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# Lactate accumulation in the intertidal hermit crab, *Pagurus samuelis*, in response to burial-induced hypoxia

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**Abstract.—** We subjected the intertidal hermit crab, *Pagurus samuelis* (Stimpson, 1857), to various treatments to determine physiological responses of this species to the environmental stress of burial. Hermit crabs were buried with 6 cm of sediment and excavated at 2 h intervals up to a maximum of 12 h. Duration of burial and state (alive or dead) of the crab were analyzed for effects on lactate accumulation in hemolymph. Hermit crab weight, shell weight, weight ratio, lactate, and burial duration were analyzed for their influence on survival. As expected, lactate levels, as well as incidence of death, rose with duration of burial. Significant interaction, however, was also found between burial duration, crab state, and lactate concentration. There was a trend for lactate concentration to be low for surviving crabs, yet higher for dead crabs during shorter burial durations. Conversely and surprisingly, lactate concentrations were very high in surviving crabs, yet lower in dead crabs during long burial durations. Since some surviving crabs were able to develop very high lactate levels, we suggest that lactate buildup itself is unlikely to be the sole cause of death. Further studies are needed to identify factors affecting crab resilience during the stress of burial.

***Key words*:** *rapid sedimentation, flooding, anaerobic conditions, intertidal zone, L-Lactate, survival, hypoxia, anoxia*

## ■ Introduction

Hermit crabs can be found inhabiting intertidal zones panglobally. Because of physiological sensitivity of some species to freshwater they can be used as ecological indicators of hyposaline conditions (Dunbar, 2001; Dunbar & Coates, 2004; Dunbar *et al.*, 2003; Hall *et al.*, 1990). Freshwater inflow due to flooding is a common threat to animals living near the sediment-water interface and can include with it a significant amount of sediment (Maurer *et al.*, 1981; McCall, 1978; Niedoroda *et al.*, 1989). Organisms can also become buried by sediment from river deposits (McKnight, 1969), tides (Grant, 1983), bioturbation (Thayer, 1983), terrestrial runoff (Edgar & Barrett, 2000) dredging (Messieh *et al.*, 1991), fishing (Hall *et al.*, 1990; Jackson & James, 1979), wind erosion, or trampling (Chandrasekara & Frid, 1997).

Such burial events can impose physical constraints on the buried organisms, restricting their motility and ability to stir the local material. Burial also dramatically reduces diffusion rates of liquids and gasses, so that the supply of oxygen becomes limited to that accessible only in the immediate surroundings. As a result, burial by sediment may cause the immediate environment to become hypoxic (Nichols *et al.*, 1978; Shives & Dunbar, 2010; Valére-Rivet *et al.*, 2017). Several decapod species exhibit various physiological adaptations to short-term hypoxia, including increased scaphognathite

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(Anderson *et al.*, 1991; Paterson & Thorne, 1995) or pleopod (Felder, 1979; Torres *et al.*, 1977) beat frequency, and increased cardiac output (Thompson & Pritchard, 1969). If hypoxic conditions persist, concentrations of urate (Dykens, 1991) and the respiratory pigment, hemocyanin (deFur *et al.*, 1990) will increase. Urate allosterically binds to hemocyanin causing a greater affinity for oxygen and increased delivery to the tissues (Menze *et al.*, 2005). In field conditions, if oxygen concentrations are reduced, crustaceans may respond behaviorally by moving in or out of the area (Matabos *et al.*, 2012), engaging in atypical interspecific interactions (Pretterebner *et al.*, 2012), or having reduced ability for handling prey items (Mistri, 2004). If ambient oxygen concentration drops below a critical level, decapods compensate physiologically by decreasing oxygen consumption (Grieshaber *et al.*, 1994) and energy expenditure (Hand, 1998; Hochachka & Lutz, 2001), including decreasing pleopod beat frequency (Felder, 1979; Torres *et al.*, 1977), followed by a shift to anaerobic respiration (AR) to meet energetic demands (Grieshaber *et al.*, 1994).

