Mar Biol (2016) 163:193
DOI 10.1007/s00227-016-2963-2

ORIGINAL PAPER

A diet rich in polyunsaturated fatty acids improves the capacity for HSP70 synthesis in adult scallop *Argopecten purpuratus* and their offspring

Hernán M. Pérez[1] · Katherina Brokordt[1] · Alvaro Gallardo[1] · Igor Vidal[1] · Helga Guderley[2]

Received: 30 March 2016 / Accepted: 4 August 2016

Abstract In scallops, aerobic power requirements of gonad maturation reduce HSP70 synthesis capacity in response to thermal or hypoxia stress. As dietary lipid quality is crucial for membrane function, we predicted that supplementing *Argopecten purpuratus* broodstock with essential polyunsaturated fatty acids (PUFA) during gonadal maturation would mitigate the loss in adult performance while favoring that of larvae. Scallops were fed during reproductive conditioning with microalgal diets with high and low PUFA contents, while a control group was maintained in natural conditions. After maturation, scallops of each treatment were kept at normal temperature (18 °C) or stressed by increasing the temperature (+6 °C). Mature scallops fed each diet were stimulated to spawn, and the ensuing larvae were exposed to handling and thermal stress. Relative transcription of *hsp*70 mRNA was assessed by RT-qPCR, and HSP70 proteins were quantified by ELISA. Feeding a high-PUFA diet during reproductive conditioning enhanced HSP70 induction (at transcription and protein levels) by mature *A. purpuratus* in response to thermal stress. By contrast, neither scallops matured in the natural environment nor those fed the low-PUFA diet increased HSP70 levels after thermal stress; *hsp*70 mRNA levels were also reduced. Offspring from broodstock fed a high-PUFA diet increased induction of HSP70 after exposure to handling and handling plus thermal stress relative to offspring of scallops fed a low-PUFA diet. Survival of these larvae was also enhanced. Feeding adult scallops with appropriate PUFA during reproductive conditioning could improve adult stress responses and favor stress responses and survival of ensuing larvae.

Responsible Editor: H. Pörtner.

Reviewed by J. Miao and an undisclosed expert.

✉ Katherina Brokordt
kbrokord@ucn.cl

[1] Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Universidad Católica del Norte, 1281 Larrondo, Coquimbo, Chile

[2] Department of Biology, Université Laval, Quebec City PQ G1K 7P4, Canada

Introduction

Broadcast spawning organisms invest massively in reproduction, producing great numbers of energy-rich gametes to assure fertilization success and to allow early planktonic development without external food. In bivalve molluscs, aerobic power requirements of gonad maturation slow vital processes such as growth (Iglesias and Navarro 1991), escape performance (Brokordt et al. 2000a, b, 2003, 2006; Kraffe et al. 2008), and immune responses (Li et al. 2007, 2010). In many scallop species, macromolecular requirements of gonad maturation deplete reserves in digestive gland and muscle (Barber and Blake 1983; Brokordt and Guderley 2004). For the scallop *Argopecten purpuratus*, gonad maturation even decreases a crucial element of cellular stress responses, heat shock protein (HSP) production (Brokordt et al. 2015). Effectively, the capacity of mature and spawned scallops to increase mRNA and protein levels of 70 kDa HSPs following thermal or hypoxia stress is markedly reduced compared to that of adult scallops with immature gonads. Furthermore, the capacity for mitochondrial ATP production, as assessed by citrate synthase activity, decreases with gonadal maturation and spawning (Brokordt et al. 2015). Similar results are found for the oyster *Crassostrea gigas* in which spawning reduces

Published online: 23 August 2016

adenylate energy charge, glycogen levels and heat shock-induced levels of HSP72 and HSP69 (Li et al. 2007). In summary, for oysters and scallops, reproductive investment decreases metabolic capacities and reduces the capacity for HSP70 induction during stress responses. In many bivalves, reproductive maturation and spawning occur during periods of strong fluctuations in temperature and oxygenation (Cheney et al. 2000; Tomaru et al. 2001; Cabello et al. 2002; Xiao et al. 2005; Li et al. 2007; Zhang et al. 2010), and under these conditions, a decrease in stress response capacity would be particularly problematical. It is revealing that mass mortalities often coincide with periods of reproductive activity for broadcast spawning bivalves (Tremblay et al. 1998; Xiao et al. 2005; Samain et al. 2007).

The production and activity of HSPs require considerable metabolic support (Hofmann and Somero 1995; Somero 2002; Sharma et al. 2010). As molecular chaperones, HSPs assist refolding of misfolded proteins, decrease aggregation of unfolded proteins and facilitate channeling of irreversibly damaged proteins toward proteolytic degradation (Parsell and Lindquist 1993). The impact of reproductive investment on aerobic power budgeting and energy availability could reduce stress response performance (Li et al. 2007; Guderley and Pörtner 2010; Brokordt et al. 2015). Hence, physiological mechanisms that enhance metabolic capacities could mitigate the impact of reproductive investment on cellular stress tolerance in the broodstock and potentially improve offspring performance. One such mechanism involves adjustments in lipid composition.

Lipids store energy and provide the matrix of cellular membranes. Lipid quality is crucial for both roles, as both membrane function and energy mobilization require dynamic transformations of lipid structure (Pernet et al. 2007). Phospholipids are the major component of biological membranes and their molecular composition sets membrane properties (Hulbert et al. 2005). When phospholipids contain higher levels of polyunsaturated fatty acids (PUFA) especially n-3 PUFA, such as 22:6n-3 or 20:5n-3, membrane proteins show increased molecular activity. In parallel, cellular metabolic rates are higher in cells with more polyunsaturated membranes (reviewed by Hulbert et al. 2005). This could facilitate metabolic support of vital activities including stress responses.

Many animals, particularly filter feeding bivalves, are unable to synthesize n-6 or n-3 PUFA de novo and rely heavily on dietary PUFA. In bivalves, as in mammals (reviewed by Hulbert et al. 2005), fatty acid composition of storage and membrane lipids is strongly influenced by the abundance of dietary n-6 and n-3 PUFA (Delaporte et al. 2003, 2006; Martínez-Pita et al. 2014). Dietary PUFA levels are positively correlated with PUFA content in gonads, digestive gland, gills and eggs (Caers et al. 2003; Delaporte et al. 2006; Martínez-Pita et al. 2014). Mitochondria from *A. purpuratus* fed a microalga rich in PUFA show increased oxidative capacities compared to those fed a diet lacking in PUFA (Guderley et al. 2011). Dietary lipids accumulated in eggs provide material for growth and energy until larvae are able to feed (Ehteshami et al. 2011; Martínez-Pita et al. 2012a, b). In bivalves, PUFA levels during reproductive conditioning or in the larval diet can strongly influence larval survival (Berntsson et al. 1997; Martínez et al. 2000; Hendricks et al. 2003). Thus, providing adult scallops with the appropriate dietary PUFA during reproductive conditioning could improve adult stress responses and favor stress responses and survival of the ensuing larvae.

