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# Lethal and sublethal implications of low temperature exposure for three intertidal predators

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#### ABSTRACT

Benthic invertebrate predators play a key role in top-down trophic regulation in intertidal ecosystems. While the physiological and ecological consequences of predator exposure to high temperatures during summer low tides are increasingly well-studied, the effects of cold exposure during winter low tides remain poorly understood. To address this knowledge gap, we measured the supercooling points, survival, and feeding rates of three intertidal predator species in British Columbia, Canada - the sea stars Pisaster ochraceus and Evasterias troschelii and the dogwhelk Nucella lamellosa — in response to exposure to sub-zero air temperatures. Overall, we found that all three predators exhibited evidence of internal freezing at relatively mild sub-zero temperatures, with sea stars exhibiting an average supercooling point of -2.50 °C, and the dogwhelk averaging approximately -3.99 °C. None of the tested species are strongly freeze tolerant, as evidenced by moderate-to-low survival rates after exposure to -8 °C air. All three predators exhibited significantly reduced feeding rates over a two-week period following a single 3-h sublethal (-0.5 °C) exposure event. We also quantified variation in predator body temperature among thermal microhabitats during winter low tides. Predators that were found at the base of large boulders, on the sediment, and within crevices had higher body temperatures during winter low tides, as compared to those situated in other microhabitats. However, we did not find evidence of behavioural thermoregulation via selective microhabitat use during cold weather. Since these intertidal predators are less freeze tolerant than their preferred prey, winter low temperature exposures can have important implications for organism survival and predator-prey dynamics across thermal gradients at both local (habitat-driven) and geographic (climate-driven) scales.

#### 1. Introduction

Predators play a pivotal role in determining the distribution, abundance, and diversity of species in benthic marine ecosystems. Distribution limits of prey, most notably lower intertidal zonation limits, are frequently set by predators (Connell, 1972; Paine, 1974). Furthermore, predators can maintain higher species richness by preferentially consuming competitively dominant prey, a phenomenon that is well-represented by the keystone ecological role of the sea star *Pisaster ochraceus* (hereafter, *P. ochraceus*) as a consumer of intertidal mussels (Paine, 1966; 1969). Predators exert additional indirect control over ecosystems via trophic cascades, as in the iconic example of sea otters maintaining kelp forests through the suppression of herbivorous sea urchins (Watson and Estes, 2011). Given their ecological importance, changes in the abundance and per capita effects of predators by external factors such as species invasions (Rossong et al., 2006), disease (Miner et al., 2018), recovery from human harvest (Gregr et al., 2020), and shifts in their abiotic environment (Gooding et al., 2009) can have far-reaching implications for benthic marine ecosystems.

Temperature is one such important factor that influences how predators impact the ecosystems of which they are a part. Most predators in benthic marine systems are ectotherms, and thus temperature exerts substantial effects on their feeding, growth, and reproduction (Angilletta, 2009). Ecological theory generally predicts that consumer pressure is positively related to temperature within non-stressful thermal ranges (O'Connor, 2009). For example, on rocky shores in the

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northeast Pacific where upwelling dynamics control water temperature, the feeding rates of predators like P. ochraceus and the dogwhelk Nucella canaliculata are higher when temperatures are warmer (Sanford, 1999; 2002). When temperatures are extremely high or low, however, predators can become physiologically stressed and exhibit decreased feeding rates either through behavioural changes, sublethal damage, or predator mortality (Kidawa et al., 2010; Arribas et al., 2022). Ecological theory (e.g., environmental stress models) thus predicts that the importance of predation may decline at thermal extremes due to the increased susceptibility of predators to thermal stress relative to their prey (Menge and Olson, 1990). As with the preceding examples, much of the emphasis on temperature effects in the ecological literature has been on either non-stressful thermal variation (e.g., metabolic scaling) or on extreme high temperatures (e.g., ecophysiological studies couched in a global warming framework). Although extreme low temperatures are also ecologically and physiologically significant, they are much less commonly studied, and their ecological implications in intertidal environments remain particularly poorly understood.

During low tide, intertidal organisms frequently encounter extreme air temperatures that may significantly affect their survival and performance. Air temperature can be considerably warmer than seawater in the summer and considerably colder than seawater in the winter, meaning that intertidal species are frequently subject to temperature stress when emersed (Helmuth et al., 2006; Stickle et al., 2015). Air temperatures above 35 °C can cause heat stress mortality in P. ochraceus (Pincebourde et al., 2008), and mass mortalities of intertidal invertebrates are frequently observed following heatwaves (Harley, 2008; Raymond et al. 2022). Similarly, intertidal mass mortality events have been observed in response to freezing temperatures; for instance, extensive mortality was observed in the intertidal mussel Mytilus trossulus after a three-week period of extreme freezing temperatures (Carroll and Highsmith, 1996). Additionally, multiple species of oysters, mussels and barnacles experienced mass mortality after the severe winter of 1962 in Britain (Crisp, 1964). Further, low temperature thermal safety margins, which impact the degree of mortality observed in response to cold stress, can vary between populations of an organism. For example, these thermal safety margins are slim near the poleward range limit of an intertidal gastropod (Nerita yoldii), and lethal impacts of cold temperatures are thus predicted to be extensive for this species at higher latitudes (Wang et al., 2020). Milder cold temperatures can also induce sublethal effects, reducing the performance of intertidal species. The littorine snail, Littorina scutulata, displays reduced activity when exposed to temperatures below -3 °C for extended periods (Reid and Harley, 2021), which could impair their grazing ability. Clearly, extremes in air temperature can lead to negative downstream impacts for intertidal invertebrate species; however, how these impacts vary between species, and the strategies that intertidal species may utilize to mitigate low temperature stress remain understudied.

To avoid or reduce exposure to thermal stress in the intertidal zone, motile organisms can behaviourally thermoregulate as a first line of defence (Hews et al., 2021) by relocating to more thermally benign areas of shore, utilizing thermal gradients present in both summer and winter (Helmuth et al., 2006; Stickle et al., 2017; Reid and Harley, 2021). One gradient is vertical: higher shore levels remain emersed for longer and therefore reach temperatures that are both hotter - during summer and colder — during winter — than lower shore levels (Reid and Harley, 2021). Smaller-scale thermal gradients can be caused by topographic variability, substratum heterogeneity, or the presence of foundation species; examples of these thermal refugia in the intertidal zone include crevices, undersides of boulders, and areas beneath algal canopy (Helmuth and Hofmann, 2001; Reid and Harley, 2021). Although behavioural thermoregulation is a viable option to mediate the effects of extreme temperatures, particularly for motile animals, there are additional physiological mechanisms that may be utilized to combat the effects of extreme temperatures.

