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Effects of handling during experimental procedures on stress indices in the green shore crab, *Carcinus maenas* (L)

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ABSTRACT

Stress due to handling is often an unavoidable feature of experimental investigations. In some cases, appropriate settling times are not considered, and as such, physiological responses caused by handling may become additive with those of experimental treatments. This study investigated the effect of different handling procedures on the acute physiological responses of green shore crab (*Carcinus maenas*). Handling, such as would occur during transport around a research facility or transfer during experimental procedure, was designated as light (10 min emersion) or severe (10 min emersion with shaking). Oxygen consumption (MO_2) and haemolymph glucose and haemolymph L-lactate concentrations were elevated post-handling, the magnitude of the change related to the severity of handling stress. Glucose and L-lactate concentrations peaked within 1 h and returned to basal levels within 6 h, but MO_2 remained elevated for 10 h, reflecting the additional energy required to oxidize L-lactate and replenish energy reserves. Differences between light and severe handling treatments showed that vibration (shaking) was a major contributor to the stress response, rather than the experimental emersion. This was confirmed in a second experiment where crabs were handled without emersion, and MO_2 remained elevated for 14 h. In this experiment, the most pronounced increase in MO_2 and metabolic parameters occurred in crabs that were physically touched and moved rapidly from the holding to experimental tanks. Here the touch, as well as vibration and visual stimuli, provoked a fight-flight response in the crabs. Stress responses were also evident in crabs gently transferred by containers. The fact that transferring crabs with no physical touching and minimal visual and vibrational stimuli still evoked a stress response, albeit less pronounced, supports a recommendation that crustaceans should be left to settle in the apparatus for at least 12 h after handling before experimental procedures are initiated.

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Introduction

Fisheries and laboratory practices frequently inflict stress on marine organisms, which from the perspective of systems biology, may be defined as ‘when a biological control system detects a failure to control a fitness-critical variable, which may be either internal or external to the organism’ (Del Giudice et al. 2018). Handling occurs during capture processes and transport for the live fisheries trade, and is frequently an unavoidable feature of experimental investigations in a research environment. Handling stressors may be chronic or repeated, influencing the longer-term fitness of the animal (Calow and Forbes 1998) or an acute, short-term challenge causing a temporary change in homeostasis. Laboratory handling of marine animals may be classified as an acute stress (rather than chronic) because the stimulus is often removed before the individual’s response is complete (Pickering et al. 1982). Despite this, defining handling stress remains complex, with many methodologies describing handling procedures in different ways. Differentiating between ‘laboratory handling’ and ‘live fishery transport and handling’ may be desirable to avoid confusion.

Crustaceans are used extensively as model organisms for research. Compared to other crustacean taxa, decapods may be more tolerant to handling stress; some are facultative air breathers (Taylor 1982), and many have extra protection from their rigid exoskeletons (Broadhurst and Uhlmann 2007). In scientific research, it is common practice to acclimate organisms in laboratory conditions prior to experimentation to eliminate the effects of their previous environment on their behaviour and physiology. However, exact guidelines for appropriate settling periods following handling stress (i.e. transport and handling immediately prior to and during the actual experimental procedure) in crustaceans have received little attention (Carvalho and Phan 1997). Acute handling during experimental investigations can be stressful, but because these handling procedures vary greatly it is difficult to draw firm conclusions. Some articles do not specify handling procedures (e.g. McMahon et al. 1978; Vinagre et al. 2012). When reported, some procedures could be classified as mild disturbances, such as repeatedly picking the animal up (e.g. Telford 1968), or emersion during transfer between areas (e.g. Wilkens et al. 1985; Crear and Forteach 2001). Elaborate handling procedures incorporating numerous stressors could be described as more severe (e.g. Mercier et al. 2006, 2009; Aparicio-Simón et al. 2010). At the far end of the spectrum, practices such as declawing (Patterson et al. 2007) or electrical shocks (Elwood and Adams 2015) appear to cause extreme discomfort and pain to crustaceans.

A number of metrics are used to evaluate stress responses in crustaceans. Stress and metabolism are highly interrelated; therefore, measuring the oxygen consumption rate (MO_2) of a crustacean is an excellent indicator of physiological condition (Jouve-Duhamel and Truchot 1985; Childress and Seibel 1998; Haukenes et al. 2009). In crustaceans, a rapid release of crustacean hyperglycaemic hormone mobilizes glucose, which acts to meet the increasing demand for energy in response to a wide variety of stressors (Chang et al. 1999; Webster 2015). Thus, measuring haemolymph glucose levels is another important indicator (Telford 1968; Santos and Keller 1993; Hall and Van Ham 1998; Racotta and Palacios 1998). Finally, elevated energy requirements may also induce a shift to anaerobic metabolism, resulting in the accumulation of metabolic end-products

such as L-lactate, indicative of environmental and handling stress (Whiteley and Taylor 1992; Santos and Keller 1993; Crear and Forteath 2001; Ridgway et al. 2006).

Knowing the magnitude of a stress response after handling procedures and the duration of post-stress recovery is necessary to inform appropriate settling periods. Without consideration of settling periods, changes in physiology or behaviour due to handling may add to those resulting from the experiment proper (Pickering et al. 1982). Settling periods of 8 h have been advised post-handling in lobsters, based on elevated concentrations of glucose and L-Lactate in the haemolymph together with increased oxygen consumption (Crear and Forteath 2001). A settling time of 12 h for crabs (*Cancer gracilis*, *Cancer magister*, *Carcinus maenas*) is based on oxygen consumption and heart rate (McGaw 2007; McGaw and Nancollas 2018). Although these give a time frame on which to base settling periods, they may not be applicable to all decapod species, as many have evolved different magnitudes of responses and tolerances to stress (Bridges and Brand 1980; Bergmann et al. 2001).