Therefore, the objectives of this study were (1) to evaluate whether *A. purpuratus* fed a diet rich in PUFA during reproductive conditioning will improve their capacity to respond to stress through HSP70 synthesis (at transcription and protein levels) and (2) to examine whether larvae produced from scallops fed a diet rich in PUFA will have improved survival and synthesis of HSP70 in response to hatchery manipulations (i.e., handling and thermal stress).

Materials and methods

Animal procurement and holding conditions

Adult *A. purpuratus* (70–80 mm shell height; $n = 300$) with immature gonads were obtained from the aquaculture concession belonging to Universidad Católica del Norte at Tongoy Bay in Coquimbo, northern Chile (30°16′ S; 71°35′ W), during the summer 2011 (where seawater temperature ranged from 16 to 18 °C). The flaccid and pale gonads of immature individuals were identified using a visual scale following Disalvo et al. (1984) and Martínez and Pérez (2003) for this hermaphrodite scallop. The scallops were transported to the Universidad Católica del Norte's laboratory in Coquimbo. To recover from transport, the scallops were placed in 1000-L tanks supplied with filtered, aerated and running seawater (16–18 °C) and fed a diet of 50 % *Isochrysis galbana* and 50 % *Chaetoceros calcitrans* for 3 days. The stress trial (see below) was given to 30 scallops to evaluate stress response capacity before gonad maturation. One group of 90 scallops was returned to the aquaculture concession at Tongoy Bay for gonad maturation; here they had access to a natural diet and no additional feeding. This group was termed the "natural environment" treatment. The other 180 scallops were conditioned until maturation (25 days) under laboratory conditions with two microalgal diets, one high and the other low in PUFA ($n = 90$ each). Scallops were haphazardly assigned to a dietary treatment. Each treatment group was split into three 200-L tanks ($n = 30$ scallops per tank) in which they were fed (see below for details) by a continuous drip system. The

Table 1 Proportion (%) of total lipids and fatty acids in microalgae used in diet treatments, and total lipids and fatty acids (mg g^{-1}) in the microalgal mixture used for the high- and low-PUFA diets during reproductive conditioning of *A. purpuratus*

Diet	Microalgae	Lipids	SFA	MUFA	PUFA
High PUFA	*Isochrysis galbana*	25.0	28.3	23.4	47.6
	M35	14.1	60.8	27.8	7.75
	M53	18.7	19.0	48.1	29.5
	Microalgal mix[a]	233	70	60	110
Low PUFA	*Nanocloropsis oculata*	22.0	35.7	41.9	19.6
	M19	1.3	56.7	37.4	2.58
	M22	0.8	59.4	31.2	7.08
	Microalgal mix*	222	80	93	43

[a] For each diet, microalgae were mixed in the proportions given in "Materials and methods" section

water was changed daily, and temperature was between 17 and 18 °C.

Microalgal diet composition

The diets were isocaloric and isoproteic mixtures of laboratory and endemic microalgae. The microalgae, *I. galbana* (*var* T-iso) and *Nanocloropsis oculata*, were obtained from our culture facility in exponential growth phase at 5–6 × 10^6 cells mL^{-1}. Endemic microalgae were obtained in exponential growth phase at 5–6 × 10^6 cells mL^{-1} from Chañar Blanco S.A. As the identity of the endemic microalgae is an industrial secret, they are identified with a code (Table 1). Scallops were fed approximately 6 % of their dry mass per day. The high-PUFA diet contained 80 % *I. galbana* and 10 % each of M35 and M53. For the low-PUFA diet, 84 % *N. oculata* was combined with 8 % each of M19 and M22. Table 1 shows the proportions (%) of total lipids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in each microalga, as well as the levels of total lipids and total SFA, MUFA and PUFA (mg g^{-1}) in the diets. Both diets contained similar concentration of lipids (mg g^{-1}), but the high-PUFA diet contained 2.6 times more PUFA than the low-PUFA diet (Table 1).

Experimental design

Dietary conditioning lasted 25 days, the period necessary for gonad maturation. After this, 30 scallops per treatment were given the stress trial (where 15 were stressed and 15 not stressed), and 60 scallops per treatment were stimulated to spawn for the larval experiments. We did not observe mortality among adult scallops during the experimental period.

Of the scallops per condition (i.e., immature; matured in the natural environment; and matured with a diet high or low in PUFA), 15 were subjected to a sudden temperature increase from 18 to 24 °C and then maintained at 24 °C for 6 h (i.e., stressed scallops). A previous study indicated that this change in temperature increases HSP70 levels 1.5-fold in muscle, 2.0-fold in mantle and threefold in gills, compared with controls from unstressed *A. purpuratus* scallops (Brokordt et al. 2015). Therefore, here we focused on gills. An additional 15 scallops per dietary condition were maintained at 18 °C over the same 6 h and served as unstressed scallops. Following the stress trials, each individual's gills were removed; one portion was frozen in liquid nitrogen and stored at −80 °C for later HSP70 quantification. Another portion was stored in RNA later (Ambion) at −20 °C until processing for *hsp*70 gene transcription determinations. Gonads from mature scallops were dissected into male and female portions. The female portion was frozen at −80 °C for lipid quantification and fatty acid profile determination.

The remaining mature scallops from each dietary condition ($n = 60$) were stimulated to spawn by adding excess microalgae. Oocytes from spawned scallops were fertilized with sperm from other individuals fed the same diet, in a 1:50 proportion oocyte/sperm. Resulting embryos per treatment were distributed in three larval-growing tanks of 200 L each, with filtered (1 μm ø) sea water at 18 °C and gentle aeration, at a density of 100 embryo mL^{-1}. After 48 h of development, when embryos attained the D-shaped veliger stage, each tank was completely sieved through a 50-μm mesh filter. As for all analyses, the larvae need to be concentrated by sieving, it was not possible to measure unstressed larvae. Three samples of 0.5 mL (concentrated larvae) per tank were taken and immediately frozen in liquid nitrogen and stored at −80 °C for later HSP70 quantification. We considered that these larvae had been exposed to handling stress. Heat shock was applied to three samples (0.5 mL concentrated larvae per sample) per tank per diet. Each sample was transferred to a 250-mL glass container filled with filtered sea water and held at 24 °C for 30 min. Preliminary experiments showed that 30 min was enough to stimulate a significant increase in HSP70, while avoiding larval mortality. We considered that these larvae had been exposed to handling and thermal stress.

Finally, to evaluate larval survival, three samples of fertilized oocytes (2 mL each at 100 eggs mL^{-1}) per tank per diet treatment were transferred to 500-mL glass containers (i.e., three replicates per treatment) filled with filtered sea water and held at 18 °C with gentle aeration. No food was provided to the embryos to evaluate the effect of parental dietary condition on survival at different larval stages. Larval survival was measured at 48, 72 and 96 h

post-fertilization, by taking three 1 mL samples per container and counting larvae under a microscope.

