



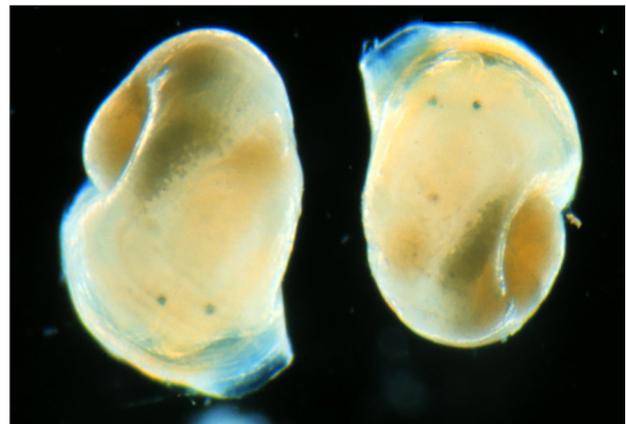
FEATURE ARTICLE

Role of initial energy reserves in stress tolerance thresholds during the early benthic phase in intertidal invertebrates

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ABSTRACT: Although depleted energy reserves are not a major direct cause of early benthic phase mortality for many species of marine invertebrates, initial energy reserves might nevertheless play a role in early benthic phase mortality through indirect effects on physiological tolerance thresholds, a mechanism that has not been tested. We therefore examined the extent to which energy reserves affect acute tolerance thresholds of early benthic phase individuals of 2 species (*Balanus glandula* and *Nucella ostrina*) to 2 of the most challenging intertidal stressors: desiccation and high emersion temperature. Energy reserve levels were estimated by maintaining individuals without food for different durations of time or using individuals of different body mass. Individuals in each energy level treatment were then exposed to a range of desiccation periods and emersion temperatures. Lower estimated energy levels had no effect on tolerance to emersion temperature, but did significantly reduce the ability to tolerate desiccation. This suggests the survival of individuals through the early benthic phase is likely dependent on initial energy content through an indirect effect on tolerance to desiccation. Also, habitats experiencing intense desiccation likely impose a selective pressure on intertidal invertebrates favoring the evolution of greater energy reserves at the onset of the early benthic phase. These findings suggest depleted energy reserves at the onset of the early benthic phase can influence early benthic phase mortality through indirect effects by making individuals more vulnerable to environmental stressors. In this way, energy reserves may impact recruitment rates and thus influence population dynamics and intertidal community structure.



Recently hatched early benthic phase snails (*Nucella ostrina*) are more vulnerable to desiccation stress when their energy reserves are depleted.

Photo: Louis A. Gosselin

KEY WORDS: Early benthic phase · Juvenile intertidal invertebrates · Mortality factors · Stress · Emersion temperature · Desiccation · Energy reserves · Tolerance thresholds · Recruitment

1. INTRODUCTION

Intertidal invertebrates are extremely vulnerable during their early benthic phase (EBP), the period immediately following settlement or hatching into the benthic environment and which can encompass both pre- and post-metamorphic life stages (Santee

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et al. 2016, Mendt & Gosselin 2021). Most new cohorts of intertidal invertebrates suffer mortality rates of 30 to 100% in the first few days of the EBP (Gosselin & Qian 1997, Hunt & Scheibling 1997). This high level of mortality can drastically impact recruitment rates and thus influence overall population abundance and distribution as well as intertidal community structure (Connell 1985, Stoner 1990, Hunt & Scheibling 1997). Though generally high, EBP mortality is also highly variable not only among species but also among cohorts of a single species and even among individuals within a cohort (Jarrett 2000, Phillips 2002). Many factors can cause mortality during the EBP; however, the mechanisms responsible for the high degree of variation in mortality are still not fully understood.

Among the most important factors contributing to EBP mortality for intertidal invertebrates are those associated with aerial exposure at low tide and with the unique challenges associated with these periods of emersion. Two of the most significant of these environmental stressors are desiccation and elevated emersion temperature, both of which have been shown to cause significant mortality in many species of intertidal invertebrates (Foster 1971, Gosselin & Chia 1995, Helmuth & Hofmann 2001, Miller et al. 2009, Jenewein & Gosselin 2013a). Indeed, these 2 stressors define the upper limit of the vertical range for many intertidal species (Connell 1961, Foster 1971). Loss of moisture from body tissues (i.e. desiccation) and elevated temperatures both have the potential to disrupt a range of physiological processes including protein stability, organ function, and gas exchange (Buckley et al. 2001, Sokolova & Pörtner 2001, Miller et al. 2009). EBP individuals are particularly vulnerable to these stressors due to their very small body size, and thus large surface area:volume ratio (SA:V) and small reservoir of water in their tissues, which greatly reduces their ability to tolerate adverse environmental conditions (Lowell 1984, Gosselin 1997).

Individuals fed reduced rations throughout the larval phase often have lower success rates through the EBP than those reared on full rations (Phillips 2002, Thiyagarajan et al. 2003, Emlet & Sadro 2006). This suggests that low initial energy reserves might lead to higher EBP mortality; thus, energy reserves at the onset of the EBP have been proposed as a factor that may play a major role in determining whether an individual will survive the critical first days of early benthic life (Gosselin & Qian 1997, Hunt & Scheibling 1997, Jarrett & Pechenik 1997). This hypothesis is supported by evidence that individuals within a

cohort differ considerably in size and amount of organic content held at the start of the EBP, both of which are proxies for energy reserves (Marshall et al. 2003, Emlet & Sadro 2006, Phillips 2006), with some individuals possessing relatively low energy reserves at the onset of the EBP (Lucas et al. 1979, Jarrett & Pechenik 1997, Thiyagarajan et al. 2003). Additionally, limiting larval diet can negatively impact growth rate during juvenile life (Woodcock & Benkendorff 2008, Pechenik & Tyrell 2015, Gilman & Rognstad 2018), leaving individuals more vulnerable to mortality factors for a longer period of time. In wild populations, differences in energy reserves among EBP individuals could be due to different rates of feeding in the larval stage or delayed metamorphosis (Thiyagarajan et al. 2003). Many species also transition through energy-intensive stages or non-feeding periods prior to entering the EBP, during which initial energy reserves can become depleted (Lucas et al. 1979, Wendt 2000, Bryan 2004, Bennett & Marshall 2005). Recent research, however, has revealed that the vast majority of individuals entering the EBP likely have adequate energy reserves to survive the critical first days to weeks of early benthic life and that depleted energy reserves are not an important direct cause of EBP mortality (Mendt & Gosselin 2021). Initial energy reserves might nevertheless have indirect effects on EBP mortality by altering tolerance thresholds to environmental stressors; these indirect effects of initial energy reserves are therefore the focus of the present study.

