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Seawater temperature, but not pH, affects startle response behaviour in a wide-ranging marine mollusc

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ABSTRACT

Under the risk of predation, the first response of bivalves is to close their shells. The strength and duration of valve closure can influence the probability of predator-related mortality. The behavioural ecology of valve closure responses, however, is 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 (i.e., startle response) following a tactile cue over a series of four consecutive trials. Time to open was highly repeatable on the short-term (adjusted R = 0.56) and decreased across the four trials from an average of 390.0 ± 493.6 secs in Trial 1 (mean ± SD) to 252.6 ± 421.4 secs in Trial 4. On average, individuals from the larger size class had a shorter time to open (154.1 ± 236.0 secs) than their smaller-sized counterparts (453.4 ± 449.9 secs). High temperature significantly increased time to open by 230%, on average, 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). For predators, the successful capture and consumption of prey is important for growth and survival. For prey, defence and avoidance against a predator's attack is critical for survival.

To combat predation, prey can employ a tremendous variety of defenses, including chemical, morphological, and behavioural defenses (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). 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; A. A. Robson et al., 2010; Smee & Weissburg, 2006), particularly given that inducible 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 close their valves and 'hide' (Carroll & Clements, 2019; A. Robson et al., 2007; A. A. 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). As such, the effectiveness of this strategy will depend on the strength of valve closure (weaker closure would allow predators to detect cues and open shells more easily) and the (Wilson et al. 2012)duration of closure (opening too soon would increase the probabilities of detection and the predator preventing further closure and successfully consuming the bivalve). While the strength of valve closure is predominantly dictated morphologically by adductor muscle strength, the duration of valve closure is determined by an individual's behavioural decision. Under the threat of predation, the duration of valve closure thus represents a startle response in semi-sessile bivalves (as measured and defined in previous studies; e.g. Rudin and 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) and Sundin et al. (2017) 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, 2019a). 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* × *trossolus*). We predicted that: 1) startle responses would be repeatable; 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 collection and husbandry

Adult mussels (Figure S1a) were hand-collected from the side of a nearshore pier at a depth of 0–1 m in the Gullmar Fjord, adjacent to the Kristineberg Marine Research and Innovation Centre (KMRIC; 58.250 °N, 11.447 °E). 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 1a). The animals were then placed into their experimental replicate tanks (Figure S1b,c) upon which exposure to temperature and pH treatments commenced (see below).

Experimental design and setup

A 2×2×2 design was employed with two size classes (small and large; as above), two pH levels (ambient and –0.7 units), and two temperatures (16 °C and 20 °C [+4 °C]) crossed in a fully-factorial manner. Size classes were chosen based on vulnerability to sea star predation in a comparable biological community (Baltic Sea), whereby the small size class was at the upper end of sizes consumed by sea stars, while the large size class was well above a size refuge threshold and are considered safe from sea star predation (Sommer et al., 1999). We used a small size class that was at the upper end of the size refuge threshold reported for the Baltic Sea because invertebrates in the Baltic Sea are generally smaller than on the west coast of Sweden due to low salinity conditions in the Baltic (Westerbom et al., 2002). Although these specific thresholds may not directly translate to the Gullmar Fjord system, we assumed that the relative vulnerability to predation (not only from sea stars, but from other predators such as crabs and fish) would be greater for the smaller size class. Furthermore, while increasing valve closure times may not be an efficient strategy for avoiding sea star predation (based on sea stars' mode of feeding), it would be for other predators in the Gullmar Fjord system such as crabs and fish. Temperature and pH treatments were designed to simulate deviations from ambient conditions in the fjord according to near future projections. We used a temperature offset of +4°C following ambient temperatures until they

147 reached 16 °C after which temperature conditions were kept constant at 16 °C (low) and 20 °C
148 (high) (Figure S2). We capped temperature manipulations at 16 °C and 20 °C to avoid temperature-
149 related mortality that can occur during long exposures to temperatures above 20°C (Clements et
150 al. 2018) and to avoid spawning. A pH offset of –0.7 units was employed, which represented an
151 ocean acidification scenario corresponding to the extreme of the natural variability expected by
152 2100. This scenario was based on a –0.3 unit differential from the minimum pH currently observed
153 in the fjord (0.4 units; low pH ≈7.6 from a mean of ≈8.1 according to Dorey et al. 2013).

