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Ocean acidification decreases mussel byssal attachment strength and induces molecular byssal responses

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ABSTRACT: Ocean acidification (OA) is a term describing the uptake of CO_2 from the atmosphere, decreasing seawater pH and altering carbonate chemistry. Mussels are an ecologically and economically important taxon that attach to solid surfaces via the byssus. To date, little is known about the effects of OA on mussel byssal attachment and the underlying molecular byssal responses. This study demonstrated that after 1 wk of exposure to acidified seawater, both mechanical properties (such as strength and extensibility) and the numbers of byssal threads produced by *Mytilus coruscus* were significantly reduced, leading to a 60 to 65% decrease in mussel byssal attachment strength. Real-time PCR results suggested that OA also altered the expression of genes encoding the proximal thread matrix protein (PTMP), precursor collagen proteins (preCOL-P, -NG and -D) and mussel foot proteins (mfp-1, -2, -3, -4, -5 and -6). The down-regulation of some specific byssal proteins may be one of the reasons for the weakened mechanical properties of individual byssal threads under OA conditions. In contrast, the up-regulation of some other specific byssal proteins may be adaptive responses to minimize the adverse effect of OA on byssal attachment. OA may weaken mussel byssal attachment by reducing the production and mechanical properties of byssal threads and by inducing byssal molecular responses. The weakened byssal attachment induced by OA therefore could pose a substantial threat to both mussel aquaculture and mussel-bed ecosystems.

KEY WORDS: Elevated pCO_2 · *Mytilus coruscus* · Byssal thread · Gene expression · Mechanical properties

INTRODUCTION

Mussels (particularly those in the genus *Mytilus*) living on rocky shores in the intertidal zone are ecologically and economically important oceanic organisms. Many mussels have been used as human food for thousands of years and are important aquaculture species. These sessile bivalves attach to a wide array of substrata by means of their strong holdfast structure, the byssus (Waite 1983). Mussels can also attach to one another via the byssus, thereby forming aggregates. The aggregation of mussels provides stability and protection against predation and environmental perturbations and increases fertilization success (Liu et al. 2011, Christensen et al. 2015). Therefore, strong byssal attachment is critical for mussel survival, self-defence and reproduction (Bandara et al. 2013). Morphologically, the mussel byssus is a bundle of radially distributed threads divided into 3 sections: (1) the root, which connects the whole structure to the byssus retractor muscles and is embedded in the byssus gland at the posterior basal

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region of the foot; (2) the stem, which extends from the root and links the byssal threads; and (3) the byssal threads, which originate from the stem in many directions and anchor to foreign surfaces (Brown 1952). The byssal thread can be further subdivided into 3 parts: (1) the proximal region, which is highly extensible and has a corrugated surface; (2) the distal region, which is smoother, stiffer and approximately twice the length of the proximal region; and (3) the adhesive plaque, which adheres the byssal thread to the substrate (Bell & Gosline 1996). *Mytilus* byssal threads contain 3 main groups of proteins: catechol oxidase, collagenous proteins and polyphenolic proteins, which are produced by the foot organ of the animal (Silverman & Roberto 2007).

To date, 1 proximal thread matrix protein (PTMP), 3 precursor collagen proteins (preCOL-P, -NG and -D) and 6 different families of mussel foot proteins (mfp-1, -2, -3, -4, -5 and -6) have been identified and characterized (Silverman & Roberto 2010); their locations in the byssal thread are illustrated in Fig. 1. PreCOL-P is essential for the elasticity and extensibility to the proximal region, whereas preCOL-D is required for the stiffness and strength to the distal region of the byssal thread (Bandara et al. 2013). preCOL-NG, which is evenly distributed throughout the proximal and distal region of the byssal thread, is believed to mediate the interaction between preCOL-P and preCOL-D (Bandara et al. 2013). The non-collagenous PTMP binds preCOL-P and plays a role in stiffening in the proximal region of the byssal thread (Silverman & Roberto 2010). mfp-1 is distributed throughout the byssal thread and forms a protective cuticle covering the inner core, which is mainly made of collagenous preCOLs (~81% of the dry weight) and non-collagenous TMPs (~9% of the dry weight) (Silverman & Roberto 2010), whereas the other 5 mfps (mfp-2, -3, -4, -5 and -6) are confined exclusively to the adhesive plaque (Lee et al. 2011). All mussel foot proteins contain a high concentration of 3,4-dihydroxyphenylalanine (DOPA), which plays an important role in mussel adhesion (Qin & Buehler 2014).

Since the Industrial Revolution, the atmospheric carbon dioxide (CO_2) concentration has increased by nearly 40%, from a partial pressure (pCO_2) of approximately 280 µatm to a present level of nearly 400 µatm (Solomon et al. 2007). Approximately one-third to one-half of the CO_2 released into the atmosphere is eventually absorbed by the ocean, lowering the pH of the surface seawater and causing wholesale shifts in seawater carbonate chemistry, a process known as ocean acidification (OA; Caldeira & Wickett 2003, Feely et al. 2004, Doney et al. 2009). Over