Dietary and tissue lipid composition

Fatty acid composition of the gills, female gonads (three pools of five samples each per treatment) and diets were obtained from the Algae Center of Research and Technological Development (CIDTA) using gas chromatography and standard protocols for lipid (Folch et al. 1957) and fatty acid extraction. Fatty acid methyl esters (FAMEs) of total lipid were prepared by transmethylation with 14 % BF_3MeOH for 10 min at 60 °C. FAMEs were then obtained by a liquid–liquid extraction with hexane and washed with 20 % NaCl. The organic phase was roto-evaporated and resuspended with 1 mL hexane. FAMEs were analyzed with a Clarus 600, PerkinElmer gas chromatograph.

Total RNA extraction, cDNA synthesis and mRNA transcription analysis with quantitative real-time PCR

For this, we followed Brokordt et al. (2015). Total RNA was isolated from gill tissues using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RQ1 RNase-Free DNase (Promega, USA) was used to eliminate DNA contamination. Equal amounts of RNA from three individuals per treatment were pooled (thus the 15 individuals per treatment became five pools of three individuals per treatment). Each RNA pool was reverse-transcribed using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. We used 200 ng of total RNA for the RT-PCR analysis. *Hsp*70 gene transcription was performed using specific *A. purpuratus* primers (*Hsp*70F 5′GAGGCCGTCGCCTATGGTGC3′; *Hsp*70R 5′GCGGTCTCGATA-CCCAGGGACA3′) (GenBank accession number FJ839890); with *EF*1*α* (GenBank accession number ES469321.1.) as an endogenous control (primers: *EF*1*α*F 5′CATGACACAGGAACTCCC3′; *EF*1*α*R 5′GGTCTGACCGTTCTTGGAAA3′). In preliminary studies, the stability of this endogenous gene was tested for our species and tissues. For both genes, RT-qPCR efficiency was previously set between 90 and 110 % through serial dilution of cDNA. The designed primers were selected from a conserved region that does not discriminate between genes encoding for different *Hsp*70 isoforms and thus between constitutive and inducible ones. However, in preliminary studies using these primers, *Hsp*70 mRNA showed a strong increase after stress, which indicates that we are measuring the *Hsp*70 inducible isoforms (Brokordt et al. 2015).

All qPCR were performed in triplicate in a 20-μL reaction mixture containing 5 μL cDNA, 0.2 mM of each primer, using the Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (Fermentas). qPCR were run in a StepOne Plus Real-Time PCR System (Applied Biosystems, USA). Efficiency of *Hsp*70 amplification was similar to that of the housekeeping gene, so the comparative method ΔΔ CT (Livak and Schmittgen 2001) was applied for relative quantification of *A. purpuratus Hsp*70. For both the target and housekeeping genes, qPCR initial denaturing time was 10 min at 95 °C, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, with a ramp rate of the melt curve of 95 °C (15 s), 55 °C (15 s) and 95 °C (15 s).

Extraction and quantification of total protein for HSP70 determination

Total protein was quantified for 0.03 g of gill from each individual and for each larval sample. Gill and larval pellets were homogenized in 150 μL of homogenization buffer (32 mM Tris–HCl at pH 7.5, 2 % SDS, 1 mM EDTA, 1 mM Pefabloc and 1 mM protease inhibitor cocktail; Sigma). The homogenate was incubated for 5 min at 100 °C, then resuspended in 100 μL of homogenization buffer and re-incubated at 100 °C for 5 min. The homogenate was centrifuged at 10,600*g* for 20 min. Total protein was quantified in an aliquot of the supernatant with a Micro-BCA kit using a microplate spectrophotometer EPOCH (BioTek).

Quantification of HSP70 protein levels

HSP70 was measured in the gill tissue of each individual and in pooled larvae by enzyme-linked immunosorbent assay (ELISA), which was validated in previous tests by comparing ELISA results with immunoprobing of Western blots (Brokordt et al. 2015). Western blot analyses (using the same antibodies described later) showed only one band at the level of 70 kD-HSP. Total protein (30 μg/mL) was diluted in 0.05 M carbonate–bicarbonate buffer at pH 9.6, and 50 μL of sample per well was incubated in an ELISA plate overnight at 4 °C with three blanks (containing buffer only) and various concentrations of cognate HSP70 (H8285, Sigma) to generate a standard curve. The plate was washed twice with phosphate-buffered saline (PBS) (200 μL per well). Next, 200 μL of blocking buffer (PBS + 5 % skim milk) was added to each well and incubated for 2 h. The wells were washed again with PBS. Subsequently, 100 μL of the primary antibody [polyclonal mono-specific anti-epitope that recognizes the inducible and constitutive forms of HSP70 specific for *A. purpuratus*, developed in immunized mice with a synthetic peptide epitope (group of immunological markers on aquatic organisms, Catholic University of Valparaiso)] diluted 1:400 in blocking buffer + 0.05 % tween-20 was added to each well, and the plate was incubated overnight at 4 °C. The plate was then washed four times with PBS, incubated

with goat anti-mouse IgG (Thermo Scientific) secondary antibody, diluted in blocking buffer + 0.05 % tween-20 for 2 h at 25 °C and washed again four times with PBS. Next, 100 µL of substrate solution (10 mg *o*-phenylenediamine dihydrochloride in 25 mL of 0.05 M citrate phosphate buffer) was added, followed by incubation of the plate for 30 min at 25 °C. Finally, the plate was read at 450 nm in a microplate spectrophotometer. The absorbance of the sample was corrected by the mean absorbance of the blanks and divided by a conversion factor, estimated from a linear regression curve of cognate HSP70. The calculated result was the concentration of HSP70 in µg/mg total protein.

Statistical analyses

To evaluate the effect of dietary condition on fatty acid contents and profiles in gills and female gonad of mature *A. purpuratus*, we performed a one-way ANOVA. To evaluate the effect of dietary condition on *hsp*70 mRNA levels and HSP70 abundance in mature *A. purpuratus* exposed to thermal stress, we performed two-way ANOVA. Model predictors were dietary treatments (with three levels: high PUFA, low PUFA and natural environment) and absence/presence of thermal stress (i.e., unstressed vs. stressed). To compare these results with those observed before gonad maturation, *hsp*70 mRNA levels and HSP70 abundance in immature scallops were introduced in the analysis as a fourth level of dietary treatment.

To evaluate the effect of parental dietary condition on HSP70 abundance in stressed larvae, we performed a two-way ANOVA. Model predictors were parental diet (with three levels: high PUFA, low PUFA and natural environment) and stress intensity (i.e., handling vs. handling plus thermal stress). To evaluate the effect of parental diet on larval survival, we performed a one-way ANOVA for each post-fertilization time (i.e., 48, 72 and 96 h post-fertilization). For each ANOVA, normality of the dependent variable was tested using the Shapiro–Wilks test (SAS 1999) and homogeneity of variances using the Levene test (Snedecor and Cochran 1989) to verify that the data met model assumptions. A posteriori tests for specific differences were conducted via the multiple pairwise comparisons least-square means (Lenth and Hervé 2015), with significance evaluated at $P \leq 0.05$.