154 A flow-through seawater system was constructed to expose animals to experimental temperature
155 and pH conditions (Figure S1b). Ambient seawater was continuously pumped directly from the
156 Gullmar Fjord into each of 12 header tanks (n = 3 header tanks per temperature×pH treatment).
157 The water entered the lab through one of two lines, each of which were equipped with in-line
158 temperature controllers to maintain the desired conditions for each temperature treatment.
159 Seawater pH was manipulated in six of the header tanks via pure CO_2 injection which was
160 maintained with a pH-stat control system (Aqua Medic, Bissendorf, Germany); pH was left at
161 ambient conditions in the other six header tanks. Salinity was left uncontrolled in all treatments
162 and varied naturally with ambient conditions in the fjord (Figure S2). The water in all 12 header
163 tanks was continuously aerated to ensure proper mixing, oxygenation, and gas equilibration.

164 Seawater from each header tank was gravity fed into two exposure tanks (4 L) where the animals
165 were held, one exposure tank for each size class (Figure S1b,c; n = 5 mussels replicate tank^{-1}; N =
166 120 mussels). Flow rate to the exposure chambers was adjusted to ≈3 ml s^{-1} (≈22.2 min for one
167 volume turnover). Mussels fed on the natural diet of plankton available in the seawater and were
168 exposed to a 12:12 light:dark cycle as above. Filtration and ingestion of food was confirmed by
169 the continual presence of faeces and pseudofaeces throughout the experiment, which was siphoned

off to clean the exposure tanks as needed. Mortality, which was negligible (n = 4/120, 3%), was checked daily and dead mussels were immediately removed from the exposure tanks. The animals were exposed to experimental conditions for 88–93 days 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 (2μm) 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 above provided highly consistent temperature and carbonate system offset conditions (Figure S2); mean values are provided in Table 1.

Table 1. Abiotic parameters of the experimental treatments. Data are means ± standard deviation. Raw data can be found in in Supplementary file 3 ('Carbonate chemistry' sheet) and full CO2SYS results are in Supplementary file 4; temporal trends are presented in Figure S2.

	Measured				Estimated			
Treatment	Salinity	Temp (ºC)	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 separate, flow-through experimental tanks (same style as the exposure tanks) 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 with treatment order randomized. Prior to each assay, mussels were removed from their exposure tank and each one placed into 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 and the time to visually re-open was recorded (in seconds) with a stopwatch for each individual. This process was repeated for each individual over four consecutive trials every 30 mins from the previous re-opening, allowing us to determine the short-term repeatability in time to open and to compute individual valve closure coefficients of variation. If an animal did not open within 30 mins, the observations for that individual ceased, the individual was assigned a value of 1800 secs, and the animal was given an additional 30 mins to re-open before starting the next trial. Data were discarded from the analysis if an animal was not open at the start of a trial, which only occurred for individuals that did not

open at all in any trials (n = 44 observations from 11 individuals; see Supplementary File 3). 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 are reported as means ± one standard deviation.

Linear models were used to determine if shell length and wet weights differed between size classes both prior to and after the exposure period using the lm() function followed by the anova() function to determine significance. Wet weights and post-exposure shell lengths were natural log transformed prior to analysis to assume normality (see Supplementary File 2). Generalized linear models (GLMs) were used to test for the fixed effects of initial size (continuous), pH_T (categorical, 2 levels), temperature (categorical, 2 levels), and their interactions on % changes in shell length and wet weight; GLMMs 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.

