Animal size and seawater temperature, but not pH, influence a repeatable startle response behaviour in a wide-ranging marine mollusc

Jeff C. Clements[1*], Kirti Ramesh[2], Jacob Nysveen[2,3], Sam Dupont[2], Fredrik Jutfelt[1]

[1] Department of Biology, Norwegian University of Science and Technology, Høgskoleringen 5, 7491 Trondheim, Norway

[2] Department of Biological and Environmental Sciences, University of Gothenburg, Kristineberg Marine Research Centre, Kristineberg 566, 45178 Fiskebäckskil, Sweden

[3] Havets Hus, Strandvägen 9, 45330 Lysekil, Sweden

Abstract

Startle response behaviours are important in predator avoidance and escape for a wide array of animals. For many marine invertebrates, however, startle response behaviours are understudied, and the effects of global change stressors on these responses are unknown. We exposed two size classes of blue mussels (*Mytilus edulis* × *trossulus*) to different combinations of temperature (15 and 19 °C) and pH (8.2 and 7.5 pH_T) for three months and subsequently measured individual time to open following a tactile predator cue (i.e., startle response time) over a series of four consecutive trials. Time to open was highly repeatable on the short-term and decreased linearly across the four trials. Individuals from the larger size class had a shorter time to open than their smaller-sized counterparts. High temperature increased time to open compared to low temperature, while pH had no effect. These results suggest that bivalve time to open is repeatable, related to relative vulnerability to predation, and affected by temperature. Given that increased closure times impact feeding and respiration, the effect of temperature on closure duration may play a role in the sensitivity to ocean warming in this species and contribute to ecosystem-level effects.

Keywords: anti-predator response | carbon dioxide | environmental stress | global change biology | ocean acidification | ocean warming

Introduction

Predator-prey interactions have long been considered a fundamental component of animal ecology, as the ways in which predators and their prey interact is a major driving force shaping the ecology and evolution of biological systems (Connell, 1961; Dawkins & Krebs, 1979; Klompmaker et al., 2019; Paine, 1966). To combat predation, prey can employ a tremendous variety of defenses, including chemical, morphological, physiological and behavioural defenses (Antoł et al., 2018; Harvell, 1990; Kats & Dill, 1998; Lima & Dill, 1990). One of the more common ways of avoiding an immediate predator attack is to simply move away from a predator. For semi-sessile animals such as many bivalves, however, this defense is not an option for immediate attacks, although these animals can move and aggregate given sufficient time to do so (Reimer and Tedengren 1996; Côté and Jelnikar 1999; Casey and Chattopadhyay 2008). Instead, bivalves rely heavily on a suite of inducible morphological defenses, often related to shell morphology and substrate attachment strength (Christensen et al., 2012; Lord & Whitlatch, 2012; Scherer et al., 2018; Smith & Jennings, 2000; Trussell & Smith, 2000). Some species have also been documented to reduce physiological (i.e., metabolic) rates in response to predator presence – a mechanism that is suggested to reduce detection by predators (Antoł et al., 2018). Additionally, behavioural responses such as burrowing, aggregating, and valve closures play an important role in predator avoidance in these animals (Casey & Chattopadhyay, 2008; Côté & Jelnikar, 1999; Flynn & Smee, 2010; Nicastro et al., 2007; Reimer & Tedengren, 1996; Robson et al., 2010; Smee & Weissburg, 2006), particularly given that inducible morphological defenses can take long periods of time to accrue whereas many behavioural defenses are instantaneous.

Although the repertoire of anti-predator behaviours for semi-sessile bivalves is more limited than vagile species, often the most immediate response to the threat of predation is to partially or fully close their valves and 'hide' (Carroll & Clements, 2019; Clements et al., 2020; Robson et al., 2007; Robson et al., 2010). This avoidance strategy is thought to reduce the probability of being detected by predators (as hiding would reduce the emittance of chemical cues that predators could detect) and can reduce the probability of predators successfully accessing and consuming the tissue (Barbeau & Sceibling, 1994; Carroll & Clements, 2019). While full or partial closures can help reduce detectability prior to an attack, effective valve closure during an attack can also reduce the probability of a predator-driven mortality. The effectiveness of this strategy during an attack will depend largely on adductor muscle strength, as stronger adductor muscles would provide a stronger closure (i.e., more difficult for predators to open shell) for a longer duration of time, both of which would incur a reduced probability of mortality (Wilson et al. 2012). While the strength of valve closure is solely dictated by adductor muscle strength, the duration of valve closure would also be influenced by an individual's behavioural decision. The duration of valve closure in response to olfactory and/or tactile cues can thus represent a startle response functionally applicable to predator avoidance and escape in semi-sessile bivalves (as measured and defined in previous studies; e.g. (Carroll & Clements, 2019; Rudin & Briffa, 2012; Wilson et al., 2012).

While it is known that semi-sessile bivalves close their shells and exhibit a startle response under the threat of predation, aspects of the behavioural ecology of this response are understudied. Living in clusters as opposed to being solitary can reduce time to open in freshwater mussels (Wilson et al., 2012), likely resulting from reduced vulnerability to predation for group-living bivalves, and hence representing a measure of boldness in these animals (Côté and Jelnikar 1999; Casey and Chattopadhyay 2008; Kobak and Ryńska 2014). Wilson et al. (2012) also suggested that startle responses were repeatable in freshwater bivalves, but did not directly quantify the repeatability of this measure. Recent evidence also suggests that cue type can affect valve closure responses to predators (Dzierżyńska-Białończyk et al., 2019). Startle responses could be affected by other factors as well, such as size (larger mussels are less vulnerable to predation than smaller mussels; Sommer et al. 1999) and time (time to open may change over short- and long-time scales due to fatigue or habituation). Such aspects of the behavioural ecology of valve closure responses to predator attacks, however, remain unexplored.

It is widely documented that predator-prey dynamics can be affected by global change stressors (Bretagnolle & Terraube, 2010; Romero et al., 2018). In the marine realm, studies report that both ocean warming and acidification can affect predator-prey interactions in fish (Allan et al., 2017) and invertebrates (Sanford et al. 2014; Wright et al. 2018; Lord et al. 2019; but see Landes and Zimmer 2012, Sundin et al. 2017, and Clark et al., 2020 for contrasting results). With respect to invertebrates, however, much of this work has focused on alterations in predator-prey dynamics resulting from morphological effects. As such, only a handful of studies regarding prey defenses, including startle responses, are available (Clements & Comeau, 2019). For example, in hermit crabs, exposure to increased temperature reduced the mean time to open and increased inter-individual variation, which was suggested to be a function of temperature effects on metabolism (Briffa et al., 2013). A few studies have also tested for effects of seawater pH on prey defenses, reporting varied effects (Bibby et al. 2007; Manríquez et al. 2013; Watson et al. 2014; Turra et al. 2019). However, the combined effects of temperature and pH on invertebrate startle responses are absent from the literature. Such studies are important since alterations to animal behaviour under global change are predicted to drive ecosystem-level impacts (Kroeker et al., 2014; Nagelkerken & Munday, 2016).