Results

Effect of dietary treatment on fatty acid profiles and contents in gills and gonads

After 25 days of reproductive conditioning with distinct diets, both gills and gonads of mature scallops showed contrasting proportions (%) of PUFA and contents (mg g^{-1}) of total fatty acid (FA), PUFA and lipids (Tables 2 and 3). Gills from scallops fed the low-PUFA diet had lower % PUFA, compared with gills from scallops fed the high-PUFA or the natural diet (Table 2). In the gills, total FA and PUFA, and lipid concentrations were higher in scallops fed the high-PUFA diet than in those fed the low-PUFA or the natural diet. Gonads of scallops fed the high-PUFA diet showed the highest % PUFA, while those fed the low-PUFA diet showed the lowest % PUFA. Gonadal contents of lipids, FA and PUFA were equivalent in scallops fed the high-PUFA and the natural diets and higher than in scallops fed the low-PUFA diet (Table 3). For both gills and gonads, levels of 20:5n-3 and 22:6n-3 in scallops fed the high-PUFA diet were closer to those of scallops matured in the natural environment than to those fed the low-PUFA diet.

Effect of dietary condition on hsp70 mRNA levels and HSP70 abundance in mature scallop exposed to thermal stress

Dietary conditions during gonad maturation significantly affected the heat shock response in scallop gills (Table 4). While under all dietary conditions, thermal stress led scallops to increase *hsp*70 mRNA levels over those of unstressed scallops (Fig. 1a), the intensity of this response varied with dietary condition. Scallops with immature gonads more than doubled relative levels of *hsp*70 mRNA in response to heat shock. After gonadal maturation in the natural environment, the production of *hsp*70 mRNA in response to heat stress was reduced, with an induction of only 0.5-fold relative to the unstressed individuals. Scallops fed the high-PUFA diet during gonad maturation raised *hsp*70 mRNA levels as much if not more than scallops with immature gonads, i.e., an induction of 2.6-fold compared with the respective unstressed scallops. Finally, stressed scallops fed the low-PUFA diet increased 0.75-fold their levels of *hsp*70 mRNA in response to heat stress in comparison with the respective unstressed scallops.

Following thermal stress, scallops with immature gonads and scallops fed the high-PUFA diet had significantly higher HSP70 protein levels (2.4- and 1.6-fold, respectively) than unstressed scallops (Fig. 1b). In contrast, neither scallops matured in the natural environment nor those fed the low-PUFA diet were able to increase HSP70 protein levels after thermal stress. Interestingly, basal levels of HSP70 were highest in gills of scallops fed the high-PUFA diet.

Effect of parental dietary condition on larval levels of HSP70

In general, both parental dietary condition and exposure to different levels of stress affected protein levels of HSP70 in D-shaped veliger larvae (i.e., at 48 h post-fertilization)

Table 2 Fatty acid contents and profiles in gills of mature *A. purpuratus* fed microalgal diets containing different levels of PUFA during reproductive conditioning

Fatty acids	High-PUFA diet	Low-PUFA diet	Environmental diet
SFA			
14:0	1.57 (0.08)^{c}	2.21 (0.03)^{b}	3.11 (0.13)^{a}
15:0	0.76 (0.18)	0.97 (0.09)	0.83 (0.06)
16:0	19.1 (0.87)	21.9 (1.09)	22.4 (0.25)
17:0	ND	0.66 (0.07)	ND
18:0	16.7 (0.63)	18.0 (0.24)	15.8 (0.66)
MUFA			
16:1	3.69 (0.18)	4.77 (0.16)	3.82 (0.49)
18:1n-9c/18:1n-9t	2.49 (0.01)^{a}	2.05 (0.01)^{b}	1.70 (0.06)^{c}
20:1n-9	5.98 (0.26)	5.48 (0.15)	5.22 (0.11)
PUFA			
20:2	1.70 (0.13)^{a}	1.11 (0.05)^{b}	ND
20:3n-3	7.93 (0.04)^{a}	7.38 (0.10)^{b}	5.16 (0.04)^{c}
20:5n-3	10.3 (0.08)^{a}	8.72 (0.25)^{b}	10.7 (0.18)^{a}
22:6n-3	29.8 (0.17)^{a}	26.7 (0.81)^{b}	31.3 (0.72)^{a}
ΣSFA	38.1 (0.44)^{b}	43.8 (0.30)^{a}	42.1 (0.38)^{a}
ΣMUFA	12.2 (0.15)^{a}	12.3 (0.14)^{a}	10.7 (0.25)
ΣPUFA	50.0 (0.51)^{a}	44.0 (1.21)^{b}	47.2 (1.41)^{ab}
22:6n-3/20:5n-3	2.89 (0.04)^{b}	3.06 (0.03)^{a}	2.93 (0.02)^{ab}
Tissue contents (mg g^{-1})			
ΣFA	8.10 (0.41)^{a}	5.90 (0.19)^{b}	5.70 (0.18)^{b}
ΣPUFA*	4.10 (0.12)^{a}	2.54 (0.07)^{b}	2.68 (0.13)^{b}
Lipids	13.9 (0.07)^{a}	10.2 (0.07)^{b}	9.79 (0.10)^{b}

Values are expressed as % of the total fatty acids, except for tissue contents of fatty acids (ΣFA) and PUFA (ΣPUFA). Values are shown as mean (standard error). $n = 3$ (three pools of five individuals per treatment). Data followed by different letters differ significantly between dietary treatments (ANOVA; L-S means test; $P < 0.05$)

ND not detectable

(Table 5). Larvae from broodstock fed the low-PUFA diet showed the lowest HSP70 levels after handling plus thermal stress (Fig. 2). Larvae from broodstock maintained in the natural environment and those from scallops fed the high-PUFA diet during gonad maturation did not differ in their HSP70 levels after the combined stresses, although a tendency toward higher HSP70 levels was observed for larvae from scallops fed the high-PUFA diet (Fig. 2).

Effect of parental dietary condition on larval survival

Survival of larvae from broodstock fed the high-PUFA diet was consistently significantly higher than that of larvae from broodstock maintained in the natural environment or those fed the low-PUFA diet (ANOVA; L-S means a posteriori test; $P < 0.0001$ for each comparison) (Fig. 3). Survival of larvae from broodstock fed the low-PUFA diet was not different from that of larvae from broodstock maintained in the natural environment, except at 72 h post-fertilization, where the former showed lower survival.