Repeatability (R) of time to open was estimated using generalized linear mixed models (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 $R \geq 0.37$). Two GLMMs were constructed: one to estimate agreements repeatability (R_{agree}; repeatability without accounting for any fixed effects) and another to estimate adjusted repeatability (R_{adj}; repeatability accounting for fixed effects of size class, pH_T, and temperature). The GLMMs were build using the MCMCglmm() function in the *MCMCglmm* R package (Hadfield, 2010). Estimates and 90% confidence intervals for R_{agree} and R_{adj} were obtained using the posteriormode() and HPDinterval() functions, respectively, with code adapted from Roche et al. (2016).

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). 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 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. 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() function to determine significant effects.

RESULTS

Shell length and wet weight

Following exposure, shell lengths and wet weights were significantly different between the two size classes, with mean shell lengths of 67.5 ± 5.1 mm, 50.9 ± 4.1 mm, and mean wet weights of 40.8 ± 8.9 g and 17.6 ± 4.2 g, in the large and small size classes, respectively (Figure 1a-c; Table S1). For growth rates (i.e., changes in shell length and wet weight), initial size had a significant

independent effect on growth rate, with smaller animals growing showing larger increases in both shell length and wet weight than larger individuals (Figure 1d,e). There were no significant effects of temperature, pH_T, or any interactions on growth rates (Table S2).

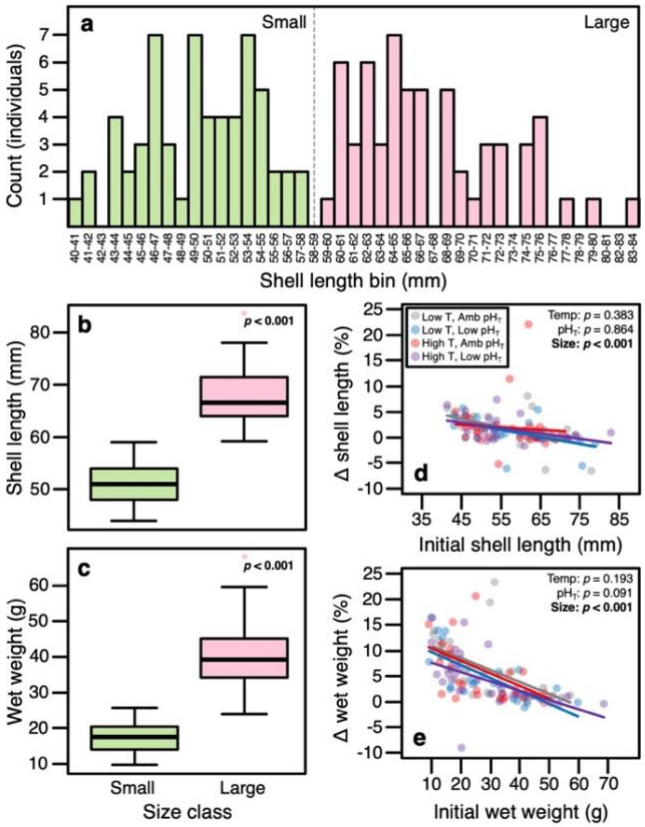

Figure 1. (a) Frequency distribution of shell lengths used to define mussel size classes at the beginning of the experiment. Green bars are the small size class and pink bars are the large size class (n = 60 for each size class). **(b-c)** Boxplots of post-experiment shell length (a) and wet weight (b) for each of the two size classes (n = 58 for each size class). **(d-e)** Scatterplots of changes in (Δ) shell length (d) and wet weight (e) 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). *P*-values represent results of linear models (see Table S1 in Supplementary file 2).

Short-term repeatability in time to open

Time to open was highly repeatable. Agreement repeatability (R_{agree}; repeatability without accounting for any fixed effects) was estimated to be 0.64 [0.56–0.77, 95% CI]. Similarly, adjusted

repeatability (R_{adj}; repeatability accounting for fixed effects of size, pH_T, and temperature) was 0.56 [0.43–0.63, 95% CI].

