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Disruption of amylase genes by RNA interference affects reproduction in the Pacific oyster *Crassostrea gigas*

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Abstract :

Feeding strategies and digestive capacities can have important implications for variation in energetic pathways associated with ecological and economically important traits, such as growth or reproduction in bivalve species. Here, we investigated the role of amylase in the digestive processes of *Crassostrea gigas*, using *in vivo* RNA interference. This approach also allowed us to investigate the relationship between energy intake by feeding and gametogenesis in oysters. Double-stranded (ds)RNA designed to target the two α-amylase genes A and B was injected *in vivo* into the visceral mass of oysters at two doses. These treatments caused significant reductions in mean mRNA levels of the amylase genes: −50.7% and −59% mRNA A, and −71.9% and −70.6% mRNA B in 15 and 75 µg dsRNA-injected oysters, respectively, relative to controls. Interestingly, reproductive knock-down phenotypes were observed for both sexes at 48 days post-injection, with a significant reduction of the gonad area (−22.5% relative to controls) and germ cell under-proliferation revealed by histology. In response to the higher dose of dsRNA, we also observed reductions in amylase activity (−53%) and absorption efficiency (−5%). Based on these data, dynamic energy budget modeling showed that the limitation of energy intake by feeding that was induced by injection of amylase dsRNA was insufficient to affect gonadic development at the level observed in the present study. This finding suggests that other driving mechanisms, such as endogenous hormonal modulation, might significantly change energy allocation to reproduction, and increase the maintenance rate in oysters in response to dsRNA injection.

Keywords : alpha-Amylase, dsRNA, Gametogenesis, Marine bivalve, Dynamic energy budget

1. Introduction

Energy allocation in marine bivalves is of major concern for aquaculture production of these species as it directly governs growth and reproduction. Knowledge of feeding strategies and digestive capacities can provide an understanding of energetic pathways and their effects on many aspects of bivalve physiology. Digestive enzyme activities and their controlling mechanisms are important for maximizing absorption and food conversion efficiencies (Bayne, 1976; Ibarrola et al., 1998, 2000). Among digestive enzymes, α-amylase (α-1,4 glucan-4-glucanohydrolase) is a key enzyme for carbohydrate digestion and is possibly a limiting factor for the absorption efficiency of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) (Moal et al., 2000; Sellos et al., 2003). More broadly, α-amylase can represent individual energy status, as observed in *Drosophila* when starch is the only carbohydrate source in the substrate (Powell and Andjelkovic, 1983). α-amylase catalyzes the hydrolysis of internal (α-1,4) glucoside bonds in starch of related polysaccharides and oligosaccharides. In *C. gigas*, two genes (*AMYA* and *AMYB*) have been characterized. These code for two amylase mRNAs that encode mature proteins of 500 and 499 amino acids, respectively, and which have 94% similarity (Sellos et al., 2003). Both genes are transcribed at different levels depending on the tissue. A strong preferential expression of these amylase genes was observed in the digestive gland, leading to high amylase activities that correspond to the digestive function of this organ (Huvet et al., 2003), while significant expression of *AMYB* was also detected in the labial palps (Sellos et al., 2003). Food was demonstrated to constitute an external regulatory factor for amylase expression in oyster digestive glands, differently affecting the mRNA expression of both genes. Indeed, AMYA was observed to be regulated at the mRNA level when food varied quantitatively (Huvet et al., 2003), whereas AMYB was regulated by starch concentration in the food (Huvet et al., 2012); these variations affected amylase enzymatic properties, activity and apparent Michaelis-Menten constant. Associations between amylase expression, growth and consumption-related traits were previously established by studying contrasting genotypes (Prudence et al., 2006; Huvet et al., 2008). The polymorphism of *AMYA* and *AMYB* was analyzed, revealing that the fastest growing genotypes had the highest values of ingestion and, to a lesser extent, absorption efficiency.

The rate of energy intake and subsequent energy allocation has fundamental consequences at individual and population levels through growth and reproduction processes. Reproduction is indeed of particular concern for marine bivalves, and especially oysters, as they display very high fecundity, a characteristic feature of the “r” demographic strategy (Williams, 1975). The high fecundity of *C. gigas*, which produces large numbers of propagules and broadcasts them into a hostile environment, is commonly viewed as an evolutionary response to intense and unpredictable mortality at early life history stages. As a result, gametogenesis has a major impact on several physiological functions, generating phenotypic and genetic trade-offs with growth and survival (Ernande et al., 2004; Huvet et al., 2010). From a physiological point of view, reproduction and especially the number of gametes produced are already known to be positively regulated by food availability and by energy reserves made prior to the completion of gametogenesis (Cannuel and Beninger, 2005; Chavez-Villalba et al., 2002a,b; Muranaka and Lannan, 1984).

A mechanistic understanding of energy allocation and of the effects of disturbances on bioenergetics, such as on energy intake, is therefore of primary interest. Energy budget models can help in this respect by describing physiological processes linked with the energetics of marine bivalves in response to environmental fluctuations (see Alunno-Bruscia et al., 2011, for a summary). Among the energetic models developed and used for bivalves, dynamic energy

budget (DEB) models have provided rates at which organisms assimilate and use energy for maintenance, growth and reproduction (*e.g.*, Pouvreau et al., 2006; Alunno-Bruscia et al., 2011). The recently improved version of the DEB model for *C. gigas* focused on the description of reproduction-related features and permitted a description of energy allocation to the gametogenesis and resorption processes (Bernard et al., 2011). Beyond the description of effects of variability in environmental forcing on energy intake and use, the DEB model appears to be a valuable tool for studying disturbance in individual energetics (Kooijman and Bedaux, 1996). For instance, in the DEB model framework, toxicants may impact an individual‟s energetics by changing one or more parameter values and consequently modulate energy fluxes (Kooijman, 2010; Muller et al., 2010).