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result in potentially lethal freezing injuries (Lee, 2010; Loomis, 1995). These injuries can include mechanical damage from the ice crystal lattice and cell dehydration induced by disruptions of cell osmotic gradients from ice formation, among other types of cellular damage (Storey and Storey, 2002). Many species of intertidal invertebrates are thought to be freeze tolerant (Aarset, 1982; Loomis, 1995), meaning that they can withstand and mediate these potential freezing injuries when subjected to freezing temperatures. One such strategy to avoid and/or cope with freezing damage is employing cryoprotectant molecules, which function to reduce internal injuries caused by freezing (Storey and Storey, 2002). In several Alaskan Nucella spp., higher cryoprotectant concentrations corresponded to a higher degree of freeze tolerance at the species level (Stickle et al., 2015). Even mild sub-zero temperatures, while they may not cause extensive internal ice formation, can still lead to negative physiological impacts such as reduced ATP availability, as has been found in terrestrial insects (Churchill and Storey, 1989), or reduced digestive function as genes required for stress response and basal organismal processes are upregulated (Koštál et al., 2007; Zhang et al., 2011).

The physiological effects of cold temperatures may have ecological consequences. Metabolic rate declines exponentially with decreasing temperature (Cossins and Bowler, 1987; Sinclair et al., 2015), which may have implications for feeding rates. Reduced predation rates have been documented in *P. ochraceus* and *Nucella lamellosa* (hereafter, *N. lamellosa*) exposed to cool water in both laboratory (Sanford, 2002) and field (Sanford, 1999; Stickle et al., 2010) studies, with Alaskan *Nucella* spp. ceasing feeding entirely when air temperatures drop below freezing (Stickle et al., 2010). Decreased predation under cold temperatures, coupled with increases in predator mortality, may substantially alter intertidal community structure as prey increase in abundance (Saier, 2001). This reduction in predation may have further implications for intertidal biodiversity, particularly if the preferred prey is a competitive dominant or foundation species (Paine, 1966; Lafferty and Suchanek 2016).

In this study, we examined both the sublethal and lethal consequences of exposure to sub-zero air temperatures for three common, ecologically important predatory intertidal invertebrates in the northeast Pacific: the sea stars P. ochraceus and Evasterias troschelii (hereafter, E. troschelii), and the dogwhelk N. lamellosa. Using a combination of laboratory studies, field surveys and opportunistic field measurements (when low tides and cold temperatures allowed) we examined the physiological and behavioural responses of these species to low temperature exposure and investigated how their responses may affect their ecological role within the intertidal community. Our main objectives were to: 1) determine the relative freeze tolerance of these predator species by measuring their supercooling points and survival after cold and freezing temperature exposure, 2) determine whether feeding rates are affected by sublethal cold exposures through laboratory experiments, and 3) complement lab findings with in situ assessments of field body temperatures and factors that influence predators' experienced temperatures, including microhabitat types and organismal aggregations.

# 2. Methods

#### 2.1. Study species and study areas

We conducted this work in  $s_{\partial i l i w_{\partial t}}$ , of the  $s_{\partial i l i i w_{\partial t} a A}$  (Tsleil-Waututh) people, otherwise known as the Burrard Inlet, which is an extension of the Salish Sea abutting Vancouver, British Columbia, Canada. We visited two sites to measure environmental conditions and organismal body temperatures:  $\chi w \dot{a} \dot{y} \chi w ay$ , in the vicinity of the Girl in a Wetsuit statue in Stanley Park, Vancouver, BC (latitude: 49.302528°, longitude: -123.126083°), and Smelákw'a, otherwise known as Sharon Cove in

When extreme low temperatures cannot be avoided, intertidal

West Vancouver, BC (latitude: 49.33927°, longitude: -123.22277°). Both sites experience mixed semidiurnal tides, with lower low tides occurring almost exclusively at night during the winter (Reid and Harley, 2021). Stanley Park features small (0.5 m diameter) and large (2 m diameter) boulders interspersed with sand and shell hash (see Reid and Harley, 2021 for site photos), and Sharon Cove is a granitic bedrock shore with some cobble sections lower on shore (see Kay et al., 2019).

*P. ochraceus* is a keystone predator distributed from southern California to southeast Alaska in the Northeast Pacific (Lambert, 2000) and drives patterns in species diversity and intertidal zonation (Paine, 1966; 1974; Rogers and Elliott, 2013). *E. troschelii* ranges from California to Southern Alaska (Lambert, 2000), and commonly co-occurs with *P. ochraceus* in the low intertidal zone of the Salish Sea (Kay et al., 2019; Mauzey et al., 1968). *N. lamellosa* is a predatory gastropod found from central California to southwest Alaska that preys upon barnacles and mussels and occupies the mid-to-low intertidal zone (Stickle et al., 2015, 2017).

For the experiments described below, we collected animals during low tides on 11 and 28 January 2021, with additional *P. ochraceus* collected on 11 February 2021. Animals were transported to the University of British Columbia and placed in aerated aquaria (32–34 PSU, see Table S12) within 2 h of collection. We collected all organisms under a Fisheries and Oceans Canada scientific collecting permit (license number XMCFR 21 2020).

# 2.2. Supercooling point determination and survival after cold exposure

For laboratory experiments to measure supercooling points and survival after cold exposure, we housed organisms in 20 L aerated tanks filled with natural seawater sourced from the Vancouver Aquarium (34 PSU, 10 °C) and held within a recirculating sea table (with water tank changes every three days). We acclimated all animals to laboratory conditions for at least two weeks prior to experiments, during which time they were starved, with the maximum starvation time lasting two and a half weeks. Prior to the commencement of any lab experiments, we removed sea stars showing lesions, necrosis, or other signs of sea star wasting disease, an ongoing epidemic disease in the Northeast Pacific that affects sea stars (Hewson et al., 2014; Miner et al., 2018; Kay et al., 2019). We made every attempt to discard sea stars which demonstrated evidence of sea star wasting disease from lab data, however, there was always a chance of infection amongst the housed individuals.