The green shore crab (*Carcinus maenas*, Linnaeus, 1758) is a 'hardy' decapod species, resilient to many stressors, such as emersion, low salinity, temperature change, and decreased food availability (Taylor 1982; Klassen and Locke 2007; Leignel et al. 2014). Its relatively large size, ease of capture and ecological importance as an invasive species has made it one of the most studied crabs in the world, particularly regarding its adaptive physiology (Leignel et al. 2014; Simonik and Henry 2014). Because this species experiences many environmental challenges in its intertidal habitat (Crothers 1967), one might predict that it should be relatively unperturbed by environmental and laboratory stressors. Other decapods that have been studied (lobsters and prawns) appear to be more sensitive to environmental and handling stressors (Mercier et al. 2006, 2009; Aparicio-Simón et al. 2010). Therefore, data from green shore crabs will provide a good baseline for minimal responses to stress, from which handling and recovery practices for other species could be based. Investigation of the potential stress of handling decapod crustaceans during experimentation will likely become more pertinent in the coming years. Recently, there has been debate regarding if/how decapod crustaceans experience pain and stress, and there is continuing discussion about re-classifying them as 'animals,' thereby requiring animal welfare protocols for scientific experiments (Elwood et al. 2009; Stevens et al. 2016; Weineck et al. 2018; Diggles 2019; Drinkwater et al. 2019; Elwood 2019).

Repeated handling of animals may be an inevitable feature of some laboratory experiments. Some recent work in our lab showed that repeated short-term handling (10–15 min every 2 weeks for 6 months) increased mortality up to 60% for handled green shore crabs, compared to 3% for unhandled counterparts (Wilson et al. In prep.). Thus, the primary aim of the present study was to determine how handling might influence short-term changes in oxygen consumption and concentrations of glucose and L-lactate in the haemolymph. Even where experiments do not require repeated handling, at the very least animals need to be transferred between holding tanks and experimental apparatus, and in some cases, experimental design does not allow for a settling period after handling. Therefore, the second aim of the study was to use oxygen consumption rates to determine the least stressful way to transport animals, and to develop an idea of the time span and the degree to which metabolic parameters remain elevated.

Material and methods

Animal collection

Intermoult male green shore crabs (*Carcinus maenas*) of 60–80 mm carapace width, were trapped during July–September 2018 at Fox Harbour, Newfoundland, Canada. Crabs were transferred to the Department of Ocean Sciences, Memorial University (St. Johns, NL), and kept in a tank (220 × 95 × 45 cm depth) continuously supplied with flow through seawater ($T = 10\text{--}12^\circ\text{C}$, $S = 30\text{--}32$). The water was aerated using air stones, and the crabs were fed a mixed diet of fish, mussels and seaweed twice weekly. Plastic tubes were added to provide shelter and reduce aggressive interactions. Crabs were maintained in these conditions for at least 2 weeks prior to use in any experiment. All crabs were fasted for at least 3 d prior to experimentation to eliminate the risk of postprandial increases in oxygen consumption (McGaw and Reiber 2000; McGaw 2007), and only crabs with all limbs and no visible damage to the carapace were used.

Acute responses to handling/transport

The first series of experiments determined the short-term responses of crabs to acute transport/handling stress and their subsequent recovery. Crabs were separated into three groups: a non-handled control group, a light handling/transport treatment and a severe handling/transport treatment. For the control treatment, the crabs were left undisturbed in the holding tank and not handled. The light handled crabs were gently removed from the tank (by hand) and transferred to a plastic container where they were left emersed in air ($T = 20^\circ\text{C}$) for 10 min. For the severe handling treatment, the crabs were removed from the tank, placed into a plastic container (in the air) and shaken for 10 min. This handling, while emersed, was designed to be analogous to the transport of animals during collection or around a research facility. A number of metrics were used to evaluate stress responses: these included measurements of oxygen consumption rate (MO_2) and haemolymph glucose and L-lactate concentrations. Separate groups of crabs were used to measure MO_2 , and for glucose and L-lactate concentrations.

Oxygen consumption

Rates of oxygen consumption (MO_2) were measured at hourly intervals using an L-DAQ intermittent flow respirometry system (Loligo systems, Tuborg, Denmark). This fully automated system is equipped with two pumps. The first pump continually flushed aerated seawater ($T = 10\text{--}12^\circ\text{C}$, $S = 30\text{--}32$) through a number of cylindrical chambers (20 cm diameter × 12 cm depth), each containing a single crab pre-exposed to one of the three treatments ($n = 13$ per treatment). Crabs were introduced into the chambers immediately after the treatment and the first measurement period started straight away. The control crabs were gently transferred from the holding tank to the measurement chamber, as per the ‘minimal stress’ steps outlined in the experiment two below. During measurements, the first pump was automatically turned off, preventing newly oxygenated water from entering the sealed chamber. A second pump recirculated the water through the chamber (10 L min^{-1}). Oxygen consumption was measured during this 20 min recirculation phase while the oxygen was depleted, after which time the

chamber was continuously flushed for 40 min with fresh seawater before the next reading began. The oxygen consumption of individual crabs was measured for a total period of 20 hours. Data were recorded on a Loligo data acquisition system (Tuborg, Denmark) which expressed oxygen consumption as $\text{mg O}_2 \text{ kg.h}^{-1}$. The apparatus was surrounded by black plastic sheeting to remove visual disturbance to the animal and reduce any influence of diurnal rhythms. The first measurement period started at approximately 10:00 for all trials to reduce the influence of circadian rhythms (Scott et al. 2018) on oxygen consumption.

In addition to recording changes in MO_2 over time, the following metabolic parameters were calculated for each individual crab. (a) Resting metabolic rate (RMR) – mean of the lowest five MO_2 values, (b) maximal MO_2 (MMR) – highest-recorded oxygen consumption, (c) Aerobic Scope (AS) – MMR/RMR , (d) ‘Duration’ – time for MO_2 to reach stable (variance of $<5 \text{ mg O}_2 \text{ kg.h}^{-1}$ for 5 h) values and (e) equivalent energy expenditure (EEE) of each individual, calculated using the total increase in MO_2 above RMR and standardised to kJ using the conversion factor of $1 \text{ mg O}_2 = 0.014 \text{ kJ}$ (Secor 2009).

Haemolymph glucose and L-lactate

New groups of crabs ($n = 48$ per treatment) were used to measure the effects of the different handling protocols on the concentrations of glucose and L-lactate in the haemolymph. Following either the light or severe handling treatment, as described above, crabs were placed (as a group) into a new holding tank supplied with seawater ($T = 10\text{--}12^\circ\text{C}$, $S = 30\text{--}32$). Control crabs were not moved from their original holding tank. Individuals were removed at 0 h, 0.5, 1, 2, 4 and 6 h post treatment ($n = 8$ per time point, per treatment), and 1 mL of haemolymph was extracted using a needle (18 g) attached to a syringe (1 mL) that was inserted into the infrabranchial sinus via the arthroal membrane at the base of the third walking leg (pereopod). A different crab was used at each time point to ensure any stress caused by haemolymph extraction would not influence the blood chemistry at subsequent time points. Extracted haemolymph was transferred to a microcentrifuge tube (Eppendorf, vol. = 1.8 mL) and frozen immediately at $T = -80^\circ\text{C}$ for subsequent analysis. Once sampled, the crabs were not returned to the main holding tank. As haemolymph glucose and haemolymph L-lactate concentrations can vary over the course of 24 h (Scott et al. 2018), all trials began at approximately 10:00 am.