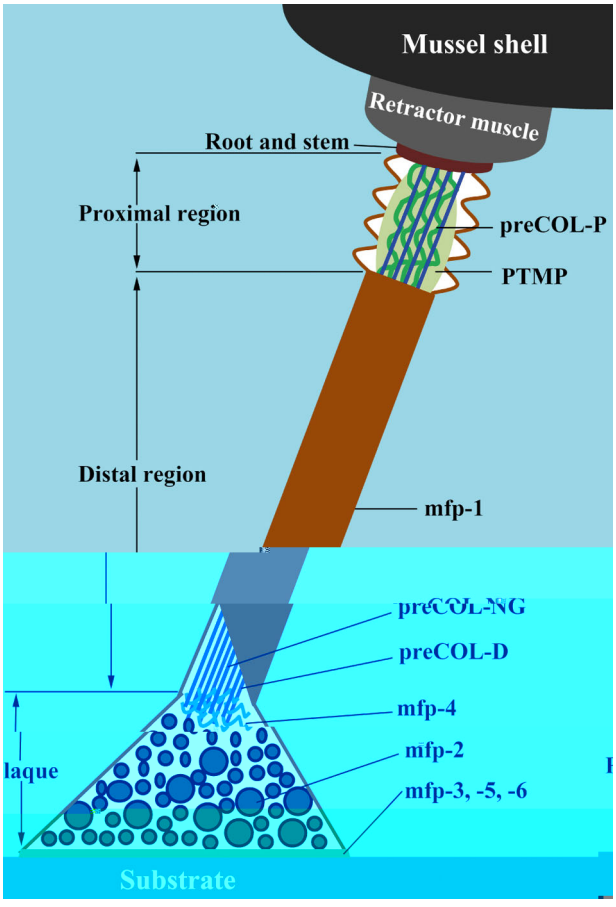

Fig. 1. Distribution of byssal proteins in the byssus of mussels in the genus *Mytilus*

the past 2 centuries, the average surface seawater pH has decreased by approximately 0.1 units, from approximately 8.21 to 8.10. It has been predicted to drop another 0.3 to 0.4 units by the end of the 21st century and 0.7 units around the year 2300 (Caldeira & Wickett 2005, Orr et al. 2005). This poses a great threat to marine organisms and ecosystems, especially those in coastal regions (Fabry et al. 2008). During the past decade, a wide variety of organisms and biological processes have been found to be vulnerable to OA (Kurihara 2008, Wood et al. 2008, Sanford et al. 2014, Prazeres et al. 2015). However, biological responses to OA are species-specific and vary with life stages (Kurihara 2008, Ries et al. 2009, Kroeker et al. 2010). To our knowledge, only 1 publication has described the effects of OA on mussel byssal attachment, showing that a pCO_2 increase from 300 to 1500 µatm reduced the mechanical performance of byssal threads secreted by *M. trossulus* and could subsequently decrease the overall byssal attachment strength by 35 to 41% (O'Donnell et al. 2013). How-

ever, the effect of OA on mussel byssal thread production was not investigated. In addition, the effect of OA on the overall byssal attachment strength was estimated based on a model (Bell & Gosline 1996) assuming mussels attach on substratum with a constant number (50) of byssal threads. Since the number of byssal threads positively correlates with the overall byssal attachment strength (Babarro et al. 2008), the conclusion that OA reduced the overall byssal attachment strength in the previous study may not hold true if more byssal threads were produced under the OA scenario. Moreover, the molecular byssal responses, if any, of mussels under OA conditions remain to be explored.

Therefore, we investigated the effects of OA on the mechanical properties and byssal thread production of the hard-shell mussel *M. coruscus*. The expression of genes encoding PTMP, preCOLs (-P, -NG and -D) and mfps (-1 to -6) were analysed to examine the molecular byssal responses of this mussel to OA.

MATERIALS AND METHODS

Animal collection and acclimation

Mytilus coruscus was sampled from Dongtou Island (seawater pH ranges from 8.0 to 8.4), Zhejiang, China, in August 2013 and transported to Qingjiang Station of the Zhejiang Mariculture Research Institute, China. Mussels of similar size (mean ± SD shell length of 97.75 ± 7.95 mm) were selected, epibionts were gently cleaned off, and mussels were acclimated in a 1000 l aquarium in filtered seawater (pH = 8.10 ± 0.05, temperature = 28 ± 0.5°C, salinity = 21 ± 0.3‰) with continuous aeration for 1 wk prior to experiments. Animals were fed twice daily with microalgae (*Platymonas subcordiformis*) to satiation. Excess food and faeces were removed daily by siphoning from the bottom of the aquarium, followed by refilling with fresh filtered seawater.

Experimental set-up and seawater parameters

After acclimation, the mussels were initially stripped of old byssal threads and randomly transferred to 1 l experimental chambers nested in the custom-made system including 4 pH values, with 1 mussel in each chamber. Each pH level consisted of 10 replicate experimental chambers. The pH levels of seawater were set at the ambient pH value (8.10) and 3 lowered pH values (7.80, 7.60 and 7.40), which were projected values for the year 2100 and beyond (Caldeira & Wickett 2005, Solomon et al. 2007). Following methods described by Shi et al. (2016) and Liu et al. (2016), experimental chambers were filled with filtered seawater with continuous ambient air or a CO_2 gas mixture bubbled in to set the pH to the desired value and to maintain the dissolved oxygen (DO) concentration to near saturation. The CO_2 gas mixture was obtained by mixing CO_2-free air and pure CO_2 gas at known flow rates using flow controllers (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m565p067_supp.xlsx). The experiment lasted for 1 wk. Mussels were fed twice daily with microalgae (*P. subcordiformis*) to satiation. Two-thirds of the seawater in each chamber were removed daily by siphoning, followed by refilling with seawater pre-equilibrated to the appropriate pH. During the 1 wk incubation, no dead mussels were detected.

To ensure no substantial fluctuations in seawater chemistry throughout the incubation period, pH, salinity, temperature and total alkalinity (TA) were measured daily before and after each water change. Each pH level was monitored by a Sartorius PB-10 pH meter calibrated with standard US National Bureau of Standards buffers. Salinity was determined using a conductivity meter (Multi 3410 WTW). Temperature was measured with a mercury thermometer. The TA was obtained by potentiometric titration (Anderson & Robinson 1946). The carbonate system parameters were calculated from the measured pH, salinity, temperature and TA values using the open-source program CO2SYS (Pierrot et al. 2006), with the constants supplied by Mehrbach et al. (1973) and refitted by Dickson & Millero (1987) and the KSO_4 dissociation constant of Dickson (1990). Both measured and calculated seawater parameters of the experimental trials are summarised in Table 1.

Byssus collection and quantitative analysis

At the end of the experiment, newly secreted byssus was carefully removed at the interface between the adhesive plaque and substrate by a scalpel. Mussels were dissected with a scalpel by severing the adductor muscles. The full-length byssal threads were then excised carefully at their points of attachment to the stems.