The physiological mechanisms and adaptations utilized by EBP individuals to tolerate adverse conditions are thought to be energy dependent, as energy demand increases during exposure to environmental stress (Sokolova et al. 2012). These mechanisms can include increasing the production of protective proteins such as heat shock proteins (HSPs), synthesizing new proteins to replace those that have been damaged, restructuring cellular membranes, and shifting gene expression to accommodate these adaptive mechanisms (Sokolova & Pörtner 2001, Hofmann et al. 2002, Somero 2002, Berger & Emlet 2007). Many of these mechanisms have an associated energetic cost; thus, individuals with limited energy reserves may be unable to adequately respond to the physiological stressors they encounter. In this way, low energy reserves could indirectly lead to increased EBP mortality by reducing the ability of the individual to respond to environmental stressors such as desiccation and high emersion temperature.

Consequently, the purpose of this study was to assess the indirect impacts of depleted energy

reserves on EBP mortality by testing the hypothesis that depleted energy content affects vulnerability to physiological stress during the EBP. The specific goals of this study were to examine the extent to which initial energy content affects acute tolerance thresholds of EBP individuals to the 2 most challenging environmental stressors in the intertidal zone: (1) desiccation and (2) high emersion temperature. Two species of intertidal invertebrates, the barnacle *Balanus glandula* and the snail *Nucella ostrina*, were used as model organisms. Since direct measurement of energy content in live individuals was not possible, 2 proxy methods were used to estimate energy reserves: duration of starvation prior to experimentation and initial body mass. Organic content of EBP individuals is strongly correlated with both duration of starvation (Mendt & Gosselin 2021) and initial body mass at the beginning of the EBP (Moran & Emllet 2001). Organic content, as measured by ash-free dry weight, is an effective indicator of energy reserves (Moran & McAlister 2009); thus, both duration of starvation and body mass were used as suitable proxy estimates of energy reserves in the present study.

2. MATERIALS AND METHODS

2.1. Study site and species collection

This research was conducted at the Bamfield Marine Sciences Centre from May to September in 2018 and 2019. Specimens of 2 species of intertidal invertebrates were collected from rocky intertidal shore sites in Barkley Sound, on the west coast of Vancouver Island, Canada: *Balanus glandula* were collected from Wizard Islet (48° 51.19' N, 125° 09.56' W) and Dixon Island (48° 51.06' N, 125° 07.20' W), and *Nucella ostrina* were collected from Dixon Island, Prasiola Point (48° 49.05' N, 125° 10.12' W), and Ross Islets (48° 52.23' N, 125° 09.67' W). Sites were selected based on availability of EBP individuals of these 2 species.

The 2 species examined in this study differ in their source and quantity of initial energy reserves at the beginning of the EBP. *N. ostrina* are lecithotrophic and thus depend entirely on maternal provisioning, primarily via ingestion of nurse eggs in the egg capsule. Uneven division of nurse eggs among capsule mates results in some individuals having greater energy reserves and larger body size at hatching (Moran & Emllet 2001, Lloyd & Gosselin 2007, Marko et al. 2014). *B. glandula* have planktotrophic larvae,

so the majority of their EBP energy reserves are obtained from the food they consume during the larval stage. They also transition through a non-feeding cyprid stage and undergo an energy-intensive metamorphosis, such that some individuals may have more depleted energy reserves at the onset of the EBP (Wendt 2000, Thiyagarajan et al. 2003).

EBP *B. glandula* were collected as described by Sandee et al. (2016). Small (5–10 cm diameter) rocks were collected from the intertidal zone and cleared of all small barnacles, and a perimeter was marked around the cleared area with nail polish before returning the rocks to the intertidal zone. The rocks were then recovered 48 to 72 h later and examined for newly settled barnacles; rocks containing new settlers were retained for experiments. EBP *N. ostrina* were collected by removing ripe egg capsules from intertidal substrata using fine-tipped forceps, as in Gosselin & Chia (1995). Egg capsules were returned to the laboratory and held in small cages in aerated seawater for 72 h; all newly hatched individuals were then collected and retained for experiments.

2.2. Establishing treatment levels of estimated energy reserves

To assess how the vulnerability of EBP invertebrates to physiological stressors is affected by the individual's energy reserves, the first step was to establish groups of individuals with different levels of energy content. The measurement of energy content, for example by quantifying organic content, in such small animals is a destructive process, such that individuals in which energy content had been quantified could not then be used for experiments. Consequently, 2 proxy methods were used to obtain relative estimates of energy reserves in live EBP individuals: duration of starvation and body mass. Energy reserves were estimated by duration of starvation by rearing groups of individuals of a similar body size without food for different periods of time. Organic content, an effective measure of energy content (Moran & McAlister 2009), predictably decreases as a function of the duration of starvation (*N. ostrina*: Moran & Emllet 2001, Mendt & Gosselin 2021; copepods: Helland et al. 2003). The relationship between duration of starvation and organic content has not been quantified for EBP *B. glandula*, nor has it been described, to our knowledge, in other barnacle species. Nevertheless, duration of starvation is a suitable proxy for energy reserve content. All

newly metamorphosed *B. glandula* obtained for these experiments were 0.60 to 0.68 mm in shell diameter, a very narrow range of body sizes. *N. ostrina*, however, vary considerably in size at hatching (Lloyd & Gosselin 2007, Marko et al. 2014). All newly hatched snails were thus measured and sorted into 3 size classes based on shell length (SL): small (0.8–1.10 mm SL), medium (1.11–1.40 mm SL), and large (1.41–1.80 mm SL). Only medium size class *N. ostrina* were used when testing the influence of duration of starvation on tolerance thresholds. All EBP individuals were held in water that was filtered to 1.0 μm , aerated, and maintained at 15 to 17°C. The water was replaced every second day, and containers and cages were rinsed and scrubbed to prevent the growth of biofilm, thus ensuring no food particles were available. A sample of individuals was removed from the starvation holding cages every 10 d, beginning on Day 0 and continuing up to Day 60, and used in tolerance experiments. Accordingly, groups of individuals representing 7 distinct energy level treatments were obtained: 0, 10, 20, 30, 40, 50, and 60 d durations of starvation.