Anaerobiosis was shown to occur in crustaceans in conditions, such as exercise (Briffa & Elwood, 2001; Full & Herreid, 1984; Henry *et al.*, 1994; McDonald *et al.*, 1979; Smatresk *et al.*, 1979), aerial exposure (Ridgway *et al.*, 2006), and hypoxia (Butler *et al.*, 1978; Hervant *et al.*, 1995; Holman & Hand, 2009). The crustacean physiological response to functional and environmental hypoxia is similar to that in vertebrates. When the intracellular phosphagen buffer (arginine phosphate in crustaceans) is exhausted, anaerobic glycogenolysis is employed, and lactic acid fermentation occurs (Booth & McMahon, 1985; England & Baldwin, 1983; Gornik *et al.*, 2008; Johnson *et al.*, 2004; Morris & Adamczewska, 2002). Conversion of pyruvate to lactic acid enables the restoration of $NAD^+$, which is essential for continued glycolysis under hypoxic conditions. Anaerobic glycolysis appears to be the only source of ATP in the absence of oxygen in the Crustacea (Albert & Ellington, 1985; Grieshaber *et al.*, 1994), evidenced by a significant increase in hemolymph lactate levels (Bridges & Brand, 1980; Sneddon *et al.*, 1999). Fumarate, alanine, aspartate, glutamate, succinate, and malate also increased during hypoxia (Hill *et al.*, 1991). However, the amounts detected indicate that alternative metabolic pathways are of very little significance in decapods (Hill *et al.*, 1991; Pritchard & Eddy, 1979; Zebe, 1982). These results point to lactate as the indicator of anaerobic respiration occurring within crustaceans. Regular encounters with hypoxic conditions appear to be associated with some physiologic adaptations. It was reported that, during recovery from hypoxia, lactate declines more rapidly in burrowing crustaceans compared to non-burrowing species (Bridges & Brand, 1980).

Investigations of anaerobic respiration in hermit crabs are relatively few. However, Briffa & Elwood (2001); (2005) studied AR in aggressive contacts in specimens of *Pagurus bernhardus* by analyzing the concentration of lactate and glucose in the hemolymph of individuals immediately following agonistic interactions over the ownership of gastropod shells. Still, no reported studies investigating hypoxia-induced AR during burial in Paguroidea have previously been undertaken.

The aim of the current investigation was to determine the extent to which the hermit crab, *Pagurus samuelis*, undergoes anaerobic respiration while experiencing burial with sediment. We hypothesized that lactate concentrations in the hemolymph would increase in relation to burial duration in crabs found alive at excavation. Secondly, we expected to find a critical level of lactate, above which the hermit crabs could no longer survive.

## Materials and Methods

### *Collection and care of hermit crabs*

*Pagurus samuelis* individuals of body weight ranging from 0.61–1.94 g were collected from tide pools at Shaw's Cove (33°32′43″N, 117°47′57″W) and Little Corona del Mar (33°35′21″N, 117°52′05″W) in Southern California from March–May, 2007. Gravid females were excluded from collection. Hermit crabs were kept in aquaria in the laboratory where salinity was maintained at 36 ± 3 ppK and temperature at 24 ± 2°C with ambient light. Hermit crabs were fed frozen commercial salad shrimp once a week, and water was changed every three weeks.

### *Burial methods for lactate experiments*

We randomly selected hermit crabs for treatments from the aquarium, shook them gently, and blotted them with paper towel to remove excess water. The total wet weight of each crab inside the gastropod shell was measured and recorded to ± 0.001 g. Hermit crabs were then placed into plastic containers previously filled with 3 cm of sand, one crab per container. In each treatment, a plastic mesh was placed above the crab to allow sand grains to pass through to bury the crab, but ensured the crab could not crawl upward through the sand. Crabs, with their shells in the aperture down position, were slowly buried with water saturated sand, until 6 cm of sediment covered them, and there was 1 cm of standing water.

Hermit crabs were buried at the deepest of the three depths used in a previous investigation (6 cm), because this depth resulted in the greatest proportion of mortality (Shives & Dunbar, 2010). In treatments for time, we excavated crabs at 2 h increments until the maximum burial time of 12 h. Whether the crab was alive or dead was recorded and hemolymph was collected.