Discussion

In broadcast spawning animals, nutritional quality during gonadal maturation is crucial for the health and survival of the offspring as well as the subsequent performance of the adults (Martínez et al. 2000). In scallops, the mature gonad can become the largest organ in the body. Gonadal growth and gamete maturation lead to significant depletion of energetic reserves in other organs, including muscle and digestive gland (Barber and Blake 1983; Brokordt and Guderley 2004) and lead to significant functional compromises in escape response performance (Brokordt et al. 2000a, b, 2006; Kraffe et al. 2008) and stress responses (Brokordt et al. 2015). The mechanism underlying this generalized loss of adult performance may well be the decrease in available aerobic power, as the costs of gonad maturation increase standard metabolic rates leaving less aerobic power available to mount stress responses or to carry out repeat escape responses (Guderley and Pörtner 2010). Mobilization of essential fatty

Table 3 Fatty acid composition in female gonads of mature *A. purpuratus* fed with microalgal diets with different levels of PUFA during reproductive conditioning

Fatty acids	High-PUFA diet	Low-PUFA diet	Environmental diet
SFA			
C14:0	4.88 (1.77)	6.67 (0.01)	4.27 (0.39)
C16:0	15.1 (1.03)^{b}	18.4 (0.34)^{a}	17.0 (0.60)^{ab}
C18:0	6.82 (1.03)	8.56 (0.06)	7.05 (0.03)
MUFA			
C16:1	5.21 (1.10)	7.07 (0.08)	7.88 (0.49)
C17:1	2.47 (0.14)	ND	3.64 (2.82)
C20:1n-9	1.63 (0.57)	1.25 (0.23)	1.47 (0.16)
PUFA			
C18:2n6c	1.63 (0.57)	1.19 (0.04)	0.73 (0.08)
C18:3n3	1.63 (0.45)	0.92 (0.11)	0.57 (0.01)
C20:2	1.25 (0.86)	ND	0.39 (0.01)
C20:3n-6	1.17 (1.00)	ND	0.42 (0.01)
C20:4n-6	2.94 (0.52)	ND	2.34 (0.13)
C20:5n-3	29.9 (0.17)^{a}	24.6 (0.65)^{b}	27.0 (0.91)^{a}
C22:6n-3	23.8 (0.20)^{b}	21.7 (0.30)	25.6 (0.13)^{a}
ΣSFA	26.8 (1.28)^{b}	33.6 (0.14)^{a}	28.3 (0.34)^{b}
ΣMUFA	9.31 (0.60)^{b}	8.32 (0.16)^{b}	12.9 (1.16)^{a}
ΣPUFA	62.4 (0.74)^{a}	48.4 (1.28)^{c}	57.1 (1.18)^{b}
22:6n-3/20:5n-3	1.25 (0.09)	1.13 (0.06)	1.05 (0.14)
Tissue contents (mg g^{-1})			
ΣFA	30.0 (1.50)^{a}	21.1 (1.06)^{b}	30.4 (1.52)^{a}
ΣPUFA*	18.6 (0.93)^{a}	10.1 (0.55)^{b}	17.3 (0.87)^{a}
Lipids	55.9 (0.26)^{b}	47.5 (0.81)^{c}	60.6 (1.42)^{a}

Values are expressed as % of the total fatty acids, except for tissue contents of total fatty acids (ΣFA) and total PUFA* contents (ΣPUFA) expressed in mg g^{-1}. Values are shown as mean (standard error). $n = 3$ (three pools of five individuals per treatment). Data followed by different letters differ significantly between dietary treatments (ANOVA; L-S means test; $P < 0.05$)

Table 4 Two-way ANOVAs comparing *Hsp*70 mRNA and HSP70 protein levels between *A. purpuratus* exposed to different dietary conditions during gonad maturation, and subjected to thermal stress

Source	*df*	*F*	*P*
*Hsp*70 mRNA levels after stress			
Model	1	573.8	0.000000
Condition (C)	3	43.60	0.000000
Stress level (SL)	1	90.82	0.000000
C × SL	3	10.90	0.000026
Error	38		
HSP70 protein levels after stress			
Model	1	607.79	0.000000
Condition (C)	3	17.42	0.000000
Stress level (SL)	1	26.86	0.000002
C × SL	3	6.37	0.000623
Error	81		

Condition: feeding high-PUFA and low-PUFA diets, natural environment food; and reproductive immaturity. *Stress level*: unstressed (18 °C), stressed (24 °C). For gene expression, $n = 5$ replicates per condition (each replicate includes the total RNA of three individuals). For protein levels, $n = 8$–12 individuals per condition

acids from somatic to reproductive tissues may underlie some of this loss of performance. Our current study was predicated upon the assumption that supplementing broodstock with essential PUFA during gonadal maturation could mitigate the loss in adult performance and favor that of the larvae. Our results support this concept. We found that feeding a diet rich in polyunsaturated fatty acids (PUFA) during reproductive conditioning enhanced the capacity of adult *Argopecten purpuratus* to induce HSP70 in response to thermal stress. Further, early larvae from broodstock fed a diet rich in PUFA showed an increased capacity to induce HSP70 after exposure to handling and handling plus thermal stresses relative to offspring of scallops fed a low-PUFA diet. These larvae also showed enhanced survival compared with larvae from broodstock matured in the natural environment and those fed a low-PUFA diet.

In *A. purpuratus,* gonadal maturation decreases branchial capacity to induce HSP70 upon stress exposure. A loss of aerobic capacity in gills accompanies this loss of HSP70 induction (Brokordt et al. 2015). A reduction in aerobic power due to metabolic requirements of gonadal maturation may account for some of the loss of performance in other traits such as escape responses (Guderley and Pörtner 2010). Our results suggest that depletion of essential PUFA from gill membranes during gamete maturation may underlie the reduced heat stress response. A deficiency of PUFA could reduce gill aerobic capacity, decreasing ATP availability for HSP production and action. Herein, we observed that scallops fed a mixture of microalgae high in PUFA during gonad maturation showed 60 % more PUFA in their gills (mg g^{-1}) than those fed a mixture of microalgae low in PUFA, and 40 % more PUFA than those maintained in the natural environment during the same period. Heat shock response intensity paralleled gill PUFA contents, with the strongest response occurring in scallops fed the high-PUFA diet. Higher proportions of PUFA in cell membranes should augment the activity of membrane-linked cellular processes that are major contributors to energy metabolism (Hulbert et al. 2005). The membrane pacemaker theory (Hulbert and Else 1999, 2000) suggests that higher levels of PUFA increase the rates at which the membrane proteins catalyze their reactions, due to a greater transfer of energy during collisions between membrane proteins and the more freely