Haemolymph glucose concentration was quantified using a method adapted from Morris et al. (2010). Thawed haemolymph samples were deproteinised using 6% perchloric acid with a dilution ratio of 1:1. Samples were then mixed and centrifuged at 15,000 g for 10 min. The subsequent supernatant was extracted and 50 μL of the solution added to 450 μL 0.2 M phosphate buffer to produce a 20-fold dilution of glucose. Samples were mixed, and 100 μL of the resultant haemolymph-buffer solution was added to 200 μL reagent medium, which contained Horseradish Peroxidase (Sigma, P6782), Glucose Oxidase (Sigma, 7016), ABTS (Sigma, AA1888), and 0.2 M phosphate buffer. Absorbance was determined at $\lambda = 405 \text{ nm}$ using a DTX 880 microplate reader (Beckman Coulter, Ontario, Canada). Glucose concentrations were interpolated from a standard curve.

Haemolymph L-lactate concentration was quantified using an assay adapted from Clow et al. (2016), after being thawed and then deproteinised using 6% perchloric acid with a dilution ratio of 1:10. The samples were mixed and centrifuged at 15,000 g for 10 min. The resultant supernatant was extracted and 25 μL of this extract added to 200 μL of assay medium containing glycine buffer (Sigma, G5418) and 2.5 mmol L^{-1} NAD^+ , pH 9.0. Absorbance was determined at $\lambda = 340 \text{ nm}$ using a DTX 880 microplate reader (Beckman Coulter, Ontario, Canada) before the addition of 10 IU mL^{-1} L-lactic dehydrogenase (Sigma, L2500). Absorbance was then read after 30 min or every 30 min until stable. L-lactate concentrations were interpolated from a standard curve.

Survival

A separate group of crabs ($n = 15$ per treatment) were used to estimate longer-term effects of handling. Following the light or severe handling treatment, they were placed back into the holding tanks and monitored daily for a total period of 14 d, and limb loss and mortalities recorded.

Transfer methods during experimentation

The above experiments indicated that actual handling of the crabs, rather than simple transfer between media (i.e. aerial exposure only) caused significant stress. In some experimental protocols it is necessary to transfer individuals between apparatuses without a subsequent settling time. Therefore, this experiment was designed to determine the least stressful handling method to transfer individuals from the holding tanks to experimental chambers. Twenty-four hours before the trial, crabs were removed from the holding tank and weighed, measured and labelled. Half of the crabs were then placed individually into perforated plastic containers (15 cm x 9 cm x 6 cm deep) that allowed free-flow of oxygenated water, and placed back into in the holding tank. The remaining crabs were placed, unrestrained, back into the holding tank.

Changes in MO_2 were used to determine the least stressful way to transfer crabs from the holding tank into the respirometry apparatus, rather than haemolymph glucose or L-lactate, which tended to be more variable among individuals. MO_2 of individual crabs were recorded every hour (immediately after handling) for a total period of 24 h at $T = 14\text{--}15^\circ\text{C}$, using the methods described above. For all crabs, transfer occurred in three steps, and they were not emersed while handling to enable us to examine the effects of handling alone. Three different handling stresses (minimal, moderate, and maximal) were applied, each with a three-step transfer method described below. All steps were conducted whilst the crabs were submerged in buckets containing tank water.

Minimal Stress:

Step 1: Crabs were gently and slowly moved from the holding tank into the bucket < 5 sec.

Step 2: The bucket was very carefully moved and submerged into the experimental tank < 8 sec.

Step 3: Individuals were carefully taken out of the bucket and placed slowly into the respirometry chamber < 5 sec.

Moderate Stress:

Step 1: The crabs were moved from the holding tank into the bucket <3 sec.

Step 2: The bucket was submerged in the experimental tank for 5 sec.

Step 3: Individual crabs were taken out of the bucket and moved into the respirometry chamber <3 sec.

Maximal Stress:

Step 1: The crabs were quickly moved from the holding tank into the bucket (<1 sec.).

Step 2: The bucket was submerged in an experimental tank <3 sec.

Step 3: The crabs were taken out of the bucket, shaken (underwater for 5 sec.) before being placed in the respirometry chamber, and the transfer process took <6 sec.

Additionally, within these three steps there were two different handling protocols: hand transfer and container transfer. For hand transfer, the crab was manually picked up from behind by pinching the abdomen and the top of the carapace. For individuals in a container, no physical touching of the crab was required; the entire container was moved into the bucket, the container was then placed into the respirometry chamber, where one side was opened to allow the crabs to leave the container and enter the chamber of their own accord. Once this occurred, the plastic container was removed, the chamber sealed, and recordings began immediately. Eight individuals were tested for each of the six transfer combinations ($n = 8$ per treatment, 48 individuals in total). While it is possible that unrestrained crabs used for the hand transfer treatment may show a higher stress response due to their interaction with other crabs, steps to minimize this were taken, including a low stocking density, ample provision of shelter, and feeding 3 d prior to the experiment.

Statistical analysis

To establish how different handling procedures influenced oxygen consumption rates over time (experiments one and two), two-way repeated measures ANOVAs were performed, followed by Tukey HSD post-hoc tests, where significant differences were detected. The metabolic parameters (RMR, MMR, AS, 'duration,' EEE) were analysed using 1-way ANOVAs, or ANOVA on ranks if the data were not normally distributed, followed by Tukey HSD post-hoc tests when significant differences were obtained. The effect of handling treatment and time on haemolymph glucose and L-lactate concentrations was compared using a two-way ANOVA. Significant pairwise differences were identified using a Tukey HSD post-hoc test. Data that were not normally distributed were normalized by ranking responses or using box-cox transformations. Statistical analysis was conducted using SigmaStat and $P < 0.05$ was considered as the significance level for all analyses.