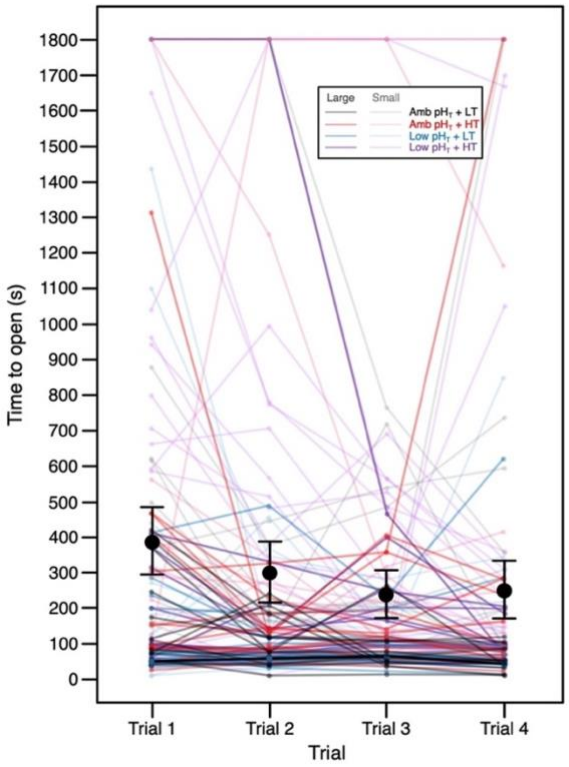

Figure 2. Individual (n = 105) time to open across the four trials. Black points and error bars represent the pooled mean ± 95% CI for time to open time in each trial.

Effects of size, pH_T, temperature, and trial on time to open

Time to open was independently affected by size class, temperature, and trial (Table S4). 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 3a,c, S3). 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 3b,c, S3). 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 3d, S3; Table S6).

291 Seawater pH_T had no effect on time to open (Amb. pH: 290.2 ± 426.4 secs, Low pH: 302.0 ± 442.6

292 secs) and there were no interactive effects (Figure S3; Table S4).

