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Effects of emersion and re-immersion on physiological and immunological variables in creel-caught and trawled Norway lobster *Nephrops norvegicus*

C. J. Bernasconi, R. F. Uglow*

Department of Biological Sciences, University of Hull, Hull HU6 7RX, UK

ABSTRACT: Immune defence in creel-caught and trawled *Nephrops norvegicus* was investigated to assess a possible relationship between phenoloxidase (PO) activation and the total haemocyte count (THC). Capture, capture method and emersion evoked physiological and immunological responses that may have implications for the ability of *N. norvegicus* to survive the effects of such stressors. Haemolymph THC was always negatively related to PO activity in the trawled samples, suggesting a decreased level of the plasma serine proteinase inhibitors which reportedly regulate the ProPO system (Le Moullac et al. 1998; Fish shellfish Immunol 8:621–629). In contrast, creel-caught samples showed increased levels of both PO and THC (cf. control *N. norvegicus*), after a 12 h emersion period. Trawling and emersion evoked progressive and significant increases ($p < 0.05$) in the mean levels of haemolymph L-lactate, glucose and total ammonia. The evidence of overt activity and measured haemolymph parameters suggest that creel fishing yields *N. norvegicus* that are more likely to survive post-harvest treatments than those that are trawled.

KEY WORDS: *Nephrops norvegicus* · Immune defence · Catching methods · Emersion · Immersion · Intrinsic quality maintenance

INTRODUCTION

Crustaceans lack immunoglobins, but they have phagocytic haemocytes that aid wound closure and clotting (Bauchau 1981) and a humoral system, both of whose activities are coordinated by a cell-based phenoloxidase (PO) enzyme cascade system. In crustaceans, mechanical injuries or the presence of foreign objects result in melanin deposition around the damaged tissue or intruding object. This acts as a physical shield against an intruder, preventing or retarding its growth but more importantly, resulting in the formation of highly reactive and toxic quinine intermediates.

'Non-self' recognition is via the haemocyte-borne prophenoloxidase (ProPO) activating system (Söderhäll 1982). The active enzyme PO (*o*-diphenol-oxygen oxidoreductase) is produced during activation of the immune system by microbial cell wall components. It is proposed to be a self-recognition system because ProPO conversion to PO can be induced by minuscule amounts of the stimulating molecules (Söderhäll & Cerenius 1998, Yildiz & Atar 2002). Morphologically different haemocyte types like hyalinocytes, semigranulocytes and granulocytes (Bauchau 1981, Söderhäll & Smith 1983) are attributed with specific functional activities (e.g. ProPO activation, Cerenius & Söderhäll 1995). Phagocytosis, coagulation, release of agglutinins and synthesis of melanin may also be evoked by abnormal environmental stressors (Jussila et al. 1997).

Current models of the crustacean PO system suggest that ProPO is released as a zymogen from the semigranular and granular cells into the plasma where it is activated by a serine proteinase. Söderhäll & Smith (1983) first isolated ProPO in the granulocytes, but found no ProPO activity in the plasma. In contrast, Per-

*Corresponding author. Email: r.f.uglow@hull.ac.uk

azzolo & Barraco (1997) found plasma ProPO activity to be <10% of total activity in *Penaeus paulensis* plasma, while Hernandez-Lopez et al. (1996) found 83% of the PO activity to be located in plasma in the spiny lobster *Panulirus interruptus.*

The commercial transportation of live crustaceans is now common in the highly specialised live seafood trade that demands quality products to be delivered consistently and cost-effectively. The transportation phase may be of short distance or duration (10s of km or hours, e.g. to home port) or long (days or 1000s of km, e.g. from the UK to Spain or Russia). Economic considerations dictate exclusion of seawater and long emersion times for longer journeys. In many instances, the delivered animals are sold rapidly as 'fresh' products, but there is increasing demand for animals that could be husbanded following delivery so that the sale period can be extended to match consumer demand. Unfortunately for *Nephrops norvegicus*, the practices and methods used along such marketing chains — from capture to final sale — are not ideally suited to survival or general quality maintenance, thus tending to jeopardise commercial goals. Frequently encountered stressors include extended periods of emersion, desiccation, rapid temperature and/or salinity changes and supranormal internal and/or external ammonia levels. In addition, physical damage particularly to limbs, occurs frequently as a consequence of catching and postharvest handling, and this results in debilitating blood loss (Uglow et al. 1986).