The overarching goal of this study was thus two-fold: 1) to assess aspects related to the behavioural ecology of bivalve startle responses including short-term repeatability, changes over time, and body size; and 2) to test the combined effects of pH and temperature on bivalve startle responses. To address these research goals, we conducted laboratory experiments using an ecologically and economically important bivalve (*Mytilus edulis* × *trossulus*). We predicted that: 1) startle responses would be repeatable based on previous yet unquantified reports that time to open following various stimuli in freshwater bivalves is repeatable (Wilson et al. 2012); 2) individual time to open would decrease over time (trials) due to either fatigue or habituation; 3) larger animals would have a shorter startle response because they are less vulnerable to predation (Sommer et al., 1999); 4) higher temperature would reduce time to open because of higher metabolism and an increased need for oxygen and nutrient uptake (Briffa et al., 2013); and 5) low pH would affect the startle response as CO_2-induced pH declines are reported to have wide-ranging behavioural effects (Clements & Hunt, 2015).

Materials and methods

Animal ethics and data accessibility

Ethical approval was not required for the species used in this experiment. Nonetheless, the study was strictly conducted under the premise of the three Rs of animal ethics. For transparency, all accompanying data and analysis code are provided in the online Supplementary Material accompanying this article. All supplementary figures (Figures S1–S5) and tables (Table S1) are contained in Supplementary File 1, annotated analysis code (in R) is in Supplementary File 2, raw data are in Supplementary File 3, and the source data files for analysis are contained in Supplementary Files 4–8.

Animal collection and husbandry

Adult mussels (Figure S1a) were hand-collected from the Gullmar Fjord (located in Skagerrak in the North Sea on the southwest coast of Sweden) in late May 2018. The animals were collected from the side of a nearshore pier at a depth of 0–1 m adjacent to the Kristineberg Marine Research and Innovation Centre (KMRIC; 58.250 °N, 11.447 °E). During collection, seawater temperature was ≈14 °C, salinity was ≈22, and pH_T was ≈8.1 (Figure S2). The mussels were transported to a temperature-controlled wet lab at the KMRIC where they were cleaned of epibionts. The animals were then placed in flow-through aquaria with ambient surface seawater (filtered to remove rocks, sediment, and larger animals while allowing plankton to pass) from the fjord for 12–14 days prior to experimentation to allow acclimation to laboratory conditions. During the acclimation period, mussels fed on a natural diet of plankton from the fjord and were subjected to a 12:12 light:dark cycle (08:30–20:30 light). Mortality was checked every two days and any dead mussels were removed from the acclimation chambers; mortality was minimal (<3%) and mussels fed as evidenced by the consistent production of both faeces and pseudofaeces. Following acclimation, the mussels were weighed (wet weight), measured (shell length), individually labelled (with nail polish), and separated into two distinct size classes based on pre-exposure shell length: small (<58 mm; mean ± SD = 49.6 ± 4.4 mm shell length; 16.3 ± 4.4 g wet weight) and large (>59 mm; 67.1 ± 5.5 mm shell length; 40.0 ± 9.8 g wet weight) (Figure S3a). The animals were then placed into replicate exposure chambers (Figure S1b, c) upon which exposure to temperature and pH treatments commenced (see below). The exposure chamber surface served as substratum for the mussels. Animals that did not attach within two days of being placed in tanks were removed and replaced such that all mussels used in experiments had attached within two days of being placed in acclimation tanks.

Experimental design and setup

A 2×2×2 design was employed with two size classes (small and large; as above), two pH levels (total scale; ambient [≈8.1] and –0.7 units; see Table 1 and Figure S2), and two temperatures (16 °C and 20 °C [+4 °C]) crossed in a fully-factorial manner. Size classes were chosen based on dominant sizes of mussels at the collection site and are reflective of the natural population in which predators are active. The size classes are thus associated with relative vulnerability to predation; although specific size refugia thresholds of these mussels are not quantitatively defined for the various predators in the Gullmar Fjord system, the smaller size class can be considered more vulnerable to predation from various predators including sea stars, crabs and fish would be greater for the smaller size class *sensu* Sommer et al. (1999).

Temperature and pH treatments were designed to simulate deviations from ambient conditions in the fjord according to near future projections. For the "low" temperature treatment, we followed ambient temperatures until 16 °C after which temperature conditions were manipulated to remain constant at 16 °C. For the "high" temperature treatment, temperatures were manipulated to provide a temperature offset of +4°C (maximum temperature of 20 °C; Figure S2). We capped temperature manipulations at 16 °C and 20 °C to avoid temperature-related mortality that can occur during long exposures to temperatures above 20°C (Clements et al. 2018) and to avoid spawning. For pH, ambient conditions were followed for the duration of the exposure period for the "ambient" pH treatment. For the "low" pH treatment, a pH offset of –0.7 units was employed, which represented an ocean acidification scenario based on a –0.3 unit differential (IPCC RCP8.5 ocean acidification projection; Hoegh-Guldberg et al., 2014) from the minimum pH currently observed in the fjord (0.5 units; low pH ≈7.6 from a mean of ≈8.1 according to Dorey et al. 2013).

A flow-through seawater system was constructed to expose animals to experimental temperature and pH conditions (Figure S1b). Ambient seawater was continuously pumped directly from the Gullmar Fjord into each of 12 header tanks (50 L; n = 3 header tanks per temperature×pH treatment). The seawater entered the lab through one of two lines, each of which were equipped with customized in-line heat exchangers controlled by an automated computer system which heated the seawater to desired temperatures prior to being delivered to the header tanks. Temperature settings in the automated computer system were adjusted such that conditions in the experimental tanks reflected the desired temperature conditions for each treatment. Seawater pH was manipulated in six of the header tanks via pure CO_2 injection which was maintained with a pH-stat control system (Aqua Medic, Bissendorf, Germany); pH was left at ambient conditions in the other six header tanks. Salinity was left uncontrolled in all treatments and varied naturally with ambient conditions in the fjord (Figure S2). The water in all 12 header tanks was continuously aerated to ensure proper mixing, oxygenation, and gas equilibration.

Seawater from each header tank was gravity fed into two exposure chambers (IKEA® SAMLA container; 28×20×14 cm length×width×depth; 5 L volume) where the animals were held, one exposure chamber for each size class (Figure S1b,c; n = 5 mussels replicate tank^{-1}; N = 120 mussels). Each exposure chamber contained a drainage hole near the top to provide ≈4 L seawater volume, ensure mixing, and provide flow-through conditions in each tank. Flow rate to the exposure chambers was adjusted to ≈3 ml s^{-1} (≈22.2 min for one volume turnover). Mussels fed on the natural diet of plankton available in the seawater and were exposed to a 12:12 light:dark cycle as above. Filtration and ingestion of food was confirmed by the continual presence of faeces and pseudofaeces throughout the experiment, which was siphoned off to clean the exposure chambers as needed. Mortality, which was negligible (n = 4/120, 3%), was checked daily and dead mussels were immediately removed from the exposure chambers. The animals were exposed to experimental conditions for 88–93 days (late May – late August 2018) depending on the day in which individuals were subjected to behavioural assays (behavioural assays took six days to complete and different mussels were tested on each day).

