Mar Biol (2013) 160:211–219
DOI 10.1007/s00227-012-2079-2

ORIGINAL PAPER

Sensitivity of gametes, fertilization, and embryo development of the Japanese pearl oyster, *Pinctada fucata martensii*, to the harmful dinoflagellate, *Heterocapsa circularisquama*

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Received: 1 March 2012 / Accepted: 18 September 2012 / Published online: 12 October 2012

Abstract Blooms of the toxic dinoflagellate *Heterocapsa circularisquama* cause massive bivalve kills in Japan. Mariculture of the Japanese pearl oyster, *Pinctada fucata martensii*, is the industry most affected by these blooms, especially in Ago Bay, Mie Prefecture, where they are frequent, cause mass mortality of oysters, and overlap with their spawning season. The goal of this August 2009 study was to assess the effects of a toxic strain of *H. circularisquama* isolated from Ago Bay on gametes, fertilization, and embryo development of pearl oysters. Spermatozoa, eggs, spermatozoa and eggs, and fertilized eggs of pearl oysters from Ago Bay were exposed to *H. circularisquama* at cell densities reported during the bloom (10–10^4 cells mL^{-1}) for different periods of time. The concentration of *H. circularisquama*, exposure duration, and their interactions all had significant effects on gamete quality, fertilization, and embryo development. The motility and swimming velocity of spermatozoa, egg viability, fertilization, and embryo development rate were significantly reduced in all concentrations, with a cell density of 10 cells mL^{-1} determined to be the critical density of *H. circularisquama* for deleterious effects. This is the first evidence of inimical effects of an HAB species on bivalve spermatozoa upon direct exposure. Further field and laboratory studies are required to investigate the potential effects of *H. circularisquama* blooms on the reproduction and recruitment of Japanese pearl oysters and other bivalves.

Communicated by J. P. Grassle.

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Introduction

Harmful algal blooms (HABs) are a worldwide concern due to the increase in occurrence, severity, and diversity of the causative agents and the bloom impacts (Zingone and Oksfeldt Enevoldsen 2000). Several factors, including climate change (Moore et al. 2008; Roger and Laffoley 2011), eutrophication and utilization of coastal waters for aquaculture (Heisler et al. 2008), and shellfish transportation (Hégaret et al. 2008) have been suggested as causes of the increase in HABs.

Several HAB species cause mass mortality in shellfish, including bivalves (Shumway 1990). In addition, toxic algae negatively affect feeding (Hégaret et al. 2007), respiration (Shumway et al. 1985), and behavior (Tran et al. 2010), and induce histopathological lesions (Pearce et al. 2005; Galimany et al. 2008a, b) and impair immune responses in several bivalve species (Hégaret and Wikfors 2005a, b).

Most studies on the impacts of HABs on bivalves have focused on the juvenile or the adult life stages of several commercially important species. However, the effects of HABs on gametes, fertilization, and early life stages of bivalves are largely unknown, despite the fact that gametes and planktonic stages of marine invertebrates are considered relatively critical phases in their response to biotic and

abiotic environmental stressors (Pechenik 1987; Przeslawski et al. 2008; Padilla and Miner 2006).

In Japan, shellfish kills, notably bivalves and gastropods, are caused mainly by blooms of *Prorocentrum* sp., *Heterosigma akashiwo*, *Karenia digitata*, *Noctiluca scintillans*, *Gonyaulax polygramma*, and *Alexandrium* spp. (Matsuyama 2003a). However, since the late 1990s, the harmful dinoflagellate *Heterocapsa circularisquama* (Horiguchi 1995) has been causing mass mortality of numerous bivalve species with no documented effects on marine vertebrates and no reported human poisoning (Matsuyama et al. 1995). Along with *Chattonella antiqua* and *Cochlodinium polykrikoides*, *H. circularisquama* is rated as an extremely harmful species that can easily reach the warning level of 5×10^2 cells mL^{-1} for fishery damage even at low nutrient levels (Imai et al. 2006).