Results

Acute responses to handling stress (Experiment 1)

Oxygen consumption rates (MO_2) were the highest immediately after introduction into the respirometry chambers (ranging between 72.23 ± 7.20 and 114.13 ± 9.90 $\text{mg O}_2 \text{ kg. h}^{-1}$) and declined significantly over time (Figure 1; RM ANOVA, $F_{20} = 45.10$, $P < 0.001$). The MO_2 declined most rapidly during the first 6 h of the experiment, slowing somewhat

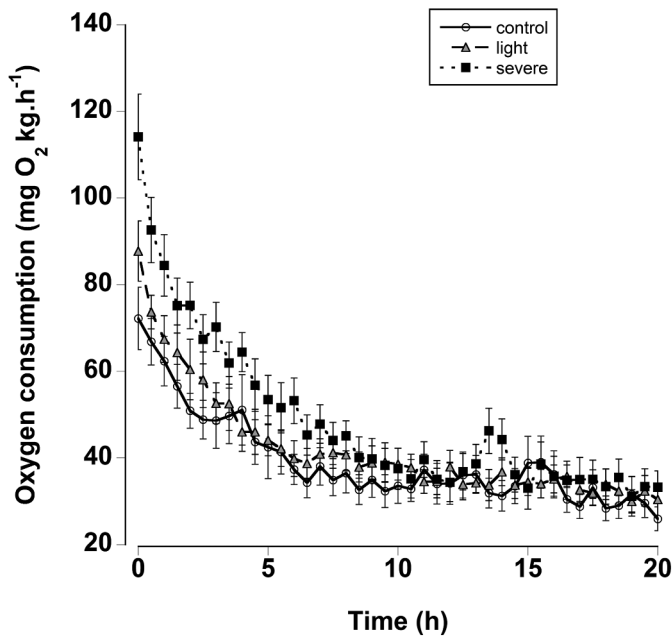


Figure 1. Oxygen consumption rates ($\text{mg O}_2 \text{ kg.h}^{-1}$) of green shore crabs, (*Carcinus maenas*), following a 10 min period of emersion (light handled), or a 10 min period of emersion with shaking (severe handled) and a control group with no emersion or shaking. The data represent the mean (\pm SEM) of 13 animals for each treatment.

between 6 and 10 h. After 10 h, MO_2 reached stable levels and did not change significantly thereafter (Figure 1). Analysis of the whole 20 h experimental period indicated that the nature of the handling stress (light or severe) had no significant effect on MO_2 (RM ANOVA, $F_2 = 2.19$, $P = 0.126$), and no significant interaction was detected between treatment type and time (RM ANOVA, $F_{40} = 1.27$, $P = 0.125$). Because MO_2 stabilized after 10 h and did not change significantly thereafter, changes in MO_2 during the first 10 h were then analysed separately. Here, in addition to a significant decline in MO_2 with time (RM ANOVA, $F_{10} = 43.15$, $P < 0.001$), there was also a significant effect of handling severity (RM ANOVA, $F_2 = 4.99$, $P = 0.012$). The MO_2 rates of the crabs exposed to severe handling stress were significantly higher than controls (Tukey HSD, $P < 0.01$), although not significantly different from that of the lightly handled crabs (Tukey HSD, $P > 0.05$). There was no significant difference between the MO_2 values of lightly handled crabs and controls (Tukey HSD, $P > 0.05$). No significant interaction was detected between the treatment type and time during the first 10 h (RM ANOVA, $F_{20} = 0.84$, $P = 0.667$).

While there was no significant difference in the AS, RMR, or EEE among the three treatments, there were significant differences in MMR and ‘duration’ (Table 1). Both the MMR and the ‘duration’ of severely handled crabs were greater than the control values, though there were no significant differences between the control and lightly handled or lightly handled and severely handled crabs.

There was a clear effect of handling stress on the concentration of glucose in the haemolymph (Figure 2); the concentration changed over time (RM ANOVA, $F_5 = 4.97$,

Table 1. Metabolic parameters for oxygen consumption data from the acute responses to handling stress experiment (Figure 1). Resting metabolic rate (RMR) – lowest five MO_2 datum points, maximal metabolic rate (MMR) highest MO_2 , aerobic scope – (MMR/RMR), ‘duration’ – time in h that MO_2 remained elevated and equivalent energy expenditure (EEE) – calculated as total MO_2 and converted to kJ as a function of crab mass. The data represent the mean \pm SEM of 13 crabs for each treatment. Different letters denote significant differences among treatments ($P < 0.05$).

	Control	Light handling	Severe handling	Statistics
RMR ($\text{mg O}_2 \text{ kg.h}^{-1}$)	26.3 ± 2.5	29.5 ± 2.7	31.6 ± 3.8	$H = 1.06, P = 0.590$
MMR ($\text{mg O}_2 \text{ kg.h}^{-1}$)	78.6 ± 6.8^a	93.9 ± 6.4^{ab}	113.1 ± 8.9^b	$F = 5.38, P = 0.009$
Scope	3.2 ± 0.3	3.4 ± 0.3	4.2 ± 0.5	$F = 1.61, P = 0.214$
Duration (h)	9.0 ± 1.1^a	11.2 ± 1.0^{ab}	13.8 ± 0.8^b	$F = 6.10, P = 0.005$
EEE (kJ)	0.30 ± 0.05	0.29 ± 0.03	0.41 ± 0.06	$H = 4.28, P = 0.118$

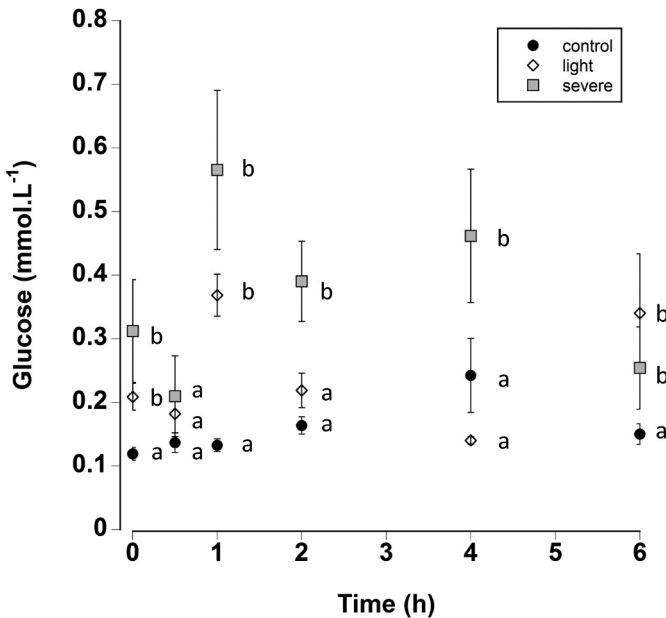


Figure 2. Haemolymph glucose concentrations (mmol.L^{-1}) of green shore crabs, (*Carcinus maenas*) following a 10 min period of emersion (light handled) or a 10 min period of emersion with shaking (severe handled), with subsequent return to seawater. Control animals were not handled or emersed. The data represent the mean (\pm SEM) of eight different animals at each time point. Different letters denote significant differences ($P < 0.05$) among the three treatments at each time point.