The second approach used to establish distinct energy reserve treatments involved sorting individuals by initial body size and using initial body mass as a proxy for energy reserves. Body mass and quantity of organic matter are strongly correlated among EBP invertebrates (Moran & Emlet 2001), with larger individuals having greater stores of lipids, which are the primary source of metabolizable energy for intertidal species (Phillips 2002, Thiyagarajan et al. 2002, Emlet & Sadro 2006, Whitehill & Moran 2012). Given that organic matter is an effective indicator of energy content (Moran & McAlister 2009), larger individuals had greater energy reserves than smaller individuals. To test the influence of body size on tolerance thresholds, *N. ostrina* individuals in the small and large size classes were used. These SL measurements were then converted to wet weight body mass (Table 1) using a regression equation for *N. ostrina* from Hamilton & Gosselin (2020). *N. ostrina* hatchlings frequently do not begin feeding until up to 10 d following emergence from the egg capsule (Gosselin

& Chia 1994), so all individuals were held without food in the laboratory for 10 d before tolerance experiments were conducted. Using body size as an index of energy reserves was not feasible for *B. glandula*, as there was insufficient variation in body size (0.60–0.68 mm shell diameter) of newly metamorphosed *B. glandula* at our field sites. Broader variation in EBP body size can occur in *B. glandula* when larvae are reared on different food rations (Emlet & Sadro 2006), but such variation was not detected in the settlers we collected from our field sites.

2.3. Emersion temperature tolerance

To determine if emersion temperature tolerance varies as a function of energy reserve, individuals in each energy level treatment (i.e. duration of starvation or size class) were exposed to 5 temperature treatments (Table 2). The range of temperatures to which each species was exposed was determined in preliminary trials and included temperatures that resulted in 0 to 100% mortality; the low end of the temperature range resulting in no mortality served as a control for the experiment. Immediately before each experiment, all individuals were examined under a dissecting microscope to ensure they were alive and also that there were no cracks in their shells, as this might have affected their tolerance. Rocks containing newly settled *B. glandula* were then blot dried and transferred to sealed 591 ml plastic Ziploc® containers. *N. ostrina* hatchlings were first placed in small cages made of plastic microcentrifuge tubes with the lower half removed and covered with 600 μm mesh to prevent their escape. These small cages were then placed within separate sealed 591 ml containers, 1 small cage per container. A sheet of paper towel saturated with seawater was also placed in each container to maintain a high humidity level (>90% relative humidity [RH]) as well as a data logger (Hygrochron iButton® model DS1923) that recorded temperature and RH every 15 min. All replicate containers were then placed in an incubator set at the lowest temperature in the experimental range for 6 h. Following the 6 h temperature treatment, all rocks and small cages were removed from containers and submerged in 15 to 17°C seawater for a 6 h recovery period and then examined again under a dissecting microscope to assess survivorship. The 6 h treatment and recovery intervals were chosen to approximate a natural tidal cycle, so that animals were exposed to emersion conditions for a duration similar to that experienced in the inter-

Table 1. Shell length (SL) and corresponding body mass for *Nucella ostrina* hatchlings. The conversion equation is wet wt = $2.98 \times \log \text{SL} - 3.84$, from Hamilton & Gosselin (2020)

Size class	SL (mm)	Body mass (wet wt, mg)
Small	0.8–1.10	0.074–0.192
Large	1.41–1.80	0.402–0.833

Table 2. Experimental range of emersion temperatures and desiccation periods used for each species. Ranges were set to include treatments that result in 0 to 100% mortality

Species	Experimental emersion temperature treatment (°C)	Experimental desiccation duration treatment (h)
<i>Balanus glandula</i>	36, 38, 40, 42, 44	4, 6, 8, 10, 12, 14, 16, 18, 20
<i>Nucella ostrina</i>	26, 28, 30, 32, 34	0.25, 0.5, 1.0, 1.5, 2.0, 3.0

tidal zone. Dead individuals were recorded and removed; rocks containing the surviving *B. glandula* were blot dried, and surviving *N. ostrina* were placed back into small cages. All rocks and small cages were transferred back into their respective containers and placed into the incubator set to the next experimental temperature (i.e. increased by 2°C). This process was repeated until the final experimental temperature was reached. Temperature within the incubators was stable throughout all experiments and stayed within $\pm 0.5^\circ\text{C}$ of the intended temperature for each treatment. It took up to 30 min for the internal temperature of each container to reach equilibrium with the incubator, but once the target temperature was reached, it remained stable for the remainder of the 6 h treatment. RH remained above 90% for the duration of the experiment in all treatments. The temperature at which each individual died was recorded, and an average temperature at death was calculated for all the individuals in each replicate container.

For the experiments using duration of starvation as a proxy for energy reserves, a single collection of at least 200 individuals of each species was used, and animals were maintained as stated above. All individuals in this experiment were collected from Dixon Island; *B. glandula* and *N. ostrina* were collected on 21 May 2019 and 14 June 2019, respectively. Using only individuals from a single collection for each species ensured that they had all experienced similar environmental conditions prior to collection. At the start of the experiment (Day 0), and then again after each 10 d interval, 30 individuals were removed from the starvation holding tanks and used in the emersion temperature experiments as described above. The experimental design for each of the 2 species was 7 durations of starvation \times 3 replicate groups of individuals per duration of starvation \times 10 individuals per replicate group.

