Baseline measurements of physiological and behavioural stress markers in the commercially important decapod Cancer pagurus (L.)

Baseline measurements of physiological and behavioural stress markers in the commercially important decapod *Cancer pagurus* (L.)

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Abstract

Increasing human activities in marine environments pose possibilities of new stressors affecting marine invertebrates important for fisheries. However, assessment of such stressors is hampered by lack of baseline information on stress markers in relevant species, particularly relating to potential diel rhythms. *Cancer pagurus* is the second most important crustacean for UK fisheries and, owing to its migratory habits, is likely to encounter anthropogenic stressors both inshore and offshore. However, there are no baseline measurements of commonly used stress markers in this species, particularly on juveniles, nor data on diel variations. This study aimed to establish baseline data for several stress parameters in *C. pagurus*: haemolymph L-Lactate, D-Glucose, Haemocyanin, haemolymph density and respiration rate, as well as behavioural indicators (activity level, antennular flicking rate) during day and night. L-Lactate and D-Glucose concentrations were positively related to crab size, larger crabs having higher concentrations. L-Lactate and D-Glucose levels followed similar circadian rhythms increasing towards dusk, coinciding with higher locomotor activities. L-Lactate levels in juvenile crabs showed a significantly different pattern compared to larger crabs, with lower concentrations maintained throughout the day without any

47 significant increase at dusk. Haemocyanin concentration and
48 haemolymph density were not affected by crab size, sex or time.
49 However, females (0.06 ± 0.01mg/g/h) consumed significantly more
50 oxygen than males (0.04 ± 0.01mg/g/h). Activity levels increased
51 significantly at night when foraging mainly occurs. Small crabs were
52 more active, but had lower antennular flicking rates compared to
53 medium and large crabs during both day and night. The present
54 work shows that crab size and sampling time influence the value of
55 commonly used crustacean stress markers, suggesting that these
56 factors should be incorporated into any studies monitoring stress
57 responses of *Cancer pagurus*.

58

59 **1. Introduction**

60 It is generally accepted that there are increasing stresses on the
61 marine environment (Crain et al., 2008; Gunderson et al., 2016),
62 including many of anthropogenic origin, such as plastic waste, noise,
63 excess nutrients, thermal effluents, pollutants, acidification and
64 electromagnetic fields (Scott et al., 2018), amongst others.
65 Consequently, there are likely to be negative impacts on marine
66 organisms manifested through stress responses (Chang, 2005),
67 which may ultimately affect organism fitness (Calow and Forbes,
68 1998). Stress responses in crustaceans have been sporadically
69 investigated over the last 20 years, generally in relation to transport
70 of commercial species (Taylor et al., 1997 ; Lorenzon et al. 2008;
71 Barrento et al., 2010, 2011). However, there remains a dearth of

baseline data on stress responses relevant to environmental stressors, particularly in relation to behavioural correlates of stress.

The European edible crab or brown crab (*Cancer pagurus* L.) is a commercially important brachyuran decapod, being exploited throughout Western Europe, from Norway to northern France (Edwards, 1979; Karlsson and Christiansen, 1996). It was worth £13.8million in 2013 in Scotland alone (Marine Scotland 2015) and is the most valuable crab fishery in UK waters (Haig *et al.* 2015). FAO (Food and Agriculture Organization of the United Nations) statistics report increased landings of *C. pagurus*, around Britain and Ireland from 10,000t in 1950 to 29,793t in 2014 (Bannister 2009; The Scottish Government 2015).

Cancer pagurus are both active predators and scavengers, consuming a wide range of prey items, and are found from shorelines to depths of 90m, with larger mature specimens being found offshore (Fish and Fish 2011). Predominantly they are found in the sub-littoral zone where they experience a relatively narrow seasonal temperature range of 4-15°C (Cuculescu *et al.* 1998). Characteristically, they exhibit a nocturnal activity cycle, whereby during daytime they tend to hide with reduced movement levels (Skajaa *et al.* 1998; Scott et al., 2018). Their behavioural repertoire also includes sensing of the environment through flicking of their

antennules, which have been shown to be involved in olfaction (Ref needed).

Several stimuli such as food, pheromones, predators, vibration and sound are known to elicit antennular flicking responses in several crab species (refs needed). Previous studies have utilized antennular flicking rates as a response to a stimulant and/or stressor in hermit crab (Snow 1975), spiny lobster (Daniel & Derby 1991), American lobster (Berg *et al*. 1992), crayfish (Mellon 1997) and Dungeness crab (Woodruff *et al.* 2013).

Respiration rates in marine organisms have been shown to be a reliable indicator of certain environmental stressors (Brown *et al.* 2013; Doney *et al.* 2012; Paterson and Spanoghe 1997). In a paper by Bradford and Taylor (1982) it was demonstrated that *Cancer pagurus* has a high degree of respiratory independence in that, during hypoxic conditions they can maintain a constant oxygen consumption rate until an air saturation percentage of around 38% is achieved, after which their respiration rate dramatically decreases. However, there is a lack of information about juvenile edible crab respiration rates.