To document the effects of feeding strategy and digestive capacity on growth and reproduction, which are of great importance for aquaculture production, we aimed to study the consequences of disruption of the amylase genes in the Pacific oyster *Crassostrea gigas*, one of the most important aquaculture species worldwide. To do so, we performed short- and long-term RNA interference (RNAi) experiments on *C. gigas*, targeting the *amylase* genes so as to evaluate their role in oyster digestive processes and improve knowledge on the relationships between food, absorption processes and reproduction. For our long-term experiment, double-stranded (ds)RNA designed to target both the amylase genes (*AMYA* and *AMYB*) was injected *in vivo* into oysters at the onset of the gametogenesis. These were then analyzed for amylase expression at the mRNA and enzymatic levels and for gametogenic development using histological methods forty-eight days post injection, which is the period needed to achieve experimentally a complete gonadal development (see Fabioux et al., 2005). Then, to investigate the influence that amylase genes might have on ecophysiological traits, oysters were monitored in a second, short-term, RNAi experiment on ecophysiological behavior. In this context and based on literature (*e.g*. Savina & Pouvreau, 2004; Huvet et al., 2008), a 12-day experiment was conducted to record repeated measurements of ecophysiological traits. This second experiment made it possible to run the oyster DEB model and compare experimental data and model output to evaluate how physiological changes induced by RNA interference might cause changes in energy fluxes and explain phenotypic changes (*i.e.*, growth, reproduction) observed in the first RNA interference experiment.

2. Results

2.1. Exp1: Effects of dsRNA injection on growth and reproduction

2.1.1. Gene expression

To validate our *in vivo* amylase dsRNA administration procedure and confirm its effectiveness in oyster digestive glands, we assayed *AMYA* and *AMYB* mRNA levels using real-time PCR. Significant mean reductions of *AMYA* mRNA levels of 50.7 ± 30.8 and 59.0 ± 33.8% relative to *actin* mRNA level were observed in 15 and 75 µg dsRNA-injected oysters, respectively, compared with Tris-injected controls ($F = 5.4$; $P = 0.012$; Fig. 1). For *AMYB*, we observed mean significant reductions of 71.9 ± 21.9 and 70.6 ± 26.5% in mRNA level, relative to *actin* mRNA level, in 15 and 75 µg dsRNA-injected oysters, respectively, compared with the controls ($F = 5.61$; $P = 0.0108$; Fig. 1). A 70% inhibition of mRNA levels after dsRNA treatment is commonly considered as a threshold indicating effective RNAi (as proposed by Jiang et al., 2006). For *AMYA*, this threshold was attained in 6 out of the 9 analyzed oysters injected with 15 µg dsRNA,

and for 7 out of the 8 oysters injected with 75 µg dsRNA. For *AMYB*, the 70% threshold was attained for 8 out of the 9 oysters injected with 15 µg dsRNA and for 7 out of the 8 oysters injected with 75 µg dsRNA.

2.1.2. Amylase activity

For specific amylase activity, significant differences were observed between treatments, giving two distinct statistical groups ($F = 3.71$; $P = 0.04$; Fig. 2). Activity estimates were significantly lower by 53% for the 75 µg dsRNA-injected oysters (0.17 ± 0.13 IU.mg^{-1} protein) compared with the 15 µg dsRNA-injected oysters (0.36 ± 0.13 IU.mg^{-1} protein) and the control oysters (0.40 ± 0.19 IU.mg^{-1} protein). Activity estimates were positively correlated with the sum of mRNA levels of *AMYA* and *AMYB (*$R^2 = 0.24$; $P = 0.023$), and individually with *AMYB* ($P = 0.012$) but not with *AMYA* at the 5% level ($P = 0.09$).

2.1.3. Reproductive characteristics

Mean gonad area measured 48 days post-injection fell into 3 statistical groups ($F = 10.7$; $P < 0.001$). The oysters injected with 75 µg amylase dsRNA showed a significantly lower mean gonad area (17.3 ± 15.6%) than oysters injected with 15 µg amylase dsRNA (28.0 ± 14.0%) and controls, which showed the highest mean gonad area (53.3 ± 18.8%). At the individual level, close to 90% (7 out of 8) of the 75 µg dsRNA-injected oysters showed a strongly reduced gonad area compared with the control oysters. Indeed, values ranged from 3.3 to 22.7% of the total area of the visceral mass on histological sections, which is below the smallest value of gonad area observed in the controls (29.5%). Furthermore, for oysters injected with amylase dsRNA, a significant correlation was observed between gonad area and *AMYA* mRNA level ($R^2 = 0.22$, $P = 0.02$) and a correlation that was close to significant was found with *AMYB* mRNA level ($R^2 = 0.12$, $P = 0.06$).

The distribution of reproductive stages appeared significantly different between the three groups (Chi-square test = 15.1, $P = 0.004$). Most of these control oysters (80%) were in stage 3, corresponding to ripeness, whereas the two thirds of the 15 µg amylase dsRNA-injected individuals were in stage 2 (germ cell post-mitotic stage) and half of the 75 µg amylase dsRNA-injected individuals were in stage 1 (germ cell proliferation stage) (Table 1).

We also examined sex-ratio (males / males + females) through histological analysis. Even though some variation in sex distribution was noticeable between groups (Table 1), no significant differences were observed between control and injected groups at the 5% level.

Finally, visual examination under a microscope showed that the cytological aspect of the germ cells themselves appeared normal and was not modified by dsRNA injection (Fig. 3). Similarly, the cellular structure of the digestive gland did not appear to be modified in dsRNA-injected oysters compared with control oysters.