One way to investigate the degree of freeze tolerance of invertebrates is to determine their supercooling point (SCP). As body temperature drops below the melting point, the fluids in an animal remain unfrozen in a state referred to as supercooled (Zachariaseen and Kristiansen, 2000). The SCP is defined as the lowest temperature before ice formation occurs (or the temperature at which supercooling ends; Lee, 2010). Comparing a species' SCP to the environmental temperatures that they experience can indicate whether they are likely to freeze in a given environment and may shed light on their overall cold tolerance strategy.

To determine SCPs, we subjected predators to a simulated cold low tide for 2 h in Panasonic MIR-154 incubators. Incubators were set to -8°C, which represents a rare but realistic winter air temperature for the Vancouver area (Kennedy et al., 2020). Predators were placed directly into the incubator set to -8 °C, mimicking the relatively rapid emersion and cooling of predators in the intertidal zone that occurs as the tide drops on a cold night (Fig. S2). We placed all animals on  $10 \times 10$  cm ceramic tiles to mimic the surface of boulders in the intertidal zone. To measure the body temperature of each sea star during freezing, we injected each individual with a Type-T thermocouple, connected to a Picolog TC-08 interface (Pico Technology, Cambridgeshire, UK), in the central disc of the body near the madreporite using a hypodermic needle and secured these thermocouples with a rubber band. In contrast with sea stars, thermocouples could be inserted into N. lamellosa less invasively by pushing the thermocouple into the foot of the snails, which caused the animals to attach to the thermocouple and retract into their

shell, taking the thermocouple tip with them. When dogwhelks subsequently relaxed their feet, the thermocouple remained in their shell, after which we placed them into the incubator. We continuously recorded body temperature during freeze exposures using PicoLog 6 beta software for MacOS (Pico Technology, Cambridge, UK). Preliminary data indicated that body temperature fluctuated substantially (up to  $\sim$ 1.5 °C) during cooling, driven by incubator thermal cycling. To mitigate this experimental artefact, we covered the animals with 0.94 L Ziplock bags of pre-frozen peas (hereafter, ice packs) which were placed in the incubator 10 min prior to the beginning of sea star exposure, which reduced exposure to over-cooled air and reduced thermal fluctuations by increasing thermal inertia (i.e., the pace with which an object's temperature equilibrates with its environment). The SCP of each specimen was determined by visual inspection of the temperature trace and recorded as the lowest temperature measured before exothermic release, or a sharp increase in body temperature, which indicates the onset of freezing (Sinclair et al., 2015; see Figs. S3-S5 for temperature traces for each species). For our control group, we placed animals in an incubator set at 8 °C for 2 h. Following the 2-h exposure to -8 °C or 8 °C, we immediately returned animals to their aquaria. We also measured the size of each individual using calipers as either the distance from the mouth to the tip of the longest arm for sea stars or the height of the shell for whelks.

We used a sample size of ten individuals for each treatment group for all species, and the experiment was performed twice (19 January and 1 February 2021). For *P. ochraceus*, we repeated the freezing treatment one additional time with four replicates (25 February 2021), resulting in total sample sizes of 44 for *P. ochraceus* and 40 for *E. troschelii* and *N. lamellosa*. Two additional traces and SCPs for *N. lamellosa* are included in the SCP data (snail 21 & 22, Fig. S5), which were from an earlier trial conducted on 10 January 2021, but these data were not included in the survival analysis.

Following SCP determination, we monitored individual survival daily for two weeks after exposure to each treatment. No food was provided to animals during this period. Sea stars sometimes exhibited signs of white spotting at their arm tips — which may have been due to freezing injuries, stress, or wasting disease — although not all of these sea stars died. We only recorded sea stars as dead if they were lying motionless on their aboral side on the bottom of the aquarium and were unable to attach to the aquarium walls. Additionally, some sea stars shed some of their limbs prior to death. We recorded *N. lamellosa* individuals as dead if they had not emerged from their shell or attached to the aquarium for at least a day or if there was white discharge coming from inside their shell.

#### 2.3. Sublethal cold exposure effects on feeding rate

To determine how exposure to low temperature affects feeding rate, we simulated a single low tide by placing individuals of all three species on ceramic tiles in Panasonic MIR-154 incubators set to either -0.5 °C or 8 °C air temperatures for 3 h and 2 h, respectively. Following incubation, we placed predators directly into separate plastic mesh-sided containers – 35  $\times$  20  $\times$  13 cm, 12  $\times$  9.5  $\times$  9.5 cm, and 11  $\times$  8  $\times$  5.5 cm in size for P. ochraceus, E. troschelii, and N. lamellosa, respectively - within a wellaerated recirculating seawater table. Due to space limitations and container availability, each container housed two P. ochraceus (except for two lone individuals, one from each treatment), while each container housed three or four E. troschelii or N. lamellosa. We fed each predator Mytilus trossulus mussels collected from Tower Beach, Vancouver (11-42 mm shell length) ad libitum. We monitored the number of mussels consumed daily for two weeks and then standardized the total number of mussels eaten per container to the number of predator individuals in the container; therefore, the level of replication was the container. We repeated this feeding experiment twice (one trial every month) with 9-10 P. ochraceus, 7-10 E. troschelii, and 9-10 N. lamellosa per trial (depending on availability). A third trial was performed for

*P. ochraceus* to increase the sample size for this species because it suffered high rates of mortality from sea star wasting disease. After omitting containers with dead or diseased predators, which were not included in analysis, we obtained a total sample size of 22 containers for *P. ochraceus* (11 each of 8 °C control and -0.5 °C exposures) 31 for *E. troschelii*, and 38 for *N. lamellosa* across the trials. Separate individuals were used for each trial. *E. troschelii* ate mussels ranging from 22 to 42 mm in size, *P. ochraceus* ate mussels ranging from 21 to 40 mm in size, and *N. lamellosa* ate mussels ranging from 11 to 32 mm in size.

#### 2.4. Effects of microhabitat on predator body temperatures

To assess general thermal patterns in the intertidal zone during times of extreme cold, we conducted thermal surveys in Stanley Park on a cold night (10–11 February 2021, Vancouver airport air temperature = -2 °C) using a e40 FLIR thermal camera. Because the camera was set to its default settings (emissivity = 0.95, a reasonable but imprecise approximation for *N. lamellosa* shell surfaces and sea star dermal tissue), the absolute temperatures presented will be slightly inaccurate, particularly as dogwhelk and sea star emissivity are likely to differ somewhat. We therefore intend the FLIR imagery to be used primarily for qualitative visualization of the general thermal environment, and restrict our inference to assessments of thermal differences among microhabitats on a species by species basis (e.g., *P. ochraceus* in a crevice vs. *P. ochraceus* out in the open) as recommended by Caddy-Retalic et al. (2011).

