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

1. Introduction

It is generally accepted that there are increasing stresses on the marine environment (Crain et al., 2008; Gunderson et al., 2016), including many of anthropogenic origin, such as plastic waste, noise, excess nutrients, thermal effluents, pollutants, acidification and electromagnetic fields (Scott et al., 2018), amongst others. Consequently, there are likely to be negative impacts on marine organisms manifested through stress responses (Chang, 2005), which may ultimately affect organism fitness (Calow and Forbes, 1998). Stress responses in crustaceans have been sporadically investigated over the last 20 years, generally in relation to transport of commercial species (Taylor et al., 1997; Lorenzon et al., 2008; 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, 2017) 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 10,000 t in 1950 to 29,793 t 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 90 m, 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

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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 (Stensmyr et al., 2005).

Several stimuli such as food, pheromones, predators, vibration and sound are known to elicit antennular flicking responses in several crab species (Keller et al., 2003; Stensmyr et al., 2005). Previous studies have utilized antennular flicking rates as a response to a stimulant and/or stressor in hermit crab (Snow, 1975), spiny lobster (Daniel and 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 in internal chemical processes. In previous studies (Bergmann et al., 2001; Durand et al., 2000; Lorenzon et al., 2011; Taylor et al., 1997) it was shown that L-Lactate, D-Glucose and haemolymph densities are useful components in measuring stress levels in crustaceans.

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

2. Materials and methods

Intermoult crabs were obtained from local fisherman and via the St Abbs and Eyemouth Voluntary Marine Reserve (St Abbs, Berwickshire, UK). Prior to experimentation each crab was sexed, carapace width (CW; mm) measured and weighed (g). All crabs were categorized into CW size groups (small: 10-79 mm; medium: 80-119 mm; large: 120 mm +). Crabs chosen for experimentation had no damage to the carapace and were missing no more than two legs (i.e., classified as good or perfect condition based on Scott et al.'s, 2018 condition index). Crabs were kept in a 1000 l flow through system at ambient sea temperature (range 13.7–14.5 °C) and natural photoperiod (range 12–14 hl) for a minimum acclimation period of 1 week and fed on frozen rag worm and live mussel during the acclimation period. Food was withheld for 24 h prior to experimentation.

2.1. Behavioural and response analysis

2.1.1. Activity level

Four 70 l tanks were connected to a 1000 l 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 3 l/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 h 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 h (08:00 am-16:00 pm)
2. Night conditions – 8 h (20:00 pm-04:00 am)

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-min 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 12 l glass tank, containing a perforated plastic adjustable arena, was set up on a temperature controlled recirculation system, with a 40 l sump tank containing 45 μm 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 min prior to testing after which the camera was set to record via a remote. The trials were recorded for a total of 10 min. 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. A total of 10 individuals were used for each of the three size groups ($N = 30$).

2.2. Haemolymph analysis

Crabs were placed individually in temperature controlled (TECO TK1000), experimental tanks for a period of 24 h, and sampled at 0 h (9:00 am), 2 h (11:00 am, large crabs only), 4 h (13:00 pm), 6 h (15:00 pm, large crabs only), 8 h (17:00 pm) and 24 h (9:00 am). Samples were staggered with 5 min 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 1 ml syringes with 25G needles. To reduce handling stress this procedure didn't take longer than 60 s. Approximately 250 μl, 300 μl and 700 μl were collected from the small, medium and large size groups respectively. The haemolymph was transferred into 1.5 ml cryogenic vials (Nalgene) and 50 μl 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 Patersonand and Spanoghe (1997). Proteins were inactivated by adding an equal volume of 0.6 M perchloric acid then separated by centrifugation. The supernatant was neutralized with 3 M potassium hydroxide. Samples were then stored at −25 °C for further analysis. A total of 53 crabs were used for D-Glucose trials and 54 for L-Lactate, comprising of all three size groups.

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 540 nm 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.