$P < 0.001$), and there was a significant treatment effect (RM ANOVA, $F_2 = 29.71$, $P < 0.001$). Because the changes over time depended on the type of treatment, there was also a significant interaction effect (RM ANOVA, $F_{10} = 3.94$, $P < 0.001$). The highest glucose concentrations of $0.56 \pm 0.13 \text{ mmol.L}^{-1}$ were measured in the severely handled crabs. Here, glucose concentrations remained elevated above control levels at 0, 1, 2, and 4 h (Tukey HSD, $P < 0.05$). Crabs in the lightly handled treatment exhibited a greater concentration of glucose than control crabs at 0, 1, and 6 h (Tukey HSD, $P < 0.01$), whereas glucose concentrations in the severely handled crabs were significantly higher than those in lightly handled crabs at 2 and 4 h (Tukey HSD, $P < 0.05$).

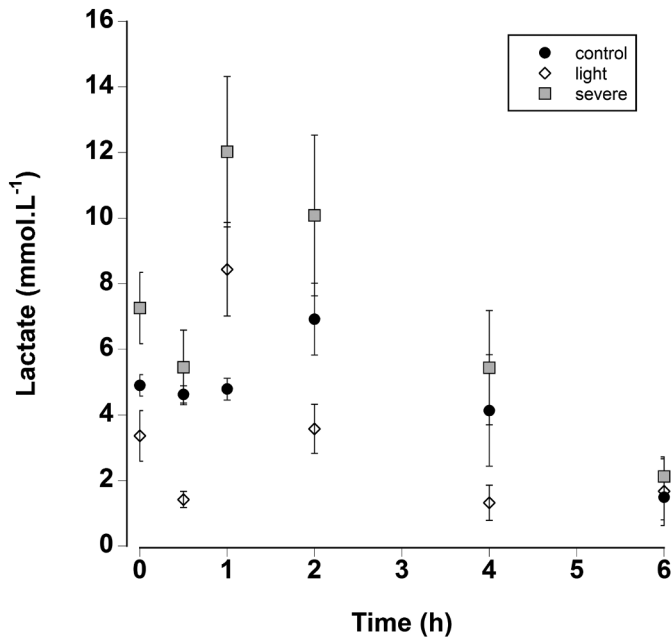


Figure 3. Changes in haemolymph lactate concentrations (mmol.L⁻¹) of green shore crabs, (*Carcinus maenas*), following a 10 min period of emersion (light handled), or a 10 min period of emersion with shaking (severe handled), with subsequent return to seawater, control animals were not handled or emersed. The data represent the mean (\pm SEM) of eight different animals at each time point.

The haemolymph L-lactate concentrations were also significantly affected by both the handling treatment and time (Figure 3), although there was no interaction effect (RM ANOVA, $F_{10} = 1.79$, $P = 0.07$). Generally, L-lactate concentrations in the haemolymph of both severely and lightly handled crabs increased rapidly, reaching the highest values 1 h after the handling stress (RM ANOVA, $F_5 = 16.71$, $P < 0.001$). The concentrations decreased slowly thereafter, and the lowest concentrations of L-lactate for the severe treatment and control crabs were measured at 6 h. Overall the L-lactate concentration also differed among the treatment types (RM ANOVA, $F_2 = 15.06$, $P < 0.001$). The highest overall mean lactate concentration of 7.01 ± 1.45 mmol.L⁻¹ was recorded from the severely handled crabs. Interestingly, the lowest mean concentration (3.31 ± 1.10 mmol.L⁻¹) was measured in haemolymph from crabs that had undergone the light handling stress, with the control levels intermediate to these two mean values. Each of these three treatments was significantly different from one another (Tukey HSD, $P < 0.05$).

Fifteen crabs from each treatment group were monitored in the tanks for 14 d after experiments to determine if these procedures led to an increased mortality. During this time one crab from the control treatment died after 10 d, and one crab from the severe handling treatment died after 12 d. None of the crabs lost any limbs during this period.

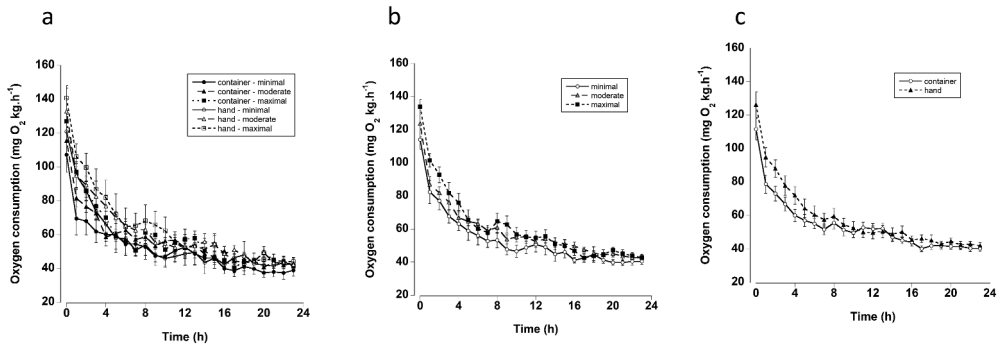


Figure 4. (a) Mean oxygen consumption rates ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$) of green shore crabs, (*Carcinus maenas*), following exposure to six different handling treatments ($n = 8$ per treatment) during transfer from holding tanks to respirometry apparatus. Recording began immediately after transfer to the apparatus and continued for 24 h, (b) Oxygen consumption rates ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$) grouped by severity of transfer stress (minimal, moderate, maximal) ($n = 16$ per treatment) and (c) MO_2 data showing the effects of container or hand transfer by combined the 3 stress level transfer methods, ($n = 24$ per treatment). The data represent the mean \pm SEM at each time point.