To investigate the effect of microhabitat type on the in situ body temperatures of our target sea star species, we documented microhabitat type, body temperature, and arm size for E. troschelii and P. ochraceus in the field monthly from October 2020 to February 2021 in Stanley Park (see Table S11 for sampling date details). We classified microhabitats as one of four types: sediment (sand or shell hash); base of boulder; side of boulder; and crevice (generally between two or more boulders). Sea star sizes were measured from the tip of the longest arm to the centre of the oral disk. Body temperatures were measured using a FLUKE 62 Max infrared thermometer aimed at the centre of the aboral side of the animal. We used air temperature data from the nearby Vancouver International Airport weather station as a proxy for air temperature experienced by organisms at low tide (Environment and Climate Change Canada, n.d.). To obtain a representative sample of body temperatures, we placed a 50 m transect line parallel to the shore at approximately 0.6 m above Canadian chart datum. We then placed  $4 \times 4$  m quadrats at randomly selected locations within each of the four 12.5 m segments of the transect. We measured the temperature and size of 15-20 randomly selected E. troschelii and P. ochraceus within each quadrat, with a goal of obtaining at least 50 measurements for each species during each survey. We also recorded the total number of P. ochraceus and E. troschelii found in each microhabitat type within each quadrat.

To ascertain how body position influences the temperature of *E. troschelii*, we measured the body temperature of a) individuals found under boulders (n = 10), b) individuals fully attached to the sides of boulders (n = 5), and c) individuals dangling from boulders, attached by only one or two arms (n = 5) using a thermometer pressed into the centre of the oral side. This survey, which occurred on 10 February 2021, opportunistically targeted *E. troschelii* as this species is more often found dangling from boulders than *P. ochraceus*.

We additionally explored the effects of microhabitat selection in *N. lamellosa* by measuring the body temperature of individuals across their vertical distribution on shore within four microhabitat types: top of boulder (n = 19); side of boulder (n = 17); base of boulder (attached to the very base of the boulder and in contact with the surrounding sand; n = 16); or on broken clam shells interspersed on the sand between boulders (n = 15). We used an Omega thermocouple thermometer to measure *N. lamellosa* body temperatures, which was performed by placing the thermocouple on an animal's foot and allowing it to draw the thermocouple into its shell as it retracted. At the same time, we recorded the shore level of each individual with the aid of a laser level and

measured their shell length with calipers. Surveys occurred on 3 February 2015 and 3 March 2015 in Stanley Park.

We note here that the field comparisons described above were made using different methodologies (indirect methods like the FLIR camera and infrared thermometer vs. direct, contact-based methods like thermometers and thermocouples). Our choice of method depended on the question being asked (e.g., visualization with FLIR vs. quantitative replication using other methods) and logistical constraints including time available and the desire to not disturb/injure serially sampled sea star populations by removing individuals from the rock for contactbased oral-side measurements. Infrared-based thermal measurements and direct contact-based measurements, while generally wellcorrelated, are expected to differ slightly from one another (e.g., Caddy-Retalic et al. 2011). We therefore restricted our quantitative comparisons to data collected with a single methodology.

# 2.5. Effects of aggregation and body position on sea star body temperature

To examine trends in body temperature related to sea star aggregation, we haphazardly selected six aggregations of both *P. ochraceus* and *E. troschelii* (>10 individuals in contact with one another) and measured the body temperature of a) an individual at the centre of the aggregation, b) an individual at the edge of the aggregation, and c) a nearby isolated individual not part of the aggregation using a thermometer pressed into the centre of the oral side. The selected individuals had an arm length of 51 ± 11.3 mm (mean ± SD, *E. troschelii*) and 97.4 ± 28.9 mm (mean ± SD, *P. ochraceus*). This survey was performed on 13 December 2016 at Sharon Cove.

# 2.6. Statistical analyses

We performed all statistical analyses using R (v. 4.1.1; R Development Core Team, 2021). Unless otherwise stated, we modelled response variables without transformation with a Gaussian error distribution, and tested models with Type I, II, or III ANOVA/ANCOVA tests depending on whether models were univariate (Type I) or, if multivariate, if interactions between main effects were hypothesized (Type III) or not (Type II) using the car package (Fox and Weisberg, 2019). To determine if SCPs differed between predator species, we constructed a linear model with species as a fixed effect. We used a Tukey post hoc analysis to compare SCPs between species. The effect of body size (arm length or shell height) on SCP was tested by constructing separate linear models for each species with size as a fixed effect. To determine if the probability of mortality differed for organisms exposed to a control condition (8 °C) or a lethal freezing stress (-8 °C) or sublethal freezing stress  $(-0.5 \degree C)$ , we performed separate univariate Cox regression analyses for each predator species and used Wald tests to evaluate the statistical significance of treatment using the survival package (Therneau and Grambsch, 2000). To ensure that the models for P. ochraceus and N. lamellosa survival in response to freezing stress converged, we added one mortality event at time = 14 to each experimental treatment. We generated survival plots using the survminer package (Kassambara et al., 2021). For the analysis of feeding activity following sublethal cold temperature exposure, we created linear models of the number of mussels eaten over the two-week observation period as a function of treatment, with separate models for each species, using the package glmmTMB (Brooks et al., 2017). In the case of P. ochraceus, variance in the response variable was considerable for sea stars kept at control conditions in comparison to those exposed to cold stress, so a dispersion formula was added to account for heteroskedasticity across treatments. We tested for differences in the sizes of mussels consumed across experimental treatments, using both treatment and trial as explanatory variables.