The experiment using body mass as a proxy for energy reserves was designed slightly differently. *N. ostrina* were collected from the field on 3 separate dates, twice from Dixon Island and once from Prasi-

ola Point, and each of these 3 batches of individuals was used in a different trial. Comparisons of tolerance thresholds between size classes were only carried out between groups from the same trial, thus accounting for temporal and spatial differences among groups. Following the 10 d starvation period, 30 to 50 individuals of each size class from each trial were used in

the emersion temperature experiments as described above. The experimental design for this experiment was 1 species \times 3 trials \times 2 size classes per trial \times 3 to 5 replicate groups of individuals per size class \times 10 individuals per replicate group.

2.4. Desiccation tolerance

The effect of energy reserves on tolerance to desiccation stress was determined by exposing the various energy treatment groups to a low-humidity environment for set periods of time. The durations of exposure to desiccation used in this experiment were determined in preliminary trials and set to include treatment periods that result in 0 to 100% mortality (Table 2). Desiccation conditions were quantified using vapor pressure deficit (VPD) as in Jenewein & Gosselin (2013a,b). VPD accounts for both temperature and RH and is an effective measure of desiccation potential. Given that *B. glandula* and *N. ostrina* occupy different microhabitats and levels in the intertidal zone, they normally experience different degrees of desiccation stress. Additionally, EBP *B. glandula* are known to have a higher desiccation tolerance threshold than EBP *N. ostrina* (Hamilton & Gosselin 2020). Thus, the 2 species were exposed to different VPD levels in the present experiment; *B. glandula* were exposed to a VPD of 2.0 kPa, whereas *N. ostrina* were exposed to the less stressful VPD of 1.6 kPa. Both VPD measurements are common in the intertidal zone during low tide throughout the summer months (Jenewein & Gosselin 2013b). To ensure this experiment tested desiccation tolerance and not temperature stress, desiccation trials were performed in a temperature-controlled incubator set well below the temperature tolerance threshold for each species, as determined in the emersion temperature experiment described above: *B. glandula* trials were carried out at 22°C, and *N. ostrina* trials were carried out at 19°C. At those temperatures, the RH needed to obtain the above VPD levels (20–28% RH for *B. glandula* and 25–31% RH for *N. ostrina*) was then achieved by plac-

ing a large pan of silica desiccant in the bottom of the incubator as well as 2 small fans to create a continuous air flow. A data logger (Lascar Electronics, model EL-USB-2) was also placed in the incubator to record temperature and RH every 10 min, allowing the calculation of VPD. For all desiccation tolerance experiments, VPD remained stable and close to the target set for each species, although there were slight fluctuations among trials. Additionally, it took up to 10 min for the internal RH of the incubator to stabilize after the door had been closed; thus, the first RH reading was omitted from the average VPD calculation. Throughout experiments involving *B. glandula* and *N. ostrina*, VPD ranged from 1.91 to 2.10 kPa and from 1.56 to 1.69 kPa, respectively.

Before each experiment, all individuals were examined under a dissecting microscope to ensure they were alive and that there were no cracks in their shells, as this may have impacted their ability to tolerate desiccation conditions. Rocks containing *B. glandula* were blot dried and then placed in a small desiccator with silica desiccant crystals for 10 min to remove any remaining water on the rock surface. *N. ostrina* were placed in small dry cages using a pair of flexible insect forceps. The rocks or small cages were then transferred into open 591 ml containers and placed into the prepared incubator for a set period of time. Following the desiccation treatment, animals were removed from the incubator and immediately submerged in seawater for 12 h to recover, after which they were checked for survival, and the number of live and dead individuals in each treatment was recorded. Contrary to the emersion temperature experiments, individuals that survived a desiccation treatment were not used again in further treatments; a different set of individuals were used for each desiccation duration treatment.

For the experiment using duration of starvation as a proxy for energy reserves, more individuals were required than could be obtained from a single field collection. Accordingly, the experiment involved 2 to 4 trials per desiccation period; each trial used EBP individuals from 1 field site collected on the same day, but the collection sites and days differed among trials. Data from all trials were combined for analysis, and trial was included as a fixed factor. At 10 d intervals, beginning on Day 0, groups of 7 to 12 individuals were removed from holding tanks and assigned to one of the desiccation duration treatments. The durations of starvation differed between the species: for *B. glandula*, starvation durations lasted up to 30 d, as the trend of the relationship was evident after 30 d of starvation, but for *N. ostrina*, it was necessary to

extend the experiment to 50 d of starvation to gain an understanding of the relationship between starvation and desiccation tolerance. The experimental design for each of the 2 species was 4 to 6 durations of starvation \times 6 to 9 desiccation duration treatments per starvation duration \times 2 to 4 replicate groups of individuals per desiccation treatment \times 7 to 12 individuals per replicate group.

For experiments using body mass, as determined from SL, 3 replicate trials were conducted, each using *N. ostrina* hatchlings from separate collections (i.e. collected from different locations on different days). Immediately prior to a desiccation trial, 6 groups of 5 to 10 individuals of each size class were removed from holding tanks and assigned to one of the 6 desiccation duration treatments. Thus, the experimental design was 2 size classes \times 6 desiccation duration treatments per size class \times 3 replicate groups of individuals per desiccation treatment \times 5 to 10 individuals per replicate group.

2.5. Data analysis

Data from the emersion temperature experiments were not normally distributed and so did not meet the requirements for ANOVA. Thus, the Kruskal-Wallis non-parametric ANOVA was used to compare temperature at death among the various energy reserve treatments (i.e. durations of starvation). When the Kruskal-Wallis test revealed a significant difference, a Dunn's multiple comparisons test was then used to compare each starvation treatment to the Day 0 control to assess how tolerance was impacted by starvation. To compare emersion temperature tolerance between size classes, a randomized block ANOVA was used, with trial as the blocking factor. Although the data did not meet the requirements for ANOVA, the p-value was deemed sufficiently low as to be unaffected by using a parametric test (Blanca et al. 2017). A significance value of 0.05 was used for both the Kruskal-Wallis and ANOVA statistical tests.