Temperature and pH conditions in the exposure and header tanks were measured every 1–6 days to ensure that offsets were consistent (Figure S2). Temperature was measured with a high precision digital thermometer (± 0.1 °C accuracy; testo-112, Testo, Lenzkirch, Germany). Seawater pH was measured on the total scale (pH_T) with a benchtop pH meter (Metrohm 827 pH lab, Metrohm, Herisau, Switzerland) calibrated with TRIS (Tris/HCl) and AMP (2-aminopyridine/HCl) buffers. The pH stat systems were adjusted accordingly whenever seawater parameters were measured for temporal pH offset consistency. Salinity was also recorded at the time of temperature and pH measurement from the KMRIC website (https://www.weather.loven.gu.se/kristineberg/en/data.shtml) with the exception of measurements on and after 27 July, which were measured directly with a handheld salinity meter (WTW, Weilheim, Germany) due to a lack of data availability on the KMRIC website. Total alkalinity (A_T) was measured weekly by titration of 25 mL filtered (2 μm) samples using a SI Analytics Titroline potentiometric titrator. Carbonate system parameters (TCO_2, pCO_2, $\Omega_{calcite}$, and $\Omega_{aragonite}$) were estimated in CO2SYS v2.1 (Pierrot et al., 2009) for each measurement of temperature, salinity, and pH_T above, using the A_T value from the closest day and the first and second dissociation constants of Mehrbach et al. (1973) refit by Dickson and Millero (1987). The methods provided highly consistent temperature and carbonate system offset conditions (Figure S2, Table 1).

Table 1. Abiotic parameters of the experimental treatments. Data are means ± standard deviation across tanks and time. Raw data and full CO2SYS results can be found in in Supplementary file 3; temporal trends are presented in Figure S2. Abbreviations: Temp = temperature; pH_T = pH on total scale; A_T = total alkalinity; TCO_2 = total carbon dioxide; pCO_2 = partial pressure of carbon dioxide; $\Omega_{calcite}$, $\Omega_{aragonite}$ = saturation states of calcite, aragonite

	Measured				**Estimated**			
Treatment	**Salinity**	**Temp (°C)**	**pH_T**	**A_T (µmol kg^{-1})**	**TCO_2 (µmol kg^{-1})**	**pCO_2 (µatm)**	**$\Omega_{calcite}$**	**$\Omega_{aragonite}$**
Amb pH + Low temp	24.4 ± 2.3	15.1 ± 1.3	8.17 ± 0.10	2101.6 ± 124.8	1908.8 ± 113.3	298.8 ± 76.9	3.9 ± 1.0	2.4 ± 0.6
Low pH + Low temp	24.4 ± 2.4	15.0 ± 1.3	7.49 ± 0.09	2139.7 ± 74.7	2162.2 ± 85.1	1694.0 ± 383.1	0.9 ± 0.2	0.6 ± 0.1
Amb pH + High temp	24.4 ± 2.5	19.3 ± 1.2	8.14 ± 0.10	2110.6 ± 82.1	1900.4 ± 83.9	332.1 ± 85.2	4.1 ± 0.9	2.6 ± 0.6
Low pH + High temp	24.4 ± 2.6	19.3 ± 1.2	7.47 ± 0.10	2127.8 ± 85.0	2138.0 ± 84.7	1793.3 ± 403.6	1.1 ± 0.3	0.7 ± 0.2

Behavioural assays

Behavioural assays were conducted in August, 2018 in separate, flow-through experimental tanks (exactly the same style as the exposure chambers) under the same abiotic seawater conditions experienced in the exposure period (i.e., low temperature mussels were tested under low temperature conditions, high temperature mussels under high temperature, and so on). A total of 10 experimental tanks were used in a given assay and we were able to conduct two assays per day; all assays took place between 9:00 and 15:00 each day (to avoid potentially confounding effects of daily biological activity patterns) with treatment order randomized. Again, the tank surface served as substratum for the mussels and all mussels included in the analysis had attached prior to assays. Prior to each assay, individual mussels were detached and transferred from their exposure chamber to an individual experimental tank where they were left for one hour prior to experimentation. The mantle tissue of the mussels was then gently touched with the round end of a wooden skewer until they closed (to simulate a predator attack) and the time to visually re-open was recorded (in seconds) with a stopwatch for each individual. The process was repeated for each individual mussel over four consecutive trials (i.e., tactile cues were administered to each individual mussel four times) every 30 mins from the previous re-opening. This allowed us to measure short-term repeatability in time to open and to compute individual valve closure coefficients of variation. Due to time constraints in keeping experiments between 9:00 and 15:00, mussels that were open at the start of the trial but did not open within the 30 min observation period were assigned a value of 1800 secs (i.e., 30 mins); the animal was given an additional 30 mins to re-open before starting the next trial. Data were only included if an individual was open at the beginning of a trial. Eleven individuals were not open at the beginning of any of their respective trials and were thus removed from the analysis (see raw data in Supplementary File 3 for details). In addition, four animals died during the exposure period. Thus, the total number of individuals for behavioural assays was reduced by 15, from 120 to 105 (leaving 50 small and 55 large individuals). We also dichotomously scored each individual trial according to whether or not the mussel opened within 30 mins after being startled.

We ensured that all behavioural observations were fully blinded by having one person place the mussels in the experimental tanks prior to behavioural observations by a different person; the observer was also naïve to the goals and hypotheses of the experiment until after behavioural assays were completed. Once behavioural assays were concluded, the mussels were once again weighed and measured, and individual changes in shell length and wet weight were calculated as a percentage of the starting length and weight.

Statistical analyses

All statistical analyses were conducted in R version 3.6.3 (R Core Team, 2020). Normality was visually assessed with Q-Q plots and histograms, and homoscedasticity was visually assessed using fitted-residual plots; all plots for assumptions, and the decisions made based on them, can be found in Supplementary File 2. Main and interactive effects were considered significant at $\alpha = 0.05$. Pairwise comparisons for significant interactive effects or significant independent effects of factors with more than two levels were determine using Tukey HSD post hoc tests with the glht() function from the *multcomp* package (Hothorn et al., 2008). Supplementary figures and tables are contained in Supplementary File 1. Annotated R script can be found in Supplementary File 2 and all raw data are contained in Supplementary file 3. Original R datafiles used in the analyses are also provided as Supplementary files 4–8. All data within the text are reported as means ± one standard deviation computed from raw data; likewise, figures reflect raw data.

Generalized linear models (GLMs) were used to test for the fixed effects of initial size (continuous), pH_T (categorical, two levels), temperature (categorical, two levels), and their interactions on % changes in shell length and wet weight; GLMs were selected because data transformations were unsuccessful at fixing initial violations of normality and homoscedasticity). We initially built generalized linear mixed models with tank as a random variable, but singularity errors suggested that these models were overfitting the data and we therefore chose to drop the random effect. GLMs were constructed using the glm() function in the *lmerTest* package (Kuznetsova et al., 2017) using a Gamma distribution (for continuous, right skewed data);. Significant effects were determined using the Anova() function in the *car* package (Type II).