After collection, the numbers of newly produced byssal threads were counted. In addition, the lengths of byssal threads were measured using a Vernier calliper (precision 0.01 mm). Digital pic-

Table 1. Seawater parameters during the 1 wk incubation of *Mytilus coruscus* (mean ± SD). Partial pressure of CO_2 (pCO_2), dissolved inorganic carbon (DIC), and saturation state of aragonite (Ω_{ara}) and calcite (Ω_{cal}) were calculated from measured pH (Ambient = pH 8.1), calibrated with standard US National Bureau of Standards buffers (pH_{NBS}), salinity (Sal), temperature (T) and total alkalinity (TA) values using the open-source program CO2SYS

Target pH	T (°C)	Sal (‰)	pH_{NBS}	TA (µmol kg^{-1})	pCO_2 (µatm)	DIC (µmol kg^{-1})	Ω_{ara}	Ω_{cal}
Ambient	27.8 ± 0.1	21.47 ± 0.21	8.11 ± 0.06	2074.77 ± 19.51	555.01 ± 5.47	1912.86 ± 18.85	2.27 ± 0.02	3.60 ± 0.04
7.80	27.7 ± 0.3	21.73 ± 0.58	7.81 ± 0.03	2099.47 ± 20.90	1207.05 ± 7.41	2030.01 ± 12.46	1.24 ± 0.01	1.97 ± 0.01
7.60	27.8 ± 0.4	21.81 ± 0.63	7.59 ± 0.04	2097.82 ± 20.40	1976.03 ± 18.99	2089.81 ± 20.08	0.81 ± 0.01	1.28 ± 0.01
7.40	27.9 ± 0.3	21.09 ± 0.75	7.38 ± 0.03	2062.83 ± 28.63	3140.13 ± 45.91	2110.66 ± 30.86	0.51 ± 0.01	0.81 ± 0.01

tures of all byssal threads were obtained using a CCD camera mounted on a Nikon AZ100 microscope. The diameters were determined at 3 independent locations of byssal threads by image analysis using the free-access software ImageJ Version 1.46r (http://imagej.nih.gov/ij/), and the mean values were used as the thread diameters. Cross-sectional areas of threads were calculated from the measured diameters. Threads were assumed to be cylindrical, and their volumes were calculated using the measured lengths and calculated cross-sectional area.

Tensile tests and analysis of mechanical properties

After measuring the quantitative values, the proximal end was clamped between cardboard, and the plaque was glued to a metal stub. The cardboard and metal stub were then secured in the clamps of a universal testing machine (AGS-J, Shimadzu). Tensile tests of the full-length byssal threads were performed in air at room temperature with a constant loading rate of 10 mm min^{-1} until failure occurred.

The breaking force (thread strength) was measured as the force required to break a single byssal thread. The breaking strain (thread extensibility) was calculated as the extension at failure, divided by the unstressed thread length. Toughness was determined as the amount of energy per unit volume that a byssal thread can absorb before failure. The breaking force was converted to breaking stress by dividing by cross-sectional area which was calculated using the measured diameter (d) with the formula Area = $\pi \times (d/2)^2$. For the mechanical property analysis, the data were firstly determined for every single byssal thread of an individual mussel, and the mean value of these byssal threads was used as the data point of the mussel individual. The failure location (proximal, distal or plaque) was recorded, and the failure occurrence at each location was expressed as a percentage of the number of threads tested. A location with thread failure occurring at the thread–plaque junction and/or adhesive plaque was scored positive. Failures that occurred at the grips were discarded from analysis to avoid underestimating the actual mechanical properties (Bell & Gosline 1996, Moeser & Carrington 2006).

RNA isolation and real-time PCR

After dissection, the foot tissue of *M. coruscus* was immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA samples were extracted from the frozen foot tissues of 3 individuals, and DNA contamination was removed using the RNAprep Pure Tissue Kit (Tiangen, DP431) following the method described by Peng et al. (2016). The RNA quality was checked by gel electrophoresis, and RNA concentration was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific). For cDNA synthesis, 1 µg of high-quality total RNA was reverse-transcribed using the M-MLV First Strand Kit (Invitrogen, C28025-032) according to the manufacturer's protocol. The cDNA was diluted 5-fold before PCR amplification.

Real-time PCR experiments were performed on a CFX96^{TM} Real-Time System (Bio-Rad). The amplifications were conducted in triplicate in a total reaction volume of 10 µl containing 5 µl of 2× SsoFast^{TM} EvaGreen Supermix (Bio-Rad, 172-5201AP), 2 µl of prepared cDNA template, 0.3 µl of each primer (10 µM) and 2.4 µl of double-distilled water. The cycle conditions were as follows: 95°C for 5 min, followed by 40 cycles of 94°C for 20 s, 61°C for 20 s and 72°C for 20 s. A melting curve analysis was used to confirm the specificity of each amplification reaction. The *18S rRNA* gene was used as a reference for the calculation of the relative expression levels of target genes. The primers and accession numbers of genes used in the present study are listed in Table 2.

Table 2. Primers and GenBank accession numbers of genes used in real-time PCR

Gene	Accession no(s)	Forward primer (5′–3′)	Reverse primer (5′–3′)
18S rRNA	EF613242	CCTTGGTGCTCTTGATTGA	GAACTACGACGGTATCTGAT
mfp-1	JQ794825	TGGCTACAATTCAAGAACTG	AGAGAAGGATGAGAACGAAT
mfp-2	KP876473.1	CGGTCACAGAAGCATCAT	CATCCTCATCGTCGTCATAT
mfp-3	GQ281052, GU321200–GU321213	TTTGCTGGCTTTAGTCCTT	ACCGCTATTCCATCCCTTA
mfp-4	DQ351535.1, DQ351536.1	ACATATTCACAGCCACCAA	TCACCGTATGATTCAAGACA
mfp-5	GQ281053	TTGGTGCTCGTTCTTGTAA	TGCTACTGCCTCCATAATG
mfp-6	GU321214–GU321222	CGGTGATTATGATTACAGAGG	GAAGACAGCATCCAGCAT
preCOL-P	EU120663.1, AF015539.1, AF448525.1	GATCTTCACATGCATCAGC	CACTGCCACCTCCTAAAC
preCOL-NG	EU120662.1, AF043944.1, KC793982.1, AF448524.1	ACAAGGACCACAAGGAGAA	ACACCACCAACACCAGTT
preCOL-D	EU120661.1, AF029249.1, AF448526.1	ACCAAGAGGAGATAGAGGAG	GGCTGTTCTGAGGTCTTC
PTMP	AF414454.1, AY053390.1, AY053391.1, EF535512.1	ACGCTTCTTCAAGTATCAAC	GACAACTCCTTCCTTCCTTA