In crustaceans, haemolymph sampling, and its subsequent analysis, enables measurement of stress through detection of abnormalities

120 in internal chemical processes. In previous studies (Bergmann *et al.*
121 2001; Durand *et al.* 2000; Lorenzon *et al.* 2011; Taylor *et al.* 1997) it
122 was shown that L-Lactate, D-Glucose and haemolymph densities are
123 useful parameters in measuring stress levels in crustaceans.

124 The aims of the current work were, firstly, to establish baseline data
125 for a variety of haemolymph markers in *C. pagurus* that have been
126 widely used to measure stress responses in other crustaceans;
127 secondly, to determine baseline data for antennular flicking rate,
128 which is related to chemosensing and ventilation rates; and thirdly,
129 to gain insight into diel individual activity levels via remote camera
130 observation.

131

132 **2. Materials and Methods**

133 Intermoult crabs were obtained from local fisherman and via the St
134 Abbs and Eyemouth Voluntary Marine Reserve (St Abbs,
135 Berwickshire, UK). Prior to experimentation each crab was sexed,
136 carapace width (CW; mm) measured and weighed (g). All crabs were
137 categorized into CW size groups (small: 10-79mm; medium: 80-
138 119mm; large: 120mm+). Crabs chosen for experimentation had no
139 damage to the carapace and were missing no more than two legs
140 (i.e., classified as good or perfect condition based on Scott *et al*.'s
141 [2018] condition index). Crabs were kept in a 1000L flow through
142 system at ambient sea temperature (range 13.7-14.5°C) and natural
143 photoperiod (range 12-14 h L) for a minimum acclimation period of
144 1 week and fed on frozen rag worm and live mussel during the

acclimation period. Food was withheld for 24 hours prior to experimentation.

2.1. Behavioural and Response Analysis

2.1.1. Activity Level

Four 70L tanks were connected to a 1000L temperature controlled sump tank which received a constant supply of UV sterilised, filtered sea water. Each tank was shaded along the sides to reduce visual disturbances. A wide aperture mesh was secured over the top of the tanks during the night to prevent the crabs from escaping. A submersible pump was used to pump water via a Hozelock adjustable control panel to the experimental tanks at an equal rate of around 3L/min. A temperature and light pendant (Onset HOBO) was placed into each tank to monitor conditions. Individual crabs were placed into each experimental tank per trial and allowed to acclimate for 1 hour before the start of experiment. After each trial the tanks were drained, sterilised (Virkon aquatic) and refilled.

Four waterproof InfraRed (IR) cameras (Sannce 1080p IR surveillance DVR system) were suspended above the experimental tanks and set to record during each trial. Trials consisted of:

1. Day conditions – 8 hours (08:00am-16:00pm)
2. Night conditions – 8 hours (20:00pm-04:00am)

Footage was then organised into images at every minute elapsed and analysed using Solomon Coder (version – beta 17.03.22). Activity level was then calculated as the percentage of 1-minute

intervals where movement occurred throughout each trial. A total of 92 individuals were analysed in day conditions (small = 26, medium = 20, large = 46) and 49 in night (small = 22, medium = 11, large = 34).

2.1.2. Flicking rate

A 12L glass tank, containing a perforated plastic adjustable arena, was set up on a temperature controlled recirculation system, with a 40L sump tank containing 45µm filtered, UV sterilised seawater and an air stone. The inflow and outflow were separated from the test animal to reduce visual disturbance. The experimental tanks were behind partitions to further reduce external stimuli.

Crabs were acclimated to experimental tanks for 30 minutes prior to testing after which the camera was set to record via a remote. The trials were recorded for a total of 10 minutes. The entire experimental system was sterilised and underwent a full water change after each trial. Temperature, dissolved oxygen and salinity were monitored before and after each trial.

The video data was post-processed with the flicking rate counted for both antennules, for each trial, then converted to average flicks per minute for each crab. Each video file was counted by 3 trained persons to ensure accuracy and consistency.

2.2. Haemolymph Analysis

Crabs were placed individually in temperature controlled (TECO TK1000), experimental tanks for a period of 24 hours, and sampled at 0h (9:00am), 2h (11:00am, large crabs only), 4h (13:00pm), 6h (15:00pm, large crabs only), 8h (17:00pm) and 24 hours (9:00am). Samples were staggered with 5 minutes between each sample taken to ensure consistency with sample times throughout the experiment. The sampling protocol used was:

Haemolymph samples were collected from the fifth walking leg using 1ml syringes with 25G needles. To reduce handling stress this procedure didn't take longer than 60 seconds. Approximately 250µl, 300µl and 700µl were collected from the small, medium and large size groups respectively. The haemolymph was transferred into 1.5ml cryogenic vials (Nalgene) and 50µl of haemolymph from each stored in a separate vial for Haemocyanin analysis. All vials were frozen in liquid Nitrogen and stored in a freezer at -25°C.