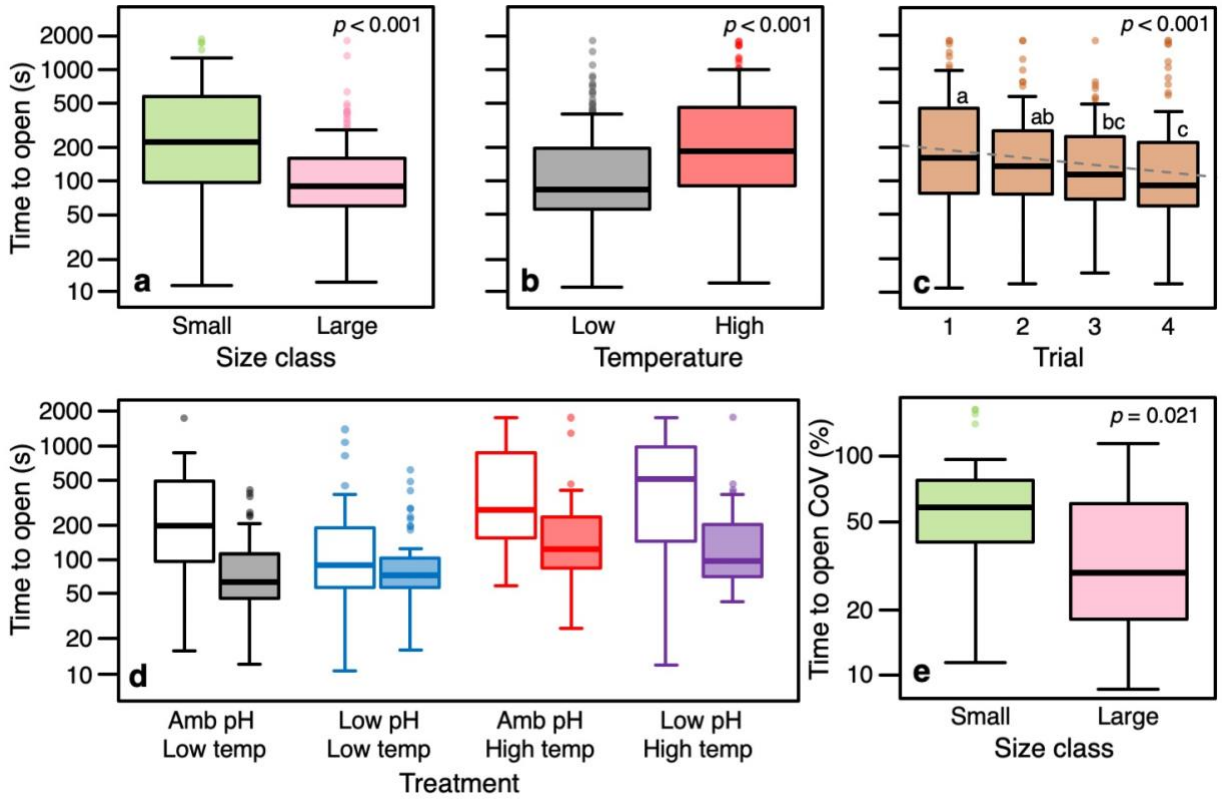

Figure 3. (a) Boxplot of time to open for each size class (n_{large} = 55, n_{small} = 50). **(b)** Boxplot of time to open for each experimental temperature (°C; $n_{control}$ = 55, n_{high} = 50). **(c)** Boxplot of startle response times for each of the four trials (n = 105 individuals per trial). Note that the y-axis is log scaled. Dashed line is the linear best fit trendline. *P*-value represents the main effect of trial from the linear mixed effects model and letters above plots denote Tukey HSD pairwise differences (see Table S3). **(d)** 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). Large and small size classes are represented by filled and open boxes, respectively. **(e)** 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*-values represent main effect results from the mixed effect Cox proportional hazards model for time to open (a-c), and linear mixed effects model for CoV (see Tables S2 and S5).

293 Alongside staying closed longer, smaller mussels also had a higher individual coefficient of

294 variation (CoV) in time to open than larger mussels (59.9 ± 37.0 % in small *versus* 41.2 ± 28.6 %

295 in large; Figure 3e). Time to open CoV was not significantly affected by any other factor (or

296 interaction) aside from size class (Table S5).

297 The propensity of individuals to remain closed for the duration of a given trial was independently
298 affect by size class and temperature but not by pH_T or trial (Figure S4; Table S7). The proportion
299 of trials in which individuals did not open was higher in the small size class and under high
300 temperatures (Figure S4). Overall, however, the percentage of trials in which individuals did not
301 open was low (13.1%).

302 **DISCUSSION**

303 This study provides novel insights into the behavioural ecology of a bivalve startle response (time
304 to open) and how this behaviour might be impacted under global changes. Results suggest that
305 startle responses in bivalves are repeatable in short-term contexts. In addition, these responses
306 appear to be a function of relative vulnerability to predation and are negatively affected by elevated
307 temperatures but not by reduced pH_T.

308 Contrary to our prediction that increased temperatures would reduce time to open, exposure to
309 elevated temperature resulted in increased time to open and drove a significantly higher proportion
310 of observations where animals did not open during a given trial. Our initial prediction was
311 generated from a physiological perspective with the reasoning that higher temperatures raise
312 metabolic rates, which increase the need for oxygen and nutrient uptake. Similar results are
313 reported for Mediterranean mussels, *Mytilus galloprovincialis*, which increased time to open under
314 higher temperature (Anestis et al., 2007). In addition, continually opening and closing would incur
315 energetic costs for individual mussels. Remaining closed for a longer period of time under the risk
316 of predation at higher temperatures (where metabolic activity, and thus basal energetic
317 expenditure, is higher) could potentially be a strategy to reduce energetic costs if the mussels would
318 have to close again after re-opening. Rather than increasing oxygen and nutrient uptake, it seems