Survivorship data from all desiccation experiments followed a binomial distribution and were thus analyzed using generalized linear models (GLMs) with a binomial link function. Each energy reserve treatment (i.e. duration of starvation or size class) was analyzed separately, and the duration of desiccation resulting in 50% mortality (desiccation LD₅₀) was determined from the resulting curve. To identify differences in acute desiccation tolerance among the various energy reserve treatments, all survivorship

data for a given species were fit to a single GLM. In all cases, trial was included as a fixed factor to account for potential differences between batches collected from different sites or at different times, and a significance value of 0.05 was used. All statistical analyses were conducted using R statistical software (version 3.5.0) (R Core Team 2018).

3. RESULTS

3.1. Emersion temperature tolerance

3.1.1. Effect of starvation duration on emersion temperature tolerance

Although there was significant variation in average temperature at death among the duration of starvation treatments in both species (Kruskal-Wallis test; *Balanus glandula*: $\chi^2 = 17.85$, $df = 6$, $p = 0.007$; *Nucella ostrina*: $\chi^2 = 15.55$, $df = 5$, $p = 0.008$), results for the non-starved control treatment were not significantly different from any of the starved treatments (Fig. 1). Starvation for periods of up to 60 d had no apparent effect on acute emersion temperature tolerance.

Results from the 30 d starvation treatment for *N. ostrina* were removed from the analysis due to uncharacteristically low temperature tolerance; 100% of individuals in that group died at 30°C, a temperature that was only lethal to a few individuals in any other starvation treatment. In addition, very high mortality also occurred among other individuals, remaining in the same holding cage, that were not used in the temperature tolerance experiment. It was apparent that the individuals in this holding cage had become unhealthy, and their ability to tolerate emersion conditions was impacted. The 40, 50, and 60 d starvation treatments involved hatchlings from the same batch of animals, collected at the same time from the same location, as those in the 0, 10, 20, and 30 d starvation treatments, but those in the 40, 50, and 60 d treatments were not held in the same holding cage as the ones used in the 30 d treatment. In addition, individuals remaining in the holding cage for the 40, 50, and 60 treatments did not experience high mortality.

3.1.2. Effect of body mass on emersion temperature tolerance

Acute temperature tolerance in EBP *N. ostrina*, as measured by temperature at death (Fig. 2), differed

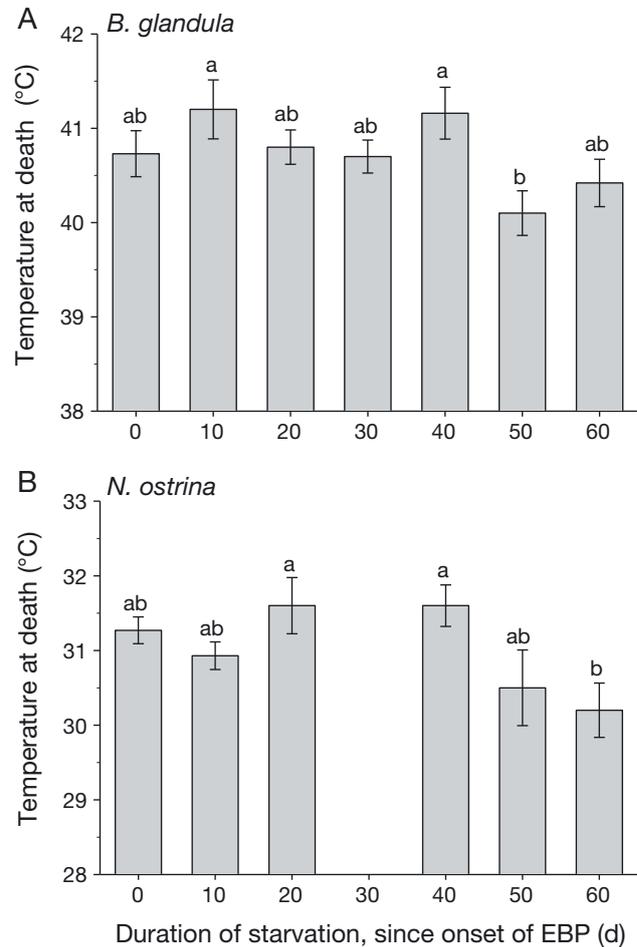


Fig. 1. Acute temperature tolerance thresholds, measured as average temperature at death, as a function of duration of starvation in early benthic phase (EBP) (A) *Balanus glandula* and (B) *Nucella ostrina*. The 30 d *N. ostrina* group was removed from this analysis due to inconsistent results for this batch of hatchlings and mass mortality among the remaining hatchlings of the same batch that were held in the same cage. In both species, starvation was initiated at the onset of the EBP. Error bars represent \pm SE. Bars with different lowercase letters are significantly different based on Dunn's multiple comparison test

significantly between the 2 size classes (randomized block ANOVA using trial as the blocking factor: $F = 71.02_{2,250}$, $p < 0.001$). In each trial, the large size class had significantly higher tolerance thresholds than the small size class; the differences between the size classes were 1.47, 0.89, and 2.12°C in trials 1, 2, and 3, respectively. This indicates that a greater initial body mass provides better tolerance to increased emersion temperatures. Additionally, within each size class (large or small), temperature at death varied significantly among trials ($p < 0.001$) (i.e. among groups collected from different sites or on different

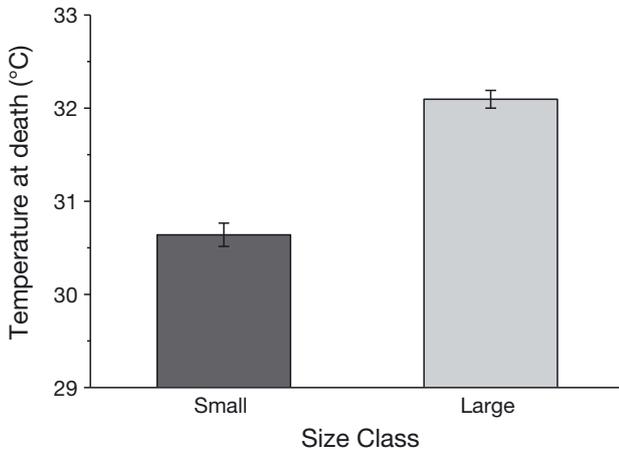


Fig. 2. Effect of initial body size on acute temperature tolerance, as measured by average temperature at death, for early benthic phase *Nucella ostrina*. Error bars represent \pm SE. These means are significantly different based on a randomized block ANOVA ($p < 0.001$)

dates). This suggests that temperature tolerance thresholds can vary among similarly sized hatchlings depending on where or when they completed encapsulated development.