Behavioural assays were limited to 30 mins and if an animal did not open its valves in that. time it was assigned a time to open of 1800 secs. As such, some observations were censored. To account for this, the effects of size class, pH_T, temperature, trial, and their interactions time to open were tested using time-to-event analysis (also known as survival analysis). This analytical approach can be applied to data that record the amount of time taken until an event of interest occurs (for our study, that event is mussel re-opening), and to account for "censored" observations in which the event of interest did not occur within a defined time period of observation (i.e., the individuals in our analysis that did not open within the 30 min observation period) (Schober & Vetter, 2018). A mixed effect Cox proportional hazard model based on Kaplan-Meier estimations was built using the Surv() function in the *survival* package (Therneau & Grambsch, 2000) and the coxme() function in the *coxme* package (Therneau, 2020) and the Anova() function (Type III) was subsequently used to test for significant effects (Fox & Weisberg, 2019).

To test for the fixed effects of size class, pH_T, temperature, trial, and their interactions on individual coefficients of variation (CoV; of time to open), we built linear mixed effects models using the lmer() function in the *lmerTest* package. Significant effects were determined using the anova() function (Type III). Data were natural log transformed prior to analysis to achieve normality and homoscedasticity (see Supplementary File 2). Independent and interactive effects of the same factors on the number of behavioural trials in which individual animals did not open were tested for with logistic regression using the glm() function with a binomial distribution family and the Anova() (Type II) function to determine significant effects. Repeatability (R) of time to open was estimated using a generalized linear mixed model (GLMMs) after (Dingemanse & Dochtermann, 2013) and interpreted in a Bayesian fashion according to Bell et al. (2009) (i.e., behaviour can be considered 'significantly' repeatable at $R \geq 0.37$). A GLMM to compute adjusted repeatability (R_{adj}; repeatability accounting for fixed effects of size class, pH_T, and temperature) was built using the MCMCglmm() function in the *MCMCglmm* R package (Hadfield, 2010). Estimates and 90% confidence intervals for R_{adj} were obtained using the posteriormode() and HPDinterval() functions, respectively, with code adapted from Roche et al. (2016).

Results

Shell length and wet weight

Following exposure, mean shell lengths were 67.5 ± 5.1 mm, 50.9 ± 4.1 mm, and mean wet weights were 40.8 ± 8.9 g and 17.6 ± 4.2 g, in the large and small size classes, respectively (Figure S3b, c). For growth rates (i.e., changes in shell length and wet weight), initial size had a significant independent effect, with smaller animals showing larger increases in both shell length and wet weight than larger individuals (Figure 1a,b). There were no significant effects of temperature, pH_T, or any interactions on growth rates (Table 2).

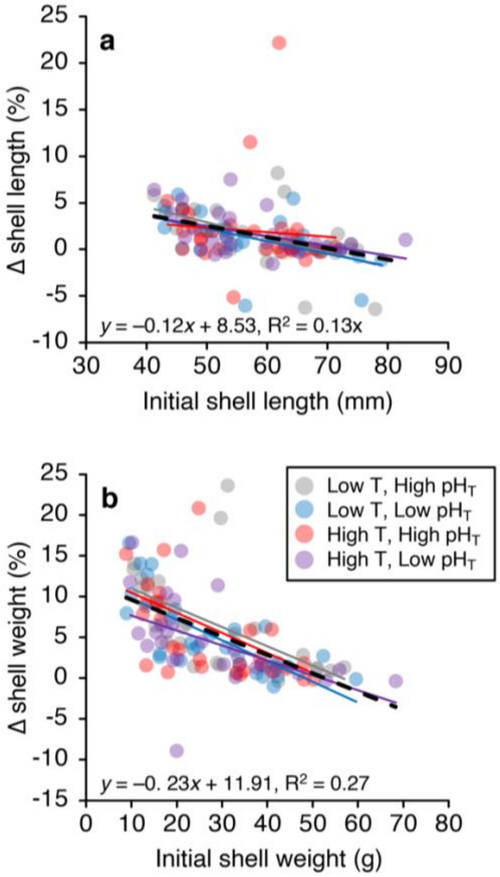

Figure 1. Scatterplots of changes in (Δ) shell length (**a**) and wet weight (**b**) as a function of initial size for each of the four treatments: ambient pH_T + low temperature (gray circles), low pH_T + low temperature (blue circles), ambient pH_T + high temperature (red circles), low pH_T + high temperature (purple circles). Equations and R^2 values represent the overall relationship for data pooled across treatments (black dashed line).

Table 2. Results of generalized linear model analyses for effects of initial size (length or weight), $\mathrm{pH_T}$, and temperature on changes in shell length (mm) and wet weight (g) between the beginning and the end of the exposure period. Bolded text denotes significant effects. χ^2 = chi-squared test statistic, df = degrees of freedom, P = p-value.

Source of error	χ^2	df	P
Shell length			
Initial length	**20.12**	**1**	**<0.0001**
$\mathrm{pH_T}$	0.03	1	0.8637
Temp	0.76	1	0.3829
Initial length × $\mathrm{pH_T}$	0.06	1	0.8004
Initial length × Temp	2.59	1	0.1073
$\mathrm{pH_T}$ × Temp	0.16	1	0.6912
Initial length × $\mathrm{pH_T}$ × Temp	0.57	1	0.4518
Wet weight			
Initial weight	**35.90**	**1**	**<0.0001**
$\mathrm{pH_T}$	**2.85**	1	0.0913
Temp	1.69	1	0.1933
Initial weight × $\mathrm{pH_T}$	0.03	1	0.8716
Initial weight × Temp	0.15	1	0.6997
$\mathrm{pH_T}$ × Temp	0.23	1	0.8716
Initial weight × $\mathrm{pH_T}$ × Temp	0.34	1	0.5589

Effects of size, $\mathrm{pH_T}$, temperature, and trial on time to open

Time to open was independently affected by size class, temperature, and trial (Table 3). The small mussels had a time to open that was, on average, ≈3× longer than their larger counterparts (453.4 ± 449.9 secs for small mussels *versus* 154.1 ± 236.0 secs for large; Figure 2a, c, S4). Likewise, mussels from the high temperature treatment remained closed ≈2× longer than those in the low temperature treatment (422.1± 535.8 secs for high temperature versus 182.6 ± 270.9 secs for low temperature; Figure 2b, c, S4). The propensity of individuals to remain closed for the duration of a given trial was independently affect by size class and temperature but not by $\mathrm{pH_T}$ or trial (Figure S4; Table 3). The proportion of trials in which individuals did not open was higher in the small size class and under high temperatures (Figure S5). Overall, however, the percentage of trials in which individuals did not open was low (13.1%). Seawater $\mathrm{pH_T}$ had no effect on time to open (Amb. pH: 290.2 ± 426.4 secs, Low pH: 302.0 ± 442.6 secs) and there were no interactive $\mathrm{pH_T}$ effects (Figure 2c; Table 3).