We used several different models to examine trends in the body temperature of predators depending upon the microhabitat they occupied. First, we modelled the dependence of sea star body temperature

(both E. troschelii and P. ochraceus) on microhabitat, air temperature, and their interaction, and water temperature (as a proxy for thermal conditions proceeding high tide), with time (the order of quadrats surveyed), and body size (arm length) as additional covariates. We detected aberrant temperature data for some E. troschelii individuals measured on 19 October 2020 using both repeated Grubbs tests and examining which data were outside of four median absolute deviations from an expected normal data distribution. These temperature data were excluded prior to generating the final model. We used a Tukey post hoc analysis to compare body temperatures among microhabitat types. We modelled whelk body temperature separately; this response, which we transformed logarithmically to meet the assumption of homoscedasticity, was modelled as a function of microhabitat and shore level above chart datum, with body size (shell length) as a covariate. To examine the effect of aggregation in sea stars, we generated linear models with planned comparisons (orthogonal design) to compare the body temperatures of a) sea stars inside versus outside aggregations, b) for sea stars in aggregations, those at the edge vs. the centre. Similarly, we compared the body temperatures of a) E. troschelii on the side of versus under boulders, and b) for *E. troschelii* on the side of boulders, those completely versus partially attached.

# 3. Results

# 3.1. Supercooling points of predators

Clear SCPs (evidence of freezing) were obtained for 14/20 of *E. troschelii*, 8/24 *P. ochraceus*, and 20/20 *N. lamellosa* individuals subjected to freezing treatments (Figs. S3–S5). Prior to reaching SCPs, the mean cooling rates for each species were  $0.60 \pm 0.051$  °C/min,  $0.59 \pm 0.082$  °C/min, and  $0.77 \pm 0.11$  °C/min (mean  $\pm$  SE) for *E. troschelii*, *P. ochraceus*, and *N. lamellosa*, respectively (Figs. S3–S5). The SCP of *N. lamellosa* differed significantly from the two sea star species (Fig. 1; p < 0.001, Tables S1 and S2), but we found no significant difference between the SCPs of the two sea stars (Fig. 1). SCP was not related to body size in any of the species (*P. ochraceus*: p = 0.30; *E. troschelii*: p = 0.55; *N. lamellosa*: p = 0.69; Table S3).



**Fig. 1.** Boxplot of the supercooling points (°C) of *E. troschelii* (n = 13), *P. ochraceus* (n = 8) and *N. lamellosa* (n = 22), as determined by two exposures to -8 °C. Only individuals demonstrating a clear supercooling point are included in these data. Letters above boxes demonstrate species that had significantly different SCPs as determined by a Tukey HSD test (p < 0.001 for both *N. lamellosa* comparisons, p = 0.889 for E. *troschelii* and *P. ochraceus* comparison). The upper edge of boxes represents the 75% quartile, the lower edge represents the 25% quartile, and the central horizontal line represents the median of the response. Upper and lower whiskers show the minimum and maximum supercooling point detected for each species, excluding outliers, which are represented by points.

#### 3.2. Survival of predators

Most *E. troschelii* (90%) and all *N. lamellosa* (100%) specimens died during the two-week monitoring period following a 2-hr exposure to -8 °C. While 40% of the *P. ochraceus* individuals survived for two weeks post-exposure, the steady loss of *P. ochraceus* through time suggests that further mortality may have occurred if the monitoring window had been extended (Fig. 2). There was a significantly lower survival probability in the freezing exposure group relative to the control groups of *E. troschelii* ( $\lambda(t) =$  Regression coefficient = 3.06, p < 0.001), *N. lamellosa* ( $\lambda(t) =$  4.60, p < 0.001), and *P. ochraceus* ( $\lambda(t) = 20.0$ , p = 0.004). The average body temperatures during the last 10 min of the -8 °C exposure for *E. troschelii*, *P. ochraceus*, and *N. lamellosa* were  $-6.06 \pm 0.46$  °C, -4.00

 $\pm$  0.69 °C, and -6.80  $\pm$  0.20 °C (mean  $\pm$  SE), respectively. Predator mortality following exposure to -0.5 °C did not differ significantly between exposure and control treatments (Fig. 2).

# 3.3. Sublethal cold exposure effects on feeding rate

All three species exhibited reduced feeding rates for two weeks following exposure to sublethal (-0.5 °C) low temperature stress relative to controls (Fig. 3: *P. ochraceus*, p < 0.001; *E. troschelii*, p < 0.001; *N. lamellosa*, p = 0.001; Table S4). The sizes of mussels consumed following exposure did not differ significantly between the cold and control exposure groups for any of the species (see Fig. S6).

#### 3.4. Effects of microhabitat on predator temperatures in situ

Thermal images of the intertidal zone reveal substantial thermal variation among microhabitats. The bases and undersides of boulders remained warmer than their sides and especially tops, and larger boulders were warmer than smaller cobbles and isolated shell fragments (Fig. 4). Body temperatures for intertidal sea stars and dogwhelks depended on microhabitat type occupied (e.g., under rocks or in crevices vs. more open areas) and position within an aggregation (Fig. 4). Note that, due to uncertainties in emissivity, absolute temperatures should be interpreted with caution; the intent of the images is to highlight amonghabitat differences and intra-specific comparisons.

Deterministic variation in body temperature was confirmed with direct measurements. The body temperatures of E. troschelii in the field reflected a complex interplay between air temperature, time since emersion at low tide, water temperature during the preceding high tide, and the microhabitat being occupied (Fig. 5). Air and water temperatures were both positively related to E. troschelii body temperature and individuals that were measured later at night (emersed for a longer period of time) were significantly colder than ones that were measured earlier on the same low tide (Fig. 5, Table S5). Microhabitat type had a significant influence on body temperature as well, with crevices providing a warmer thermal refuge than other microhabitats (p < 0.001, Table S5; Tukey HSD: p < 0.001 for all comparisons), while other microhabitats were statistically similar (Tukey HSD: p > 0.05 in all cases). A significant interaction between microhabitat and air temperature (p < 0.001, Table S5) indicated that the thermal buffering effect of refugetype microhabitats (e.g., crevices) was more pronounced on colder nights. Body size was not a significant predictor of E. troschelii temperature (p = 0.126, Table S5). As with *E. troschelii*, the body temperature of P. ochraceus varied with several attributes of the environment (Fig. 5). Body temperature was positively related to air temperature during low tide and water temperature on the preceding high tide (Fig. 5; p < 0.001 for both comparisons, Table S6). The body temperature of P. ochraceus was significantly affected by microhabitat type; animals located on the base of boulders and on sediment had warmer body temperatures (p <0.001, Table S6), whereas sampling time during low tide did not significantly affect observed body temperature (p = 0.363, Table S6). All of the temperature comparisons between microhabitat types were





**Fig. 3.** Effect of sublethal cold exposure on the feeding rate of *E. troschelii*, *P. ochraceus* and *N. lamellosa* on *M. trossulus*. Feeding was tracked for two weeks after the cold exposure treatment (*P. ochraceus*, n = 11; *E. troschelii*, n = 14; *N. lamellosa*, n = 18) and the control treatment (*P. ochraceus*, n = 11; *E. troschelii*, n = 17; *N. lamellosa*, n = 20). Boxplot format as in Fig. 1.

statistically significant (p < 0.001), except for that between crevices and the sides of boulders (p > 0.05). This trend is not evident when the data are visualised, which is due to the significant interaction of microhabitat and air temperature, with the effect of microhabitat being more pronounced on colder nights (Fig. 5; p = 0.0110, Table S6).