### *Lactate assays of hermit crab hemolymph*

After each treatment, hermit crabs were removed from their shells by cracking the shells open using a bench vise, and a 50 µl sample of hemolymph was taken from each crab. This was done by piercing the arthrodal membrane at the base of the third periopod with a 21-gauge hypodermic needle attached to a 1 ml disposable syringe. The procedure took approximately 5–15 min for each crab, but complete recovery from high lactate levels normally takes up to 24 h in crustaceans (Hervant *et al.*, 1999). Since *Pagurus samuelis* is not a burrowing species, its lactate recovery period was not expected to be rapid, although this was not tested. During this 5–15 minute period it was possible for some of the lactate to be metabolized, or that in living hermit crabs more lactate may have been produced due to stress. A control group was used to standardize for these stresses. The control group was not buried, but subjected to the stresses of cracking the shell open and collecting the hemolymph sample. All hemolymph samples were immediately deproteinized with 100 µl of cold 0.3 M perchloric acid and centrifuged. A 50 µl sample of the supernatant was stored at −20°C until the assay for lactate was conducted within 24 h.

Samples were thawed and neutralized by the drop-wise addition of potassium bicarbonate. Methyl orange was used as the indicator to identify samples with a pH of less than 4.4. The assay followed the procedure of Gutmann & Wahlefeld (1974), and Engel & Jones (1978). Samples (25 µl) were added to Eppendorf tubes containing 500 µl of hydrazine-glycine buffer, 25 µl of $NAD^+$, and 1.25 µl of lactate dehydrogenase (LDH), and incubated for 1 h at 37°C. Ethylenediaminetetraacetic acid (EDTA) was added to the hydrazine-glycine buffer to prevent interference by $Cu^{2+}$ ions associated with the hemocyanin, as recommended by Engel & Jones (1978). Diluted samples (1/10), and a series of standards were also prepared for

analysis in the same way. After incubation, 300 µl from each tube were pipetted into a 96 well microtiter plate (Nunc, Fisher Scientific, Pittsburgh, PA, USA). Standards were pipetted into the top row of wells, the neat samples in the second row, and the diluted samples in the third row. The absorbance of the standards and samples were measured at 340 nm in a plate reading spectrophotometer ($\mu$Quant, Bio-Tek Instruments, Inc., Winooski, VT, USA) and converted to lactate concentrations using a calibration curve constructed with the standards of known lactic acid concentrations. All reagents were obtained from Sigma Aldrich Inc. (St. Louis, MO, 63103).

***Statistical analyses***

Each data set was statistically analyzed together and separately using the program Statistical Package for the Social Sciences (SPSS) 14.0. After preliminary data screening, lactate values were $\log_{10}$ transformed to conform to the assumptions of parametric statistics, and 3 outliers were removed. Due to small sample sizes, duration categories were merged, 2 with 4 h, 6 with 8 h, and 10 with 12 h, leaving 3 treatment duration categories and the control. A two-way ANOVA was done to determine if the factors state (alive or dead) and time (0 h, 2–4 h, 6–8 h, 10–12 h) had an effect on the dependent variable, log lactate concentration. Additionally, a one-way ANOVA was done to determine if the factor time (0 h, 2–4 h, 6–8 h, 10–12 h) had an effect on the dependent variable, log lactate concentration, in living crabs only. To determine which variables (shell weight, crab weight, weight ratio, lactate, and burial duration) had a significant effect on state of the crab, a forward, stepwise logistic regression was conducted. All significance levels were set at $\alpha = 0.05$.

## Results

***Oxygen saturation***

In a previous study, Shives & Dunbar (2010) confirmed that percent oxygen saturation of the sediment, even without the presence of crab, dropped to a mean of 26.6 ± 4.4% within 15 minutes of burial. Percent saturation was less than 10% after 7.5 h of burial and continued to decline to a mean of 1.8 ± 0.75% after 24 h.

***Factors affecting lactate concentration***

Before logarithmic transformation of data, we obtained lactate values of 1.4–10.5 mM for control individuals and 3.2–58.4 mM for treatment individuals. The extreme values for living crabs were 1.4 and 58.4 mM and for dead crabs were 6.6 and 48.2 mM. The ranges of values over time and by state are shown in Table 1.