Haemolymph samples were deproteinated as per Paterson *et al.* (1997). Proteins were inactivated by adding an equal volume of 0.6M perchloric acid then separated by centrifugation. The supernatant was neutralized with 3M potassium hydroxide. Samples were then stored at -25°C for further analysis.

2.2.1. D-Glucose

D-Glucose concentrations were measured using a D-Glucose assay kit (Sigma GAGO20-1KT) as per Barrento *et al.* (2010). Deproteinated

samples were measured spectrophotometrically at 540nm using a microplate reader (Molecular Devices, Spectramax M5). D-Glucose concentrations (mmol l^{-1}) were calculated from a calibration curve using standards of known concentration.

2.2.2. L-Lactate

L-Lactate concentrations in deproteinated haemolymph samples were measured using L-Lactate reagent (Trinity Biotech; 735-10), per the procedure described by Barrento *et al.* (2010). Samples were transferred to 96-well flat-bottom microplates then measured spectrophotometrically at 540nm. L-Lactate concentrations (mmol l^{-1}) were calculated from a calibration curve using standards of known concentration (Trinity Biotech, Wicklow, Ireland; L-Lactate standards 735-11).

2.2.3. Haemocyanin

Haemocyanin concentrations were determined spectrophotometrically. 50µl of haemolymph was diluted with 2ml chilled distilled water and 280µL of the mixture transferred to a 96-well flat-bottom microplate and absorbances read at 335nm. Haemocyanin concentrations (mg/ml) were calculated from the molar extinction coefficient $E^{mM}_{1\,cm}$= 17.26 as previously described by Harris and Andrews (2005).

2.2.3. Refractometry

The Specific Gravity (SG) of the haemolymph was measured using a V^2 refractometer, which was calibrated before each trial with distilled water. 50µL of haemolymph was added to the refractometer and the specific gravity recorded.

2.3. Respiration

Oxygen consumption trials were performed with 15 juvenile (≤79mm carapace width) crabs. A 46L flow through tank was set up as a water bath, with filtered, UV sterilised seawater connected to a sump tank and temperature control unit to ensure temperature stability (12°C±0.1°C). A 0.3L respiration chamber was filled with UV sterilised filtered seawater and placed into the water bath. Oxygen consumption was recorded using PreSens PSt3 sensor spots (detection limit 15ppb) and an optical oxygen meter (PreSens Fibox 3). Changes in air saturation (%) in the chamber were measured continuously, with a starting O_2 concentration of 100% saturation, for approximately 30 minutes or until 60% air saturation was reached. The system was calibrated as described by Mclean and Todgham (2015) to obtain hyperoxic conditions and account for bacterial load present in the seawater. Calibration for hypoxic levels were obtained by following the manufacturers guidelines (PreSens GmbH, Regensburg, Germany).

For each trial crabs were randomly selected then sexed, weighed and carapace widths measured. The respiration chamber valve was opened and the crab was placed into the respiration chamber. After

an acclimation period of 1 hour, the valve was closed and measurements commenced. Percentage air saturation was measured for each crab and converted to oxygen consumption (mg/g/h).

2.4. Statistics

Results were expressed as mean value ± standard error (SEM). When data met ANOVA assumptions (according to Shapiro-Wilk test for normality and Levene's test for equality of error variances) multiple comparison tests (one-way ANOVA, 2-way ANOVA) were conducted to reveal differences between groups. If data could not meet ANOVA assumptions, non-parametric analysis (Wilcoxon signed rank test, Mann-Whitney, Scheirer-Ray-Hare) was performed. Post-hoc analysis for parametric (Tukey's test) and non-parametrical (pairwise Mann-Whitney) were conducted. All statistics were tested at a probability of 0.05 with the software IBM SPSS Statistics (version 23).

3. Results

3.1. Activity level

There were no significant differences in the activity levels between male and female crabs in both day (male - 7.8±1.4%, female – 7.6±1.8%) and night conditions (male - 33.9±4.1%, female – 37.3±5%). During day conditions, there was a significant difference

in activity level between all size groups ($p<0.0001$). The activity level of the crabs decreased by size (16.5±2.8% – small, 7.3±1.6% - medium, 3±0.7% - large). During night conditions, there was an overall increase in activity levels, compared to day, with size proving to be a significant factor ($p<0.05$). Activity level decreased by size from 44.7±6.8% (small) to 43.1±7.7% (medium) and finally 26.9±3.1% (large).

Figure 1.

3.2. Flicking Rate

There was a difference in flicking rate between size groups ($p=0.03$), with small crabs having significantly lower flicking rate (10.2±3.9flicks/min) compared to large (34.7±7.9flicks/min) crabs. There was no significant difference in flicking rate between males (25.9±6.7flicks/min) and females (19.2±3.8flicks/min).

Figure 2.