Statistical analysis

One-way ANOVAs were conducted to show the effects of OA on the mechanical properties, failure location and byssal thread production and morphology. Before performing 1-way ANOVA of thread diameter and length, and mechanical properties, the mean value of all threads for an individual mussel was calculated and used as the data point for that individual. As described above, tensile tests of byssal threads in which premature failure occurred at the grips were discarded from the analysis to avoid underestimating the actual mechanical properties. Therefore, mechanical properties were not obtained for every single byssal thread of an individual mussel (see Tables S1 & S2 in the Supplement), resulting in a non-1:1 relationship with morphology (diameter and length) data. The raw data used for the calculation of the mean values on different graphs came from the overlapping but not identical data sets (Tables S1 & S2). When significant differences were detected at $p < 0.05$, Tukey's post hoc test was performed to locate the differences. Gene expression levels of byssal proteins were compared using a *t*-test, and differences were considered statistically significant at $p < 0.05$. For all analyses, the assumptions of normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's tests, respectively. For cases where these assumptions were not satisfied by the raw data, the data were log-transformed prior to analysis. All data are presented as mean ± SD, and all statistical analyses were conducted using OriginPro 8.0.

RESULTS

Byssal thread production and morphology

The 1-way ANOVA showed that seawater pH had a significant impact on byssal thread production ($p < 0.05$). The number of threads newly produced by *Mytilus coruscus* of the 3 lowered pH groups (7.80, 7.60 and 7.40) was decreased by approximately 45.9, 31.1 and 56.3% in contrast with that of the ambient pH group, respectively (Fig. 2). Although no significant fluctuation of byssal thread diameter was found when seawater pH declined from ambient value

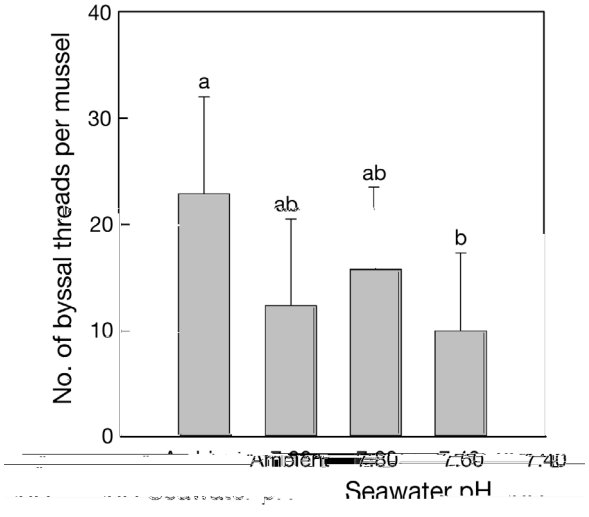

Fig. 2. Number of byssal threads newly produced by *Mytilus coruscus* after 1 wk of exposure to ambient (pH 8.1) or reduced seawater pHs (n = 8–10, mean ± SD). Means not sharing the same superscript are significantly different (Tukey's HSD, $p < 0.05$)

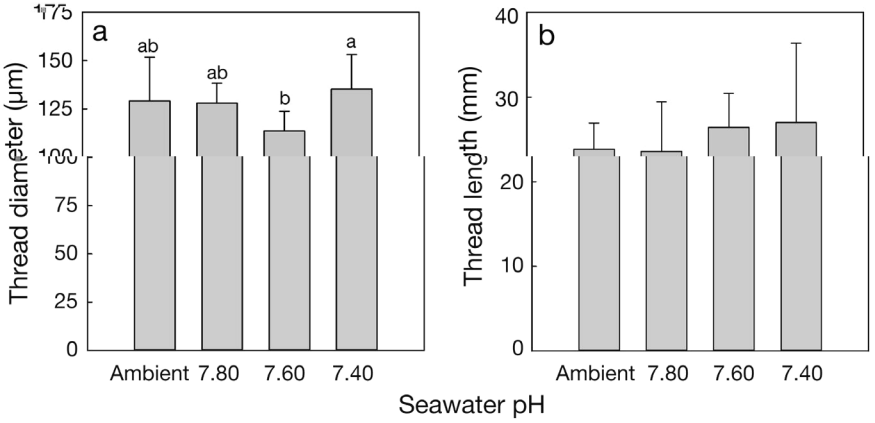

Fig. 3. Effects of ocean acidification on (a) thread diameter and (b) thread length (n = 8–10, mean ± SD) of byssal threads in *Mytilus coruscus*. Means not sharing the same superscript are significantly different (Tukey's HSD, $p < 0.05$)

(8.10) to 7.60, the byssal thread diameter of the pH 7.60 group was significantly thinner than that of the pH 7.40 group (Fig. 3a). Although the byssal thread length of the pH 7.60 and 7.40 groups was slightly greater than that of the ambient pH group, no significant variation was detected (Fig. 3b).

Mechanical properties of byssal threads

A significant effect of seawater pH on the breaking force of each byssal thread (thread strength) was detected (1-way ANOVA, $p < 0.01$). The strength of the byssal thread of the 3 lowered pH groups (7.80, 7.60 and 7.40) was significantly reduced by approximately 26, 41.8 and 23.9% compared to that of the ambient pH group, respectively (Fig. 4a).