Transfer methods during experimentation (Experiment 2)

The crabs in all six treatments exhibited the highest MO_2 rates immediately after transfer to the respiratory chambers (Figure 4(a)), and these declined during the first 14 h of the 24 h experimental period (RM ANOVA, $F_{23} = 67.62$, $P < 0.001$). After 14 h, MO_2 stabilized between 45 and 55 $\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ (Tukey HSD, $P > 0.05$). None of the six transfer methods had a significant effect on MO_2 during the 24 h experimental period (RM ANOVA, $F_5 = 1.23$, $P = 0.314$). There was also no significant interaction between the treatment (transfer method and severity of handling) and time (RM ANOVA, $F_{115} = 0.96$, $P = 0.590$). Because MO_2 stabilized after 14 h, the changes occurring during the first 14 h were then analysed separately. The decrease in MO_2 over time remained significant (RM ANOVA, $F_{14} = 73.83$; $P < 0.001$); however, there was still no significant treatment effect (RM ANOVA, $F_5 = 1.10$; $P = 0.377$), nor an interaction between treatment and time (RM ANOVA, $F_{70} = 1.19$; $P = 0.148$).

The majority of the metabolic parameters, RMR, MMR, AS and EEE were similar among treatments (Table 2). The duration that MO_2 remained elevated for the moderate and maximal hand transfer methods was longer than that recorded for the container minimal transfer method; all other durations were not significantly different from one another.

Changes in MO_2 were also analysed separately for severity of transfer (minimal, moderate, maximal) and by method of transfer stress (container, hand), by grouping data. When investigating the severity of the transfer method (Figure 4(b)), there was a significant decrease in MO_2 with time (RM ANOVA, $F_{23} = 78.59$, $P < 0.001$), but no significant effect of treatment (RM ANOVA, $F_2 = 3.09$, $P = 0.055$), and no interaction (RM ANOVA, $F_{46} = 0.86$, $P = 0.732$). This pattern was similar to the first 14 h of data analysis performed separately (RM ANOVA, time $F_{14} = 75.01$, $P < 0.001$; treatment $F_2 = 3.01$, $P = 0.059$, interaction, $F_{28} = 0.94$, $P = 0.554$).

When the metabolic parameters were analysed, no significant differences were detected for RMR, MMR, AS or 'duration' (Table 3). There was a small,

Table 2. Metabolic parameters for oxygen consumption data from the transfer methods experiment (Figure 4(a)). Resting metabolic rate (RMR) – lowest five MO_2 data points, maximal metabolic rate (MMR) highest MO_2 , aerobic scope – (MMR/RMR), ‘duration’ – time in h that MO_2 remained elevated and the equivalent energy expenditure (EEE) – calculated as total MO_2 and converted to kJ as a function of crab mass. The data represent the mean \pm SEM of eight crabs for each treatment. Different letters denote significant differences among treatments ($P < 0.05$).

	Container – Minimal	Container – Moderate	Container – Maximal	Hand – Minimal	Hand – Moderate	Hand – Maximal	Statistics
RMR ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$)	36.6 \pm 3.0	41.4 \pm 1.3	37.7 \pm 1.2	37.1 \pm 2.4	40.6 \pm 1.7	39.2 \pm 2.7	H = 4.61, $P = 0.0465$
MMR ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$)	107.2 \pm 10.4	116.5 \pm 7.2	127.0 \pm 4.0	121.6 \pm 4.8	133.0 \pm 13.6	140.7 \pm 7.4	F = 1.99, $P = 0.100$
Scope	3.1 \pm 0.4	2.8 \pm 0.1	3.4 \pm 0.2	3.4 \pm 0.3	3.2 \pm 0.3	3.7 \pm 0.3	H = 9.68, $P = 0.085$
Duration (h)	11.1 \pm 1.4 ^a	13.1 \pm 1 ^{ab}	12.1 \pm 1.0 ^{ab}	14.4 \pm 1.1 ^{ab}	16.9 \pm 1.2 ^b	16.4 \pm 1.7 ^b	F = 3.70, $P = 0.007$
EEE (kJ)	0.29 \pm 0.05	0.30 \pm 0.03	0.41 \pm 0.03	0.38 \pm 0.07	0.44 \pm 0.07	0.48 \pm 0.06	H = 9.89, $P = 0.078$

Table 3. Metabolic parameters for oxygen consumption data from the transfer stress experiment, using the three stress methods (minimal, moderate, maximal) (Figure 4(b)). Resting metabolic rate (RMR) – lowest five MO_2 datum points, maximal metabolic rate (MMR) highest MO_2 , aerobic scope – (MMR/RMR), ‘duration’ – time in h that MO_2 remained elevated and equivalent energy expenditure (EEE) – calculated as total MO_2 and converted to kJ as a function of crab mass. Data represent the mean \pm SEM of 16 crabs for each treatment. Different letters denote significant differences among treatments ($P < 0.05$).

	Minimal	Moderate	Maximal	Statistics
RMR ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$)	36.9 \pm 1.9	41.0 \pm 1.0	38.4 \pm 1.4	H = 4.24, $P = 0.121$
MMR ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$)	114.4 \pm 5.8	124.9 \pm 7.7	133.8 \pm 4.4	F = 2.54, $P = 0.090$
Scope	3.2 \pm 0.3	3.0 \pm 0.2	3.5 \pm 0.2	H = 5.37, $P = 0.068$
Duration (h)	12.5 \pm 0.8	15.0 \pm 0.9	14.3 \pm 1.1	F = 1.85, $P = 0.168$
EEE (kJ)	0.34 \pm 0.04 ^a	0.37 \pm 0.04 ^{ab}	0.45 \pm 0.03 ^b	H = 6.28, $P = 0.043$

but significant difference in the EEE among treatments, with energy expenditure being greater during maximal stress than that expended during minimal stress transfer.

Finally, the transfer method (container versus hand transfer) was analysed separately (Figure 4(c)). MO_2 dropped rapidly (RM ANOVA $F_{23} = 79.81$, $P < 0.001$), but there was no difference between container and hand transfer (RM ANOVA, $F_1 = 1.11$, $P = 0.297$), and no significant interaction was detected (RM ANOVA, $F_{23} = 1.45$, $P = 0.078$). When only the first 14 h of data were analysed, the decline in MO_2 remained significant over time (RM ANOVA, $F_{14} = 77.37$, $P < 0.001$), but still, no significant difference was detected between the container and hand transfer methods (RM ANOVA $F_1 = 0.742$, $P = 0.394$). There was, however, a significant interaction between the treatment and time (RM ANOVA, $F_{14} = 2.36$, $P < 0.003$). This occurred because during the first 2 h, MO_2 of container transferred crabs fell more rapidly compared with crabs transferred by hand.