2.1.4. Growth

At the end of the experiment, total mass was significantly lower (71.2 ± 18.0 g) for the 75 µg dsRNA-injected oysters compared with the 15 µg group (80.6 ± 8.3 g) and the controls (82.9 ± 15.8 g) ($F = 5.55$; $P = 0.01$).

2.2. Exp2: Effects of dsRNA injection on ecophysiological traits

2.2.1. Growth and condition index

No significant differences in growth were observed between 75 µg dsRNA-injected and control oysters at day 12, although a tendency was observed for dsRNA-injected oysters to have a lower wet flesh mass (-23%) and digestive gland mass (-10%; Table 2). This results in a significantly higher condition index (+12%) in control oysters (16.1 ± 1.7) compared with dsRNA-injected oysters (14.3 ± 1.6) ($F = 8$; $P = 0.009$; Table 2).

2.2.2. Ecophysiological parameters

Significant differences in absorption efficiency were observed between the treatments ($F = 4.5$; $P = 0.04$; Table 2): the values of dsRNA-injected oysters showed a reduction of 4.9% compared with the control over the experiment as a whole. A date effect was also revealed ($F = 29.4$; $P < 0.001$) showing two statistical groups: t4 (day 4) and t7 showed higher values than t9 and t11. For algal consumption, no significant differences were observed between the controls and the dsRNA-injected group; algal consumption of the dsRNA-injected group being only 2.2% higher than that of the controls over the 10 days of continuous data (Table 2).

2.3. DEB model simulations

Control oysters were simulated with standard DEB model parameters and with the absorption efficiency (AE) measured for control oysters in Exp2 (AE=81.9%; Table 2) (Fig. 4, “control”). Injected oysters were simulated with standard DEB model parameters and the absorption efficiency measured for the 75 µg dsRNA-injected treatment in Exp2 (AE = 77%; Table 2) (Fig. 4, “sim-1”). Simulated relative differences in final dry flesh mass and oocyte production between control and injected treatments were -7.5 and -10.8%, respectively, corresponding to the direct effects of a 4.9% reduction in absorption efficiency. They were therefore below the -21% and -68% measured for flesh mass and gonad area, respectively, in Exp1.

In order to make the model parameters fit with observed relative mass and reproductive effort, numerous simulations were performed with a set of parameters values (i.e. fraction of energy used for growth plus somatic maintenance Kappa from 0 to 1 and volume-specific cost of maintenance [p_M] from 0 to 200 J. $cm^{-3}.d^{-1}$). The best combination of these two parameters was selected, corresponding to the values that lead to the final dry flesh mass and oocyte number observed after 48 days (Fig. 4, “sim-2”). The best fit between observations and simulations was reached with a single set of the two parameters Kappa = 0.765 and [p_M] = 96.8 $J.cm^{-3}.d^{-1}$, which corresponds to an increase of 70 and 120% beyond standard values, respectively (*i.e.*, Kappa = 0.45 and [p_M] = 44 $J.cm^{-3}.d^{-1}$).

3. Discussion

3.1. The role of amylase in absorption efficiency and ingestion

Amylase enzyme logically plays an essential role in the digestion of *C. gigas*. However, in the present study it did not appear to be as crucial for absorption efficiency as previously

hypothesized (Moal et al., 2000; Sellos et al., 2003). Indeed, the 4.9% reduction of absorption efficiency observed in our dsRNA-injected oysters compared with the control group fed on the same monospecific diet of *Chaetoceros gracilis* can be considered low. Absorption efficiency was previously reported to vary to a greater extent between amylase genotypes (maximal variation =10%; Huvet et al., 2008) or depending on food availability. Indeed, absorption efficiency was observed up to a maximum of 75% and down to a minimum of around 20%; the latter occurring when feeding conditions are very bad or the ingested ration is dominated by mineral content (Barillé et al., 1997; Powell et al., 1992). However, neither of these situations was expected under our experimental conditions. The difference between the experiments was the addition of *T. iso* to *C. gracilis* in Exp1 (See Materials and methods), which did not change the quantity of diet. Effects arising from our experimental design are likely negligible considering that differences between such microalgal diets have only been reported to have weak effects on AE (*e.g.*, in mussel, Ren et al., 2006) and on the development of gametogenesis (*e.g.*, in flat oyster, Gonzalez-Araya et al., 2013). These assumptions about digestive mechanisms, energy budget and reproductive control do not allow us to infer what occurs in nature, which is likely to be substantially different considering the complexity of coastal food webs. In any case, we can hypothesize that disruption of 50% of amylase functioning (estimated at 53% on amylase activity) was not enough to strongly affect absorption efficiency. However, this hypothesis should be considered in the light of the fact that two successive injections of dsRNA were performed in Exp1, while there was only one in Exp2, which would also lead us to expect cumulative effects in Exp1. Ingestion did not appear affected in response to amylase disruption, whereas it has previously been shown to be affected by amylase variations linked to amylase polymorphism (Huvet et al., 2008). This was supposedly due to a compensatory effect, a decrease of energy input being offset by an increase of food intake taking into account variations in absorption efficiency.

Nevertheless, we would expect a loss of 4.9% absorption efficiency to affect energy intake and consequently oyster physiology. The total mass (Exp1) and the condition index (Exp2) of 75 µg dsRNA-injected oysters were reduced compared with the control. The condition index is related to the glycogen content, protein fraction and, to a lesser extent, to amounts of lipids, with changes in these contents being associated with the accumulation of storage or nutrients to be used during winter, or to gonad differentiation and production of gametes during spring-summer. In Exp1, disruption of reproductive parameters was observed after two dsRNA injections: a slower gametogenesis and a lower reproductive effort that may originate from a disturbance in energy balance linked to the assimilation machinery. This hypothesis is favored over a direct perturbation of the reproductive cascade because no abnormal cytological appearance of germ cells was observed here, in contrast to situations when specific reproductive genes are silenced (Fabioux et al., 2009; Huvet et al., 2012).