We found that duration of burial had a significant effect on hemolymph lactate concentration ($F_{(3,64)} = 15.032$, $p < 0.001$, partial $\eta^2 = 0.413$). Interaction between the factors state and duration was significant ($F_{(2,64)} = 5.619$, $p < 0.006$, partial $\eta^2 = 0.149$) (Fig. 1). However, the calculated effect size indicated more of the lactate variance was accounted for by duration alone.

Among hermit crabs that were found alive at the end of the treatments, ANOVA results showed a significant increase in lactate with increasing duration ($F_{(3,33)} = 14.993$, $p < 0.001$, partial $\eta^2 = 0.577$). Tukey HSD post hoc tests were run to determine which duration categories were significantly different from the others (Fig. 1). Results revealed that the control (0 h) (mean = 0.563 ± 0.087) was significantly dif-

Table 1. The minimum and maximum range values of *L*-lactate (in mM) found in each time category after burial with sediment (N = 71).

| | 0 h | 2–4 h | 6–8 h | 10–12 h |
|---|---|---|---|---|
| Alive | 1.4–10.4 | 3.2–27.9 | 3.6–34.4 | 26.2–58.4 |
| Dead | — | 6.6–44.8 | 11.7–37.9 | 5.7–48.2 |

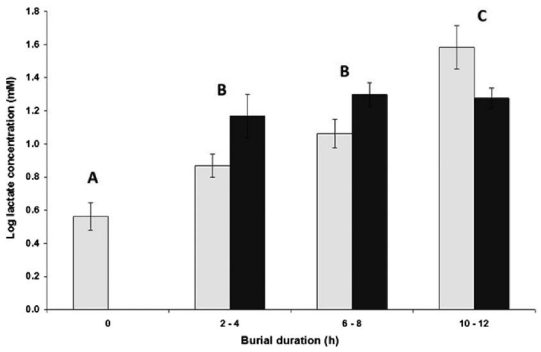


Fig. 1. The effects of state and burial duration on hermit crab lactate accumulation. Light bars represent hermit crabs found alive after excavation, while dark bars represent crabs found dead. Significant differences among surviving groups are indicated with different letters ($p < 0.05$ one-way ANOVA and Tukey's HSD). A vs B, $p < 0.045$; B vs C, $p = 0.014$; A vs C, $p < 0.001$. Error bars are ± 1 SE. N = 71.

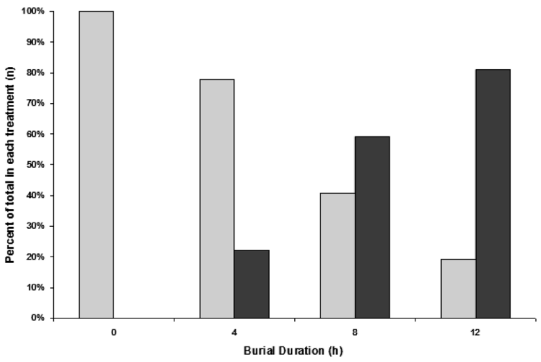


Fig. 2. The percent of hermit crabs that were found alive (light bars) or dead (dark bars) at each time interval. Burial duration was a significant factor for survival (Logistic regression, 2-Log Likelihood = 69.287, $\chi^2_{(1)} = 29.013$, $p = 0.008$). $n_0 = 10$, $n_4 = 18$, $n_8 = 22$, $n_{12} = 21$, N = 71.

ferent from all treatment groups for mean lactate concentration of the hemolymph in surviving crabs (control vs. 2–4 h alive, mean = 0.869 ± 0.066, $p = 0.045$; control vs. 6–8 h alive, mean = 1.063 ± 0.108, $p = 0.002$; control vs. 10–12 h alive, mean = 1.584 ± 0.075, $p < 0.001$). Between treatment groups of living crabs, the interval of 2–4 h (mean = 0.869 ± 0.066) was not significantly different from 6–8 h (mean = 1.063 ± 0.108), but was significantly lower than 10–12 h (mean = 1.584 ± 0.075, $p < 0.001$). The category 6–8 h (mean = 1.063 ± 0.108) was also significantly lower than 10–12 h (mean = 1.584 ± 0.075, $p = 0.014$).