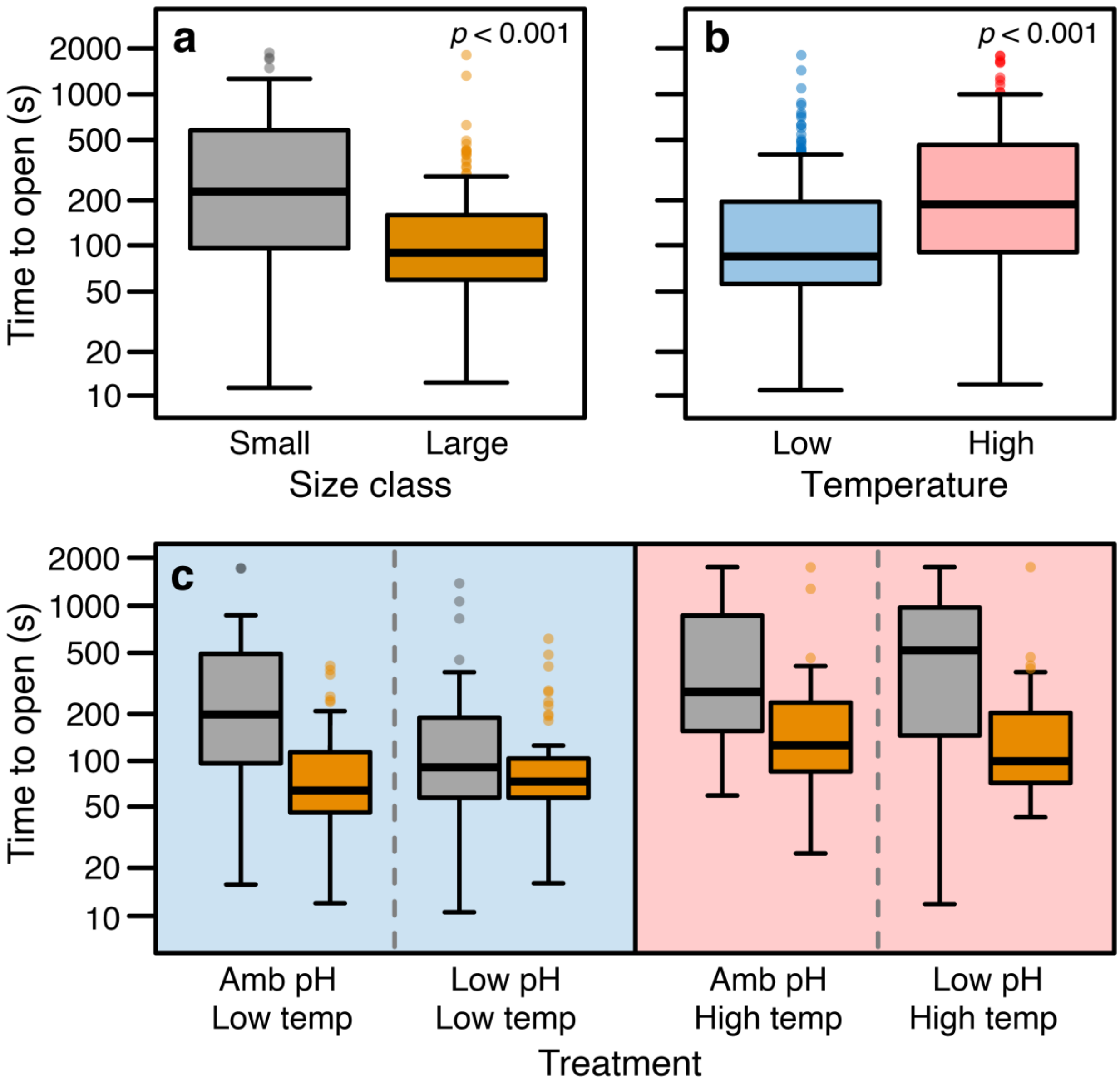

Figure 2. (a) Boxplot of time to open for each size class ($n_{large} = 55$, $n_{small} = 50$). *P*-value represents the main effect of size class from the mixed effect Cox proportional hazards model for time to open (see Table 3) **(b)** Boxplot of time to open for each experimental temperature treatment ($n_{control} = 55$, $n_{high} = 50$). *P*-value represents the main effect of temperature from the mixed effect Cox proportional hazards model for time to open (see Table 3) **(c)** Boxplot of time to open as a function of size class and experimental treatment. ($n_{amb\ pH+low\ temp,\ large} = 14$; $n_{amb\ pH+low\ temp,\ small} = 15$; $n_{low\ pH+low\ temp,\ large} = 13$; $n_{low\ pH+low\ temp\ small} = 13$; $n_{amb\ pH+high\ temp,\ large} = 12$; $n_{amb\ pH+high\ temp,\ small} = 14$; $n_{low\ pH+high\ temp,\ large} = 9$; $n_{low\ pH+high\ temp,\ small} = 15$). Small and large size classes are represented by gray- and tan-shaded boxes, respectively. Low and high temperature treatments are represented represented by blue- and red-shaded boxes, respectively. For each treatment, the short horizontal lines at the ends of each vertical line represent the maximum and minimum non-outlying values, the boxes represent the 25th to the 75th percentiles, the thick horizontal lines in each box represent the median, and the individual points represent outlying values.

Table 3. Results of Cox mixed effects regression analysis for the effects of size class, $\mathrm{pH_T}$, temperature, and trial on startle response time, and logistic regression analysis for the effects of size class, $\mathrm{pH_T}$, temperature, and trial on the proportion of individuals that either opened or closed during a given trial. Bolded text denotes significant effects. df = degrees of freedom, D df = denominator degrees of freedom, χ^2 = Chi-squared statistic, P = p-value.

		Cox model		Logistic regression	
Source of error	df	χ^2	*P*	χ^2	*P*
Size class	1	**34.56**	**<0.0001**	**15.93**	**<0.0001**
$\mathrm{pH_T}$	1	1.30	0.2540	0.98	0.3220
Temp	1	**20.52**	**<0.0001**	**16.58**	**<0.0001**
Trial	3	**35.37**	**<0.0001**	1.92	0.5901
Size class ´ $\mathrm{pH_T}$	1	1.55	0.2130	0.72	0.3962
Size class ´ Temp	1	1.11	0.2917	0.91	0.3409
$\mathrm{pH_T}$ ´ Temp	1	1.32	0.2512	3.30	0.0693
Size class ´ Trial	3	3.25	0.3546	2.12	0.5479
$\mathrm{pH_T}$ ´ Trial	3	3.82	0.2820	1.43	0.6978
Temp´ Trial	3	1.42	0.7016	1.90	0.5934
Size class´ $\mathrm{pH_T}$ ´ Temp	1	3.71	0.0540	<0.001	>0.9999
Size class ´ $\mathrm{pH_T}$ ´ Trial	3	4.07	0.2545	2.66	0.4474
Size class ´ Temp ´ Trial	3	4.81	0.1865	<0.001	>0.9999
$\mathrm{pH_T}$ ´ Temp ´ Trial	3	2.02	0.5687	<0.001	>0.9999
Size class ´ $\mathrm{pH_T}$ ´ Temp ´ Trial	3	3.43	0.3302	<0.001	>0.9999

Alongside staying closed longer, smaller mussels also had a higher individual coefficient of variation (CoV) in time to open than larger mussels (59.9 ± 37.0 % in small *versus* 41.2 ± 28.6 % in large; Figure 3a). Time to open CoV was not significantly affected by any other factor (or interaction) aside from size class (Table 4). With respect to trial, time to open decreased linearly across the four trials with the fourth trial being significantly lower than the first trial (Figure 3b, S4; Table 3, S1).