3.2. How can knockdown of reproductive effort be explained?

We suggest that the disruption of amylase genes can lead to a delayed gametogenesis with an associated reduction in reproductive effort, the latter being closely correlated to the temporal dynamics of gametogenesis when considering several modal classes of oocyte diameter through gametogenesis (Lango-Reynoso et al., 2000). On examining the median part of the visceral mass, Royer et al., (2008) found the percentage surface occupied by the gonad to be directly linked to the number and/or size of gametes produced and therefore representative of reproductive effort, which is linked to the proportion of energy allocated to reproduction (Todd and Havenhand 1983; Normand et al., 2009). In the DEB theory, oyster reproductive effort was found to depend mostly on the half-saturation coefficient for ingestion (Bernard et al., 2011). This

environment-specific coefficient reflects quantitative and qualitative variability in the oysters‟ food (Alunno-Bruscia et al., 2011). It integrates all sources of variation in the trophic environment (food sources, nutritive quality of food, fluctuations in the selection of particles before ingestion, absorption efficiency as a function of the food ingested) and the animals themselves (age and/or size of oysters, which affects the half-saturation coefficient probably because of variability in filtration, selection and/or ingestion capacities) (Alunno-Bruscia et al., 2011). In Exp2, control and dsRNA-injected oysters were fed the same monospecific diet composed of *C. gracilis*. A difference in diet cannot therefore account for these variations, which could notably be caused by differences in the energy balance or digestion machinery of the oysters. The values of absorption efficiency obtained for the dsRNA-injected oysters and the control oysters (77 and 81.9%, respectively) were used to model *C. gigas* DEB. The resulting simulations showed that the loss in energy intake implied by this difference in absorption efficiency in Exp1 did not explain the loss in mass (gap of 11.1% between sim-1 and -2) and reproductive effort (gap of 53.8% between sim-1 and -2) observed in dsRNA-injected oysters, even considering a null reserve compartment. Only a decrease of 70% in the fraction of energy allocated to reproduction and an increase of 120% in the cost of maintenance was able to reproduce the losses observed after two injections in Exp1. This result suggests that dsRNA injection causes a high level of metabolic disturbance in oysters, beyond the digestive activity managed by the α-amylase genes targeted in the present work. The fraction of energy allocated to reproduction is known to be affected by endocrine disruptors (Kooijman, 2010). It may be caused by inappropriate activation of the immune response, as the immune and the endocrine systems are intricately connected (Elenkov and Chrousos, 2002). Indeed, it was recently suggested that marine bivalves can recognize dsRNA to initiate an innate immune response that has striking similarities to the vertebrate type-1 interferon response (Choi et al., 2013; Green and Montagnani, 2013; Green et al., 2014). However, long dsRNA has been shown to sequence-specifically inhibit target gene expression in invertebrates (*e.g.*, Fire et al., 1998; Hammond et al., 2000) without any evidence of nonspecific suppression of gene expression (*e.g.*, Fabioux et al., 2009 for molecular reproductive cascade in oyster). Consequently, the present evidence that long dsRNA can trigger a type I interferon response in oysters, as already shown in vertebrates, is an interesting subject for future studies.

On the bases of our results, we can hypothesize that long dsRNA may thereby threaten the physiological integrity of oysters and consequently increase the maintenance costs. Our simulations suggest there is more than a doubling of maintenance costs to account for the phenotypic disturbances observed. Such an effect of a toxicant, related to DEB model parameters, has been described in various species (Kooijman and Bedaux, 1996; Muller et al., 2010). Previous studies have suggested a threshold effect of toxicant depending on the body burden, with a threshold limit termed “no-effect body burden” (Muller et al., 2010), which could highlight the data on knock-down phenotypes obtained in the 15 and 75 µg dsRNA-injected treatments in Exp1. Indeed, whereas reduction of mRNA levels of *AMYA* and *AMYB* appeared similar in 15- and 75-µg injected oysters, knock-down phenotypes (digestive, reproductive) were mostly observed with the highest injected dose of amylase dsRNA. Further investigations are now required to validate hypotheses resulting from the present modeling approach. The first step in validating the maintenance rate modification up to a 120% increase would be to measure respiration rate.

Food availability, energy balance and reproduction are strongly related in vertebrates through complex regulatory factors (Schneider, 2004). Among these, neuropeptide Y or gonadotropin-releasing hormones act as a switch for nutritional status and reproduction, as demonstrated in vertebrates (*e.g.*, Kalra and Kalra, 2004; Temple et al., 2003) and suggested in invertebrates (de Jong-Brink et al., 2001; Bigot et al., 2012; Bigot et al., 2014). Therefore, these neuropeptides

represent a possible line of investigation into differences in reproductive strategy depending on modification in energy input from environmental and/or endogenous variations, whether natural or induced in the laboratory. Finally, promising new powerful reverse genetics methods, such as CRISPR for RNA-guided transcriptional silencing (Qi et al., 2013), offer innovative new avenues for research on marine invertebrate biology. As shown here, reverse genetics methods are very useful for creating contrasting biological material, providing an alternative to genetically-based material for studies on the physiology of marine bivalves (*e.g.*, Huvet et al., 2008). Further developments in their application will probably also integrate the use of modeling tools.

4. Material and methods

4.1. Biological material and experiments

Experiment 1 (Exp1): Effects of dsRNA injection on growth and reproduction. Two-year-old oysters were obtained from cultured stocks in Plougastel-Daoulas (Bay of Brest, France). In February 2010, these oysters were transferred to Ifremer"s experimental facilities in Argenton (Finistère, France) at stage 0, corresponding to the undifferentiated stage according to the reproductive scale of Steele & Mulcahy (1999). They were then conditioned for 2 months in a suitable environment for germ cell maturation. Briefly, the oysters were placed in experimental raceways supplied with 1-µm filtered running seawater at 19 ± 1.0°C and fed with a mixed diet of two microalgae: 50% *Chaetoceros gracilis* (UTEX LB2658) and 50% *Tisochrysis lutea* (formerly Isochrysis sp., Tahitian clone: T. iso; CCAP 927/14) at a daily ration equal to 8% dry mass algae / dry mass oyster (see Fabioux et al., 2005 for details on oyster conditioning).