Heterocapsa circularisquama bloomed for the first time in Uranouchi Bay, Kochi prefecture, in the summer of 1988, accompanied by mass mortality of the short-neck clam *Ruditapes philippinarum* (Matsuyama et al. 1995). Since then, the geographical distribution of *H. circularisquama* has expanded to western and central Japan and north to Kamoko Lagoon, with blooms in 19 localities with associated massive kills of a dozen bivalve species (Matsuyama 2012). Depending on the locality, blooms of *H. circularisquama* occur in the early summer to late autumn (June to November), at cell densities reaching up to 2.5×10^5 cells mL^{-1}, in embayments where water temperature exceeds 23 °C and salinity 30 (Matsuyama 2003b). Factors that trigger *H. circularisquama* blooms are seawater mixing events, upwelling of nutrient-rich water, and the die-off of diatom blooms associated with an early summer rainy season (Matsuyama et al. 1996, 1997; Matsuyama 2003a).

Heterocapsa circularisquama causes several deleterious effects in adult and juvenile bivalves including changes in valve movement behavior (Nagai et al. 2006; Basti et al. 2009), reduction in clearance and respiration rates (Matsuyama et al. 1997; Basti et al. 2011a), a low hemocyte count, inflammation, degenerative pathologies and necrosis with a low mucocyte count in the gills (Basti et al. 2011b), rejection of pseudofaeces through the inhalant siphon, increased mucus secretion, contraction of the mantle and siphons, paralysis, destruction of mitochondria of the heart muscle, cardiac disorder, and death (Matsuyama et al. 1995; Nagai et al. 1996, 2006; Yamatogi et al. 2005; Matsuyama 2003a; Basti and Segawa 2010). In addition, Matsuyama (2003a) found that exposure to *H. circularisquama* at 5×10^3 cells mL^{-1} affects eggs, embryos, and trochophores of the Pacific oyster, *Crassostrea gigas*.

In all of western Japan, mariculture of the Japanese pearl oyster, *P. fucata martensii*, is the industry most devastated by *H. circularisquama* blooms, with Ago Bay, Mie prefecture, being the most productive site yet experiencing the greatest number of recurrent blooms of *H. circularisquama* since 1992 with mass mortalities of pearl oysters (Matsuyama 2012). In Ago Bay, *H. circularisquama* is first detected in late spring (May) and forms blooms from early July to early December. Its cell densities during this period range from 0.01 to $>8.7 \times 10^4$ cells mL^{-1} (Matsuyama 2012). Mortalities and adverse effects for juvenile and adult pearl oysters occur over the density range of 102–103 cells mL^{-1} and 2×10^3–6×10^3 cells mL^{-1}, respectively (Matsuyama 2003b, 2012). The density of *H. circularisquama* then decreases to less than the detection level of 1 cell L^{-1} in early winter (Shiraishi et al. 2007). In Ago bay, pearl oysters spawn from April to August with a peak between June and July (Wada 1984), overlapping with blooms of *H. circularisquama*. Thus, an understanding of the effects of *H. circularisquama* on early life stages of *P. fucata martensii* is critically important. In a recent study, we found that a few hours of exposure of trochophores and D-larvae of *P. fucata martensii* to low *H. circularisquama* cell densities of 10^2–5×10^2 cells mL^{-1} severely reduced their swimming activity and survival rate, induced several kinds of damage, and inhibited their development (Basti et al. 2011b). In the present study, we examine the effects of *H. circularisquama* on gamete quality, fertilization, and embryo development of *P. fucata martensii*, in a time- and concentration-dependent manner.

Materials and methods

Algal culture

Toxic *Heterocapsa circularisquama* (strain HC92) was isolated in August 1992 from Ago Bay, Mie Prefecture, Japan, and cultured at 25 °C, pH 7.8–8.0, salinity 33, in autoclaved (121 °C, 15 min) F/2 medium, under a 12 h L:12 h D photoperiod. Following counting, the algal cells were added to the experimental seawater at the desired densities (10–10^4 cells mL^{-1}). The seawater used to prepare the F/2 medium and to conduct the experiments was collected from Ago Bay, filtered, and then UV-treated before enrichment of the culture medium and exposure to *H. circularisquama*.