Seawater pH also had a significant effect on the breaking stress of each byssal thread (1-way ANOVA, $p < 0.01$). After 1 wk of exposure to the acidified seawater (pH 7.80, 7.60 and 7.40), the breaking stress of each byssal thread was significantly decreased to approximately 72.6, 79.1 and 58% of that of the ambient pH group, respectively (Fig. 4b).

The breaking strain of each byssal thread (thread extensibility) was significantly affected by seawater pH as well (1-way ANOVA, $p < 0.01$). The extensibility of the byssal thread of the 3 lowered pH groups

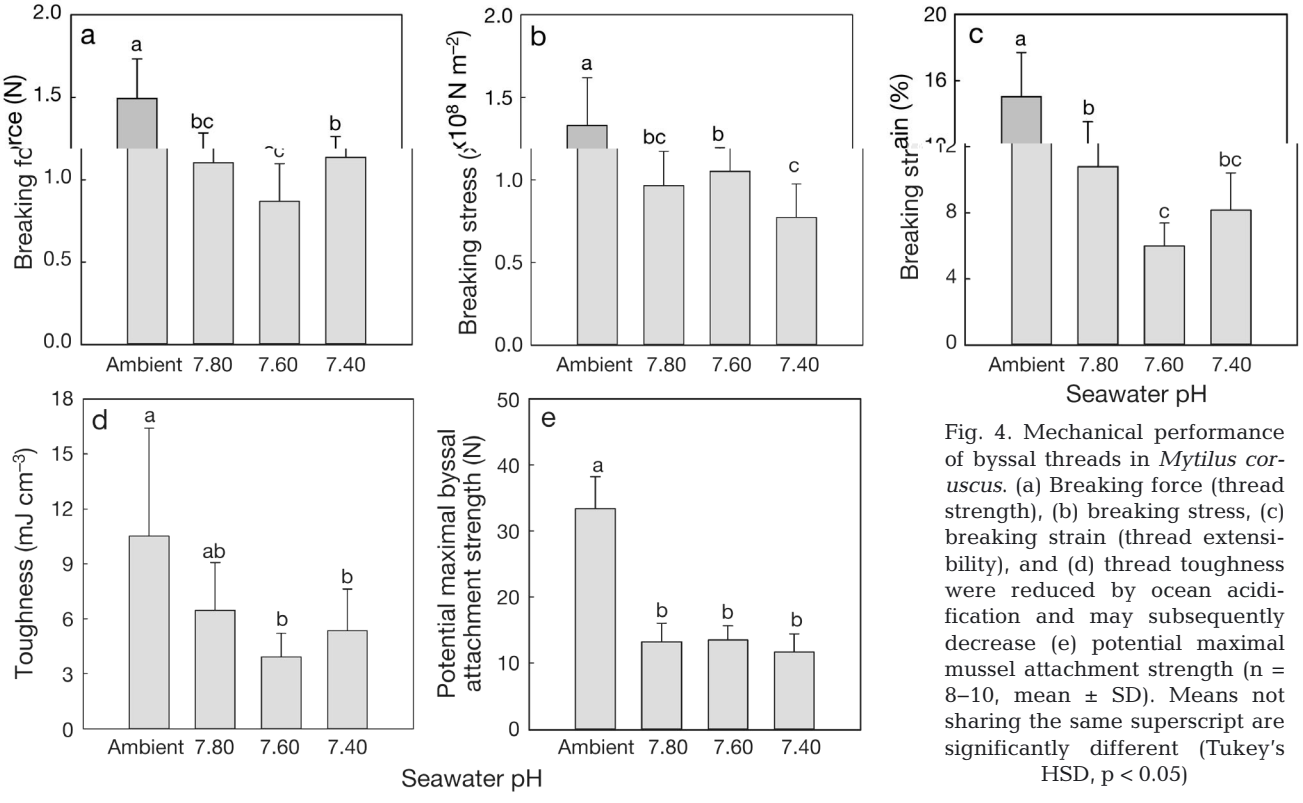

Fig. 4. Mechanical performance of byssal threads in *Mytilus coruscus*. (a) Breaking force (thread strength), (b) breaking stress, (c) breaking strain (thread extensibility), and (d) thread toughness were reduced by ocean acidification and may subsequently decrease (e) potential maximal mussel attachment strength (n = 8–10, mean ± SD). Means not sharing the same superscript are significantly different (Tukey's HSD, $p < 0.05$)

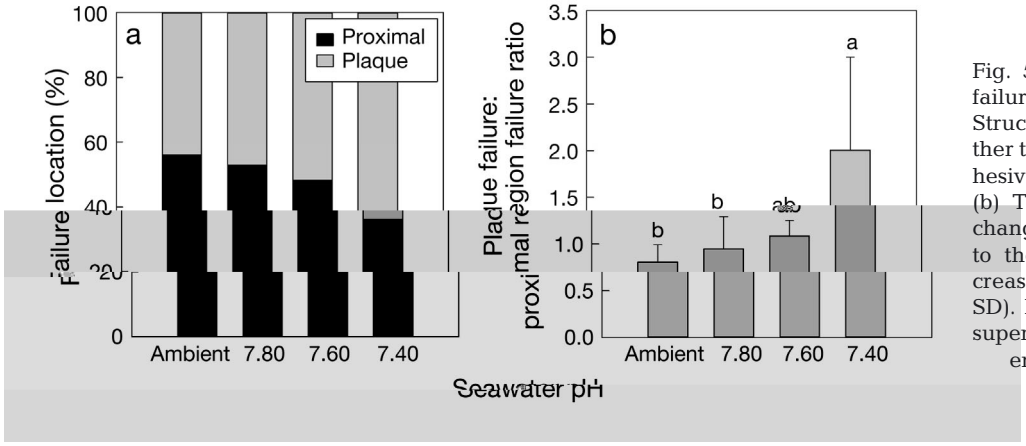

Fig. 5. Location of byssal thread failure in *Mytilus coruscus*. (a) Structural failure occurred in either the proximal region or the adhesive plaque of a byssal thread. (b) The common failure location changed from the proximal region to the adhesive plaque with increasing acidity (n = 8–10, mean ± SD). Means not sharing the same superscript are significantly different (Tukey's HSD, $p < 0.05$)

(7.80, 7.60 and 7.40) was only approximately 71.8, 39.9 and 54.4% of that of the ambient pH group, respectively (Fig. 4c).