*N. lamellosa* found at the base of boulders where they were in contact with the sandy substratum were warmest, those on the tops of boulders were coldest, and those found on empty clam shells and the sides of boulders were intermediate in temperature. These differences in body temperature were significantly different between microhabitats (p = 0.00691, Fig. 6 & Table S7). *N. lamellosa* body temperature was inversely related to shore level, with animals higher on the shore reaching significantly lower temperatures (p < 0.001, Fig. 6 & Table S7). Larger individuals were typically warmer than smaller individuals, but

**Fig. 2.** Survival probability of *E. troschelii*, *P. ochraceus* and *N. lamellosa* during a two-week monitoring period after exposure to **A.** "lethal"  $-8 \degree$ C air temperatures for 2 h (*P. ochraceus*, n = 24; *E. troschelii*, n = 20; *N. lamellosa*, n = 20) and **B.** "sublethal"  $-0.5 \degree$ C air temperature for 3 h (*P. ochraceus*, n = 31; *E. troschelii*, n = 20; *N. lamellosa*, n = 20). Control groups for both exposures were comprised of predators exposed to  $8 \degree$ C in air for 2 h ("Lethal" control group: *P. ochraceus*, n = 20; "Sublethal" control group: *P. ochraceus*, n = 20; "Sublethal" control group: *P. ochraceus*, n = 20; *E. troschelii*, n = 20; *N. lamellosa*, n = 20; *M. lamellosa*, n = 20).

the effect of body size on temperature was only marginally significant (p = 0.0332, Table S7).

Additional comparisons were made to examine the temperature of *E. troschelii* on versus under boulders and between fully and partially attached sea stars. *E. troschelii* under rocks were significantly warmer than those attached to the side of rocks (p < 0.001, Table S8). For individuals attached to the vertical or slightly overhung side of rocks, those that were fully attached by all five arms were warmer than those that were only partly attached with one or two arms with the rest of the body dangling (p < 0.001, Table S8).

#### 3.5. Effects of aggregation and body position on sea star body temperature

*E. troschelii* and *P. ochraceus* in aggregations had warmer body temperatures than solitary sea stars during cold winter low tides (Fig. 7; *P. ochraceus*: p = 0.004, Table S9; *E. troschelii* p < 0.001, Table S10). Sea stars near the centre of aggregations had significantly warmer body temperatures than those found at the periphery (Fig. 7; *P. ochraceus*: p = 0.0140, Table S9; *E. troschelii* p = 0.0127, Table S10).

#### 4. Discussion

Low temperatures during the winter have far-reaching implications for ectotherms and the ecosystems in which they occur. Here, we examined how cold and freezing temperatures affect the survival and feeding rate of three predators (sea stars *P. ochraceus* and *E. troschelii* and the gastropod *N. lamellosa*) in a temperate intertidal zone. We found that most individuals were unable to survive a freezing exposure at -8 °C. Although exposure to a less extreme low temperature (-0.5 °C) over a 3h simulated low tide did not result in substantial mortality, this sublethal exposure nevertheless suppressed feeding rates over the following two weeks. Low temperatures in our study region occasionally fall below those used in our experiments, suggesting that cold snaps may have important physiological and ecological consequences in the field. However, we found substantial and predictable spatial variation in predator body temperatures at low tide, suggesting that warmer microhabitats may mitigate the risk of low temperature extremes for



**Fig. 4.** Thermal images (taken with a FLIR E40) of the intertidal zone on a cold night (Feb 10–11, 2021) at  $\chi_{way}^{Y}_{XWay}$  (Stanley Park, Vancouver). Thermal scale bar is individualized for each image. Temperature indicated in the top left indicates the temperature at the reticule. A) Landscape-scale thermal patterns at low tide are driven by shore level and topography, with cooler temperatures higher on the shore, on upward-facing surfaces, and on smaller rocks and cobbles. *H. sapiens* (external body temperature of approximately 19 °C) pictured for thermal scale. B) Small-scale thermal heterogeneity in the intertidal zone, with several small invertebrates pictured. The pictured rock has been overturned; several *E. troschelii* are present in the warm area vacated by the rock but are not visible in this image due to their thermal similarity to the underlying shell hash and sediment. C) Exposed *E. troschelii* are much colder than their surrounding environment. D) *P. ochraceus* frequently has a thermal gradient across its body. E) When sheltering in rocky crevasses, *P. ochraceus* is generally able to maintain higher body temperatures. E) Aggregations of *N.lamellosa* create significant thermal gradients as well, with inner animals maintaining much higher body temperature than outer animals.



**Fig. 5.** Relationship between air temperature recorded during sea star distribution surveys and the body temperature of *E. troschelii* (Base of boulder, n = 161; Crevice, n = 29; Sediment, n = 66; Side of boulder, n = 73) and *P. ochraceus* (Base of boulder, n = 133; Crevice, n = 35; Sediment, n = 55; Side of boulder, n = 65) within different intertidal microhabitat types at Stanley Park in Vancouver, BC from October 2020 to February 2021. Air temperatures shown indicate the average temperature during the low tide on each night the data were collected recorded at the proximate Vancouver International Airport. Error bars about points represent standard error. Lines represent the relationships between air and body temperatures for each microhabitat predicted from multiple linear regression, not a univariate regression with air temperature, hence their imperfect fit (see text for details).