Exposure experiments

Sexually mature adult pearl oysters, *Pinctada fucata martensii*, were reared at the K. Mikimoto & Co. Ltd. farm, Ago Bay, Mie Prefecture, Japan. Oyster shells were opened and several incisions were made in the gonads. Gametes were obtained by stripping the oysters ($n = 5$, shell height = 72.8–82.0 mm) and filtering the gametes through

gauze. Maturation of eggs was induced by placing them for 45 min in 1 L of a 0.75 mM ammonia–seawater solution (dilution 1:1,000). The same solution was used to activate spermatozoa (10 min in 0.5 L, dilution 1:500). Gamete quality was assessed visually under a microscope at 200 × magnification. Only round, nontransparent eggs and motile sperm were used. Egg density was determined by taking three samples (2 mL each) under agitation and then adjusted to 10^4 eggs L^{-1}.

Preliminary experiments were performed to investigate the concentrations of *H. circularisquama* and time limits for the exposure experiments. Eggs, spermatozoa, or eggs and spermatozoa were exposed to *H. circularisquama* at 0, 10, 10^2, 5×10^2, 10^3, 5×10^3 and 10^4 cells mL^{-1} in 6-well plates to determine the effects of the toxic alga on egg viability, sperm activity rate and swimming velocity, and fertilization rate, respectively. To determine the effects of *H. circularisquama* on embryo development, eggs were fertilized with spermatozoa (10^4 spermatozoa egg^{-1}), washed with fresh filtered and UV-treated seawater, and then exposed to *H. circularisquama* before the first cleavage in 6-well plates. The experiments were run in triplicate at 25 °C.

Effects of *H. circularisquama* on spermatozoa

For each sampling time and each *H. circularisquama* density, 8-μL aliquots of the exposed spermatozoa were transferred to Teflon printed glass slides (21 wells, 4 mm diameter, Funakoshi Co., Tokyo, Japan) and spermatozoa quality was assessed using a computer-assisted sperm analysis system. The motility, which is the number of motile spermatozoa relative to the total number of spermatozoa (%), was recorded without coverslips following 5, 10, 30, and 60 min of exposure, for 2 s at 60 frames s^{-1} with high-speed videomicroscopy (Fastcam PCI 1024 note pack, Photron Co. Ltd., Japan). Spermatozoa were considered motile when the sperm showed forward head movements in 5 consecutive video frames. The motility was determined for at least 50 randomly selected spermatozoa for each measurement. The curvilinear velocity (VCL, μm s^{-1}) was determined as the sum of the incremental distances moved in each frame along the sample path divided by the total time of the track. The measurements were run in duplicate using a two-dimensional image tracking software (Image Tracker PTV, Digimo Co. Ltd., Japan).

Effects of *H. circularisquama* on egg viability, fertilization rate, and embryo development rate

Eggs, fertilized eggs, and embryos were sampled and fixed with a 5 % formalin solution for observation under a light microscope. Egg viability was expressed as the average number of round and regularly shaped eggs relative to the total initial number of eggs (%), following 30 and 60 min of exposure. Fertilization rate was expressed as the average number of eggs that were fertilized relative to the total initial number of eggs (%), following 30 and 60 min of exposure, as indicated by the presence of polar bodies, a fertilization envelope, or by cell division. The embryo development rate was expressed as the average number of regularly shaped trochophores relative to the total initial number of fertilized eggs (%), following 16 h of exposure.

Statistical analysis

Normality and homogeneity of variance were tested a priori using a Kolmogorov–Smirnov test and Bartlett's test, respectively. Data expressed as a rate were transformed by the angular transformation (arcsine $\sqrt{}$percentage) to insure normality. The curvilinear velocity of spermatozoa was Box–Cox transformed to insure normality.

The effects of *H. circularisquama* concentration and exposure duration were tested using a factorial ANOVA. To determine the concentrations at which the effects were significant, the post hoc test Fisher's LSD was performed.

Results

The concentration of *H. circularisquama*, exposure duration, and their interactions all had significant effects on the motility and swimming velocity of spermatozoa, egg viability, fertilization rate, and embryo development rate of *Pinctada fucata martensii* (ANOVA, $P < 0.01$).