***Factors associated with survival***

A forward, step-wise logistic regression was conducted to determine which independent variables (shell weight, crab weight, weight ratio, lactate, and burial duration) were predictors of survival. Regression results indicated the overall model of one predictor (burial duration) was statistically reliable in distinguishing between the hermit crab surviving the treatment or not (2-Log Likelihood = 69.287, $\chi^2_{(1)} = 29.013$, $p = 0.008$). The model correctly classified 76.1% of the cases.

The shorter the burial duration, the more likely *P. samuelis* was to survive the treatment (Fig. 2). Among the crabs that were buried for 2–4 h (n = 18) only 4 were found dead. Thirteen of the 22 crabs that were buried for 6–8 h died, and 17 of the 21 crabs that were buried for 10–12 h died.

## Discussion

As the duration of burial increased, lactate accumulation in surviving hermit crab hemolymph also increased. As previous preliminary oxygen saturation tests by Shives & Dunbar (2010) suggested, the longer hermit crabs were buried the more dissolved oxygen was consumed, causing hypoxia. These results are consistent with the rapid decrease in available oxygen during burial conditions measured by Valére-Rivet *et al.* (2017), although they additionally investigated the combined impact of temperature on oxygen availability. When available oxygen was insufficient to support aerobic respiration, crabs relied on anaerobic fermentation, which was indicated by increasing lactate levels. Several studies reported elevation in lactate with increasing duration of

hypoxia in crustaceans (Albert & Ellington, 1985; da Silva-Castiglioni *et al.*, 2010; deFur *et al.*, 1990; Gornik *et al.*, 2008; Hill *et al.*, 1991; Lallier *et al.*, 1987; Maciel *et al.*, 2008; Spicer *et al.*, 2002). This study demonstrates that increasing lactate levels in a hermit crab species is directly attributable to hypoxia. Moreover, lactate levels observed during hypoxia in this study are greater than those found by Briffa & Elwood (2001) investigating hermit crab agonistic interactions leading to functional hypoxia. Although lactate accumulates in the hemolymph of decapod crustaceans during both functional and environmental hypoxia, the extent of accumulation is much greater during long-term environmental hypoxia when lactate concentrations may exceed 40 mM in the crayfish, *Orconectes limosus* (Gäde, 1984) and the stone crab, *Menippe mercenaria* (Albert & Ellington, 1985; Gäde, 1984). Our results suggest that lactate levels may rise higher in *P. samuelis* than in either of these species, although in the former reports the organisms were likely less stressed.

The statistical variables designating crab state and duration of burial affected lactate levels in a biphasic manner. After short burial time intervals, there was a trend for lactate to be lower (8.8 ± 2.7 mM) in individuals that survived when compared with those that died (19.7 ± 5.0 mM). However, there was a trend for hemolymph lactate concentration to be higher (40.1 ± 5.0 mM) in surviving crabs than in dead crabs (22.5 ± 2.4 mM) after long burial time intervals. We found 2 hermit crabs that were dead after only 2 hours of burial, with final lactate levels of approximately 7 mM. This was not substantially different from the control mean of 4.4 ± 3.1 mM.