that bivalves generally increase the time spent closed, possibly to depress metabolism and offset the energy demand associated with higher temperature (Anestis et al., 2007; de Zwaan et al., 1980; Ortmann & Grieshaber, 2003). Such a strategy could help explain reports of reduced growth and condition under higher temperatures (Clements, Hicks, et al., 2018; Mackenzie et al., 2014), observations which have been verified in the field by mussel farmers in eastern Canada (Clements, Hicks, et al., 2018). This strategy appears ineffective for blue mussels, however, as prolonged exposure to higher temperatures is also associated with higher mortality (Clements, Hicks, et al., 2018). Given that the amount of time spent at temperatures at or above 20. °C will increase as global temperatures increase, ocean warming may pose a significant threat to these mussels unless they can adapt to increasing temperatures.

When closed, bivalve feeding activity ceases. As such, longer periods spent closed under higher temperatures have the potential to reduce energy intake if feeding rates (when open) at higher temperatures are insufficient to 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), with no effect of time up to 22 mins (estimated from Figure 3a at 22 mins using ImageJ). Based on 30 min observation periods in our experiment, 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 $hour^{-1}$). Based on our 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 testing this association more specifically for the mussel population used in this study, and a more precise metric of feeding (e.g. ingestion rate instead of filtration rate), would provide a more

definitive answer, these numbers suggest that net food intake in mussels can be reduced under high temperatures. This finding aligns well with reports of reduced glycogen content, increased mortality, and weakened byssal strength under higher temperature in previous studies (Clements, Hicks, et al., 2018).

Reduced filtration under higher temperatures not only have 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). Given that mussels remained closed for durations more than two-times longer than their control temperature counterparts, areas of high predation pressure are likely to see less effective filtering capacity, potentially affecting the effectiveness at which bivalves can 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, which 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. Such studies should be accompanied by others quantifying the capacity of various species and populations of bivalves to cope with and/or adapt to shifting temperatures in the context of predator avoidance and feeding.

Some of the most striking effects of ocean acidification have been reported on animal behaviour (Clements & Hunt, 2015) which are anticipated to drive ecosystem-level impacts under global change (Nagelkerken & Munday, 2016). Therein, behaviours involving sensory function are

thought to be highly sensitive to ocean acidification (Ashur et al., 2017; Draper & Weissburg, 2019), and anti-predator behaviours in both fish and invertebrates are reported to be impacted by acidification (Clements & Comeau, 2019a; Draper & Weissburg, 2019). As such, we predicted that exposure to low pH conditions would affect the mussels' time to open in this experiment. In contrast to this prediction, however, we observed no effect of low pH, despite employing an extreme acidification scenario (–0.7 pH_T). While clumping behaviour in *Mytilus edulis* was affected by acidification (Kong et al., 2019) and median valve openings in *Mytilus galloprovincialis* were reduced under 1200 μatm (from a 500 μatm control) (Lassoued et al., 2019), multiple studies suggest a lack of acidification effect on baseline valve gaping activity in marine bivalves (Bamber & Westerlund, 2016; Clements, Comeau, et al., 2018; Jakubowska & Normant, 2015). Furthermore, a recent study also found no effect of near-future ocean acidification (pH 7.70 from a control of 8.25) on startle responses in hermit crabs, *Pagurus criniticornis* (Turra 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 similar study on *Mytilus galloprovincialis* found no effect of low pH on valve closure responses to chemical alarm cues (Clements et al. under review). Collectively, these results suggest that ocean acidification may have a relatively weak effect on marine bivalve behaviours and perhaps a far weaker effect on animal behaviour, broadly, than currently thought (Clark et al., 2020).

We observed a high degree of behavioural repeatability in time to open following tactile predator cues in the lab, supporting our hypothesis that bivalve startle responses are repeatable. To our knowledge, only one other study has reported on the repeatability of time to open, reporting that similar responses in freshwater mussels, *Margaritifera margaritifera*, were 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 (M.A. Mallet, J.C. Clements, L.A. Comeau, unpublished data) are also repeatable on both short- and long-term 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 bivalve startle responses across different species and contexts are repeatable. The high repeatability of time to open, coupled with the ease at which they can be measured, provides for a useful behavioural model, 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).