#### intertidal species.

One important metric for understanding the consequences of physiological responses to extreme low temperature exposure for organisms is the temperature at which internal tissues freeze — the supercooling point (SCP) — which varied among species in this study. The SCP of an



**Fig. 6.** Relationship between the shore level and microhabitat of *N. lamellosa* (Base of boulder, n = 16; Empty clam shell, n = 15; Top of boulder, n = 19; Side of boulder, n = 17) and their foot temperature (°C) at Stanley Park in Vancouver, BC in 2015. Lines represent the relationship between shore level and foot temperature predicted within each microhabitat by multiple linear regression (see text for details).

organism depends primarily on three factors: solute concentrations in internal fluids; the volume of internal fluid; and the presence of ice nucleators and antinucleators (Lee, 2010). All the species studied have similar internal solute concentrations that are equal to the osmolarity of seawater because they are osmoconformers (Yancey, 2005; Deaton, 2009; Castellano et al., 2016); therefore, interspecific variation of SCPs is likely not due to this factor. Animals with a larger volume of internal fluid (i.e., larger body size) are more likely to undergo ice nucleation and begin freezing at higher temperatures because they have more fluid available to freeze (Zachariassen and Kristiansen, 2000). In our study, the SCP for the smaller *N. lamellosa* was lower than that of the two larger sea star species, indicating that dogwhelks could reach lower body temperatures before their tissues froze. The sea stars studied ranged in



**Fig. 7.** Mean body temperature of *E. troschelii* (n = 18) and *P. ochraceus* (n = 18) individuals at the edge and centre of aggregations and solitary individuals (*E. troschelii*: Edge, n = 6; Centre, n = 6; Solitary, n = 6; *P. ochraceus*: Edge, n = 6, Centre, n = 6, Solitary, n = 6). Body temperature measurements were recorded at Sharon Cove in Vancouver, BC in 2016. Asterisks show significant differences in intraspecific body temperature between positions on shore as compared using a Type I ANOVA (p < 0.05 = \*; p < 0.01 = \*\*, p < 0.001 = \*\*\*). Boxplot format as in Fig. 1.

arm length size from 22 to 103 mm (E. troschelii) to 26-126 mm (P. ochraceus), and exhibited higher SCPs than N. lamellosa, which had a shell size range of 40-60 mm; most sea stars thus likely had a higher volume of internal fluid and were therefore more likely to freeze at a higher temperature than dogwhelks. Although interspecific differences in internal fluid volume may not be accurately approximated by length due to morphological differences between species (body shape, shell presence/absence), the differences in body size are also reflected in differences in cooling rates, whereby the larger animals cool more slowly relative to smaller animals. The smaller N. lamellosa exhibited a faster cooling rate on average (-0.77 °C/min) relative to the larger P. ochraceus and E. troschelii (-0.54 °C/min and -0.60 °C/min, respectively). The lower SCP found for N. lamellosa may thus be partly due to the faster cooling rate it would experience, caused by its smaller size. However, SCPs for the two sea stars were nearly identical even though P. ochraceus tended to be larger, and body size was not a significant predictor of SCP within species. It is unclear why not all the sea stars in the study exhibited signs of freezing; we suspect this is due to methodological issues with thermocouple injection (as the tips of thermocouples would sometimes get misshapen when injected into the sea stars), or variation in freezing susceptibility amongst individuals. Finally, differences in SCP may be due to differences in ice nucleation, which can be driven by water templating activities of molecules as diverse as ice, minerals, or proteins (Zachariassen and Kristiansen, 2000). Food particles or dirt in an invertebrate's gut may also nucleate ice (Sømme, 1982); though this mechanism is unlikely in our study since all organisms were starved prior to the experiment. It is also possible that the mucus layer of the N. lamellosa could act as an inhibitor of internal fluid nucleation, similar to the protective effect of the mucus layer against freezing temperatures in Antarctic limpets (Hargens and Shabica, 1973). Ice crystals surrounding the animals could have nucleated ice in our animals, but water volumes of 1-10 mL freeze at temperatures of approximately -15 °C (Zachariassen et al., 2004), and as such it seems unlikely that this could explain SCPs of -4.5 °C. Instead, recent work has suggested that ice nucleating proteins, which allow animals to initiate freezing of their internal fluids at higher body temperatures to prevent uncontrolled ice formation at lower body temperatures (Zachariassen and Kristiansen, 2000), may be relatively common in intertidal organisms (Box et al., 2022). Given that all three predators exhibited a SCP that was relatively close to the melting point of seawater, these

predators may possess ice nucleating proteins that contribute to ice nucleation.

Regardless of the mechanisms underlying interspecific differences in SCPs, a comparison of SCPs and post-freezing survival rates across predator species suggests that while some individuals might tolerate limited freezing, none of these species are strongly freeze tolerant. Within the two-week observation period following freezing exposure, some sea stars survived, despite reaching body temperatures that were several degrees colder than their SCP. Sea star mortality in response to freezing exposure was much lower than that of N. lamellosa. Mortality may have been overestimated due to the freezing methodology employed, as the ice packs may have increased cooling rates due to conductive heat loss, leading to more lethal consequences, as previously seen in Littorina littorea (Murphy and Johnson, 1980). Additionally, these predators were subjected to the experiment after a two-week fasting period and had limited motility as they were placed in an incubator. In a real intertidal scenario, it is more likely that these predators would not be starved and would be able to move to an area of cover from the acute cold stress, and may therefore be able to mitigate the lethal effects of an acute freezing exposure. Unfortunately, we did not keep track of the survival of each predator in relation to their measured SCP (due to experiment logistics and to minimize handling). We therefore cannot confidently conclude whether sea stars and whelks are freeze tolerant since we do not know if the predators which died are only the predators which froze and vice versa (Sinclair et al., 2015). Following sublethal cold exposure (-0.5 °C), there was relatively limited mortality, suggesting that these predators demonstrate chill tolerance (Sinclair et al., 2015). A portion of the mortality seen in response to the cold exposure event was likely symptomatic of an outbreak of sea star wasting disease (since there were similar levels of mortality amongst the control group), an unavoidable consequence of housing these species in the lab. Thus, mortality due to cold exposure stress was likely even lower than observed. Further experiments that evaluate survival following documented internal freezing should be completed to establish with greater confidence whether these predators are freeze tolerant.

