4.1)^{ab}**	**90.9 (7.5)^{ac}**	**79.0 (10.2)^{a}**	**0**	**0**
D2D6	**16**	**98.5 (3.0)^{ab}**	**85.2 (11.8)^{c}**	**75.7 (5.2)^{ac}**	**80.1 (13.4)^{a}**	**92.9 (4.6)^{c}**
D6D10	**21**	**98.2 (3.5)^{ab}**	**58.5 (14.4)^{b}**	**44.8 (9.5)^{b}**	**0**	**0**
D10D14	**21**	**98.6 (2.9)^{ab}**	**64.1 (15.6)^{b}**	**62.0 (8.7)^{bc}**	**59.0 (7.2)^{b}**	**63.1 (8.4)^{ad}**
D14D18	**16**	**99.6 (1.7)^{b}**	**96.2 (7.3)^{a}**	**85.4 (8.4)^{a}**	**61.6 (9.5)^{b}**	**58.3 (8.4)^{a}**

Table 2.

Treatments	Larval life (days)	Survival (%)				
		D6	D14	D18	D22	D25
Control (TCp)	**18**	**85.0 (7.5)^{a}**	**79.7 (9.1)^{a}**	**78.3 (12.1)^{a}**	**---**	**---**
Starved larvae	**25**	**96.0 (3.5)^{a}**	**91.7 (5.0)^{a}**	**nd**	**nd**	**44.5 (7.8)^{b}**
D2D6	**22**	**90.7 (5.1)^{a}**	**93.7 (3.1)^{a}**	**88.7 (5.7)^{a}**	**86.3 (4.5)^{a}**	**---**
D6D10	**22**	**91.7 (2.5)^{a}**	**89.7 (4.9)^{a}**	**91.0 (0.0)^{a}**	**86.0 (5.7)^{a}**	**---**
D10D14	**25**	**91.7 (4.0)^{a}**	**91.0 (3.6)^{a}**	**87.7 (8.7)^{a}**	**87.7 (3.8)^{a}**	**84.3 (4.7)^{a}**
D14D18	**25**	**92.7 (2.1)^{a}**	**90.3 (7.5)^{a}**	**80.0 (4.6)^{a}**	**76.3(9.0)^{a}**	**76.3 (15.9)^{ab}**

Table 3.

Treatments	Larval life (days)	Competence (%)			Metamorphosis (%)
		D18	**D22**	**D25**	
Control (TCp)	**18**	**75.5 (6.4)^{b}**	**---**	**---**	**79.0 (7.8)^{a}**
Starved larvae	**25**	**0 (0.0)^{a}**	**0 (0.0)^{d}**	**0 (0.0)^{b}**	**0 (0.0)^{d}**
D2D6	**22**	**35.0 (4.6)^{c}**	**84.5 (10.6)^{b}**	**---**	**66.3 (10.1)^{ac}**
D6D10	**22**	**3 (1) ^{d}**	**37.3 (4.5)^{c}**	**---**	**65.3 (4.5)^{ab}**
D10D14	**25**	**0 (0.0)^{a}**	**21.0 (4.0)^{ac}**	**71.5 (20.5)^{a}**	**65.7 (1.5)^{ac}**
D14D18	**25**	**0 (0.0)^{a}**	**14.0 (9.5)^{a}**	**67.0 (18.4)^{a}**	**56.0 (6.0)^{bc}**

Table 4.

Age of larvae (days)	Control	D2D6	D6D10	D10D14	D14D18
2	**0.9 (0.1)^{a}**	**0.9 (0.1)^{a}**	**0.9 (0.1)^{a}**	**0.9 (0.1)^{a}**	**0.9 (0.1)^{a}**
6	**1.2 (0.2)^{a}**	**0.0 (0.0)^{a}**	**1.3 (0.2)^{a}**	**nd**	**nd**
10	**31.7 (4.5)^{b}**	**7.0 (0.8)^{ab}**	**0.0 (0.0)^{a}**	**24.3 (1.8)^{b}**	**nd**
14	**34.6 (0.1)^{b}**	**41.3 (3.4)^{b}**	**30.1 (0.7)^{a}**	**2.3 (0.0)^{a}**	**49.7 (3.2)^{b}**
18	**125.2 (3.6)^{c}**	**177.0 (16.0)^{c}**	**169.1 (26.4)^{b}**	**92.6 (5.8)^{c}**	**9.3 (2.1)^{a}**

Table 5.

Age of larvae (days)	Control	D2D6	D6D10	D10D14	D14D18
2	**0.3 (0.0)^{a}**	**0.3 (0.0)^{a}**	**0.3 (0.0)^{a}**	**0.3 (0.0)^{a}**	**0.3 (0.0)^{a}**
6	**0.6 (0.1)^{a}**	**0.4 (0.0)^{a}**	**0.7 (0.1)^{a}**	**nd**	**nd**
10	**3.3 (0.2)^{ab}**	**0.9 (0.0)^{a}**	**0.8 (0.1)^{a}**	**3.7 (0.0)^{b}**	**nd**
14	**7.1 (0.3)^{b}**	**6.5 (0.3)^{b}**	**3.8 (0.0)^{a}**	**2.8 (0.1)^{ab}**	**8.3 (0.1)^{b}**
18	**14.4 (3.0)^{c}**	**11.5 (0.2)^{c}**	**13.1 (2.1)^{b}**	**6.9 (1.2)^{c}**	**7.3 (1.0)^{b}**

Table 6.

4.1.1. Age of larvae (days)	Control	D2D6	D6D10	D10D14	D14D18
2	**131 (1) ^{a}**	**131 (1) ^{a}**	**131 (1) ^{a}**	**131 (1) ^{a}**	**131 (1) ^{a}**
6	**517 (30)^{ab}**	**176 (5)^{b}**	**413 (18)^{c}**	**nd**	**nd**
10	**960 (2)^{b}**	**582 (175)^{ab}**	**463 (41)^{ab}**	**1227 (90)^{b}**	**nd**
14	**1876 (43)^{c}**	**1506 (37)^{b}**	**1013 (74)^{b}**	**1279 (38)^{b}**	**2435 (16)^{b}**
18	**4776 (265)^{d}**	**3311 (583)^{c}**	**2459 (274)^{c}**	**1716 (52)^{c}**	**2370 (65)^{b}**

Table 7.

Age of larvae (days)	Control	D2D6	D6D10	D10D14	D14D18
2	**38 (3)** [a]	**38 (3)** [a]	**38 (3)** [a]	**38 (3)** [a]	**38 (3)** [a]
6	**202 (11)**[b]	**66 (5)**[a]	**129 (29)**[a]	**nd**	**nd**
10	**375 (14)**[c]	**161 (82)**[a]	**66 (23)**[a]	**381 (24)**[b]	**nd**
14	**436 (24)**[c]	**442 (46)**[b]	**311 (26)**[b]	**362 (7)** [b]	**393 (52)**[b]
18	**849 (40)**[d]	**465 (6)**[b]	**378 (2)**[b]	**457 (10)**[c]	**200 (5)**[c]