3.3. Haemolymph parameters

D-Glucose

D-Glucose concentration ranged in small crabs from 0.09mmol l^{-1} to 0.55mmol l^{-1}, in medium crabs from 0.18mmol l^{-1} to 1.10mmol l^{-1} and in large crabs from 0.05mmol l^{-1} to 1.40mmol l^{-1}. Larger crabs had significantly ($P<0.01$) higher concentrations of D-Glucose (0.62±0.05mmol l^{-1}), than the small size group (0.33±0.08mmol l^{-1}) and D-Glucose levels ($P<0.01$) varied significantly over time. For medium and large crabs mean D-Glucose levels steadily increased to

a peak at 8 hours (medium - 0.68±0.12mmol l^{-1}, large – 0.92±0.12mmol l^{-1}) after the 0h samples (medium – 0.36±0.12mmol l^{-1}, large – 0.39±0.06mmol l^{-1}). The final samples taken after 24 hours (medium – 0.44±0.10mmol l^{-1}, large – 0.66±0.11mmol l^{-1}) showed mean levels closer to baseline values. D-Glucose concentrations for small crabs showed a marginal increase from 0h to 4h but remained relatively constant throughout the 24hour sampling period. Female and male crabs' D-Glucose levels did not differ significantly in any of the size groups or at different sampling times.

L-Lactate

L-Lactate concentrations ranged from 0.09mmol l^{-1} to 7.20mmol l^{-1}in small crabs, 0.09mmol l^{-1} to 8.98mmol l^{-1} in medium crabs, and 0.03mmol l^{-1} to 12.65mmol l^{-1} in large crabs. Size had a significant effect on L-Lactate ($P<0.05$) concentration. L-Lactate ($P<0.01$) varied significantly over time. Larger crabs have higher concentrations of L-Lactate (1.98±0.21mmol l^{-1}), than the small size group (1.47±0.32mmol l^{-1}). L-Lactate concentrations for medium and large crabs remained relatively constant throughout the initial 4 hours (medium – 1.54±0.73mmol l^{-1}, large – 1.89±0.38mmol l^{-1}), after a decrease from 0h (medium – 3.10±0.96mmol l^{-1}, large – 2.19±0.46mmol l^{-1}), but showed an increase at the 8-hour mark (medium – 1.97±1.07mmol l^{-1}, large – 3.73±1.29mmol l^{-1}). In small crabs, there were significant decreases from 0h (3.34±0.78mmol l^{-1}) to 4h (0.44±0.19mmol l^{-1}). The levels then remained constant

including the sample taken at 24hours (0.48±0.13mmol l^{-1}). L-Lactate concentrations did not significantly vary between sexes.

Haemocyanin

Haemocyanin concentrations ranged from 10.04mg/ml to 96.10mg/ml in small crabs, 23.80mg/ml to 89.48mg/ml in medium crabs, and 9.40mg/ml to 79.48mg/ml in large crabs. Haemocyanin levels showed individual variability but were not variable over time. Small (52.2±10.2mg/ml), medium (52±8.6mg/ml) and large (43.5±2.7mg/ml) size groups show that the average Haemocyanin concentration does not significantly differ by size. Sex has no effect on the Haemocyanin concentrations with males (49.2±3.5mg/ml) and females (43.1±4.1mg/ml) recording similar mean values.

Haemolymph Specific Gravity

The Specific Gravity (SG) of haemolymph remained relatively constant over time with no significant differences between sample times. SG values ranged from 1.01 to 1.10 in small crabs, 1.01 to 1.12 in medium crabs, and 1.01 to 1.13 in large crabs. Small (SG=1.058±0.006) and medium (SG=1.048±0.007) crabs had similar average SG whilst large individuals (SG=1.073±0.008) had significantly higher average SG ($P<0.01$). There were no significant differences between average haemolymph SG of male (SG=1.059±0.006) and female (SG=1.058±0.006) crabs.

Figure 3.

Figure 4.

3.4. Respiration rate

Respiration rates of juvenile crabs ranged from 0.013mg O_2/g/h to 0.091mg O_2/g/h. Respiration rates were significantly different between males and females ($p<0.05$) with females (0.06±0.008mg O_2/g/h) consuming more oxygen than males (0.04±0.006mg O_2/g/h).

4. Discussion

4.1. Activity level

Small crabs inhabiting the sub-littoral zone would be subjected to increased predation, environmental factors (freshwater influx, variable temperatures and salinities) and competition for shelter. This suggests that the increase in activity level found in small crabs could aid the crab in survival by actively seeking a secure shelter area to avoid predators. Shelter utilisation is common in many decapod crustaceans (Chapman and Rice 1971; Hockett and Kritzler 1972; Hazlett and Rittschof 1975; Hill 1976) and provides refuge from predation. *Cancer pagurus* has been shown to inhabit pits in the sand when inactive (Hall *et al.* 1991) and were observed utilising rocks, crevices and kelp holdfasts during sample collection for this experiment. Larger crabs that tend to inhabit deeper areas offshore and are likely to experience a decrease in predation rate due to