The motility of both the control and exposed spermatozoa was maximal following 5 min of activation, and then decreased with time (Fig. 1). Exposure to *H. circularisquama* induced a significant decrease in the motility of spermatozoa for all experimental concentrations and exposure durations (ANOVA, $P < 0.01$). In particular, the motility of spermatozoa exposed to *H. circularisquama* at 5×10^3 and 10^4 cells mL^{-1} for 5 min was approximately 0.09 and 0.03 % of the control motility, respectively. Following 10 min of exposure to these two concentrations, all spermatozoa ceased moving. Exposure to lower concentrations of *H. circularisquama* induced a significant decrease in the motility by as much as 49.9–99.9 % of control motilities.

The swimming velocity of control spermatozoa reached its maximum of 99.27 ± 6.54 μm s^{-1} following 10 min of activation (Fig. 2) and then decreased significantly (regression analysis: $n = 60$, $r^2 = 0.93$, slope $P < 0.001$, intercept $P < 0.01$). Exposure to *H. circularisquama* induced a significant decrease in the swimming velocity of

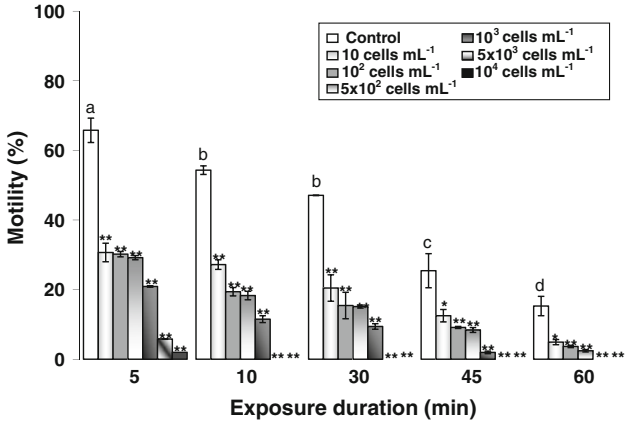

Fig. 1 Motility of *Pinctada fucata martensii* spermatozoa exposed to *H. circularisquama*. (*) indicates significant effect (ANOVA, Fisher's LSD test, $P < 0.05$). (**) indicates significant effect (ANOVA, Fisher's LSD test, $P < 0.01$). *Different letters* indicate significant difference between controls (ANOVA, $P < 0.05$)

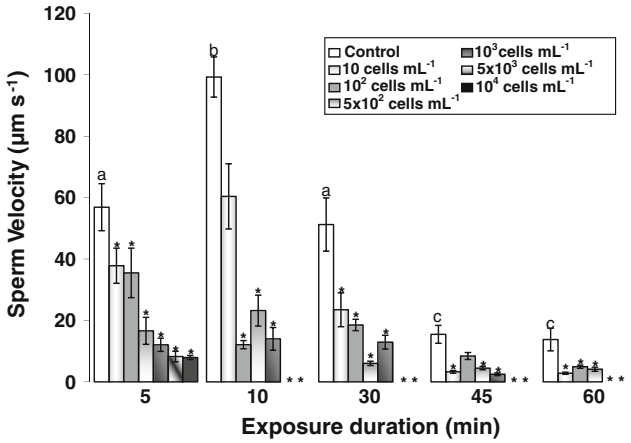

Fig. 2 Swimming velocity of *Pinctada fucata martensii* spermatozoa exposed to *H. circularisquama*. (*) indicates significant effect (ANOVA, Fisher's LSD test, $P < 0.05$). *Different letters* indicate significant difference between controls (ANOVA, $P < 0.05$)

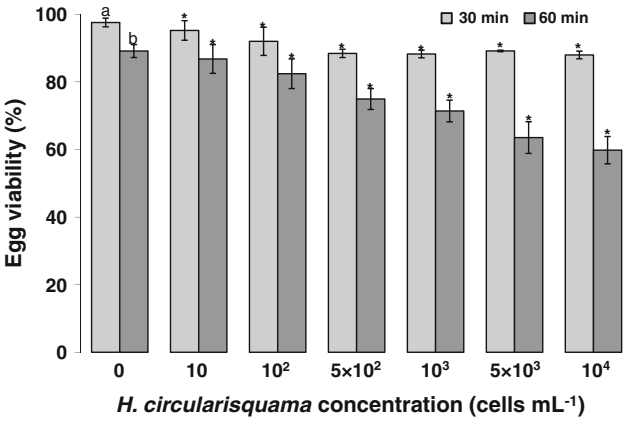

Fig. 3 Viability of *Pinctada fucata martensii* eggs exposed to *H. circularisquama*. (*) indicates significant effect (ANOVA, Fisher's LSD, $P < 0.01$). *Different letters* indicate significant difference between controls (ANOVA, $P < 0.05$)