Experiment 2 (Exp2): Effects of dsRNA injection on ecophysiological traits. For each treatment, 60 two-year-old oysters were conditioned for 12 days (October 2012) in triplicate tanks under the same conditions as described above, with the exception of the diet, which was mono-specific *Chaetoceros gracilis*. Two days after their placement, seawater sampling was started twice a day (morning and afternoon) at the inlet and outlet of each experimental tank and in a control raceway without oysters. Phytoplankton counts were made using an electronic particle counter (Multisizer 3, Beckman Coulter International S.A., Nyon, Switzerland) to provide 10 days of continuous data (from t2 to t12). Algal consumption (C) was expressed in cell volume per oyster per day ($\mu m^3.oyster^{-1}.d^{-1}$) as $C = [(C_e-C_s-C_b) \times D] / n$; C_e being the cell volume at the inlet, C_s the cell volume at the outlet, C_b the cell volume remaining in the raceway without oysters obtained by subtracting outlet from inlet, D water flow ($mL.min^{-1}$) and n = number of oysters per raceway. Four times, at 4, 7, 9 and 11 days after starting the experiment, biodeposits were collected from each tank to calculate the absorption efficiency (%) of organic matter from food ingested, as described in Savina and Pouvreau (2004) according to the Conover"s method (1966).

4.2. dsRNA synthesis

A fragment from positions 478 to 1130 of amylase cDNA (GenBank accessions AF320688 and AF321515) targeting both *AMYA* and *AMYB* mRNA was amplified by RT-PCR (primers in Huvet et al., 2003) using total RNA extracted from digestive gland as template. Along this 653 bp cDNA fragment, amplified to produce a single dsRNA, some variations exist between *AMY* alleles due to existing polymorphism (Huvet et al., 2008). The lowest identity was 88%, found between the amylase dsRNA and the *AMYB2* allele, which is insufficient to make the dsRNA nonspecific. At

least five intervals of more than 21 identical nucleotides exist in a row along the sequence, which is sufficient to produce an effective silencing of the target mRNA. Furthermore, short interfering RNA (siRNA) experiments have reported that siRNAs can silence target genes with partially complementary nucleotides (Doench et al., 2003) or non-target genes containing as few as 15 consecutive complementary nucleotides (Semizarov et al., 2003). Then, the PCR fragment was sub-cloned into the pCR4-TOPO vector (Invitrogen, Paisley, UK) and sequenced. Recombinant plasmid was purified using the Plasmid midi kit (Qiagen, Venlo, Limburg, Netherlands), linearized with either NotI or SpeI (Promega, Madison, WI, USA) enzymes (4 h at 37°C using 5 $U.\mu g^{-1}$ plasmid), phenol-chloroform extracted and finally ethanol-precipitated and suspended in RNAse-free water (Fabioux et al., 2009). Both strands of the purified plasmid were transcribed *in vitro* using T7 and T3 MEGAscript® Kits (Ambion, Austin, TX, USA) to produce amylase sense and antisense single-stranded RNA (ssRNA). The ssRNA was phenol-chloroform extracted, ethanol-precipitated and suspended in RNAse free saline solution (10mM Tris/10mM NaCl) to obtain a final concentration of 0.5 $\mu g.\mu L^{-1}$. Equimolar amounts of sense and antisense ssRNA were heated to 100°C for 1 min and left to cool at room temperature for 10 hours for annealing. One µg of dsRNA was analyzed by 1% agarose gel electrophoresis to ensure that it consisted of a single band of 653 bp.

4.3. dsRNA administration and sampling

Oysters were anesthetized in $MgCl_2$ solution (3/5 fresh water, 2/5 seawater and 50 $g.L^{-1}$ $MgCl_2$) for 5 hours. In Exp1, anesthetized oysters were injected at t0 and t19 (19 days) in the visceral mass (digestive gland surrounded by mantle-gonad tissue) with 15 or 75 µg amylase dsRNA diluted in 100 µL saline solution (N = 10 each treatment) or with the same volume of saline solution for the control (N = 10). The highest dose, 75 µg, of dsRNA injected per oyster, corresponds to a mean concentration of 10 µg $dsRNA.g^{-1}$ of oyster body mass, which is in the range of the optimal quantity of dsRNA recommended for *in vivo* RNAi experiments in adult oysters (Fabioux et al., 2009). The choice of two successive injections was made with the aim of increasing RNAi efficiency in the case of our long-term experiment (Araujo et al., 2006). After injection, oysters were maintained in raceways under optimal gonad maturation conditions. At t48, all oysters (n = 30, *i.e.*, 10 per treatment) were sampled and weighed (total mass). The digestive glands were immediately dissected from these oysters: a transversal section of the visceral mass was made for histological examination and the rest of the digestive gland was immediately frozen in liquid nitrogen, crushed to a fine powder at -196°C with a Dangoumau mill (Prolabo, Nogent-sur-Marne, France) and stored in liquid nitrogen until the RNA extraction and enzymatic assay were performed. In Exp2, the procedure was similar to that used in Exp1, except that the anesthetized oysters were only injected in the visceral mass at t0 with 75 µg amylase dsRNA (N = 60) or with the same volume of saline solution for the control (N = 60). After 12 days of conditioning (for 10 days of which the stabilized ecophysiological parameters were monitored), 5 oysters were randomly sampled per raceway (*i.e.*, 15 oysters per treatment). Collected oysters were measured in length, weighed (total and wet flesh mass) and their digestive glands dissected and immediately weighed. The condition index MI, as specified by the French norm AFNOR (Bodoy et al., 1986), was estimated using the following equation: (wet flesh mass × 100) / total mass.