There were also a number of significant differences in the metabolic parameters of container versus hand transfer crabs (Table 4). MMR, EEE and

Table 4. Metabolic parameters for oxygen consumption data from the transfer experiment analysing two transfer methods only (container, hand) (Figure 4(c)). Resting metabolic rate (RMR) – lowest five MO_2 data points, maximal metabolic rate (MMR) highest MO_2 , aerobic scope – (MMR/RMR), ‘duration’ – time in h that MO_2 remained elevated and the equivalent energy expenditure (EEE) – calculated as total MO_2 and converted to kJ as a function of crab mass. The data represent the mean \pm SEM of 24 crabs for each treatment. Different letters denote significant differences among treatments ($P < 0.05$).

	Container	Hand	Statistics
RMR ($\text{mg O}_2 \text{ kg.h}^{-1}$)	38.6 \pm 1.2	39.3 \pm 1.3	F = 0.05, P = 0.830
MMR ($\text{mg O}_2 \text{ kg.h}^{-1}$)	116.9 \pm 4.5 ^a	131.8 \pm 5.4 ^b	F = 4.51, P = 0.039
Scope	3.1 \pm 0.2	3.4 \pm 0.2	H.3.60, P = 0.058
Duration (h)	12.1 \pm 0.6 ^a	15.7 \pm 0.8 ^b	F = 13.19, P < 0.001
EEE (kJ)	0.42 \pm 0.08 ^a	0.58 \pm 0.15 ^b	F = 5.01, P = 0.030

‘duration’ were significantly higher for the hand transferred crabs, versus those transferred by a container (Table 4). The RMR and AS were similar between the two treatments.

Discussion

Stress responses, as indicated by an increase in MO_2 , and haemolymph glucose and L-lactate concentrations, were observed in the crabs after handling. The magnitude of these responses increased with the severity of the handling procedure.

Rates of oxygen consumption (MO_2)

The mean MO_2 of severely handled crabs immediately after handling was approximately 4-fold higher than basal levels. This increase was greater than the 1.75-fold change measured in rock lobsters (*Panulirus cygnus*), after 30 min of intermittent handling in air (Crear and Forteach 2001). The increases in MO_2 recorded here for green shore crabs might be expected to be less pronounced than those of lobsters, as the duration of handling stress used was shorter, and lobsters are not an emersion tolerant species (Whiteley and Taylor 1992; Crear and Forteach 2001; Lorenzon et al. 2007). This suggests that a shorter, yet more severe handling procedure is more detrimental to the animal. This was also apparent in the second experiment, where the shorter transfer duration, but more vigorous handling, resulted in a greater increase in metabolic parameters. In support of this, Norway lobsters (*Nephrops norvegicus*), exhibit higher stress indicators when caught by trawling, as opposed to being hauled in creels (Spicer et al. 1990), although Ridgway et al. (2006) found the response to be the same whether they spend 1 h or 5 h in the trawl. Crabs that were both lightly and severely handled were more active during the procedure and displayed a flared leg posture, which has been interpreted as a behavioural stress response (Stoner 2012). When placed into the respirometry apparatus, the higher MO_2 seemed largely a result of increased activity, which is a typical escape response in stressed crustaceans (Paterson 1993). It was notable that despite the crabs being emersed during the first experiment (Figure 1), but not in the second (Figure 4), a similar increase in

MO₂ occurred. This suggests that the handling process (shaking), rather than emersion, was the main cause of the subsequent elevated stress response. This is supported by Johnson and Uglow (1985), who also suggested that vibrations during experiments could account for the discrepancy in MO₂ levels reported for green shore crabs by different authors.

Oxygen consumption rates took 10 h to return to basal levels in the handling stress experiment and 14 h in the transfer method (second) experiment. The longer time in the second experiment, however, could be attributed to the higher water temperature (14°C vs. 11°C), and thus the metabolic rate of the animal being slightly elevated (Crear and Forteach 2001). The time for settling after handling was similar to other crab species (8–12 h) (McGaw 2006, 2007; McGaw and Curtis 2013), but longer than spiny lobster (*Sagmariasus verreauxi*, 2.5 h, Jensen et al. 2013), rock lobster (*Panulirus cygnus*, 5–8.5 h, Crear and Forteach 2001), and for American lobster (*Homarus americanus*, 6–8 h, Nielsen and McGaw 2016). Although our initial aim was to use the ‘hardy’ green shore crab to generate baseline data for handling protocols and settling times, it is suggested, at least with regard to MO₂, a species-specific approach might be more appropriate.

Haemolymph glucose concentration

Hyperglycaemia occurred in both groups of handled crabs, but was more pronounced in severely handled crabs. The maximum mean glucose concentration of severely handled crabs was approximately 3 to 5-fold higher than control levels, and that of lightly stressed crabs, but only at some time points. Intraspecific variations in glucose concentrations are often reported, and because different animals were used for each reading, this may explain the variance over time observed here (Telford 1968; Paterson and Spanoghe 1997; Matsumasa and Murai 2005). The increase in haemolymph glucose concentration for the light handling treatment was similar to previous levels measured during emersion in green shore crabs (Johnson and Uglow 1985). The vibration (shaking), which would be sensed by statocysts and contact sensillas on the carapace (Mellon 2014), obviously induced a pronounced stress response in the crabs. This type of handling and vibration induces a clear increase in haemolymph glucose in spiny lobsters as well (Paterson and Spanoghe 1997; Paterson et al. 1997).

The glucose (and L-lactate) concentrations in the haemolymph returned to basal levels before the MO₂. Gluconeogenesis requires increased oxygen utilisation once normal conditions are restored, and could explain this delay in recovery of the metabolic rate compared to biochemical indicators (Herreid 1980).