3.2. Desiccation tolerance

3.2.1. Effect of starvation duration on desiccation tolerance

Within each starvation treatment, survivorship was significantly impacted by the duration of the desiccation treatment in both species (Fig. 3 and Figs. S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m686p001_supp.pdf), as determined by separate GLM analyses of each treatment (Table S1). The desiccation LD₅₀ decreased with increasing duration of starvation (Fig. 4).

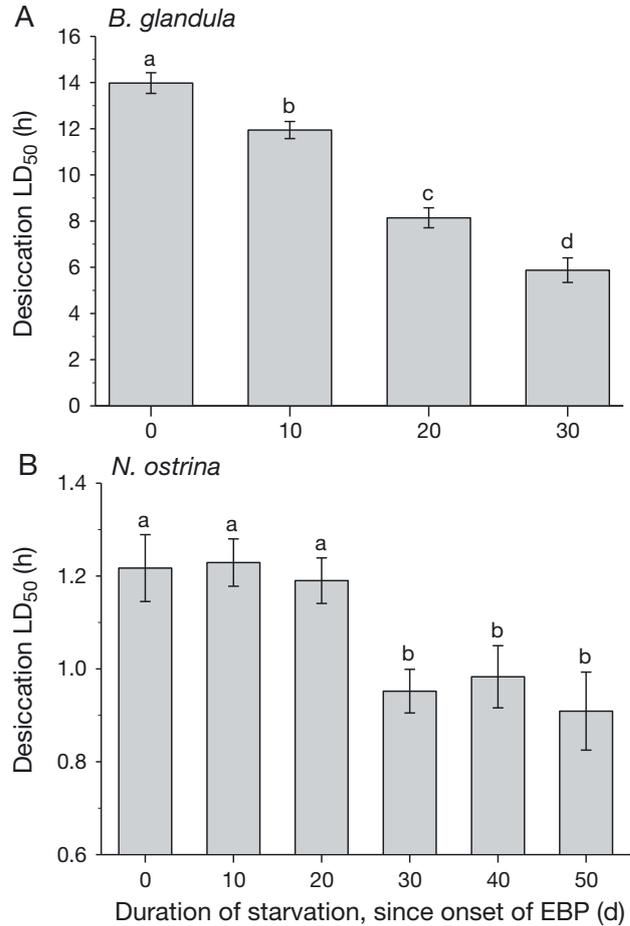
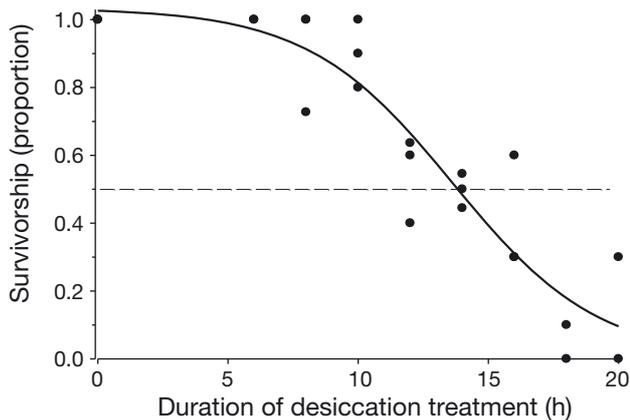


Fig. 4. Acute desiccation tolerance thresholds, measured as the duration of desiccation resulting in 50% mortality (desiccation LD₅₀), as a function of duration of starvation in early benthic phase (EBP) (A) *Balanus glandula* and (B) *Nucella ostrina*. Error bars represent \pm SE. Bars with different lower-case letters are significantly different based on generalized linear model analysis

In both species, acute desiccation tolerance thresholds differed significantly among the starvation treatments, as determined by GLM analysis of the combined survivorship data from all starvation treatments of a given species. For *B. glandula*, desiccation tolerance differed significantly among each starvation treatment, with the Day 0 control group having the highest tolerance threshold (pairwise comparisons

Fig. 3. Effect of exposure to desiccation conditions on the survivorship of newly metamorphosed (not starved) early benthic phase *Balanus glandula*. Each point represents the proportion of individuals from a group of 10 that survived the desiccation treatment for a given duration (h). The point where the curve intersects the dashed line represents the duration of desiccation resulting in 50% mortality (desiccation LD₅₀), in this case, LD₅₀ = 13.98 h

among starvation treatments: z -values < -3.36 , $df = 92$, $p < 0.001$) and tolerance decreasing gradually for each sequential starvation treatment period (Fig. 4A). For *N. ostrina*, however, the trend towards lower desiccation tolerance was not gradual. There was no significant difference in desiccation tolerance among 0, 10, and 20 d starvation treatments (pairwise comparisons: z -values > 0.001 , $df = 90$, $p > 0.71$). Desiccation tolerance was, however, significantly lower for longer starvation treatments (30, 40, and 50 d) when compared to shorter starvation treatments (pairwise comparisons: z -values < -1.76 , $df = 90$, $p < 0.005$) (Fig. 4B). Thus, duration of starvation had a significant effect on desiccation tolerance in both species.

3.2.2. Effect of body mass on desiccation tolerance

Within each *N. ostrina* size class, survivorship decreased significantly as a function of the duration of the desiccation treatment (Fig. 5), as determined by GLM analysis (small size class: z -value = -5.46 , $df = 17$, $p < 0.001$; large size class: z -value = -6.26 , $df = 17$, $p < 0.001$). In addition, when comparing size classes, the desiccation LD_{50} was significantly shorter for the small size class than for the large size class, as determined by GLM analysis of combined survivorship data from both size classes (z -value = -5.46 , $df = 23$, $p < 0.001$). Small individuals had a desiccation LD_{50} of 0.71 ± 0.05 h, whereas the desiccation LD_{50} of the large size class was 132% longer, at 1.65 ± 0.10 h.