Short-term repeatability in time to open

Adjusted repeatability (R_{adj}; repeatability accounting for fixed effects of size, $\mathrm{pH_T}$, and temperature) computed using generalized linear mixed modelling was estimated to be 0.56 [0.43–0.63, 95% CI]. Based on the Bayesian cut-off of $R>0.37$ denoting significant repeatability (Bell et al. 2009), time to open was deemed significantly and highly repeatable (Figure 4).

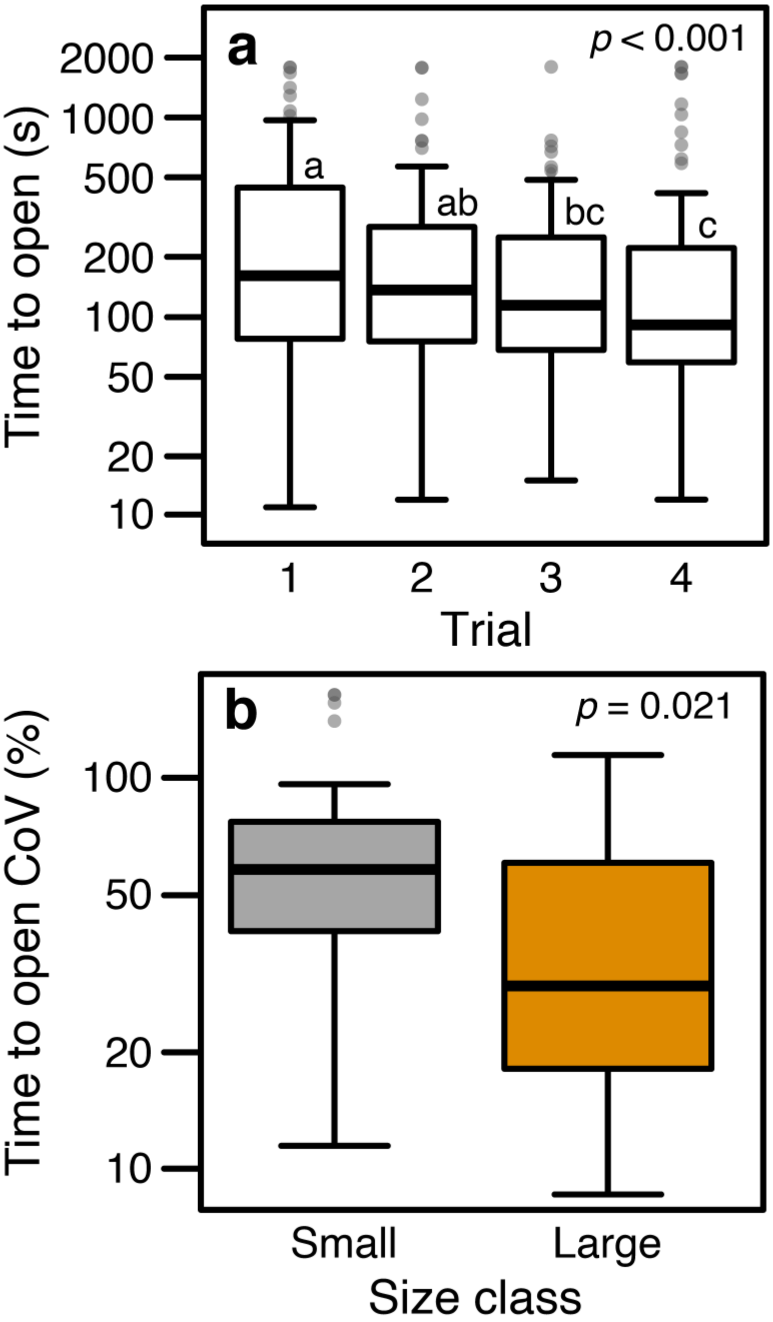

Figure 3. (a) Boxplot of startle response times for each of the four trials (*n* = 105 individuals per trial). *P*-value represents the main effect of trial from the mixed effect Cox proportional hazards model for time to open (see Table 3) and letters above plots denote Tukey HSD pairwise differences (see Table S1). **(b)** Boxplot of time to open coefficient of variation (CoV) for each size class (n_{large} = 55, n_{small} = 50). Note that all y-axes are log scaled. Sample sizes are number of individuals. *P*-value represents main effect of size class from the linear mixed effects model for time to open CoV (see Table 3).

Table 4. Results of linear mixed effects model analysis for the effects of size class, pH$_{\mathrm{T}}$, temperature, and trial on startle response time coefficient of variation. Response variable data were log transformed prior to analysis. Bolded text denotes significant effects. SS = sum of squares, MS = mean of squares, N df = numerator degrees of freedom, D df = denominator degrees of freedom, F = Fisher's F statistic, *P* = p-value.

Source of error	SS	MS	N df	D df	F	*P*
Size class	**2.30**	**2.30**	**1**	**15.84**	**6.54**	**0.0212**
pH$_{\mathrm{T}}$	0.28	0.28	1	15.84	0.80	0.3859
Temp	0.05	0.05	1	15.84	0.14	0.7155
Size class × pH$_{\mathrm{T}}$	0.45	0.45	1	15.84	1.29	0.2733
Size class × Temp	0.19	0.19	1	15.84	0.55	0.4692
pH$_{\mathrm{T}}$ × Temp	0.63	0.63	1	15.84	1.80	0.1991
Size class × pH$_{\mathrm{T}}$ × Temp	0.10	0.10	1	15.84	0.28	0.6026