Haemolymph L-lactate concentration

Haemolymph L-lactate concentration is also an important stress indicator in crustaceans (Spicer et al. 1990; Whiteley and Taylor 1992; Santos and Keller 1993; Crear and Forteach 2001; Ridgway et al. 2006). Although L-lactate is typically regarded as an end-product of anaerobic respiration, it can also be used by crustaceans as an energy source (Gladden 2004; Jayasundara and Somero 2013). A 5-fold increase in mean haemolymph L-lactate concentrations occurred in severely handled crabs; these values fall within the range reported for other decapod species exposed to exhaustive handling and emersion (Crear

and Forteach 2001; Ridgway et al. 2006; Aparicio-Simón et al. 2010). Interestingly, overall L-lactate concentrations were lowest in the lightly handled crabs, despite the fact that individuals were emersed for 10 minutes. The green shore crab is an effective bimodal breather, maintaining MO_2 levels in air between 50% (Simonik and Henry 2014) and 120% (Taylor and Butler 1978) of those measured in water, with no accumulation of L-lactate (Taylor and Butler 1978; Johnson and Uglow 1985; Nancollas and McGaw accepted). This suggests that emersion alone cannot account for elevated L-lactate concentrations. Further support for this suggestion comes from the second experiment where the crabs were not emersed, but MO_2 was elevated to a similar magnitude of the lightly handled crabs. Although the emersion may have played a minor role (as evidenced by a transient increase in L-lactate concentration after 1 h in the lightly handled crabs), this increase in L-lactate was more likely due to the stress induced by physical contact with the animal (Figure 4(c)), whereby crabs were transferred by hand back into the holding tank after the treatment period. In line with changes in MO_2 and haemolymph glucose, the greatest increase in L-lactate concentration occurred in the severe handling treatment and remained elevated above control levels for 4 h. The vibrations caused by shaking clearly stressed the crabs, elevating metabolism to a degree where energetic requirements could only be met through anaerobic means. This was illustrated in the higher MO_2 levels of the severely handled crabs when returned to water, reflecting the repayment of oxygen debt in order to metabolize L-lactate and restore oxygen levels within the body tissues (Jensen et al. 2013).

It could be argued that because the crabs were transferred from 12°C water to 20°C air, it was the increase in temperature that was responsible for the increase in L-lactate production (Lorenzon et al. 2007). However, the crabs were in air for only 10 min, and crustacean body temperature can take over 20 min to equilibrate. Even then, evaporation from the body surface means it is always cooler than the surrounding air (McGaw 2003; Payette and McGaw 2003). In addition, if this were the case, a similar rise in L-lactate might be expected in the lightly handled crabs.

Transfer methods during experimentation

Most studies on handling stress have involved commercially important crustaceans and methods to minimize loss of product during transport (reviewed in Fotedar and Evans, 2011; Woll et al. 2010; Stoner 2012). Because nearly all these crustaceans are from subtidal habitats and cannot maintain oxygen supply in air (DeFur 1988), the research has focused on the effects of emersion during transport, rather than the actual handling of animals. The results of the first experiment showed emersion did not have a large effect on green shore crabs; therefore, in the second experiment we focused on handling and transfer methods because these are typically a crucial part of any experimental protocol. When initially transferred to the respirometers (even for control animals), green shore crabs increased their MO_2 , with rapid movement and transfer by hand invoking the highest magnitude responses. In this case, the crab would probably perceive these visual (Hemmi 2004; Smolka et al. 2011), vibrational (Roberts et al. 2016; Tidua and Briffa 2016; Fitzgibbon et al. 2017) and tactile cues (Crawl and Covich 1994; Mellon 2014) as a predator threat. All of these can individually, or in combination, invoke a physiological startle response (McMahon and Wilkens 1972, 1975, 1977; Florey and

Kriebel 1974; Burnovicz et al. 2009; Yang et al. 2013; Yazawa 2015). The startle response is characterized by an instantaneous stoppage of the heart and ventilation typically lasting just a few seconds, followed by a substantial increase in cardiorespiratory parameters; these prepare the animal for the fight or flight scenario (Hermitte and Maldonado 2006; Burnovicz et al. 2009; Yang et al. 2013). The first stimulus the crab would perceive is usually visual, and can be a shadow or a change in light levels that would present during approach to the holding tank (McMahon and Wilkens 1975; Forward 1976; Yazawa 2015). The instantaneous shutdown of the heart and ventilation may help crustaceans avoid being detected by predatory fish that might home in on electrical signals from the beating heart (McMahon and Wilkens 1977). Vibration through water can also be stressful for crabs, especially anthropogenic generated vibrations/noises (Florey and Kriebel 1974; Burnovicz et al. 2009; Roberts et al. 2016; Tidua and Briffa 2016), and thus shaking or rapid movement of the bucket would invoke a larger stress response. The most substantial increase in MO_2 and metabolic parameters were evident in crabs transferred by hand, versus container transfer (Figure 4(c); Table 4). Crustaceans are highly thigmotactic, using sensillas on the body surface that are sensitive to mechanical deformation, to navigate and detect predators and prey (reviewed in Mellon 2014). When picked up, the crabs exhibited leg flaring and/or tried to pinch the handler. Fish, birds and other crustaceans are the main predators that would attack crabs (Donahue et al. 2009), and as such these encounters, which are often terminal, are likely to invoke the highest stress response.

Experimental protocols

The measured stress responses were most apparent during the first 6 h of the experiment, and could be classified as temporary, because they returned to control values between 6 h (haemolymph parameters) and 14 h (MO_2) after handling. In light of current results, a settling period of at least 12 h after handling is recommended. If the experimental protocol does not allow a settling period, one should avoid physically touching the animals by using a container transfer method. Rapid movements and transfer methods are likely to induce an anti-predator-type startle response. A slow transfer avoiding as much vibration as possible will reduce, but not abolish, this response. If needed, the effects of handling can be corrected with a pre-determined stress index (Nancollas 2020), but studies should quote any settlement times and transfer/handling methods, to ensure such handling effects are acknowledged.

Despite the fact that these responses appeared transient, our recent work shows repeated handling of crabs (every 2 weeks for 10–15 min, over 6 months), results in a substantial increase in mortality of 60% for handled crabs versus 3% for non-handled crabs (Wilson et al. In prep. obs.). Therefore, although acute in nature, these repeated stress responses may have chronic downstream effects, known as an ‘allostatic load’ (McEwen and Seeman 1999). These continued periods of stress likely exhaust energy stores, as shown in white shrimp (*Litopenaeus* spp.) subjected to chronic stress (Sánchez et al. 2001; Mercier et al. 2006). Thus, handling procedures should be kept to a minimum when planning long-term, repeated measures experiments on decapod crustaceans.

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