Based on the observed ranges in hemolymph lactate levels in surviving and dead crabs we conclude that death most likely was not due to lactate toxicity. Instead, we suggest a different cause of death. Hermit crabs have two internal sources of energy, circulating glucose and stored glycogen (Briffa & Elwood, 2004). Once circulating glucose has been utilized, glycogen stored in muscles and hepatopancreas is likely the most rapidly accessible and most helpful glycogen source during severe acute demands for energy. Zou (1996) found that in the Chinese freshwater crab, *Eriocheir sinensis*, during severe hypoxia, hemolymph glucose rose quickly, with lactate rising a few hours later. They concluded that glucose was mobilized from glycogen in preparation for the switch to anaerobiosis. Inadequate glycogen stores would likely lead to a delayed, partial, or ineffective shift to anaerobic metabolism. Other studies have confirmed that glycogen stores decrease with increasing lactate concentration in the hemolymph (Hill *et al.*, 1991; Sneddon *et al.*, 1999; Taylor & Spicer, 1987), indicating glycogenolysis, rather than gluconeogenesis, as the main source of glucose for glycolysis. Moreover, diet was found to directly affect crab hemolymph glucose levels, as well as hepatopancreas and muscle glycogen concentrations (Marqueze *et al.*, 2011; Vinagre & Da Silva, 1992). Therefore, some individual *P. samuelis* crabs may have exhibited ineffective anaerobic metabolism due to insufficient glycogen stores, and subsequently died quickly with relatively moderate hemolymph lactate levels. Conversely, crabs with adequate glycogen stores may have been able to sustain their metabolic processes and survive 10–12 h of hypoxia while ATP was supplied largely by glycolysis and lactic acid fermentation.

Gastropod shells aid in survival and are known to provide protection from desiccation (Reese, 1968) and osmotic stress (Shumway, 1978). Childress (1972) calculated weight ratios for hermit crab shell weight to body weight, and found optimum ratios for growth and reproduction. Therefore, it is likely that a hermit crab in a shell that is lighter (smaller) than preferred and faced with the stress of a shallow burial event, such as those presented here, would have less capacity for water stor-

age inside the shell (Bertness, 1981). Without water and its dissolved oxygen stored in the shell, crabs may be more susceptible to death from anoxia. However, we found no effect of weight ratios on survival. This may be because an abundance of vacant shells was available in the aquaria, so it is unlikely hermit crabs inhabited smaller than optimal shells.

We hypothesized that a critical concentration of lactate would be found, above which hermit crabs would no longer survive. Surprisingly, this was not the case. The individual with the highest lactate concentration found in the current study (58.4 mM) was found alive after 12 h of burial. This suggests that *P. samuelis* can indeed undergo a significant amount of AR to survive even severe, acute hypoxia, and that it is unlikely that a moderately high concentration of lactate caused increased mortality in hermit crabs found dead in our treatments. Moreover, other studies showed that a rise in hemolymph lactate causes crustacean hemocyanin to increase its oxygen affinity, and therefore binds the less available oxygen better than under normoxic conditions (Bridges, 2001; Lallier & Truchot, 1989; Truchot, 1980).

In addition to anaerobic physiology, the preconditioning of individual crabs through prior experiences of burial events may be an important factor in survival of acute burial events under study. While this was not within the scope of the current study, it nevertheless provides another potential area of exploration into hermit crab physiology. Duration strongly influenced survival of a shallow burial event, with death positively correlated with burial duration, consistent with other burial studies (Chandrasekara & Frid, 1997; Schiel *et al.*, 2006).

As demonstrated in previous studies, mobility can decrease burial duration if a taxon is able to crawl up through the sediment (Hinchey *et al.*, 2006; Shives & Dunbar, 2010). Indeed, Shives & Dunbar (2010) demonstrated that hermit crabs may survive shallow burial events either by carrying the shell to the surface, or by abandoning the shell and reaching the surface without it. Hermit crabs are very mobile, and many of them would likely have maneuvered through the sediment had we not restricted them in the current study.

Hermit crabs that face the environmental stressor of burial have the ability to cope with decreasing oxygen saturation by using AR to meet acute energy demands, and prolong the functioning of metabolic processes. In order for hermit crabs to employ glycolysis and lactic acid fermentation they must have a supply of glucose. Previous work indicates that, in decapods, glycogen serves as the glucose source once hemolymph glucose has been depleted (Briffa & Elwood, 2004; Hill *et al.*, 1991; Sneddon *et al.*, 1999). Although aerobic respiration yields significantly more ATP molecules, anaerobiosis may be utilized in *P. samuelis* to survive acute and relatively short-term environmental hypoxia. This adaptation would prove beneficial to hermit crabs that may experience hypoxia in the dynamic environment of the rocky intertidal zone. Still, further studies are needed to characterize the roles of preconditioning and glycogen stores as contributing factors for survival of acute, extreme hypoxia in hermit crabs.

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