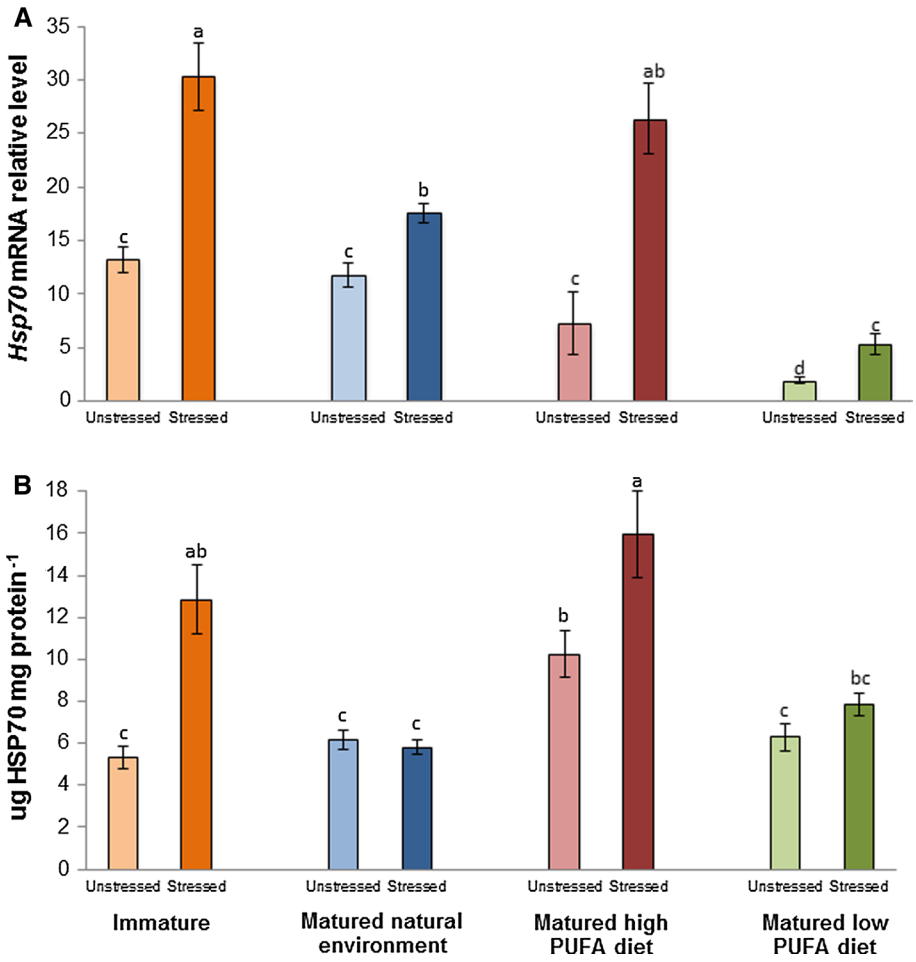

Fig. 1 *Hsp*70 gene transcription (**a**) and HSP70 protein levels (**b**) in gill tissue of *A. purpuratus* scallops exposed (stressed) and not exposed (unstressed) to thermal stress at immature and mature reproductive stages. Mature scallops were conditioned in the natural environment or fed laboratory diets high or low in PUFA. Values represent mean ± S.E. (for mRNA levels, $n = 5$ replicates per condition, each replicate includes three individuals' total RNA; for protein levels, $n = 15$ replicates per condition). Means sharing the same letter are not significantly different ($P \geq 0.05$) as indicated by a posteriori multiple comparisons (LS means)

Table 5 Two-way ANOVAs comparing the effect of parental dietary condition and stress level on HSP70 protein levels in *A. purpuratus* D-shaped veliger larvae

Source	DF	*F*	*P*
Model	1	1912	0.000000
Dietary condition (DC)	2	15.17	0.000116
Stress level (SL)	1	37.58	0.000007
DC × SL	2	0.86	0.440593
Error	16		

Parental diet levels: high PUFA, low PUFA and natural environment. *Stress level*: handling and handling plus thermal stress (24 °C). $n = 3$ larval pools per condition

moving membrane phospholipids (Hulbert and Else 1999). This would elevate aerobic capacities and may increase energy available for HSP70 synthesis. *A. purpuratus* fed mono-specific diets with higher 22:6n-3 contents increased adductor muscle and female gonad mitochondrial oxidative capacities (Guderley et al. 2011). Interestingly, we observed that basal levels of HSP70 (i.e., in non-stressed scallops) were highest in gills of *A. purpuratus* fed the high-PUFA diet. This may be associated with an increased rate of protein turnover and demand for HSP70 during translation (i.e., chaperoning).

During gonadal maturation, dietary lipids, in particular essential PUFA, are directed to the developing gametes (Marty et al. 1992; Utting and Millican 1997; Martínez et al. 2000). The integration of PUFA ingested by the parents into larval membranes seems to influence larval performance. Larvae produced by scallops fed a low-PUFA diet have reduced stress responses compared to those produced by parents fed the high-PUFA or natural diets. In contrast to the pattern observed in the gills, gonadal PUFA concentrations in animals fed the natural diet were similar to those of scallops fed the high-PUFA diet, supporting the concept that dietary PUFA are targeted to the gonad during gonadal maturation. A functional impact of this preferential integration is suggested by larval stress responses 48 h after fertilization. While the stress response in gills of scallops fed the natural diet was attenuated compared to that of scallops fed the high-PUFA diet, larval stress responses were similar in the groups fed the natural and the high-PUFA diets. As in early larval life, 22:6n-3 is maintained in

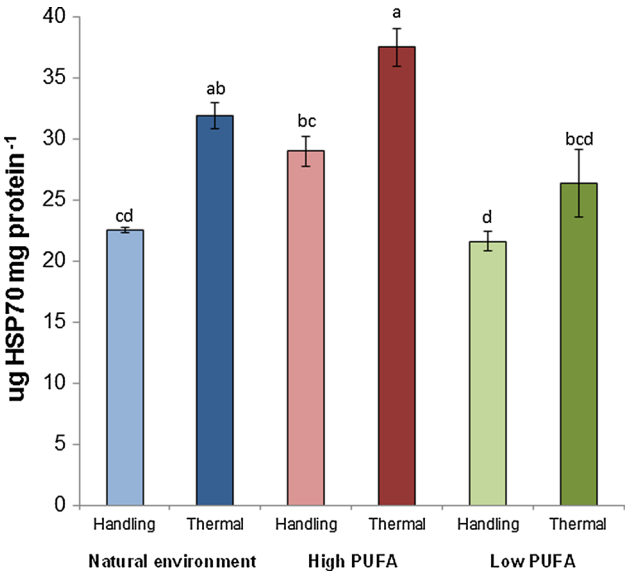

Fig. 2 HSP70 protein levels in D-shaped veliger larvae (48 h post-fertilization) after exposure to handling or handling plus thermal stress. Larvae were from *A. purpuratus* scallop broodstock maintained in the natural environment, or fed high- or low-PUFA diets during gonad maturation. Values represent mean ± S.E. ($n = 3$ pooled or concentrated larvae per condition). Means sharing the same letter are not significantly different ($P \geq 0.05$) as indicated by a posteriori multiple comparisons (LS means)