spermatozoa for all experimental concentrations and exposure durations. The swimming velocity decreased to 66.5–20.3 %, 62.2–12.2 %, 29–11.7 %, 25–14.1 %, 14.4–0.0 %, and 13.9–0.0 % of the control swimming velocity for exposure to 10, 10^2, 5×10^2, 10^3, 5×10^3, and 10^4 cells mL^{-1}, respectively.

Egg viability was significantly reduced following exposure to *H. circularisquama* at 10–10^4 cells mL^{-1} for 30 and 60 min (Fig. 3). Egg viability was 90.1 and 67.1 % of control viability following exposure to 10^4 cells mL^{-1} for 30 and 60 min, respectively. The eggs exposed to *H. circularisquama* were irregular in shape with a swollen egg membrane and *H. circularisquama* cells transformed into temporary cysts attached to the egg membranes (Fig. 4a, b).

The fertilization rate of *P. fucata martensii* was significantly decreased following 30 min of exposure to *H. circularisquama* at 5×10^3 and 10^4 cells mL^{-1} by as much as 56.5 and 60.4 % of the control, respectively (Fig. 5). Following 60 min of exposure, the fertilization rates were significantly decreased for all experimental concentrations of *H. circularisquama*, especially for 5×10^3 and 10^4 cells mL^{-1} for which the fertilization rates were reduced by as much as 57.4 and 73.9 % of the control, respectively. Fertilized egg membranes were damaged by *H. circularisquama* (Fig. 4c, d).

The developmental rate of fertilized eggs exposed to *H. circularisquama* at 10, 10^2, 5×10^2, 10^3, 5×10^3, and 10^4 cells mL^{-1} for 16 h was significantly decreased by 6.8, 8.9, 17.2, 25.5, 75.7, and 77.7 % of the control, respectively (Fig. 6). Four-cell blastomeres and blastula stages of fertilized eggs exposed to *H. circularisquama* showed abnormal swellings, cytoplasmic discharges, and bursting of outer membranes (Fig. 4e, f).

Discussion

In the present study, the harmful dinoflagellate *Heterocapsa circularisquama* significantly reduced spermatozoa motility and swimming velocity, egg viability, fertilization rate, and embryo development rate of *Pinctada fucata martensii* in a time- and concentration-dependent manner, within minutes of exposure to a low density of 10 cells mL^{-1}. The cells of *H. circularisquama* were observed attached to eggs, fertilized eggs, and embryos of pearl oysters, shedding their walls and transforming into temporary cysts. Several anomalies were observed with egg membrane, fertilization membrane, and vitelline envelope swellings, cytoplasmic discharges, and subsequent lysis.