Examining the sublethal effects of extreme temperatures are important for organisms, populations, and communities. For the predator species studied here, we found prolonged reductions in feeding rates after a single, relatively brief cold exposure event. Notably, our single low tide cold exposures produced reductions in feeding in P. ochraceus and N. lamellosa that were broadly similar to much longer exposures to reduced seawater temperatures associated with coastal upwelling (Sanford 1999; 2002). Given the paucity of studies pertaining to the effects of cold exposure on the metabolism of these species, it remains unclear whether the reduction in feeding rate was a physiological stress response or potentially an adaptive behavioural response. For instance, terrestrial insects have been found to induce prolonged fasting periods when exposed to cold winter temperatures as organisms seek to save energy while recovering their basal ATP and metabolite concentrations (Churchill and Storey, 1989; Pullin and Bale, 1989; Zhang et al., 2011; Colinet et al., 2016). Recovery of basal digestive enzymes and metabolites is energetically expensive (Sokolova et al., 2012), and thus the relatively slow feeding rate recovery after cold exposure may be a form of energy conservation for intertidal predators. However, none of the entrained physiological responses that accompany this response in insects (i.e., gut clearing, cryoprotectant production) have been demonstrated in our intertidal study organisms, and it is therefore difficult to determine from our data whether they are capable of this sophisticated response of prolonged fasting. Regardless of the underlying mechanism (s), such significant reductions in feeding following a single cold exposure event are notable given their potential implications for trophic cascades. Reduced predation rates lead to reduced top-down control of competitively dominant, habitat-forming prey species in our study system, which may have substantial implications for intertidal biodiversity (Lafferty and Suchanek, 2016; Paine, 1966). However, responses amongst intertidal predators in situ are unlikely to be uniform due to

variation in individual physiological tolerance and environmental heterogeneity.

While responses to extreme body temperatures depend on an organism's underlying physiology, the actual body temperatures experienced in the field will depend on a host of additional factors including the presence and use of thermal refugia (Helmuth and Hofmann, 2001; Pincebourde et al., 2008; Reid and Harley, 2021). Previous work has shown that crevices and the underside of rocks act as thermal refugia in the intertidal zone during the winter (Reid and Harley, 2021), meaning that if intertidal invertebrates can move to these microhabitats, they may avoid or reduce their exposure to extreme cold air temperatures. In this study, we found evidence that certain microhabitats can mitigate cold temperatures for intertidal predators. P. ochraceus and N. lamellosa were significantly warmer when situated at the base of boulders and in contact with the sediment relative to organisms situated on the side of boulders (up to 4.5 °C warmer for P. ochraceus) or on empty clam shells (up to 5 °C warmer for N. lamellosa). Similarly, E. troschelii found in crevices or underneath rocks were significantly warmer than those on the side or base of rocks. These results are comparable with a previous study, which found that during winter low tides, the underside of rocks were up to 3 °C warmer — and crevices up to 4.5 °C warmer — than the surface of rocks, with temperature differences being more pronounced on colder nights, as we observed here (Reid and Harley, 2021). Additionally, P. ochraceus and E. troschelii situated in the centre of an aggregation of conspecifics were significantly warmer than those at the edge of an aggregation, with thermographic imagery suggesting that this is also true for N. lamellosa. Given the presence of these thermal refugia, predators may behaviourally thermoregulate by migrating to warmer habitats on colder nights as has been found for Littorina spp. and N. lamellosa (Stickle et al., 2017; Reid and Harley, 2021). However, relating the proportion of sea stars found on each habitat type to the air temperature experienced during the low tides preceding each survey demonstrated that cold temperatures alone do not drive the movement of sea stars to warmer thermal refugia. Instead, it is possible that sea stars have a generalized, time- and temperature-invariant preference for thermally benign microhabitats in the winter. Unfortunately, we were unable to quantify the proportional availability of the different habitat types to determine whether certain microhabitats were occupied at frequencies higher or lower than predicted by chance. We also did not directly investigate differences in predator abundances across shore levels, which may have been an important behavioural mechanism used by predators to mediate cold exposure. For example, during the warmest months, a greater proportion of *P. ochraceus* are found lower on the shore where temperatures are cooler relative to higher in the intertidal zone, where aerial exposure, and thus potential for heat-induced stress, is greater (Pincebourde et al., 2008). The role of the vertical intertidal gradient in shaping organism body temperature, and thus distribution, should be explicitly considered in future studies.

In conclusion, our study shows that intertidal predators are at risk of lethal freezing injuries when exposed to acute freeze stress (below -8°C) that occurs, albeit rarely, in the region studied here. It is important to emphasize that the results of the acute cold stress experiment can only be interpreted in the context of a sudden cold spell, and do not necessarily reflect the mortality that would occur after a series of cold nights in the intertidal zone. Our results further suggest that more commonly encountered, milder sub-zero temperatures may have important but underappreciated effects on intertidal community structure via sublethal impacts on ecologically important foundational species. After exposure to temperatures of -0.5 °C, feeding rates were depressed in all three species examined, particularly for the keystone predator P. ochraceus. Although our data indicate that the risk of both lethal and sublethal consequences of low environmental temperatures is reduced for predators occupying thermal refugia, we found no evidence that the predators studied behaviourally thermoregulate to utilize these refugia. As a result, low temperature events can result in reductions in predation rates - by decreasing predator abundance or decreasing per capita

consumption — that may considerably outlast the duration of the cold snap. Since dominant prey like the intertidal mussel *Mytilus trossulus* are relatively more tolerant to cold exposure events (Kennedy et al., 2020; Kennedy, 2022), occasional cold snaps may result in higher prey abundance along with associated shifts in biodiversity (Paine 1966; Lafferty and Suchanek, 2016). The overall degree to which transient low temperature extremes determine longer-lasting patterns in intertidal community structure, and at what scales, will require further research on the lethal and sublethal consequences of such exposure for a broad range of species.

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# CRediT authorship contribution statement

Danja Currie-Olsen: Investigation, Methodology, Formal analysis, Project administration, Writing – original draft. Amelia V. Hesketh: Formal analysis, Visualization, Writing – review & editing. Jaime Grimm: Investigation, Methodology, Project administration, Writing – review & editing. Jessica Kennedy: Formal analysis, Methodology, Writing – review & editing. Katie E. Marshall: Resources, Methodology, Writing – review & editing, Funding acquisition. Christopher D.G. Harley: Investigation, Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Funding acquisition.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

The raw data, processed data, and novel code required to reproduce these findings are available to view from Open Science Framework: https://osf.io/uqk54/?

view\_only=6214a6eb251b4cd78d04f66c03462afb.

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# Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2023.103549.

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