The present study aimed to use total haemocyte count (THC) and PO as haematological indicators of environmentally induced immune defence in a commercially fished marine decapod crustacean, (Smith & Johnston 1992, Hauton et al. 1995, Jussila et al. 1997), so as to provide additional information on changes evoked by events associated with capture and capture methods, including emersion. Such information would supplement the quantitative data on other parameters (e.g. haemolymph lactate, glucose, and ammonia levels) that are anticipated to change with stressor intensity. Commercially, *Nephrops norvegicus* are often required to be delivered alive, in good condition, and also with high expectancy of survival on being returned to seawater holding systems. As some postharvest emersion is inevitable, particular attention has been paid to determining the modifying effects of emersion duration. Because it is generally assumed that capture methods differ in the severity of their imposed stress, these studies were made using both trawled and creel-caught *N. norvegicus.* There is now a global dimension to the trade in live crustaceans and a better appreciation of the physiological effects of capture methods and emersion duration is required to facilitate more efficient, cost-effective distribution systems and provide better insights into the responses of these animals to such stressors. Behavioural or physiological strategies that serve to increase tolerance to low oxygen events will have considerable adaptive value for the species and may offer an avenue to explore new ways of distributing these commercially valuable animals.

MATERIALS AND METHODS

Creel-caught *Nephrops norvegicus* were obtained from the NW coast of Scotland and long-trawled *N. norvegicus* from the NE coast of England. The creel-caught animals were brought from the holding ponds of a Scottish dealer to the laboratory, and the long-trawled animals were from the final trawl of the day. All *N. norvegicus* were brought to the laboratory in polystyrene boxes containing an ice gel pack. At Hull University, they were maintained in plastic, opaque tanks (1.5 m inner diameter, 500 l) supplied with biologically filtered, recirculating, aerated seawater (salinity = 35 ± 1; temperature = 5°C; a 12:12 h light:dark photoperiod and <100 µmol NH_4 l^{-1}). Prior to being used for experiments, all *N. norvegicus* were held for 24 h to allow temperature acclimation and recovery from transportation and handling stresses.

Groups of 18 adult *Nephrops norvegicus* from each capture method (mean weight = 56.40 ± 1.01 g for creel-caught and 50.35 ± 1.65 g for trawl-caught samples) were emersed for 0 h (control) and 12 h. No mortalities occurred in any group. Experimental emersion involved careful removal from the water and packing in a polystyrene box with seawater-dampened newspaper and a large gel ice pack. Each box was stored for the appropriate duration in a temperature-controlled room (5 ± 1°C). At the end of the 12 h emersion period, each box was unpacked and 6 lobsters were re-immersed for 2 h into individual, acid-washed tanks each holding 1 l seawater [salinity = 35; <10 µmol total ammonia (TA) l^{-1}]. Haemolymph samples were collected from separate groups (n = 6) prior to emersion (control), after 12 h emersion, and at the end of the 2 h re-immersion period. Emersion and re-immersion durations are based on those that occur in many consignments of *Nephrops* made daily in the UK.

Haemolymph samples (1.5 ml) were collected using a 2 ml syringe (Hamilton), the needle (23 gauge) of which was inserted either through the arthrodial membrane at the base of the 5th pereiopod, or beneath the cephalothorax and into the pericardium. Each haemolymph sample was divided into 3 aliquots and kept in labelled microcentrifuge tubes kept on ice.

Haemolymph L-lactate was measured using Trinity Biotech diagnostic kit no. 735-10 and haemolymph

glucose using Sigma diagnostic kit no. 510-A. Ammonia concentrations were measured using a flow-injection gas diffusion system (Hunter & Uglow 1993). PO activity was determined by adapting the methods of Söderhäll (1981), Smith & Söderhäll (1983), Söderhäll & Smith (1983), and Jackson et al. (1993). For this assay, all glassware and pipette tips were washed with liposol to remove any trace oxidants before being rinsed twice in deionised water and once in Milli-Q water. These were then sterilised by autoclaving at 120°C for 20 min to remove any bacterial films. All buffers and reagents were made up with Milli-Q water as required (Hauton et al. 1995).

Haemolymph samples (0.6 ml each) required for the ProPO studies were extracted using a 2 ml syringe with a 23 gauge needle (Hamilton), containing 0.4 ml of ice-cold citrate EDTA buffer (pH = 4.6) (Söderhäll & Smith 1983) as an anticoagulant. Each sample was centrifuged at 450 rpm for 15 min and the pellet of haemocytes was washed twice, without re-suspension, with 2 ml of ice-cold 0.01 M sodium cacodylate citrate buffer at pH 7 (Jackson et al. 1993). Samples were then rapidly frozen by immersion in liquid nitrogen, in 2 ml of 0.01 M sodium cacodylate buffer at pH 7, and stored frozen until analysis. Prior to analysis, each sample was slowly defrosted and homogenised using a glass piston homogeniser on ice at a constant room temperature of 5°C. Each sample was then centrifuged at 900 rpm at 3°C for a further 25 min to remove cell debris and the supernatant, designated HLS was then assayed for phenoloxidase activity.