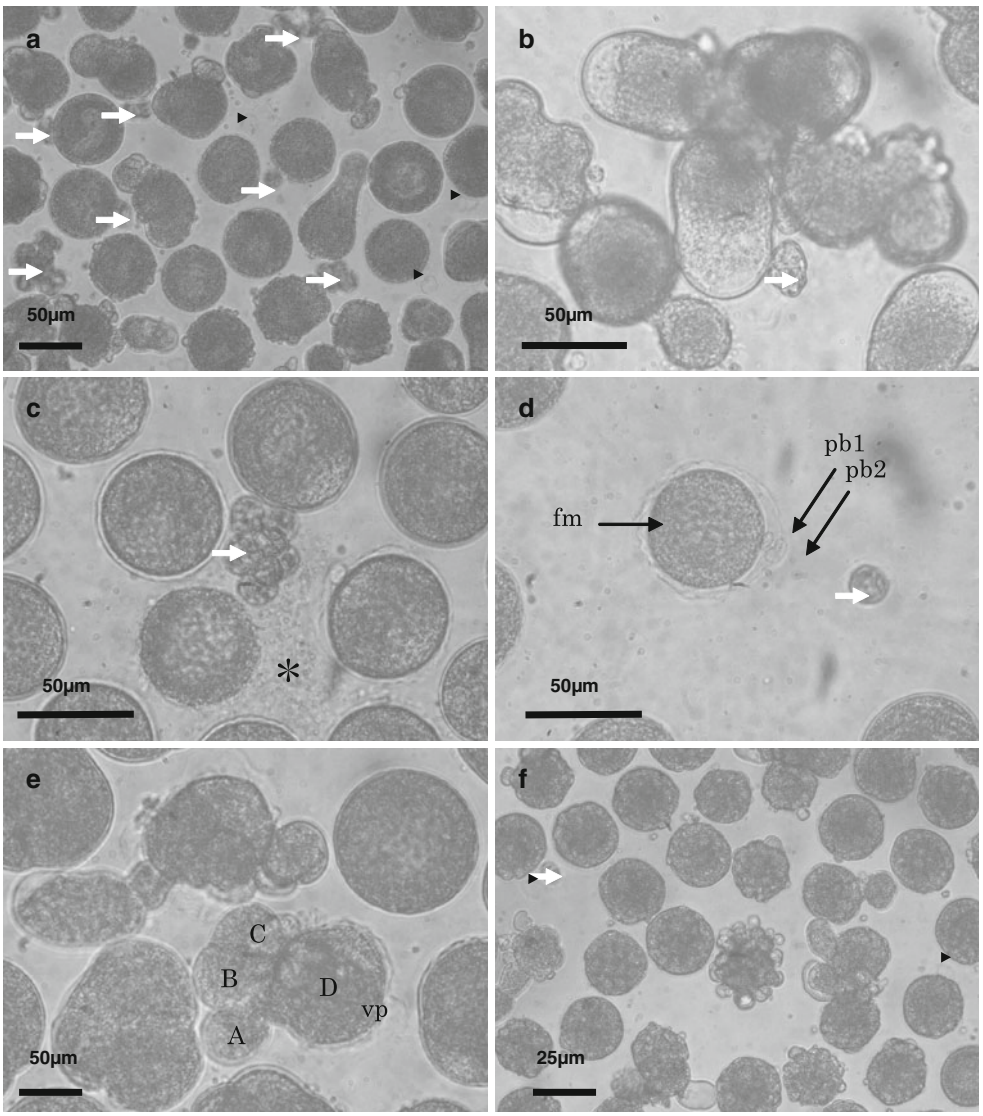

Fig. 4 *Pinctada fucata martensii*. Light micrographs. **a**, **b** Eggs exposed for 60 min to *H. circularisquama* at 10^3 cells mL^{-1} with irregular shape, cytoplasmic discharges, and *H. circularisquama* cells attached to egg membrane; **c**, **d** fertilized eggs 60 min after exposure to *H. circularisquama* at 10^3 cells mL^{-1} with swellings, cytoplasmic discharges, and disintegration of fertilization membrane; **e** 4-cell blastomeres embryo with cytoplasmic discharges; **f** blastula with severe abnormalities, swellings, cytoplasmic discharges. *White arrows* indicate *H. circularisquama* temporary cysts; *Black arrowheads* mark *H. circularisquama* cell walls; *Black asterisk* shows disintegrated fertilization membrane. *A*, *B*, *C*, *D* blastomeres; *fm* fertilization membrane; *pb1* first polar body; *pb2* second polar body; *vp* vegetal pole

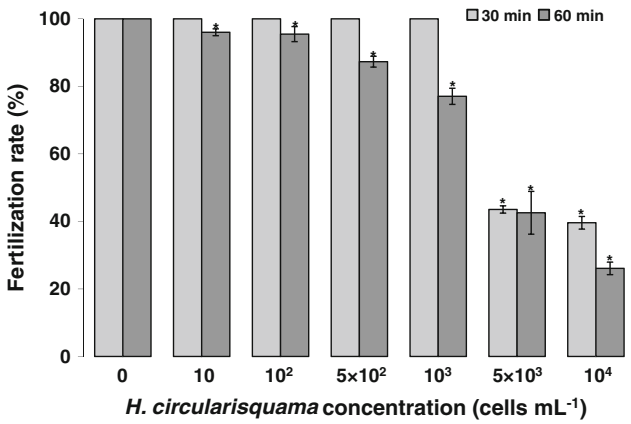

Fig. 5 Fertilization rate for *Pinctada fucata martensii* eggs and spermatozoa exposed to *Heterocapsa circularisquama*. (*) indicates significant effect (ANOVA, Fisher's LSD, $P < 0.01$)