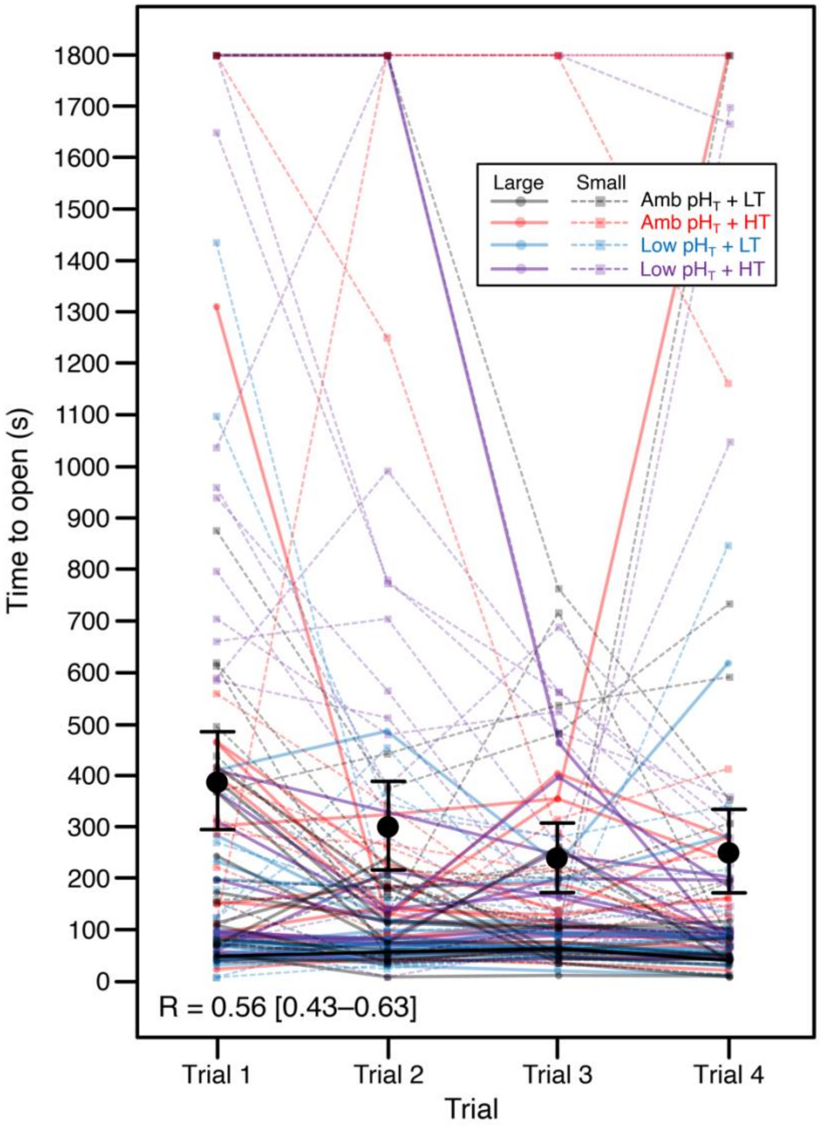

Figure 4. Individual (*n* = 105) time to open across the four trials. Black points and error bars represent the pooled mean ± 95% CI for time to open time in each trial.

Discussion

This study provides novel insights into the behavioural ecology of a bivalve startle response (time to open) and how this behaviour might be impacted under global changes. Results suggest that startle responses in bivalves are repeatable in short-term contexts, are a function of animal size, and are negatively affected by elevated temperatures but not by reduced pH_T. Given the important role of valve closures in avoiding and escaping predation (Carroll & Clements, 2019; Clements et al., 2020; Robson et al., 2007; Robson et al., 2010), our results demonstrate that animal size and seawater temperature influence behavioural predator avoidance in bivalves.

Effects of elevated temperature and low pH

Generally speaking, shifts in seawater temperatures can result in changes to organismal physiology, which can, in turn, drive behavioural changes. For prey, changes in activity patterns and metabolism under high temperatures have the potential to influence anti-predator behaviours. From this perspective, we predicted that increased metabolic rates and the need for oxygen and nutrient uptake under high temperatures would cause the mussels to open faster compared to lower temperatures. Contrary to our prediction, elevated temperature increased time to open and drove a significantly higher proportion of observations where animals did not open during a given trial. Similar results are reported for Mediterranean mussels, *Mytilus galloprovincialis*, which increased time to open under higher temperature (Anestis et al., 2007).

The reasons for longer valve closures under high temperatures are not well understood. Elevated temperatures can increase ectothermic predator activity (Wu et al., 2017). Thus, mussels may be able to anticipate periods of high predator activity using a combination of cues (tactile stimulus and high temperature in this experiment) and respond adaptively by staying closed for longer periods of time. Increased metabolic rates under higher temperatures can also increase detectability by predators and staying closed longer would thus reduce the chances of being detected by predators (Smee & Weissburg, 2006). Given that the mussels from our study site can temporarily experience the maximum temperatures used in our study, such adaptive reasons for the observed responses would not be surprising. Continually opening and closing would also incur energetic costs for individual mussels. Remaining closed for a longer period of time under the risk of predation at higher temperatures (when metabolic activity is higher) could potentially be a strategy to reduce energetic costs. Importantly, however, the abovementioned reasons remain speculative and further research is needed.

Some of the most striking effects of ocean acidification have been reported on animal behaviour (Clements & Hunt, 2015). CO_2-induced low pH is thought to impair neurological function (Tresguerres & Hamilton, 2017), alter the molecular structure of chemical cues (Roggatz et al., 2016), and influence morphological and physiological determinants of behaviour (Briffa et al., 2012). Behaviours involving sensory function are thought to be highly sensitive to ocean acidification (Ashur et al., 2017; Draper & Weissburg, 2019), and a suite of anti-predator behaviours in fish and invertebrates are reportedly impacted by acidification (see reviews by Clements & Comeau, 2019a and Draper & Weissburg, 2019 for detailed examples). In contrast, we observed no effect of low pH on time to open, despite employing an extreme acidification scenario (–0.7 pH_T). Turra et al., (2019) also found no effect of low pH (pH 7.70 from a control of 8.25) on startle responses in hermit crabs, *Pagurus criniticornis*. Clumping behaviour in *Mytilus edulis* (Kong et al., 2019) was affected by acidification; however multiple studies suggest no effect on baseline valve gaping activity in marine bivalves, even under extremely low pH conditions ($pH < 7$) (Bamber & Westerlund, 2016; Clements et al., 2018; Jakubowska & Normant, 2015). Furthermore, while valve gaping in *Mytilus galloprovincialis* was affected by acidification when food was reduced, normal feeding regimes saw no effect (Lassoued et al., 2019). While it could be argued that the lack of pH effect is due to the cue type used (i.e., tactile *versus* olfactory), a recent study on *Mytilus galloprovincialis* found no effect of low pH on valve closure responses to chemical alarm cues (Clements et al., 2020).. Coupled with recent studies suggesting that reported strong effects of acidification on fish behaviour are likely overestimated (Clark et al., 2020; Clements et al., 2020), low pH appears to have little effect on marine bivalve behaviours, and perhaps a far weaker effect on animal behaviour, broadly, than currently thought.