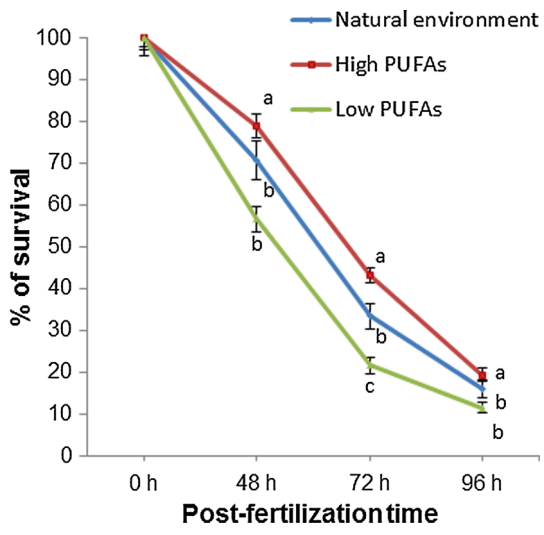

Fig. 3 Effect of parental dietary condition on larval survival at different times post-fertilization (0–96 h). Larvae were from *A. purpuratus* scallop broodstock maintained in the natural environment, or fed high- or low-PUFA diets during gonad maturation. Larvae were not fed to evaluate the effect of parental dietary condition on larval survival. Values represent mean ± S.E. ($n = 3$, one mL sample per post-fertilization time per condition). Means sharing the same letters are not significantly different ($P \geq 0.05$) indicated by a posteriori multiple comparisons (LS means)

the phospholipid fraction, whereas other PUFA including 20:5n-3 are metabolized (Marty et al. 1992), we suggest that larvae from broodstock fed the high-PUFA and natural diets had high membrane 22:6n-3 levels. Our results clearly demonstrate that the diet during broodstock conditioning has strong carryover effects that influence stress response performance of early larvae.

Poor nutritional status decreased the capacity of the snail *Concholepas concholepas* to synthesize HSP70 upon exposure to several stressors (Jeno and Brokordt 2014). Mechanisms based on availability of energy could explain differences between the performance of broodstock (and the ensuing offspring) fed high-PUFA and low-PUFA diets. Among the energy-based mechanisms, higher lipid storage in the scallops fed the diet rich in PUFA could have increased energy available for gene transcription and protein synthesis of HSP70 during stress exposure. Although the high- and low-PUFA diets did not differ in their total lipid content, the gonadal FA content of scallops fed the high-PUFA and the natural diets was greater than that of scallops fed the low-PUFA diet. Lipid content was highest in the gonads of scallops fed the natural diet, followed by those fed the high-PUFA diet and finally those fed the low-PUFA diet. However, integration of lipids from different algal food sources into gametes is unlikely to simply follow energy availability (Hendricks et al. 2003).

Cellular regulation by the more fluid membranes produced by integration of PUFA could underlie the increased capacity for HSP70 induction in scallops (and their offspring) fed the high-PUFA diet. Samples et al. (1999) observed that trout leukocytes supplemented with DHA and arachidonic acid showed a higher induction of *hsp*70 mRNA after heat shock. They propose that a change in temperature alters the physical state of membrane phospholipids, making them available to the phospholipase that generates lipid mediators that interact with the heat shock factor to drive the heat shock response (Samples et al. 1999). This could suggest that membranes with more PUFA (such as 22:6n-3 or 20:4n-6) may liberate more lipid mediators upon heat shock leading to stronger induction of HSPs.

While the exact mechanism whereby integration of PUFA such as 22:6n-3 into membranes in adult and larval scallops influences stress response capacities remains to be elucidated, parental diet has marked effects upon larval and adult performance. If membrane quality acts via an improved aerobic capacity, a more balanced microalgal diet, such as used in the current experiment, could mitigate the loss of stress response capacity observed with gonadal maturation in a range of scallop species. The strong influence of parental diet upon larval performance of D larvae

may not continue into later larval life, when larvae find their own food. Nonetheless, the increase in survival and stress response capacity during early stages, with their high larval mortality, could increase reproductive success. Positive carryover effects such as we observed would therefore be under positive selection.

Acknowledgments We gratefully acknowledge Germán Lira from Laboratorio Central de Cultivos from UCN for scallop procurement and maintenance. This study was supported by FONDECYT 3110101 funding to HP and KB.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

References

Barber BJ, Blake NJ (1983) Growth and reproduction of the bay scallop, *Argopecten irradians* (Lamarck) at its southern distributional limit. J Exp Mar Biol Ecol 66:247–256

Berntsson KM, Jonsson PR, Wängberg SA, Carlsson AS (1997) Effects of broodstock diets on fatty acid composition, survival and growth rates in larvae of the European flat oyster, *Ostrea edulis*. Aquaculture 154:139–153

Brokordt K, Guderley H (2004) Energetic requirements during gonad maturation and spawning in scallops: sex differences in *Chlamys islandica* (Muller 1776). J Shellfish Res 23:25–32

Brokordt KB, Himmelman JH, Guderley H (2000a) Effect of reproduction on escape responses an muscle metabolic in the scallop *Chlamys islandica* Muller 1776. J Exp Mar Biol Ecol 251:205–225

Brokordt KB, Himmelman JH, Nusetti OA, Guderley H (2000b) Reproductive investment reduces recuperation from exhaustive escape activity in the tropical scallop *Euvola ziczac*. Mar Biol 137:857–865

Brokordt K, Guderley H, Guay M, Gaymer C, Himmelman J (2003) Sex differences investment: maternal care reduces escape responses capacity in the whelk *Buccinum undatum*. J Exp Mar Biol Ecol 291:161–180

Brokordt K, Fernandez M, Gaymer C (2006) Domestication reduces the capacity to escape from predators. J Exp Mar Biol Ecol 329:11–19

Brokordt K, Pérez H, Herrera C, Gallardo A (2015) Reproduction reduces HSP70 expression capacity in *Argopecten purpuratus* scallops subject to hypoxia and heat stress. Aquat Biol 23:265–274

Cabello R, Tam J, Jacinto ME (2002) Procesos naturales y antropogénicos asociados al evento de mortalidad de conchas de abanico ocurrido en la bahía de Paracas (Pisco, Perú) en junio del 2000. Rev Peru Biol 9:49–65

Caers M, Coutteau P, Sorgeloos P, Gajardo G (2003) Impact of algal diets and emulsions on the fatty acid composition and content of selected tissues of adult broodstock of the Chilean scallop *Argopecten pupuratus* (Lamarck, 1819). Aquaculture 217:437–452

Cheney DP, MacDonald BF, Elston RA (2000) Summer mortality of Pacific oysters, *Crassostrea gigas* (Thunberg): initial findings on multiple environmental stressors in Puget Sound, Washington, 1998. J Shellfish Res 19:353–359

Delaporte M, Soudant P, Moal J, Lambert C, Quéré C, Miner P, Choquet G, Paillard C, Samain JF (2003) Effect of a monospecific algal diet on immune functions in two bivalve species—*Crassostrea gigas* and *Ruditapes philippinarum*. J Exp Biol 206:3053–3064

Delaporte M, Soudant P, Lambert C, Moal J, Pouvreau S, Samain JF (2006) Impact of food availability on energy storage and defense related hemocyte parameters of the Pacific oyster *Crassostrea gigas* during an experimental reproductive cycle. Aquaculture 254:571–582

Disalvo L, Alarcon E, Martinez E, Uribe E (1984) Progress in mass culture of *Chlamys* (*Argopecten*) *purpurata* (Lamarck, 1819) with notes on its natural history. Rev Chil Hist Nat 57:35–45