PO occurs as an inactive zymogen within the granulocytes of crustaceans and thus requires activating by an elicitor, in this case trypsin. Four hundred µl of HLS were incubated at 15°C with 400 µl of 0.1% trypsin (0.5 Anson units g^{-1} from beef pancreas) in cacodylate buffer. After 1 h, 400 µl of dihydroxyphenylalanine (L-dopa, 4 g l^{-1} in Milli-Q water) was added to the activated HLS and the change in the absorbance of the sample was measured over the first 5 min. A control was prepared for each individual sample to correct for any background oxidation of the L-dopa by the cacodylate buffer or the inactive HLS. The controls consisted of 400 µl each of HLS, cacodylate buffer (to replace the trypsin) and L-dopa, and similarly treated as the samples. Enzyme activity was expressed in units where 1 unit represented an increase in absorbance of the sample of 0.001 min^{-1} at 490 nm. All measurements were made on a Cecil CE 292 Digital Ultraviolet Spectrophotometer.

THCs for individual animals were estimated with a haemocytometer under 100× magnification, using 0.15 ml of haemolymph drawn from the pericardial sinus with a 2 ml syringe. The haemolymph was transferred to a 0.6 ml microcentrifuge tube containing 0.15 ml of ice-cooled anticoagulant for THC. The sample was immediately placed on ice. From this diluted sample, 10 µl were taken and transferred into a Neubauer haemocytometer (Hausser Scientific). Two replicates each of four 0.004 mm^3 sections were counted and the mean was taken as the haemocytometer count. THC (cells ml^{-1}) was calculated as:

$$THC = HC/8 \times CF/0.004 \times DF$$

where HC is the haemocytometer count, CF is the conversion factor (1000) for changing mm^3 to ml, and DF is the dilution factor (2). THC analysis was completed within 2 h; cell lysis and haemolymph coagulation occurred after 3 h.

During this investigation, the plasma was discarded during the preparation of the haemocyte lysate supernatant (HLS). As a consequence, the levels of PO activity given here indicate the total amount of ProPO being stored in the granulocytes only and do not indicate any of the activated PO that may have been present in the plasma. Preliminary tests supported the findings of Söderhäll & Smith (1983) of undetectable ProPO activity in the plasma of *Nephrops norvegicus*.

All data are represented as means ± SE. The number of lobsters measured (n) is given in parenthesis. Statistical differences between sample means were tested for significance using *t*-test or ANOVA and Levene's test for homogeneity of variances. Multiple range tests were used to identify groups that were significantly different at $p < 0.05$. For data with non-normal distribution, the less stringent, non-parametric, Mann-Whitney *U*-test was adopted.

RESULTS

No mortalities occurred during these investigations, but the trawled samples had noticeably reduced movements compared with those that were creel-caught. The haemolymph of the trawled group also had significantly higher levels of some quantifiable variables that are generally interpreted as being indicative of the impact of stressors (cf. the creeled group) (Table 1). Only haemolymph glucose levels were the same in the 2 groups.

The 12 h emersion period evoked increases in PO activity that were significant ($p < 0.05$) in the trawled group only (Fig. 1), and re-immersion did not affect the trawled group which maintained a significantly higher HLS PO activity than its control group ($p < 0.05$).

The effect of the 12 h emersion period on THCs was a significant increase in the creeled group and a significant decrease in the trawled group ($p < 0.05$ in each case). The 2 h re-immersion period had no significant effect on the THCs of either group ($p > 0.05$ in both cases) (Fig. 2).

Table 1. Concentrations (mean ± SE) of some haemolymph constituents in creeled and trawled *Nephrops norvegicus* (n = 6) before and after emersion and re-immersion (re-imm). TA: total ammonia; PO: phenoloxidase; abs: absorbance; THC: total haemocyte count

		Ammonia (µmol TA l^{-1})	Glucose (mmol l^{-1})	Lactate (mmol l^{-1})	PO activity (abs min^{-1} ml^{-1})	THC (10^6 cells ml^{-1})
0 h	Creeled	207.13 ± 63.33	0.46 ± 0.07	0.06 ± 0.02	0.14 ± 0.02	5.9 ± 0.93
12 h	Emersed	691.79 ± 83.37	0.37 ± 0.11	0.51 ± 0.26	0.20 ± 0.07	11.0 ± 2.07
+2 h	Re-imm	388.30 ± 89.75	0.20 ± 0.06	0.36 ± 0.24	0.14 ± 0.03	10.0 ± 1.37
0 h	Trawled	931.35 ± 122.18	0.55 ± 0.17	3.20 ± 1.80	0.35 ± 0.12	17.0 ± 1.25
12 h	Emersed	937.31 ± 136.77	0.79 ± 0.18	17.88 ± 3.62	0.70 ± 0.15	1.1 ± 0.16
+2 h	Re-imm	200.31 ± 37.06	1.63 ± 0.29	17.93 ± 2.47	0.70 ± 0.21	0.7 ± 0.07

In the creeled group, circulating glucose levels did not change significantly during emersion or re-immersion and this contrasted with the progressive hyperglycaemia that occurred in the trawled *Nephrops norvegicus*. The increase in the trawled group was not significant after 12 h emersion but was significantly higher ($p < 0.05$) than that of the control by the end of the 2 h re-immersion period (Fig. 3).