individuals reaching a size where the ability of a single predator to consume them is surpassed (Paine 1976). Kelp forests and algal beds have significantly higher biomass productivity than the open ocean (Mann 1973; Ricklefs and Miller 2000; Park 2001) which may result in less competition for shelter. Both a decrease in predation and competition for resources may result in lower overall activity levels. The lower activity levels in larger mature crabs were confirmed when a decrease in overall activity of approximately 13% between small juveniles and mature adults during the day and around an 18% decrease during the night were observed. Previous studies have highlighted the nocturnal behaviour of *Cancer pagurus* adults (Skajaa *et al.* 1998), however this is the first confirmation, to our knowledge, that juveniles follow similar patterns. Active foraging takes place at night (Seed 1969; Skajaa *et al.* 1998), with peaks at dawn and dusk, for all size groups. This nocturnal foraging behaviour was confirmed by an increase in activity level of at least 24% approx. at night across all size groups. Whilst ad-hoc observations in holding tanks during this experiment showed that individuals will consume food during the day with an increase in active foraging and consumption during dusk through the night until dawn. This nocturnal activity may be in response to visually receptive predators that commonly prey on *Cancer pagurus* such as birds, cod, seals and wolffish (Rae 1967, 1968; Rae and Shelton 1982; Skajaa 1998). Nocturnal foraging may also widen the food spectrum of *Cancer pagurus* and result in increased pursuit success as has been found in *Carcinus maenas* (Naylor 1960). Sex did not have a significant effect

412 on the activity levels despite previous studies suggesting that male
413 crab movement is limited whilst females migrate over large
414 distances relatively rapidly (Brown & Bennett 1980; Edwards 1979).

415 4.2. Flicking rate

416 Whilst smaller crabs tend to have higher activity levels, larger
417 mature crabs may rely more on chemical sensing through
418 antennular flicking and less on physical exploration during day
419 conditions. In work conducted by Woodruff *et al.* (2013) on the
420 closely related Dungeness crab, *Cancer magister*, the number of
421 antennular flicks per minute ranged from 5.6 to 40 which coincided
422 with earlier work undertaken by Pearson *et al.* (1979), and are in
423 line with the results obtained in this experiment of 5 to 43flicks/min
424 correlated well with these previous studies. There were no
425 significant differences between flicking rates of male and female
426 crabs of any size. Previous studies found that male helmet crabs,
427 *Telmessus cheiragonus*, can detect pheromones, released through
428 the female's urine, via their first antennae (Kamio *et al.* 2000). If
429 pheromones are utilised by *Cancer pagurus* females in mate
430 attraction then there may be seasonal variations whereby males will
431 have increased antennular flicking. This could occur during the lead
432 up to the breeding season, when mature females may release
433 pheromones intended to attract a male prior to moulting and
434 subsequent mating.

435 4.3. Haemolymph parameters

As previously reported by Barrento *et al.* 2010 there were no differences in haemolymph parameters between males and females. The D-Glucose concentrations found correspond to those in previous literature (Barrento *et al.* 2010; Lorenzon *et al.* 2008; Watt *et al.* 1999). These results correlate well with current literature which suggests that D-Glucose levels continually rise in relation to increased locomotor activity (Hamann 1974; Kallen *et al.* 1988; Kallen *et al.* 1990; Reddy *et al*. 1981; Tilden *et al.* 2001). This suggests that levels would continue to rise throughout the night, before decreasing back to original levels at the next sampling time exactly 24 hours later. D-Glucose, which is the primary fuel for ATP formation in crustaceans, is essential to maintain metabolic processes (Barrento *et al*. 2010). Activity in crabs should partially be reflected in D-Glucose concentrations (Briffa & Elwood 2001). A negative correlation between D-Glucose levels and vigour has been shown where healthy individuals have lower levels and weak and moribund crabs have become hyperglycaemic (Barrento *et al.* 2010). D-Glucose levels have been found to vary significantly in individual crabs as it is controlled by individual physiological status and reactions to external stimulus (Matsumasa & Murai 2005). L-Lactate, as a metabolite, is typically an indicator of anaerobic respiration due to impaired gill function or hypoxic/anoxic conditions (Durand *et al.* 2000). L-Lactate levels were found to be highly variable in individuals of all sizes. L-Lactate levels found during this experiment fit within the range from previous studies (Barrento *et al.* 2010; Barrento *et al.* 2011; Lorenzon *et al.* 2008;). In these studies L-Lactate

462 concentrations ranged from basal levels of 0.34±0.008mmol l^{-1} to
463 41.9±8.9mmol l^{-1} after 48-hour semi-dry transport. The change in
464 concentration over time suggests that L-Lactate levels follow the
465 same trends as D-Glucose, with higher levels during peak
466 locomotory activity, typically occurring during the night.
467 Haemocyanin levels have been shown to increase during periods of
468 hypoxia, where additional proteins are required to transport oxygen
469 (Hagerman *et al.* 1990), therefore as expected no significant
470 changes were found in this experiment.