Our hypothesis that smaller mussels would remain closed longer than larger mussels was supported as smaller mussels remained closed three times longer than the larger mussels. The hypothesis was based on the fact that individuals in the smaller size class are considered more vulnerable to predation than the large size class. This idea is also supported by the observation that freshwater mussels living in clusters had shorter time to open than their solitary counterparts (Wilson et al., 2012), since living in clusters is thought to reduce vulnerability to predation in group-living bivalves (Wilson et al. 2012). Valve closures and the cessation of feeding are also reported to be cue specific (Castorani & Hovel, 2016; Dzierżyńska-Białończyk et al., 2019). It is thus likely that time to open is at least partly dictated by relative vulnerability to predation and represents a measure of ‘boldness’ in bivalves. It is important to note, however, that feeding, and oxygen uptake, stop when a bivalve is closed. As such, while conferring a lower probability of being consumed by a predator, remaining closed for a longer period of time also means reduced filtering

411 time, which can affect the net growth of individuals (Nakaoka, 2000). Extended periods without
412 oxygen uptake slows the metabolism which can also have numerous negative impacts (Ortmann
413 & Grieshaber, 2003), including reduced growth and fecundity. Indeed, previous studies have found
414 that blue mussels and other bivalves will incur costs to growth in the interest of protection from
415 predation (Eschweiler & Christensen, 2011; Nakaoka, 2000).

416 Interestingly, we observed that individual coefficients of variation were significantly higher in the
417 smaller size class, meaning that time to open in the smaller size group were more variable that
418 those in the large size class. This may be due to the relative importance of predator avoidance and
419 feeding in the two size classes. For instance, while both size classes would benefit from
420 maximizing food intake, animals from the large size class were considered less vulnerable to
421 predation and therefore could afford to be consistently bolder (i.e., open faster) and take less risks.
422 In contrast, the smaller size class was considered vulnerable to predation and would therefore stay
423 closed longer. The smaller size class still needs to maximize food intake, however, and they may
424 thus be more likely to take more risks (i.e., sometimes open quickly) than the larger size class,
425 which may explain the higher degree of variability observed in the smaller size class. This
426 explanation thus remains speculative and more research into the mechanism and function of more
427 variable behaviour in smaller bivalves is needed.

428 Across the four consecutive trials, time to open linearly decreased as trials progressed. Such an
429 observation may indicate habituation or fatigue. If this observation represents short-term
430 habituation, such a response would likely be adaptive. For example, given the aforementioned
431 trade-offs between feeding and avoiding predation, as contextual adjustments would allow the
432 animals to minimize the risk of being consumed by a predator while maximizing energy
433 acquisition. It is important to note here, however, that we only used a single tactile predator cue in

our experiments in the absence of olfactory cues, which comes with limitations as recent evidence suggests that different cues can alter bivalve gaping behaviour in different ways (Dzierżyńska-Białończyk et al., 2019). Nonetheless, our approach does not allow us to determine if this response was habituation or simply fatigue. Since adductor muscle contractions required for shell closure would incur energetic costs, the shorter time to open in later trials may simply reflect reduced energy to sustain shell closures. This is particularly apparent given the relatively short rest period between trials. Further research is thus warranted to determine whether or not the trial effect observed here is related to habituation or fatigue.

CONCLUSIONS

The results of this study lend novel insights regarding bivalve startle response behaviour, suggesting that this behaviour is highly repeatable in short-term contexts, and are likely a function of relative vulnerability to predation. Low pH conditions simulating ocean acidification had no effect on bivalve startle responses in this study, adding to the growing body of literature suggesting that the behavioural effects of low pH on marine fauna may be less severe than previously thought. In contrast, however, our results show that these responses 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 warranted and highly 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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