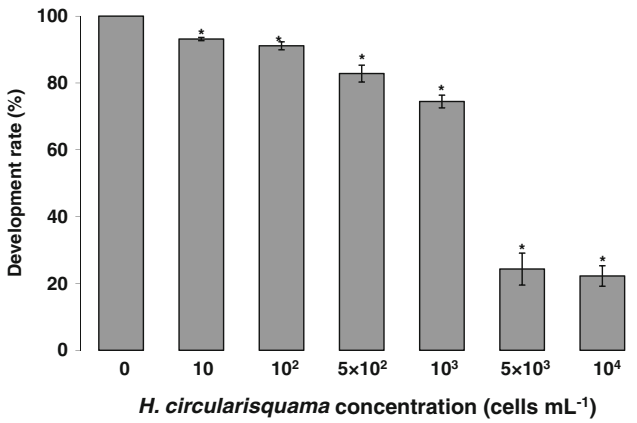

Fig. 6 Development rates of *Pinctada fucata martensii* fertilized eggs exposed for 16 h to different concentrations of *Heterocapsa circularisquama*. (*) indicates significant effect (ANOVA, Fisher's LSD, $P < 0.01$)

Eggs and embryos of *Crassostrea gigas* and *Mytilus galloprovincialis* showed similar abnormalities when exposed to another toxic strain of *H. circularisquama* at densities exceeding 5×10^3 cells mL^{-1} (Matsuyama et al. 2001; Matsuyama 2003a). Studies on the effects of HABs on eggs, fertilization, and embryos of bivalves are very limited. The embryonic development of *C. virginica* was not affected following exposure to *Karlodinium veneficum* (Stoecker et al. 2008), whereas *Prorocentrum minimum* and *Heterosigma akashiwo* caused significant mortality and inhibited embryonic development of *C. virginica* (Wikfors and Smolowitz 1995) and *Argopecten irradians* (Wang et al. 2006), respectively. Egg hatching of the blue mussel *M. edulis*, the bay scallop *Chlamys farreri*, and *A. irradians* was inhibited by *Chrysocromulina polylepis* (Granmo et al. 1988), *Alexandrium tamarense* (Yan et al. 2001), and *H. akashiwo* (Wang et al. 2006), respectively. Physical contact with *H. circularisquama* cells seems to be the mechanism by which surface-located toxins are released from the algal cells (Kamiyama and Arima 1997; Matsuyama et al. 1997, 2001) and was also described for the toxic HAB species *A. tamarense* (Yan et al. 2001, 2003) and *H. akashiwo* (Wang et al. 2006). Basti et al. (2011b) showed that direct contact with cells of the same strain of *H. circularisquama* inhibited the development, and reduced the activity and survivorship of trochophores and D-larvae of the pearl oyster *P. fucata martensii* over cell densities of 10^2–5×10^2 cells mL^{-1}. The damage in both trochophores and D-larvae included exfoliation of larval cilia, epithelial desquamation, abnormal masses in the velum, abnormal shells, and delayed mineralization of the shell (Basti et al. 2011b). Cell contact, temporary cyst formation, and production of toxins by *H. circularisquama*, as for other HAB species, could be regarded as defensive strategies to control its own population cell density (Uchida 2001), to compete with concurrent phytoplankton populations (Uchida et al. 1995), and to decrease grazing populations by reducing feeding (Kamiyama and Arima 1997) but also fecundity and hatching success (Yan et al. 2003; Wang et al. 2006).