471 4.4. Respiration rate

472 Bottoms (1977) and Burnett and Bridges (1981) described that short
473 term rhythmic patterns are present in the respiration of this species,
474 where there are alternating periods of apnoea and bradycardia in
475 the branchial chambers. It has been suggested that these apnoea
476 pauses could enable the animal to save on metabolic energy during
477 periods of inactivity by reducing the energy spent on pumping both
478 water and blood (Bottoms 1977; McMahon and Wilkens 1977;
479 Burnett and Bridges 1981). Respiration rates could therefore vary
480 quite significantly between periods of apnoea (pausing behaviour)
481 and normal pre-pause respiration. Respiration rates of mature
482 *Cancer pagurus* were reported as being 28.03mg O_2/g/h during pre-
483 pause and 4.42mg O_2/g/h post-pause (Bradford and Taylor 1982). In
484 a study by Burnett and Bridges (1981) it was found that the crabs
485 were utilising this pausing behaviour 40-50% of the time. The
486 respiration rates of juvenile crabs observed in this experiment

correspond well with those of different crab species, of similar size, in previous studies: velvet swimming crab, *Necora puber*, (0.21 ±0.119mg O_2/g/h (Small *et al.* 2010)); spider crab, *Hyas araneus*, (0.025mg O_2/g/h (Camus *et al.* 2002)); and Dungeness crab, *Cancer magister*, (0.044mg O_2/g/h (Johansen *et al.* 1970)). Respiration values found in the literature for the shore crab, *Carcinus maenas*, vary from 0.33mg O_2/g/h (Newell et al. 1972); 0.071mg O_2/g/h (Taylor & Butler 1978) and 0.071 – 0.122mg O_2/g/h (Taylor & Wheatly 1979). However, these values are still closely related to juvenile *Cancer pagurus* respiration rates found in this study,

5. Conclusions

There is a natural cycle of L-Lactate and D-Glucose concentrations in the haemolymph of *Cancer pagurus,* which must be fully understood to successfully measure the effects of anthropogenic and environmental stressors on this species. Both cycles appear to approximately follow a 24-hour period that is directly linked with activity level and nocturnal behaviour. There is a clear size-linked difference in L-Lactate and D-Glucose concentrations in crabs, with high individual variability. L-Lactate and D-Glucose concentrations in small crabs do not follow the same pattern as that found in medium and large crabs. Medium and large crabs D-Glucose and L-Lactate concentration rose during afternoon and evening hours whilst D-Glucose level remained constant and L-Lactate levels decreased in small crabs. Haemocyanin and the density of the haemolymph do

not follow a 24-hour cycle, and remain constant throughout different sizes of crab. The activity levels of this species increase significantly during the night, with peaks at dawn and dusk, when foraging takes place. There is a higher activity level in smaller crabs, but a lower antennular flicking rate, during both day and night, suggesting that smaller crabs rely more on movement and physical exploration more than the chemical and vibrational sensing that is heavily utilised in larger crabs. Respiration rate varies between male and female crabs, with female crabs consuming more oxygen per hour. The use of L-Lactate and D-Glucose concentrations, as a stress parameter, in future research must factor in the natural diel cycles and individual variation to accurately determine potential affecting factors. The sole use of adult crabs in previous studies does not necessarily best represent the potential effects on this species. Juvenile crabs are found to have higher activity levels, lower antennular flicking rates, and lower L-Lactate and D-Glucose concentrations than those found in medium and large crabs. These factors must be considered during future work with this species.

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536

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Figure Legends

Figure 1: Activity level (mean ± SEM) for the three size groups in both day and night conditions, along with combined totals.

Figure 2: Flicking rate (mean ± SEM) for the three size groups, also split by sex. A total of 10 individuals were used for each size group, n=30.

Figure 3: D-Glucose concentration (mean ± SEM) over the 24-hour sampling period for the three size groups of crabs. Samples were taken at 2h and 6h for large crabs only. n= 7 (small) 8 (medium) 38 (large).

Figure 4: L-Lactate concentration (mean ± SEM) over the 24-hour sampling period for the three size groups of crabs. Samples were taken at 2h and 6h for large crabs only. n=8 (small) 8 (medium) 38 (large).