4.4. Histology

Gonadic development was examined on histological slides of a transversal section of the whole gonadic area for dsRNA-injected and control oysters at t0 and t48 according to Fabioux et al.

(2005). For each sample, a 3-mm cross-section of the visceral mass was excised in front of the pericardic region and immediately fixed in modified Davidson's solution at 4°C for 48 h. In order to avoid any bias related to the cut of the median section through the visceral mass, these cuts were always made at the same position by the same person and the same manner (Royer et al., 2008). Sections were dehydrated in ascending ethanol solutions, cleared with the xylene substitute Microclearing (Diapath, Italy), and embedded in paraffin wax. Sections of 5 µm were cut, mounted on glass slides and stained with Harry's hematoxylin-eosin. The slides were then examined under a light microscope to determine sex and the gametogenic stage according to the reproductive scale of Steele & Mulcahy (1999). Percentage areas of gonadic tubules, conjunctive tissue and digestive gland were then determined on each histological section. Slides were scanned with a digital scanner (HP scanjet 7400c) and images recorded in TIFF format. Tissue areas were then measured using image analysis software (Imaq Vision Builder, National Instruments Corp., Austin, TX, USA). Gonad area percentage was estimated as pixel number on the gonad / pixel number on total sections, as described in Fabioux et al. (2005). Finally, special attention was paid to the cellular structure of the gonad and the digestive tissues to check for any abnormalities.

4.5. Real-time RT-PCR analysis

Total RNA was isolated using Extract-all reagent (Eurobio, Courtaboeuf, France) at a concentration of 1 mL / 50 mg powder, and treated with DNAse I (Sigma, 1U / µg total RNA). RNA concentrations were measured at 260 nm (1 OD = 40 $\mu g.mL^{-1}$ RNA) using an ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). A 1 µg sample of total RNA was reverse-transcribed following the protocol in Huvet et al. (2003) and amplified in triplicate by real time PCR according to Huvet et al. (2004), using specific amylase primers (Huvet et al., 2008). Amplifications of actin and elongation factor I cDNA were performed in order to confirm the steady-state level of reference gene expression providing an internal control for gene expression. Actin and elongation factor I primers were those used by Huvet et al. (2012) and Fabioux et al. (2004), respectively. The calculation of relative *AMYA* and *AMYB* mRNA levels was normalized to actin, as no significant differences in Cq values were observed for actin between treatments ($F = 1.65$; $P = 0.201$; CV = 2.9%) whereas some were noted for elongation factor I ($F = 3.47$; $P = 0.049$). For a studied gene “i”, results were expressed in arbitrary units as $2^{-\Delta\Delta Cq}$ (Livak and Schmittgen, 2001) with ΔCq = Cq (i) – Cq (actin) and $\Delta\Delta Cq$ = ΔCq of cDNA sample – ΔCq of the cDNA reference.

4.6. Enzymatic analysis

Amylase activity was evaluated using an autoanalyser (Technicon Instrument Corporation, Tarrytown, New York, USA) according to Samain et al. (1977). Briefly, all the samples were ground in distilled water with 0.02 M $CaCl_2$ before analysis. Amylase activity was then assayed at a substrate concentration of 1 $mg.mL^{-1}$ by determining starch hydrolysis according to the iodine reaction adapted to bivalve amylases. One unit of alpha-amylase was defined as the amount of enzyme that degrades 1 $mg.min^{-1}$ starch at 45°C. Soluble proteins were determined according to the method of Lowry et al. (1951).

4.7. DEB-model simulations

DEB model simulations were performed in order to evaluate how physiological changes induced by RNA interference may influence energy fluxes and could explain observed phenotypic changes. The DEB model describes four state variable dynamics: the energy stored in reserves, E, the energy allocated for structural growth, E_V, the energy allocated to development and reproduction, E_R, and the energy used in the construction of gametes, E_{GO} (see Bernard et al., 2011, for full description). In the DEB model, a constant fraction, named Kappa, of the energy mobilized from the reserves is allocated to growth and structural maintenance, the remainder (i.e. 1 - Kappa) being allocated to the reproduction and maturity maintenance [p_M] (Kooijman, 2010). In the DEB theory, Kappa is considered constant and species-specific. Nonetheless, depending on the different types of forcing (*e.g*. sexual dimorphism, photoperiod or endocrine perturbation), the value of Kappa may change and consequently may change the fraction of allocation to reproduction. Food uptake follows a type-II Holling functional response depending on food density (Pouvreau et al., 2006). A constant proportion of ingested energy is assimilated according to the absorption efficiency (also known as assimilation efficiency AE, %). The model formulation and parameter values used here are based on the study of Bernard et al. (2011).

The simulations were made for the control and 75 µg amylase dsRNA-injected oysters, under Exp1 conditions of food and temperature, using the absorption efficiency, AE, measured in Exp2. Oysters were fed *ad libitum* so food uptake was maximal (*i.e.*, functional response = 1). Initial state was established according to the initial biometrics measurements and maturity observations. The initial reserve buffer was assumed to be empty, as the oysters were sampled at the end of winter after a period of low food level. Oocyte production was estimated according to an energy content of 9.3×10^{-4} J.oocyte^{-1}.