Seawater pH also exerted significant effects on the toughness of each byssal thread (1-way ANOVA, $p < 0.01$). The toughness of pH 7.60 and 7.40 groups was significantly decreased to approximately 37.3 and 50.8% of that of the ambient pH group, respectively (Fig. 4d).

The potential maximal byssal attachment strength for a given mussel was estimated by multiplying the average thread strength by byssal thread number of the mussel. Before performing the estimation, the mean value of all thread strengths for a given mussel was calculated and used as the average thread strength for the mussel. The potential maximal byssal attachment strength of the 3 lowered pH groups was significantly decreased by 61, 60 and 65%, respectively, compared to that of the ambient pH group (Fig. 4e).

Location of byssal thread failure

In all tensile tests, the byssal thread failure occurred in either the proximal region or the adhesive plaque (Fig. 5a). The 1-way ANOVA showed that seawater pH had a significant effect on the location of byssal thread failure ($p < 0.05$). Compared to the ambient pH group, significantly more byssal thread failures occurred in the adhesive plaque in the pH 7.40 group (Fig. 5b).

Gene expressions of byssal proteins

Compared to the ambient pH group, the expression of *mfp-1* was significantly decreased in the pH 7.80 group, but was significantly increased by approximately 3-fold in the pH 7.60 and 9-fold in the pH 7.40 groups (Fig. 6a). Although the fold-change varied, similar expression patterns were shown by *mfp-2*, *-3*, *-5* and *-6*, which were significantly up-regulated in the pH 7.80 and 7.60 groups, while they were significantly down-regulated in the pH 7.40 group (Fig. 6b, c,e,f). In comparison with the ambient pH group, the expressions of *mfp-4* and *preCOL-NG* were significantly depressed in the lowered pH groups (7.80, 7.60 and 7.40; Fig. 6d,i). In contrast, the expressions of *preCOL-P*, *PTMP* and *preCOL-D* were significantly increased in the lowered pH groups (Fig. 6g,h,j).

DISCUSSION

The present study showed that OA exerted significant adverse impacts on the mechanical properties of byssal threads, which led to reductions in the strength, extensibility and toughness of individual byssal threads. In addition, the common failure location of byssal threads changed from the proximal region to the adhesive plaque as the seawater pH declined from the ambient value (8.10) to 7.40. Moreover, fewer byssal threads were produced by the hard-shell mussels *Mytilus coruscus* under OA conditions. Given that both the mechanical performance and byssal thread number are positively correlated with the overall byssal attachment strength (Bell & Gosline 1996, Babarro et al. 2008), the reductions in mechanical properties and byssal thread number would greatly limit the ability of mussels to attach to a substratum (a 60 to 65% decrease based on the estimation of potential maximal byssal attachment strength in the present study). These effects might be further enhanced over time, since the mechanical properties of byssal threads exhibit age-dependent variations. For example, Moeser & Carrington (2006) found that thread extensibility in blue mussels *M.*