769 **Figure 1.**

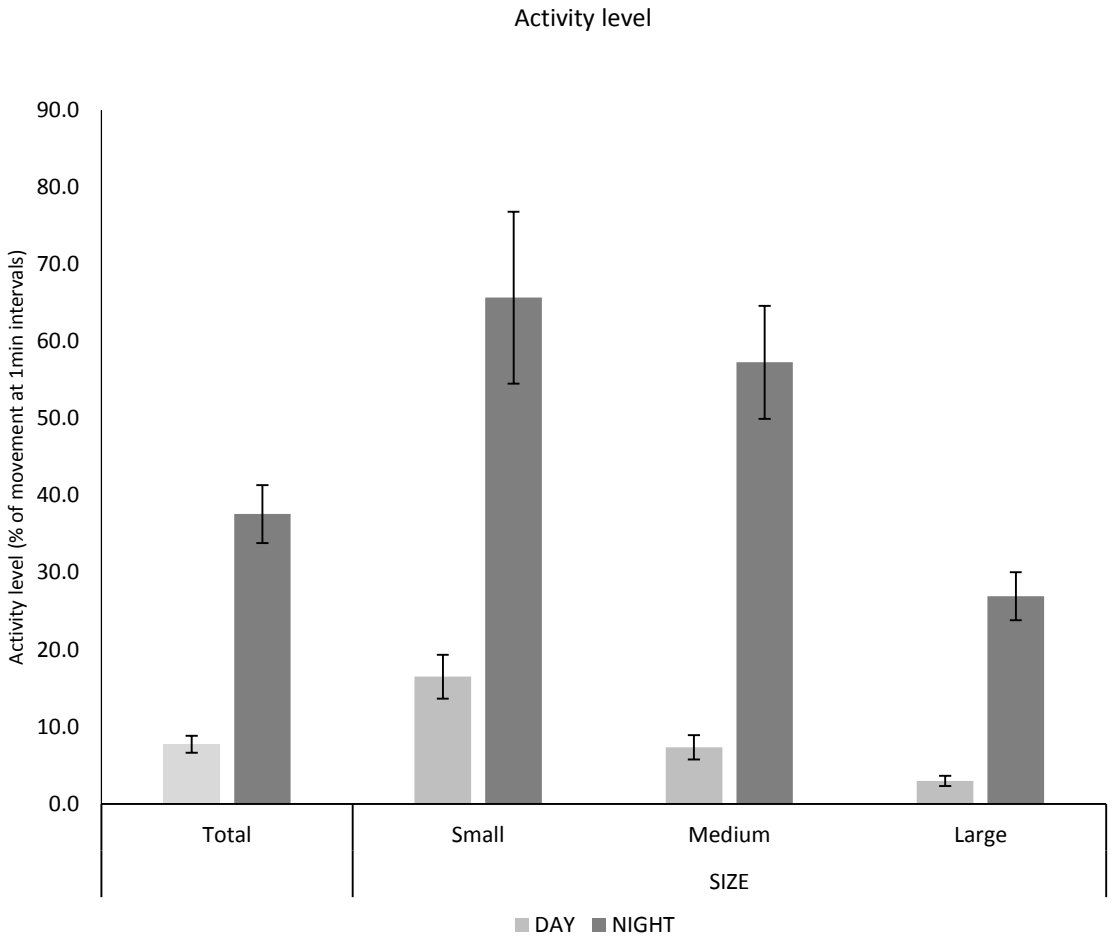

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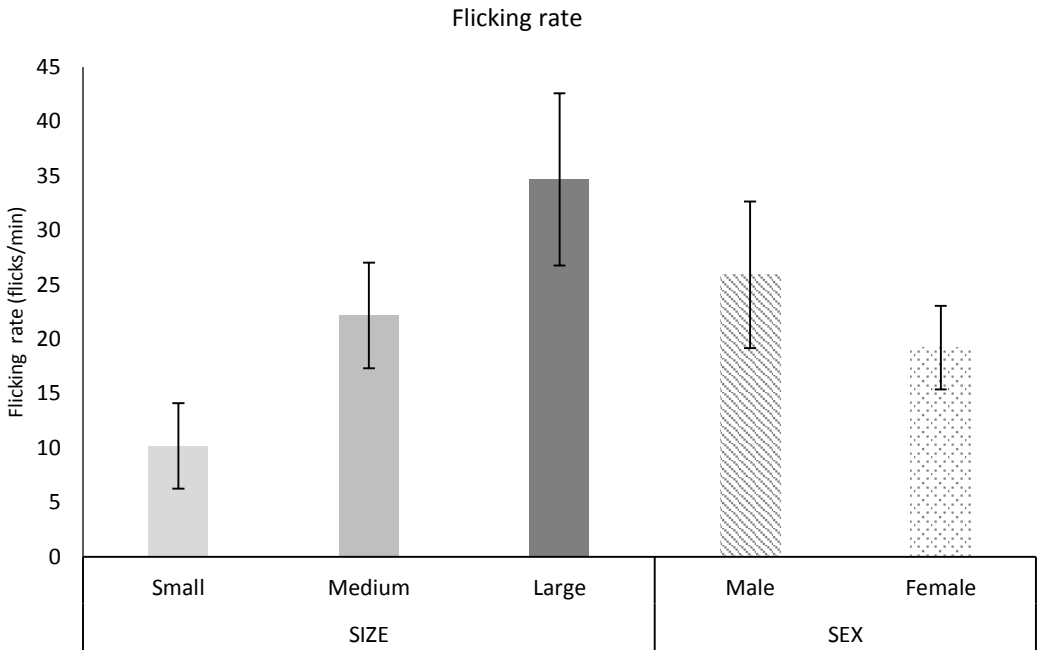
Figure 2.