4.8. Statistical analyses

Statistical analyses were performed with STATGRAPHICS software, using one-way ANOVA (mRNA level, amylase activity, gonad area and growth performance) or repeated measures ANOVA (algal consumption and absorption efficiency) after normality of the datasets was tested using Levene"s test. When differences were detected, multiple comparison tests were conducted with the Tukey"s HSD method. When necessary, data were transformed to achieve homogeneity of variances: gonad area and absorption efficiency were analyzed after angular transformation and relative *AMYA* mRNA levels were transformed using a base 10 logarithmic transformation. Results are expressed as means ± standard deviation. Comparisons of sex and gametogenic stage distributions between treatments were made using Chi-square tests. Differences were considered statistically significant at $P < 0.05$.

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Competing interest

The authors declare no competing or financial interests.

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Summary

Disruption of amylase gene expression reduces absorption efficiency and constrains reproduction in oysters; the reduction in energy intake is insufficient to affect reproduction, suggesting that dsRNA causes disturbances that increase maintenance costs.

Tables and Figures

Table 1. Distribution of gametogenic stages and sex at 48 days postinjection in control oysters and oysters injected with amylase dsRNA (experiment 1).

Treatment	N	Sex (%)		Gametogenic stage (%)		
		female %	male %	1: developing early active %	2: developing late active %	3: ripe %
control	10	50	50	0	20	80
amylase 15 µg	10	44.5	55.5	11.11	66.66	22.22
amylase 75 µg	10	75	25	50	37.5	12.5

Gametogenic stages are according to the reproductive scale of Steele and Mulcahy (1999): 1, developing early active; 2, developing late active; 3, ripe.
N, number of individuals analyzed.

Table 2. Mass and ecophysiological parameters of groups of control oysters and oysters injected with 75 µg amylase dsRNA (Exp2).

Treatment	Mass (gram)			Condition index	Ecophysiological parameters[1]	
	Total	Wet flesh	Digestive gland		Algal consumption ($\mu m^3.oyster^{-1}.day^{-1}$)	Absorption efficiency (%)
control	53.2 ± 17.1	8.50 ± 2.69	0.59 ± 0.24	16.1 ± 1.7 A	7.42 ± $1.34.10^7$	81.9 ± 1.1 A
amylase 75 µg	46.1 ± 17.4	6.66 ± 2.82	0.53 ± 0.26	14.3 ± 1.6 B	7.58 ± $1.34.10^7$	77.0 ± 1.3 B

[1]Algal consumption is expressed over the whole experiment in terms of cell volume per oyster per day; absorption efficiency is an estimated percentage.
Values are means ± s.d. with 15 animals analyzed per condition for mass and 10 days of continuous data for ecophysiological parameters. Multiple comparisons were made between treatments using Tukey"s HSD method at the 5% level; homogeneous groups share the same letter.

Fig. 1. Amylase transcript levels in the digestive gland of oysters treated with amylase double-stranded RNA. Oysters were injected with Tris NaCl (control) or with 15 or 75 µg amylase (AMY) double-stranded (ds)RNA, and transcript levels relative to actin transcripts were monitored 48 days postinjection (experiment 1) using real-time PCR. Grey bars, *AMYA*; black bars, *AMYB*. Results are expressed as means±s.d. of N=8 or 9 oysters (for the 75 and 15 µg dsRNA treatment, respectively). Multiple comparisons were made between treatments using Tukey"s HSD method at the 5% level; homogeneous groups have the same letters (the same statistical groups were obtained for *AMYA* and *AMYB*).

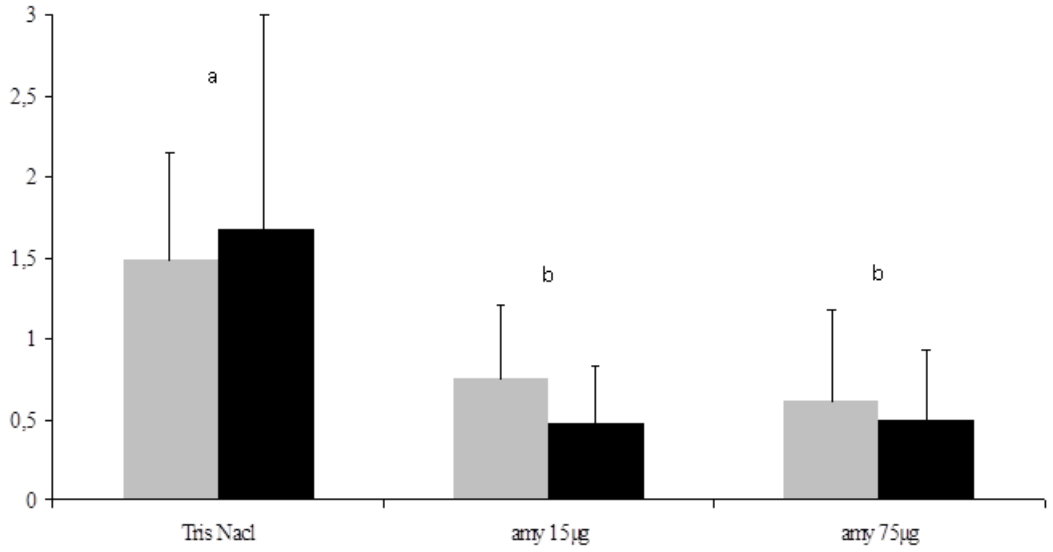

Fig. 2. Specific amylase activity in the digestive gland of oysters treated with amylase double-stranded RNA. Oysters were injected with Tris NaCl (control) or with 15 or 75 µg amylase (AMY) double-stranded (ds)RNA, and amylase activity was monitored 48 days post-injection (experiment 1). Results are expressed as means±s.d. of N=10 oysters. Multiple comparisons were made between treatments using Tukey"s HSD method at the 5% level; homogeneous groups share the same letter.