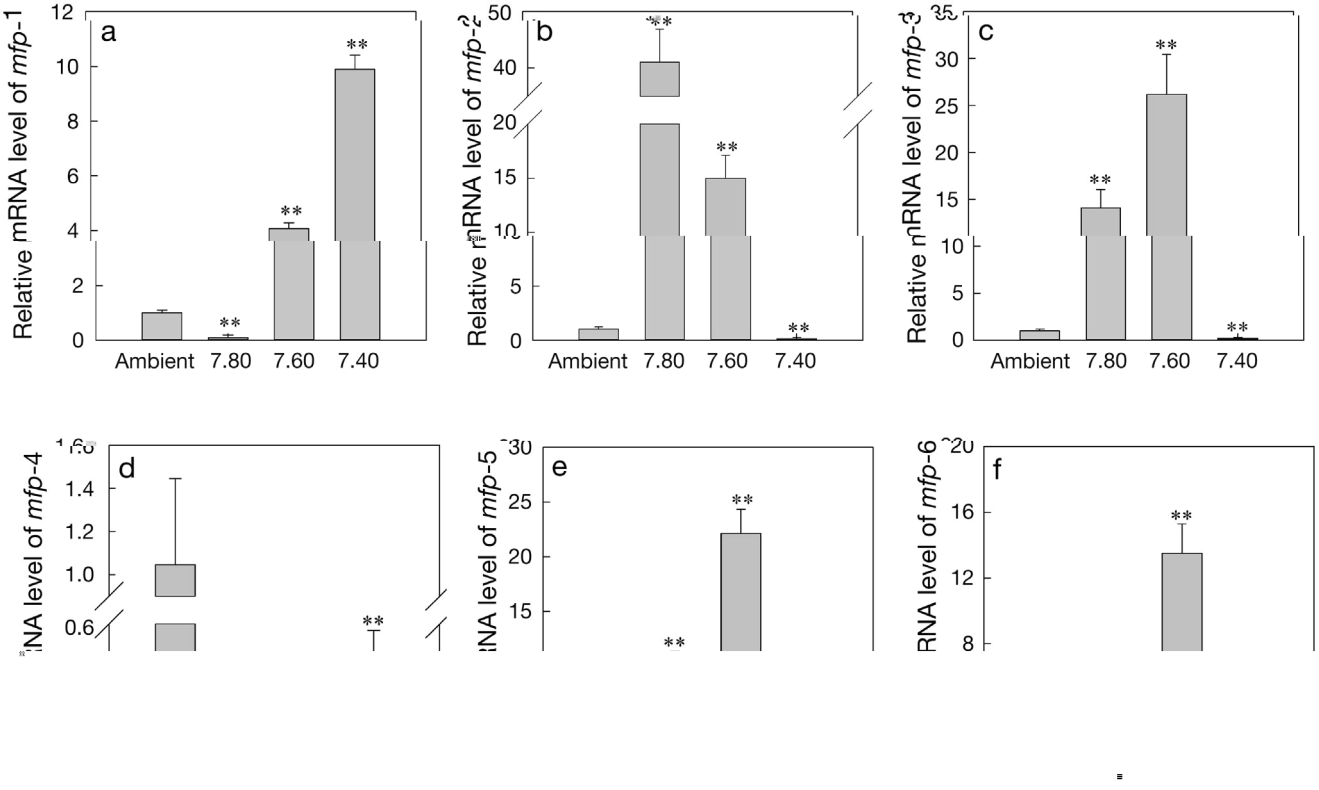

Fig. 6. Gene expression of byssal proteins in *Mytilus coruscus*. Real-time PCR of (a) *mfp-1*, (b) *mfp-2*, (c) *mfp-3*, (d) *mfp-4*, (e) *mfp-5*, (f) *mfp-6*, (g) *preCOL-P*, (h) *PTMP*, (i) *preCOL-NG* and (j) *preCOL-D* after a 1 wk experiment (n = 3, mean ± SD, *t*-test, **$p < 0.01$ relative to the ambient pH group)

edulis reduced more than 40% and thread strength declined over 29% after 4 wk of seawater exposure during spring and summer.

The weakened attachment strength would make mussels more vulnerable to environmental turbulence and subsequently increase the risk of dislodgement. Due to their important role in maintaining species richness (Borthagaray & Carranza 2007), the dislodgement of mussels may pose a great threat to coastal rocky communities. The weakened byssal attachment may also hamper the formation and maintenance of mussel aggregates, in which individuals are attached to one another via byssal threads. Since the formation of mussel aggregates increases local population density, which subsequently not only increases fertilization success but also provides protection against predators and other disturbances for the individuals (de Jager et al. 2011, Liu et al. 2011, Christensen et al. 2015), OA may impair the recruitment, anti-predation capability and survivorship of mussels by hampering byssal attachment. Therefore, OA could reduce the yields of suspension-cultured mussels and subsequently present a challenge to the mussel aquaculture industry, which was worth more than 4.0 billion USD in 2014 according to the Food and Agriculture Organization of the United Nations (FAO 2016).

A series of factors may contribute to the decrease in mussel byssal attachment strength detected under the simulated future OA scenarios. Mechanically, rather than altering the thread thickness, the OA-induced decrease in thread strength may be due to the decreased breaking stress of the byssal thread. For instance, although there was no significant difference in the diameters of byssal threads, the breaking stress of the byssal thread was significantly decreased in the pH 7.80 group compared to the ambient pH group. On the one hand, the weakened byssal attachment may be partially due to the seawater pH decline. The high concentration of DOPA in the byssal thread plays an important role in the formation of cross-links between polymer chains of individual byssal proteins (Waite 1990, Yu et al. 1999), which contributes greatly to the mechanical properties of the protective cuticle and the adhesive plaque of the byssal thread (Silverman & Roberto 2007). However, the function of DOPA and cross-links in mussel adhesion depends on seawater pH. For example, after testing the cross-links under the conditions of pH 5, 8 and 12, Holten-Andersen et al. (2011) suggested that pH is important for cross-link stability. Therefore, OA may exert adverse effects on mussel attachment by affecting the function of DOPA and cross-links.

On the other hand, due to their essential role in mechanical performance (Lucas et al. 2002, Bandara et al. 2013), the down-regulation of a series of specific byssal proteins may contribute to the weakened byssal attachment under the simulated future OA scenarios as well. For example, *preCOL-NG* and *mfp-4* were down-regulated through the 3 lowered pH levels in comparison with the ambient pH level. Due to the close relation to the mechanical properties of the inner core and the distal region-adhesive plaque junction, the decreased expression of *preCOL-NG* and *mfp-4* may partially explain the decrease in thread strength and extensibility under OA conditions.

Interestingly, the expressions of some specific byssal proteins were up-regulated in simulated future OA scenarios, which may be an adaptive response of mussels to minimize the effect of OA on byssal attachment. For example, *preCOL-P*, *PTMP* and *preCOL-D* were up-regulated in the OA challenge groups, suggesting that mussels elevate the elasticity of the proximal region and the stiffness of the distal region, and compensate for the weakened byssal threads as much as possible. However, the expressions of some specific byssal proteins showed mixed responses under OA scenarios. For example, the expression of *mfp-1* was down-regulated in the pH 7.80 group, but up-regulated in the pH 7.60 and 7.40 groups. Due to the important role of *mfp-1* in the formation of the protective cuticle, these alterations may lead to the weakening of mechanical properties of byssal thread under the pH 7.80 OA scenario and to a stiff cuticle to protect the byssal thread against environmental turbulence under pH 7.60 and 7.40 OA scenarios. Apart from the above, the expressions of *mfp-2*, *-3*, *-5* and *-6* were increased in the pH 7.80 and 7.60 groups, but decreased in the pH 7.40 group. Since these proteins (mfp-2, -3, -5 and -6) were exclusively confined to the adhesive plaque, these responses may improve the adhesion to substratum under pH 7.80 and 7.60 OA scenarios and hamper the mechanical performance of the adhesive plaque under the OA (pH 7.40) scenario. The down-regulation of *mfp-2*, *-3*, *-5* and *-6* may account for the

challenge to mussel aquaculture and mussel-bed ecosystems. With increasing acidity, more byssal thread failures occurred in the adhesive plaque. To some extent, the down-regulation of some specific byssal proteins may partially explain the weakened byssal attachment under simulated future OA scenarios due to their essential roles in mussel adhesion, while the up-regulation of some other specific byssal proteins may be an adaptive response of mussel individuals to cope with the OA conditions. However, many previous studies found that byssal threads decay over time. If OA accelerates byssal thread decay, it would further limit the ability of mussels to attach to the substratum. Therefore, further study is needed to determine whether the rate of byssal thread decay varies under future OA scenarios.