Flicking rate
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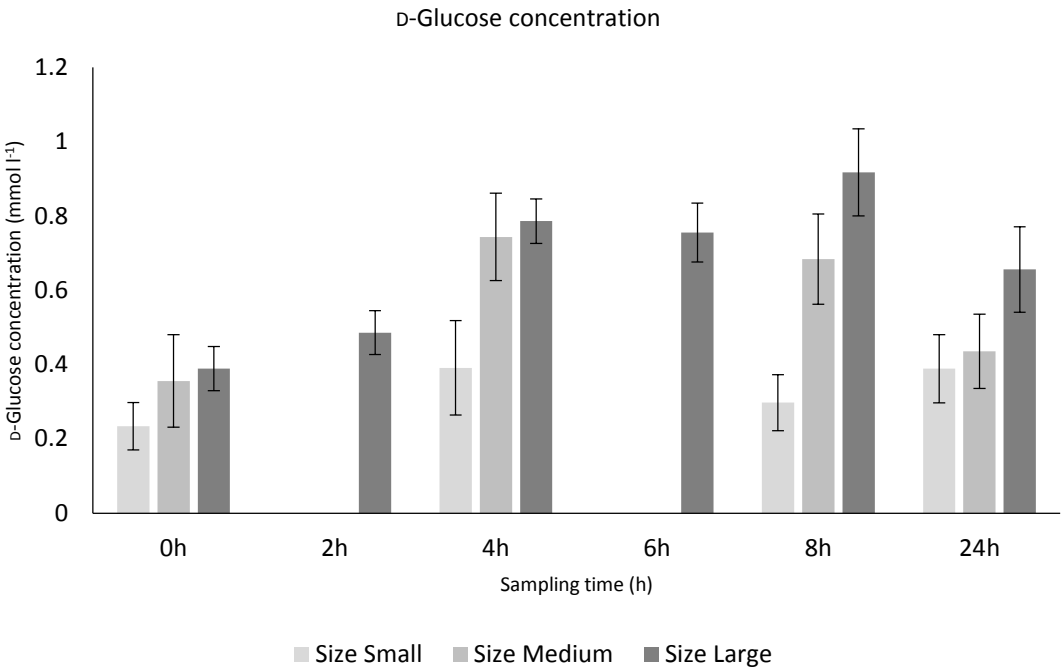
802 **Figure 3.**



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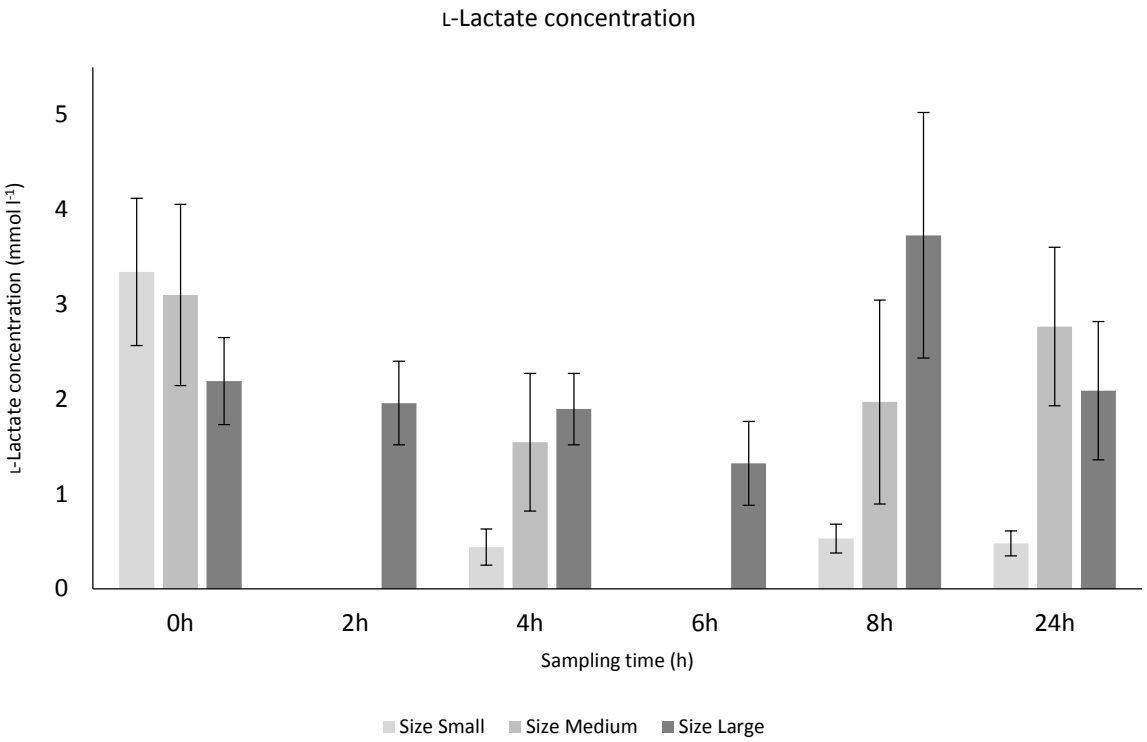
822 **Figure 4.**



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