Ehteshami F, Christianus A, Rameshi H, Harmin SA, Saad CR (2011) The effects of dietary supplements of polyunsaturated fatty acid on pearl oyster, *Pinctada margaritifera* L., gonad composition and reproductive output. Aquac Res 42:613–622

Folch JM, Lee SM, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Guderley H, Pörtner HO (2010) Metabolic power budgeting and adaptive strategies in zoology: examples from scallops and fish. Can J Zool 88:753–763

Guderley H, Brokordt K, Pérez HM, Marty Y, Kraffe E (2011) Diet and performance in the scallop, *Argopecten purpuratus*: force production during escape responses and mitochondrial oxidative capacities. Aquat Living Res 24:261–271

Hendricks IE, van Duren LA, Herman PMJ (2003) Effect of dietary polyunsaturated fatty acids on reproductive output and larval growth of bivalves. J Exp Mar Biol Ecol 296:199–213

Hofmann G, Somero G (1995) Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and Hsp70 in the intertidal mussel *Mytilus trossulus*. J Exp Biol 198:1509–1518

Hulbert AJ, Else PL (1999) Membranes as possible pacemakers of metabolism. J Theor Biol 199:257–274

Hulbert AJ, Else PL (2000) Mechanisms underlying the cost of living in animals. Annu Rev Physiol 62:207–235

Hulbert AJ, Turner N, Storlien LH, Else PL (2005) Dietary fats and membrane function: implications for metabolism and disease. Biol Rev 80:155–169

Iglesias JIP, Navarro E (1991) Energetics of growth and reproduction in cockles (*Cerastoderma edule*): seasonal and age-dependent variations. Mar Biol 111:359–368

Institute SAS (1999) SAS/STAT User's Guide release 8.02 ed. SAS Institute Press, Cary

Jeno K, Brokordt K (2014) Nutritional status affects the capacity of the snail *Concholepas concholepas* to synthesize Hsp70 when exposed to stressors associated with tidal regimes in the intertidal zone. Mar Biol 161:1039–1049

Kraffe E, Tremblay R, Belvin S, LeCoz J, Marty Y, Guderley H (2008) Effect of reproduction on escape responses, metabolic rates and muscle mitochondrial properties in the scallop *Placopecten magellanicus*. Mar Biol 156:25–38

Lenth RV, Hervé M (2015) Least-squares means: R package version 2.14. url http://cran.r-project.org

Li Y, Qin JG, Abbott C, Li X, Bekendorff K (2007) Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters. Am J Physiol Regul Integr Comp Physiol 293:2353–2362

Li Y, Qin JG, Li X, Bekendorff K (2010) Assessment of metabolic and immune changes in postspawning Pacific oyster *Crassostrea gigas*: identification of a critical period of vulnerability after spawning. Aquac Res 41:155–165

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-Delta Delta C(T) method. Methods 25:402–408

Martínez G, Pérez H (2003) Effect of different temperature regimes on reproductive conditioning in the scallop *Argopecten purpuratus*. Aquaculture 228:153–167

Martínez G, Brokordt K, Aguilera C, Soto V, Guderley H (2000) Effect of diet and temperature upon muscle metabolic capacities and biochemical composition of gonad and muscle in *Argopecten purpuratus* Lamarck 1819. J Exp Mar Biol Ecol 247:29–49

Martínez-Pita I, Hachero-Cruzado I, Sánchez-Lazo C, Moreno O (2012a) Effect of diet on the lipid of the commercial clam *Donax trunculus* (Mollusca: Bivalvia): sex related differences. Aquac Res 43:1134–1144

Martínez-Pita I, Sánchez-Lazo C, Ruíz-Jarabo I, Herrera M, Mancera JM (2012b) Biochemical composition, lipid classes, fatty acids and sexual hormones in the mussel *Mytilus galloprovincialis* from cultivated populations on South Spain. Aquaculture 358:274–283

Martínez-Pita I, Sánchez-Lazo C, García FJ (2014) Influence of microalga lipid composition on the sexual maturation of *Mytilus galloprovincialis*: a hatchery study. Aquac Nutr 22:202–216

Marty Y, Delaunay F, Moal J, Samain JF (1992) Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development. J Exp Mar Biol Ecol 163:221–234

Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437–496

Pernet F, Tremblay R, Comeau L, Guderley H (2007) Temperature adaptation in two bivalve species from different thermal habitats: energetics and remodeling of membrane lipids. J Exp Biol 210:2999–3014

Samain JF, Dégremont L, Solechnik P, Haure J, Bédier E, Ropert M, Moal J, Huvet A, Bacca H, Van Wormhoudt A, Delaporte M, Costil K, Pouvreau S, Lambert C, Boulo V, Soudant P, Nicolas JL, Le Roux F, Renault T, Gagnaire B, Geret F, Boutet I, Burgeot T, Boudry P (2007) Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture 268:227–243

Samples BL, Pool GL, Lumb RH (1999) Polyunsaturated fatty acids enhance the heat induced stress response in rainbow trout (*Oncorhyncus mykiss*) leukocytes. Comp Biochem Physiol 123:389–397

Sharma S, Singh R, Kaur M, Kaur G (2010) Late-onset dietary restriction compensates for age-related increase in oxidative stress and alterations of HSP 70 and synapsin1 protein levels in male Wistar rats. Biogerontology 11:197–209

Snedecor GW, Cochran WG (1989) Statistical methods ed 8. Iowa State University Press, Ames

Somero GN (2002) Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. Integr Comp Biol 42:780–789

Tomaru Y, Kawabata Z, Nakano S (2001) Mass mortality of the Japanese pearl oyster *Pinctada fucata martensii* in relation to water temperature, chlorophyll a and phytoplankton composition. Dis Aquat Org 44:61–68

Tremblay R, Myrand B, Sevigny JM, Blier P, Guderley H (1998) Bioenergetic and genetic parameters in relation to susceptibility of blue mussels, *Mytilus edulis* (L.) to summer mortality. J Exp Mar Biol Ecol 221:27–58

Utting SD, Millican PF (1997) Techniques for the hatchery conditioning of bivalve broodstocks and subsequent effect on egg quality and larval viability. Aquaculture 155:45–54

Xiao J, Ford SE, Yang H, Zhang G, Zhang F, Guo X (2005) Studies on mass summer mortality of cultured zhikong scallops (*Chlamys farreri* Jones et Preston) in China. Aquaculture 250:602–615

Zhang J, Gilbert D, Gooday AJ, Levin L, Naqvi SWA, Middelburg JJ, Scranton M, Ekau W, Pena A, Dewitte B, Oguz T, Monteiro MS, Urban E, Rabalais NN, Ittekkot V, Kemp WM, Ulloa O, Elmgren R, Escobar-Briones E, Van der Plas AK (2010) Natural and human-induced hypoxia and consequences for coastal areas: synthesis and future development. Biogeosciences 7:1443–1467