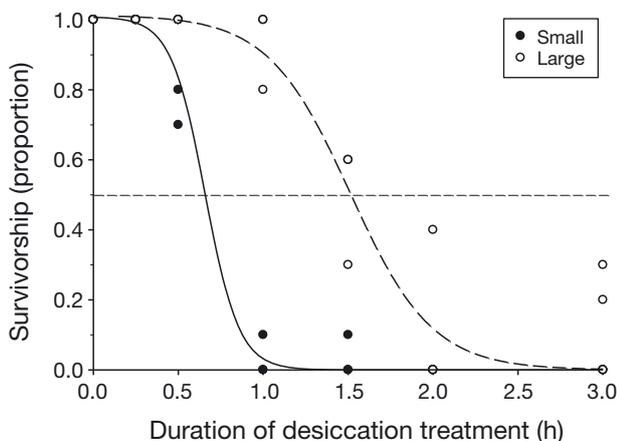


Fig. 5. Effect of exposure to desiccation conditions on the survival of newly hatched *Nucella ostrina* of 2 size classes (small = 0.074–0.192 mg; large = 0.402–0.833 mg). Each point represents the proportion of individuals from a group of 10 that survived desiccation treatment for a given duration (h). The points where the curves intersect the dashed line represent the duration of desiccation resulting in 50% mortality for each size class (desiccation LD_{50})

4. DISCUSSION

4.1. Effect of estimated energy reserves on tolerance to elevated emersion temperature

In EBP invertebrates, organic matter, a proxy for energy content, can decrease by half after only 10 d of starvation and approach a minimum after 40 to 50 d of starvation (*Nucella ostrina*: Mendt & Gosselin 2021), at which point nearly all metabolizable organic compounds have likely been utilized. Yet, the ability of EBP individuals to tolerate elevated emersion temperature was not impacted by the duration of the starvation period they had experienced. In both *Balanus glandula* and *N. ostrina*, EBP individuals starved for 50 or 60 d had comparable acute temperature tolerance thresholds to individuals that had not been starved. This suggests that the mechanisms employed by EBP individuals to tolerate acute elevated emersion temperatures are not energetically intensive. The production of HSPs is one common strategy among invertebrate species to cope with the physiological challenges of increased environmental temperatures (Sokolova et al. 2012). Since the production and activation of these specialized proteins is energetically expensive (Krebs & Loeschcke 1994, Somero 2002), individuals with more depleted energy reserves were expected to produce fewer HSPs and thus be more vulnerable to elevated temperatures. However, our results indicate that individuals with highly depleted energy reserves, as estimated by duration of starvation, were just as tolerant of acute exposure to high temperatures as individuals with a full complement of energy reserves. This suggests that temperature tolerance may be determined by other physiological and biochemical mechanisms that require minimal energy expenditure, such as metabolic rate depression (Storey 1998) and reduced ATP turnover rates (Sokolova & Pörtner 2001). Alternatively, benthic invertebrates may already possess protective mechanisms, such as a store of HSPs or fermentable substrates used as a quick energy source (Sokolova & Pörtner 2001), upon entering the EBP, protecting them from the physiological stress of elevated temperature during emersion without requiring the use of stored energy. The quantity of stored HSPs, as well as the capacity of EBP invertebrates to utilize other suggested tolerance methods, should be a focus of future research.

Emersion temperature tolerance in *N. ostrina* hatchlings was significantly impacted by their initial body mass. For *N. ostrina* hatchlings, a greater initial body mass is indicative of having consumed more

nurse eggs during embryonic development (Rivest 1983, Gallardo et al. 2004, Lloyd & Gosselin 2007), such that larger individuals have a larger reserve of stored energy (particularly lipids) upon hatching than their smaller counterparts (Moran & Emlet 2001). However, given that no relationship was detected in the previous experiment between temperature tolerance and duration of starvation, the effect of body mass on temperature tolerance was likely due to body size itself rather than to a difference in initial energy reserve. Body size can be an important determinant of tolerance thresholds, as found in this and other studies (Lowell 1984, Gosselin 1997, Hamilton & Gosselin 2020), largely because of the lower SA:V ratio and increased water reservoir in the tissues of larger individuals. These traits would enhance temperature tolerance in large hatchlings by decreasing the rate of heat exchange with the external environment and perhaps also the ability to carry out evaporative cooling, thus decreasing the rate of warming of the body. It is also possible that the capacity for other physiological or biochemical mechanisms, such as those discussed in the previous paragraph, may differ between snails of different body size. This remains speculative, however, and may be explored in future studies.

An interesting finding of the body size experiment was that tolerance thresholds also differed significantly among replicate groups of hatchlings of the same body size. These replicate groups, however, were collected from different field sites or on different dates, indicating some natural variation in temperature tolerance within and among cohorts of the same species. The mechanisms controlling this variation remain unclear but may involve geographical (i.e. interpopulation) and seasonal differences in tolerance thresholds.

4.2. Effect of estimated energy reserves on tolerance to desiccation

Unlike emersion temperature, vulnerability to desiccation was impacted by estimated initial energy reserves; in both species, EBP individuals starved for a longer duration of time had lower desiccation tolerance thresholds than those that had not been starved. This suggests the mechanisms or strategies employed by EBP intertidal invertebrates to withstand the challenge of desiccation are more dependent on energy-mediated processes than those used for acute temperature tolerance. Such physiological mechanisms of desiccation tolerance may include produc-

tion of protective proteins, such as certain HSPs (i.e. HSP70) (Hayward et al. 2004), late embryogenesis abundant proteins, and Trehalose (Hibshman et al. 2020), as well as the synthesis of new proteins to replace those that become damaged. Many of these responses have a significant energetic cost, such that individuals with reduced energy reserves may be unable to properly utilize these mechanisms and thus be less tolerant of desiccation stress (Sokolova 2013). The specific energy-dependent mechanisms of desiccation tolerance utilized by intertidal invertebrates require further research.