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LITERATURE CITED

Anderson DH, Robinson RJ (1946) Rapid electrometric determination of alkalinity of sea water using glass electrode. Ind Eng Chem Anal Ed 18:767–769

Babarro JMF, Fernández-Reiriz MJ, Labarta U (2008) Secretion of byssal threads and attachment strength of *Mytilus galloprovincialis*: the influence of size and food availability. J Mar Biol Assoc UK 88:783–791

Bandara N, Zeng H, Wu J (2013) Marine mussel adhesion: biochemistry, mechanisms, and biomimetics. J Adhes Sci Technol 27:2139–2162

Bell EC, Gosline JM (1996) Mechanical design of mussel byssus: material yield enhances attachment strength. J Exp Biol 199:1005–1017

Borthagaray AI, Carranza A (2007) Mussels as ecosystem engineers: their contribution to species richness in a rocky littoral community. Acta Oecol 31:243–250

Brown CH (1952) Some structural proteins of *Mytilus edulis*. Q J Microsc Sci 93:487–502

Caldeira K, Wickett ME (2003) Oceanography: anthropogenic carbon and ocean pH. Nature 425:365

Caldeira K, Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J Geophys Res 110:C09S04

Christensen HT, Dolmer P, Hansen BW, Holmer M and others (2015) Aggregation and attachment responses of blue mussels, *Mytilus edulis*—impact of substrate composition, time scale and source of mussel seed. Aquaculture 435:245–251

de Jager M, Weissing FJ, Herman PMJ, Nolet BA, van de Koppel J (2011) Lévy walks evolve through interaction between movement and environmental complexity. Science 332:1551–1553

Dickson AG (1990) Thermodynamics of the dissociation of boric acid in potassium chloride solutions from 273.15 K to 318.15 K. J Chem Eng Data 22:113–127

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep-Sea Res A 34:1733–1743

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO_2 problem. Annu Rev Mar Sci 1:169–192

Fabry VJ, Seibel BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J Mar Sci 65:414–432

FAO (Food and Agriculture Organization of the United Nations) (2016) FAO yearbook: fishery and aquaculture statistics, 2014. FAO, Rome

Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, Fabry VJ, Millero FJ (2004) Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 305:362–366

Holten-Andersen N, Harrington MJ, Birkedal H, Lee BP, Messersmith PB, Lee KY, Waite JH (2011) pH-induced metal-ligand cross-links inspired by mussel yield self-healing polymer networks with near-covalent elastic moduli. Proc Natl Acad Sci USA 108:2651–2655

Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol Lett 13:1419–1434

Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373:275–284

Lee BP, Messersmith PB, Israelachvili JN, Waite JH (2011) Mussel-inspired adhesives and coatings. Annu Rev Mater Res 41:99–132

Liu G, Stapleton E, Innes D, Thompson R (2011) Aggregational behavior of the blue mussels *Mytilus edulis* and *Mytilus trossulus*: a potential pre-zygotic reproductive isolation mechanism. Mar Ecol 32:480–487

Liu S, Shi W, Guo C, Zhai X and others (2016) Ocean acidification weakens the immune response of blood clam through hampering the NF-kappa β and toll-like receptor pathways. Fish Shellfish Immunol 54:322–327

Lucas JM, Vaccaro E, Waite JH (2002) A molecular, morphometric and mechanical comparison of the structural elements of byssus from *Mytilus edulis* and *Mytilus galloprovincialis*. J Exp Biol 205:1807–1817

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurement of apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol Oceanogr 18:897–907

Moeser GM, Carrington E (2006) Seasonal variation in mussel byssal thread mechanics. J Exp Biol 209:1996–2003

O'Donnell MJ, George MN, Carrington E (2013) Mussel byssus attachment weakened by ocean acidification. Nat Clim Change 3:587–590

Orr JC, Fabry VJ, Aumont O, Bopp L and others (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681–686

Peng C, Zhao XG, Liu SX, Shi W and others (2016) Effects of anthropogenic sound on digging behavior, metabolism, Ca^{2+}/Mg^{2+}-ATPase activity and metabolism-related gene expression of the bivalve *Sinonovacula constricta*. Sci Rep 6:24266

Pierrot D, Lewis E, Wallace DWR (2006) MS Excel program developed for CO_2 system calculations. ORNL/CDIAC-105a Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, TN

Prazeres M, Uthicke S, Pandolfi JM (2015) Ocean acidification induces biochemical and morphological changes in the calcification process of large benthic foraminifera. Proc R Soc Lond B Biol Sci 282:20142782

Qin Z, Buehler MJ (2014) Molecular mechanics of mussel adhesion proteins. J Mech Phys Solids 62:19–30

Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. Geology 37:1131–1134

Sanford E, Gaylord B, Hettinger A, Lenz EA, Meyer K, Hill TM (2014) Ocean acidification increases the vulnerability of native oysters to predation by invasive snails. Proc R Soc Lond B Biol Sci 281:20132681

Shi W, Zhao XG, Che ZM, Chai XL, Liu GX (2016) Ocean acidification increases cadmium accumulation in marine bivalves: a potential threat to seafood safety. Sci Rep 6: 20197

Silverman HG, Roberto FF (2007) Understanding marine mussel adhesion. Mar Biotechnol 9:661–681

Silverman HG, Roberto FF (2010) Byssus formation in *Mytilus*. In: von Byern J, Grunwald I (eds) Biological adhesive systems. Springer Vienna, Vienna, p 273–283

Solomon S, Qin D, Manning M, Chen Z and others (eds) (2007) Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Summary for policymakers. Cambridge University Press, Cambridge and New York, NY

Waite JH (1983) Adhesion in byssally attached bivalves. Biol Rev Camb Philos Soc 58:209–231

Waite JH (1990) The phylogeny and chemical diversity of quinone-tanned glues and varnishes. Comp Biochem Physiol B Comp Biochem 97:19–29

Wood HL, Spicer JI, Widdicombe S (2008) Ocean acidification may increase calcification rates, but at a cost. Proc R Soc Lond B Biol Sci 275:1767–1773

Yu M, Hwang J, Deming TJ (1999) Role of l-3,4-dihydroxyphenylalanine in mussel adhesive proteins. J Am Chem Soc 121:5825–5826

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