In contrast with a previous study reporting no effect of direct exposure to *H. circularisquama* on *C. gigas* spermatozoa motility and fertility (Matsuyama 2003a), we found that direct exposure to *H. circularisquama* at the low density of 10 cells mL^{-1} reduced the motility rate and swimming velocity of pearl oyster spermatozoa in a statistically significant concentration- and time-dependent manner. This contradiction could be related to differences either in the toxicity of *H. circularisquama* strains, in the sperm sensitivity of the bivalve species, or in the experimental protocol and data analysis used in both studies. Even though the ultrastructural features of bivalve spermatozoa are fundamentally similar (Bozzo et al. 1993; Garrido and Gallardo 1996), differential responses in the spermatozoa of the two bivalve species cannot be excluded. The strain of *H. circularisquama* used in *C. gigas* experiments also showed toxic effects on eggs, embryos, and larvae of *M. galloprovincialis*, *Ruditapes philippinarum*, and *C. gigas* with damage very similar to that reported in the present study. In the former work, however, sperm evaluation was performed subjectively, by microscopic assessment of motility and swimming vigor, which are affected by various factors including interpretation of measures using nonlinear scales, which are unsuitable for statistical analysis. In the present work, the assessment relied on quantitative, computer-assisted tracking of spermatozoa movements captured with high-speed videomicroscopy, which allowed accurate and reliable assessment of spermatozoa motility and swimming speed, and provided data fit for statistical analysis (Rurangwa et al. 2004). Additional comparative experiments investigating the effects of *H. circularisquama* on spermatozoa motility of different bivalve species may further support the results of our findings.

In a recent study, spermatozoa, obtained from mature *C. gigas* exposed to toxic *Alexandrium minutum*, showed reduced energy status and motility associated with morphological changes at cellular and subcellular levels, indirectly induced through the release of toxins upon *A. minutum* ingestion (Haberkorn et al. 2010). There are no reports of deleterious effects of direct exposure of bivalve or other invertebrates' spermatozoa to HABs, but there has been a report of inhibition of sperm motility in echinoderms (*Asteria rubens* and *Psammechinus miliaris*) and polychaetes (*Arenicola marina* and *Nereis virens*) when directly exposed to unsaturated short-chain aldehydes (SCAs), bioactive metabolites secreted by diatoms as part of an oxylipin chemical defense system activated in response to wounding (Caldwell et al. 2004). In addition to inhibiting spermatozoa motility, diatom SCAs affect oocyte maturation, fertilization, embryogenesis, and larval development of an array of invertebrate species including copepods, sea urchins, polychaetes, and ascidians. Their cytotoxicity is related to molecular targets associated with calcium signaling and cell death (Caldwell 2009). Similarly, many notoriously potent HAB toxins (such as saxitoxins, brevetoxins, domoic acid, maitotoxins, azaspiracids) affect cytosolic calcium regulation (Berman et al. 2002; LePage et al. 2003; Kakizaki et al. 2006; Pérez-Gómez et al. 2006). Reported influx of Ca^{2+} in trochophore larvae of *R. philippinarum* (Matsuyama 2003a), abnormal and delayed shell mineralization of *P. fucata martensii* trochophores and D-larvae (Basti et al. 2011b), and necrosis of HeLa cells with accumulation of *H. circularisquama* toxin extracts in the plasma membrane (Kim et al. 2008) all strongly suggest that *H. circularisquama* toxins specifically bind to membrane receptors that disrupt cellular homeostasis, especially

for Ca^{2+} (Matsuyama 2012). Indeed, calcium, both internal and external, plays a pivotal role in sperm motility (Morisawa and Yoshida 2005), the process of fertilization (both sperm–egg binding and sperm–egg fusion) (Santella et al. 2004), egg activation (Sticker 1999), and triggering early embryogenesis (Deguchi and Morisawa 2002), all of which could be related to the severe deleterious effects on sperm, eggs, fertilization, and embryos of *P. fucata martensii* exposed to *H. circularisquama* in the present study.

In conclusion, we found that *H. circularisquama* severely affects spermatozoa, eggs, fertilization, and embryos of pearl oysters, at low concentrations and within minutes of exposure. Nonetheless, inferences about the ecological implications from these findings cannot be made due to the small number of broodstock used (3 males and 2 females) and the fact that gametes and other samples were pooled. Further studies looking on morphological, ultrastructural, and physiological modifications of spermatozoa, eggs, fertilization, and embryo developmental processes upon direct exposure and exposure of broodstock to *H. circularisquama* and other toxic HABs, with improved experimental designs are required to understand the impacts of HABs on the reproduction and recruitment of bivalves.

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