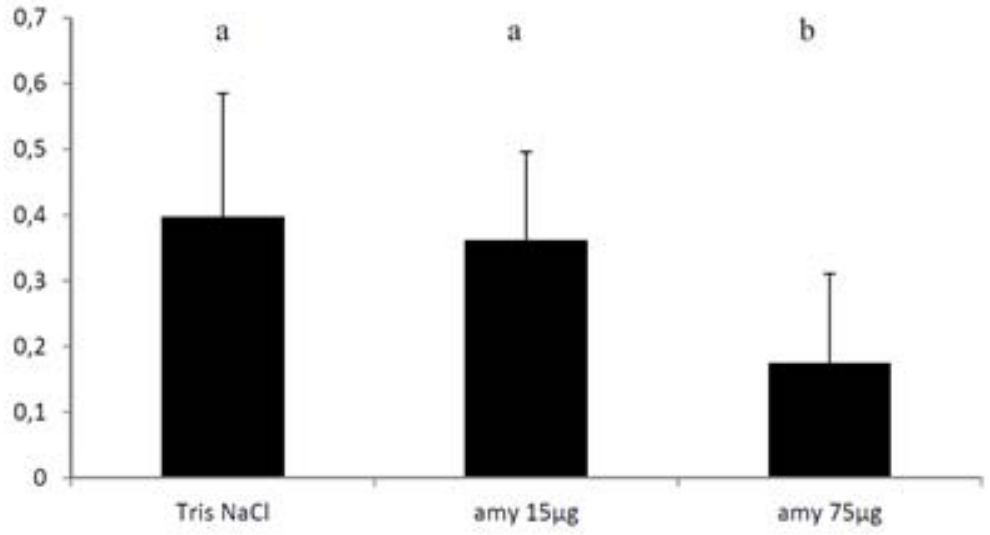

Figure 3. Under-proliferation of germ cells. One knock-down phenotype observed for in vivo amylase dsRNA-injected oysters at 48 days post injection. Images are of female tissues; similar observations were made on male slides. (A) Control female, injected with saline solution. Gonadic tubules are confluent and filled with ripe oocytes. (B and C) Females injected with 15 µg and 75 µg amylase dsRNA, respectively. A reduction of the gonadic tubule area is observed as a result of a decrease in germ cell number; this is moderate in B (15 µg) and strong in C (75 µg). GT: Gonadic Tubule; CT: Connective Tissue; DG: Digestive Gland; GC: Germ Cells. Magnification 100×. Scale bars represent 100 µm.

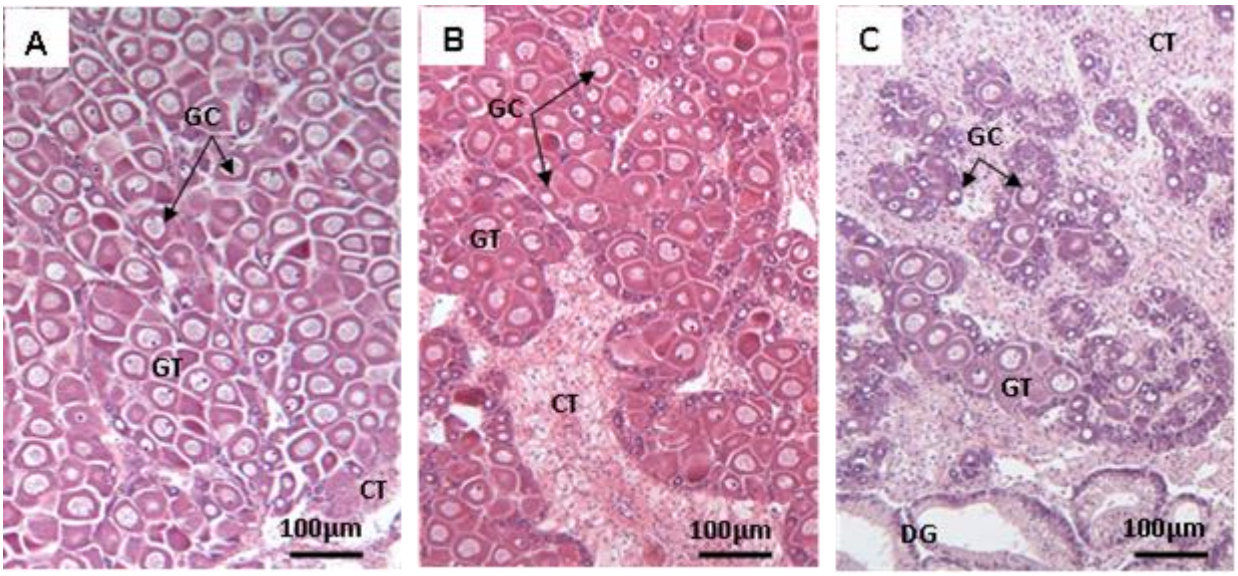

Figure 4. Oyster dynamic energetic budget model simulations. Conditions were as for experiment 1 (i.e. food, temperature) for a mean individual. (A) Dry flesh mass; (B) oocyte number. The control has standard dynamic energy budget (DEB) model parameter values (Kappa = 0.45 and $[p_M]$ = 44 J.cm^{-3}.d^{-1}) and absorption efficiency measured for the control treatment in Exp2 (AE=81.9%). Sim-1 simulates oysters with standard Kappa and $[p_M]$ values and absorption efficiency (AE) measured for the 75 µg dsRNA-injected treatment in Exp2 (AE=77%), which leads to direct effects of a 4.9% reduction of absorption efficiency. Sim-2 simulates oysters with adjusted Kappa and $[p_M]$ values (Kappa = 0.765 and $[p_M]$ = 96.8 J.cm^{-3}.d^{-1}) and absorption efficiency measured for the 75 µg dsRNA-injected treatment (AE=77%). On the plots, "Acc." corresponds to the acclimation period (no food and increased temperature) before injection. The final percentage differences between control and injected are shown on each plot.