Effects of animal size

Smaller mussels remained closed three times longer, on average, than the larger mussels, supporting our hypothesis based on the idea that the smaller mussels were more vulnerable to predation than larger ones. Along the same vein, freshwater mussels living in clusters (which reduces vulnerability to predation) open quicker than their solitary counterparts (Wilson et al., 2012) and valve closure responses can be cue-specific depending on perceived threat (Castorani & Hovel, 2016; Dzierżyńska-Białończyk et al., 2019). As such, time to open appears to be at least partly dictated by relative vulnerability to predation and thus represents a measure of 'boldness' in bivalves. Interestingly, however, we also observed that individual coefficients of variation were significantly higher for smaller mussels compared to larger ones. This may be due to differences in the relative importance of predator avoidance and feeding in the two size classes. For instance, while both size classes would benefit from maximizing food intake, larger mussels could afford to be consistently bolder (i.e., open faster) and obtain food more readily than smaller mussels. Smaller mussels still need to maximize food intake, however, and they may take more risks (i.e., sometimes open quickly) to do so, which may explain the increased variation. Since these behaviours are repeatable, it seems likely that a persistent spectrum of "shyness to boldness" can exist within size classes, but that there is a larger overlaying trend of increasing boldness with size leading to mostly bold individuals in the large size class. This explanation remains speculative and more research into the mechanism and function of more variable behaviour in smaller bivalves is needed.

Tired mussels or evidence of habituation?

Across the four consecutive trials, time to open linearly decreased as trials progressed. While such an observation could indicate either habituation or fatigue, we suggest that habituation is a more likely culprit. Under natural settings, bivalves repeatedly and unpredictably need to close for a variety of reasons. To accommodate this need to close, bivalve adductor muscles contain both striated and smooth muscle fibres, providing both rapid muscular responses and prolonged contractions. Furthermore, many bivalves, including mytilid mussels, are equipped with the unique ability to engage "catch contractions" whereby smooth muscles allow for extended periods of muscle contraction without much energy loss (Galler et al., 2010). If this observation does indeed represent short-term habituation, such a response would likely be adaptive. For example, given the aforementioned trade-offs between feeding and avoiding predation, contextual adjustments in valve gaping and pumping would allow the animals to minimize the risk of being consumed by a predator while maximizing energy acquisition. Nonetheless, our approach does not allow us to empirically determine if this response was habituation or fatigue and further research is warranted.

A highly repeatable behaviour

We observed a high degree of behavioural repeatability in time to open following tactile predator cues in the lab. Similarly, time to open in freshwater mussels, *Margaritifera margaritifera*, was repeatable across three trials with different cue types (although a quantitative estimate of repeatability was not reported; Wilson et al. 2012). Behavioural components of escape performance in scallops (Brokordt et al., 2012; Laming et al., 2013) and other aspects of valve gaping behaviour in oysters (M.A. Mallet, J.C. Clements, L.A. Comeau, unpublished data) are also repeatable on both short- (hours) and long-term (days-weeks) timescales. Furthermore, startle responses in other invertebrates such as sea anemones, hermit crabs, and squid are thought to be repeatable (Briffa et al., 2013; Briffa & Greenaway, 2011; Rudin & Briffa, 2012; Sinn et al., 2008). Our results, together with these other studies, suggest that startle responses in bivalves and other "simple" invertebrates are repeatable across different species and contexts. The high repeatability of time to open, coupled with the ease at which it can be measured, provides for a useful model for behavioural ecology, particularly with respect to theoretical questions associated with animal personality (Gosling, 2001; Roche et al., 2016), behavioural syndromes (Sih et al., 2004), temperament (Réale et al., 2007), and coping styles (Koolhaas et al., 1999).

Population- and community-level effects

When closed, bivalve feeding activity ceases. As such, longer periods spent closed under higher temperatures and predator activity have the potential to reduce energy intake if feeding rates at higher temperatures cannot compensate for the lost time feeding. Kittner and Riisgård (2005) reported that individual blue mussels increase their filtration rates from ≈5.1 L h^{-1} at 15.6 °C to ≈5.5 L h-1 at 20.3 °C (on average). Based on our results, mussels at 16 °C remained closed, on average, for 182.6 secs (≈3mins, or 6 mins $hour^{-1}$). In contrast, mussels at 20 °C remained closed for an average of 422.1 secs (≈7 mins, or 14 mins $hour^{-1}$). Using these data, some back-of-the-envelope calculations reveal that mussels at 16 °C can filter a total of 4.6 L $hour^{-1}$, while those at 20 °C only filter 4.2 L $hour^{-1}$ (≈10 % less). Furthermore, differences in baseline opening times at similar temperature reveal a similar trend (Anestis et al., 2007). While studies directly testing this association between valve closure duration and food intake would provide a more definitive answer, our data suggest that net food intake in mussels can be reduced under high temperatures and periods of high predator activity. This finding aligns well with reports of reduced glycogen content, increased mortality, and weakened byssal strength under higher temperature in previous studies (Clements et al., 2018).

Reduced filtration as outlined above not only has implications for individual bivalves and their growth but could potentially impact the ecosystem benefits provided by bivalves (Clements & Comeau, 2019b; van der Schatte Olivier et al., 2020). For example, since mussels remained closed >2× longer than their control temperature counterparts after experiencing a cue simulating a predator attack, areas of high predation pressure are likely to see less effective filtering capacity. This could in turn the ability of bivalves to clean water and cycle nutrients. Similarly, our results, coupled with others (Anestis et al., 2007), suggest that the filtering capacity of bivalves may decrease in a warmer ocean and may be amplified in areas where predators exist in high abundance. Given the ubiquitous distribution of marine bivalves and their importance to marine ecosystems globally, it is possible that ocean warming could influence benthic systems worldwide. Furthermore, our results provide a basis for informing spatial planning of shellfish restoration and aquaculture activities globally. More studies of predator encounter rates in the field in conjunction with associated ecosystem service estimates are needed to quantify the effects of temperature and warming on bivalve ecosystem services.

Conclusions

The results of this study lend novel insights regarding bivalve startle response behaviour (time to re-open following a tactile predator cue), suggesting that this behaviour is a function of relative vulnerability to predation, highly repeatable in short-term contexts, and exhibits signs of habituation. This startle response appears unaffected by low pH conditions, adding to the growing body of literature suggesting that behavioural effects of acidification may be less severe than previously reported. In contrast, however, time to open can be negatively affected by elevated temperature. Coupled with previous studies reporting similar results, ocean warming could have drastic implications for the important ecosystem services that bivalves provide globally. Future studies directly quantifying the effects of warming on these ecosystem services and bivalve populations worldwide are strongly encouraged.

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Ethics

Ethical approval was not required for the species used in this experiment. Nonetheless, the study was strictly conducted under the premise of the three Rs of animal ethics.

Data Accessibility Statement

All statistical results, raw data, R code, and original datafiles uploaded to R are available as supplementary material.

Author Contributions

JCC conceptualized the idea. JCC, FJ and KR designed the experiment. JCC and KR tended to the animals and collected abiotic seawater parameters. JCC and JN carried out the behavioural experiments. JCC analyzed data and wrote the manuscript. SD and FJ provided in-kind support and technical guidance. All authors revised and approved the manuscript.

Competing interests

We declare we have no competing interests.

Supplementary material

Supplementary file 1. Supplementary figures and tables.
Supplementary file 2. Annotated R script.
Supplementary file 3. Raw data.
Supplementary file 4. R data file: mussel.size.txt
Supplementary file 5. R data file: valvo.txt
Supplementary file 6. R data file: valvo.repeat.txt
Supplementary file 7. R data file: valvo.cov.txt
Supplementary file 8. R data file: valvo.logistic.txt

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