The effect of duration of starvation on desiccation tolerance thresholds appears to be different in the 2 species. In *B. glandula*, desiccation tolerance decreased gradually with increasing duration of starvation such that individuals in each starvation treatment had a significantly lower tolerance of desiccation than individuals starved for shorter durations. *B. glandula* transition through a non-feeding cyprid stage prior to completing an energetically expensive metamorphosis shortly after settlement (Lucas et al. 1979, Anderson 1994, Thiyagarajan et al. 2003), such that some individuals likely begin the EBP with very limited energy reserves. Any further depletion of energy, such as a delay in feeding, could also further restrict the physiological mechanisms of desiccation tolerance described above. For *N. ostrina*, desiccation tolerance thresholds were impacted differently; rather than decreasing gradually, desiccation tolerance was unchanged for groups starved for up to 20 d but was significantly reduced in groups starved for longer durations. Since *N. ostrina* hatchlings begin the EBP with relatively large energy reserves obtained through maternal provisioning (Spight 1976, Marko et al. 2014), it is likely they have enough stored energy to maintain energy-mediated mechanisms of desiccation tolerance for a more extended period. Prior research has revealed that metabolizable energy reserves in EBP *N. ostrina*, as estimated by organic matter content, decline rapidly throughout the first 20 to 30 d when starved, decreasing by 58 to 69% during this period (Mendt & Gosselin 2021). After that point, metabolizable energy reserves quickly approach a minimum and are thus likely sufficiently reduced to begin impacting any energy-dependent mechanisms of desiccation tolerance.

As was the case for emersion temperature tolerance, desiccation tolerance thresholds were also affected by initial body mass; *N. ostrina* hatchlings in the large size class had significantly higher tolerance thresholds than those in the small size class. The

higher organic content, and thus larger reserves of energy, present in larger individuals likely contributed to the increased desiccation tolerance in the larger size class in addition to the physical characteristics of larger individuals, such as having a smaller SA:V ratio and a larger reservoir of water in their tissues (Lowell 1984, Gosselin 1997). Together, these factors have a large impact on the ability of EBP individuals of different sizes to tolerate desiccation stress and likely determine tolerance thresholds.

4.3. Contribution of energy reserves to EBP mortality via effects on stress tolerance

The present study investigated the indirect effects of initial energy reserves on EBP mortality, and more specifically on the vulnerability of EBP individuals to two of the most significant abiotic stressors in the intertidal zone, desiccation and elevated emersion temperature (Gosselin & Chia 1995, Miller et al. 2009). One approach used to obtain groups of individuals with distinct levels of initial energy reserve was to sort them according to initial body size, and vulnerability to both environmental stressors did vary as a function of body size, with larger individuals being more tolerant of desiccation and elevated temperatures than smaller individuals. This outcome, however, was likely primarily due to the physical characteristics of a larger body size, such as a smaller SA:V ratio, rather than the difference in energy stores between the size classes, especially in the case of temperature tolerance.

The most interesting finding of this study is that depleted energy reserves, as estimated by duration of starvation, can indirectly lead to EBP mortality by reducing tolerance to desiccation. Desiccation stress is likely a more important selective pressure on EBP individuals than emersion temperature, regardless of energy reserves. Acute temperature tolerance thresholds of EBP individuals of these 2 species are higher than most temperatures experienced in the field during the summer. All temperature treatments in this study were within the range of low tide summer temperatures recorded in the intertidal zone in Barkley Sound (Jenewein & Gosselin 2013b). However, the temperatures necessary to cause significant mortality, even in the most vulnerable experimental groups, were at the high end of the natural temperature range ($>30^{\circ}\text{C}$) in the intertidal zone, and those high temperatures usually persist only for short periods of time (<2.5 h) before the surfaces are immersed by the incoming tide (Jenewein & Gosselin 2013b).

Desiccation tolerance thresholds, however, were lower than durations of aerial exposure occurring in most middle and upper intertidal habitats. The duration of desiccation required to cause significant mortality among individuals in all starvation treatments of both species was shorter than durations regularly experienced in middle and upper intertidal habitats in the study area, such that EBP individuals would be vulnerable to desiccation stress during many daytime summer low tides. This indicates EBP individuals are substantially more vulnerable to desiccation conditions in their natural habitats than to the temperature conditions in those same habitats. In addition, individuals with low energy reserves would be most vulnerable to desiccation stress, and thus less likely to survive the EBP, whereas those entering the EBP with greater stores of energy are more likely to survive desiccation during the critical first few days of the EBP. In this way, desiccation may be acting as a selective pressure favoring the evolution of greater energy reserves at the onset of the EBP, especially in species that inhabit areas of the intertidal with the greatest level of desiccation stress, such as the upper intertidal zone.

The findings of this study suggest energy reserves at the start of the EBP may be an important indirect cause of EBP mortality by reducing desiccation tolerance thresholds. An additional consequence of this indirect effect is that variation in initial energy reserves would likely be a contributing factor in the extensive variation in intraspecific EBP mortality rates. In all the experiments of this study, however, tolerance thresholds to both stressors also varied among individuals and replicate groups within the same starvation treatment (i.e. with the same energy reserves). Replicate batches of individuals were obtained from different locations or at different times of the spring and summer, suggesting differences in tolerance thresholds may be linked to the location or time when each batch of animals was collected. This could have resulted from differences among populations or temporal differences in environmental conditions experienced by individuals from different batches (Jarrett & Pechenik 1997, Jarrett 2003). Additionally, in the experiment examining the relationship between duration of starvation and emersion temperature tolerance, significant differences in tolerance thresholds also occurred among starvation treatments, but those differences were random with regard to the duration of starvation each group had experienced (i.e. the amount of remaining energy reserve). Together, these findings suggest tolerance thresholds are also influenced by factors other than

initial energy reserves. Such factors could include latent effects resulting from the environmental conditions experienced during the embryonic and larval stages, altering their ability to tolerate similar stressors during the subsequent EBP (Pechenik 2006, Nasrolahi et al. 2016), or transgenerational effects resulting from the conditions experienced by the parents (Agrawal et al. 1999, Marshall 2008, Donelan & Trussell 2015). Additionally, parasites or bacterial infections might negatively impact tolerance thresholds. A better understanding of the mechanisms controlling EBP mortality requires further research on how these additional factors influence acute tolerance thresholds in EBP intertidal invertebrates.

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