Emersion resulted in significant increases ($p < 0.05$) in the mean concentration of haemolymph L-lactate in both groups (Fig. 4, Table 1). That in the creeled group, however, remained significantly lower than in the trawled group (0.51 ± 0.26 and 17.88 ± 3.62 mmol l^{-1}, respectively). The 2 h re-immersion period evoked further increases in both groups but this only reached statistical significance ($p < 0.05$) in the trawled group.

After 12 h emersion, the blood ammonia level in the creeled group was significantly higher ($p < 0.05$) than that of its control group (Fig. 5, Table 1). However, this was still significantly lower than the level in the corresponding trawled group, which showed no significant effects attributable to emersion ($p > 0.05$). Blood ammonia levels decreased significantly in both groups during the 2 h re-immersion period ($p < 0.05$ in both cases) but this change was particularly prominent in the trawled group ($p < 0.05$), which had a final value significantly less than that of the creeled group and its own control group ($p < 0.05$ in each case). The creeled group had a final blood ammonia level that was significantly higher than that of its own control group.

DISCUSSION

These findings indicate that *Nephrops norvegicus* have different behavioural strategies and physiological tolerances, and thus show different responses to altered environmental variables such as emersion. Such environmental changes alter the immune status of crustaceans (Perazzolo & Barracco 1997, Le Moullac et al. 1998, Söderhäll et al. 2003). Here we have shown that commercial fishing methods for decapod crustaceans (e.g. creeling and trawling) are also stressful and result in a diminished immune vigour as measured

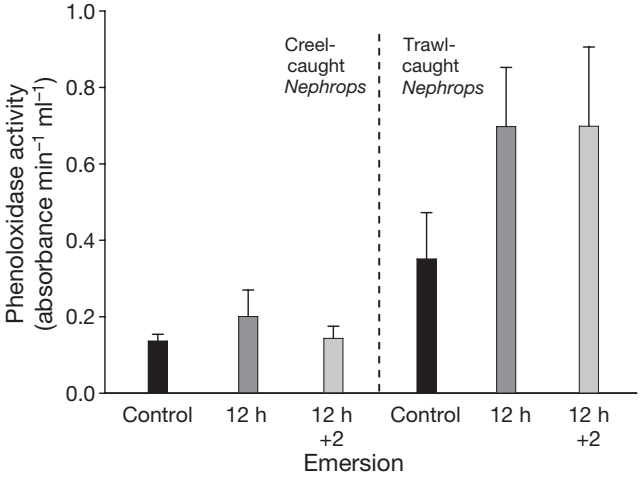

Fig. 1. *Nephrops norvegicus*. Haemolymph phenoloxidase (PO) activity (mean + SE) in creeled and trawled specimens after 0 h (control), 12 h emersion, and 12 h emersion and 2 h re-immersion (n = 6)

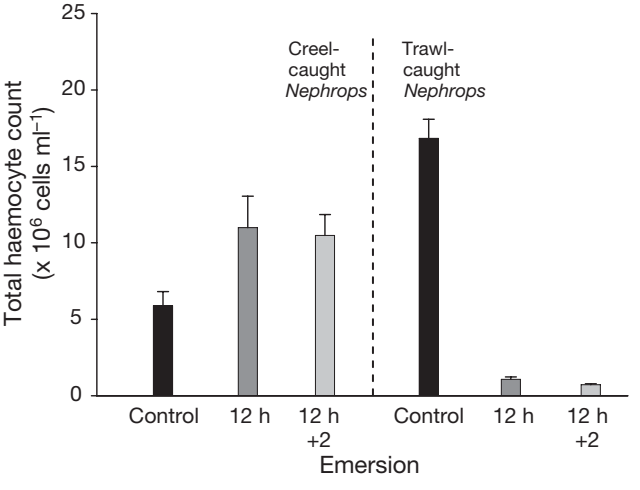

Fig. 2. *Nephrops norvegicus*. Haemolymph total haemocyte count (mean + SE) in creeled and trawled specimens after 0 h (control), 12 h emersion, and 12 h emersion and 2 h re-immersion (n = 6)

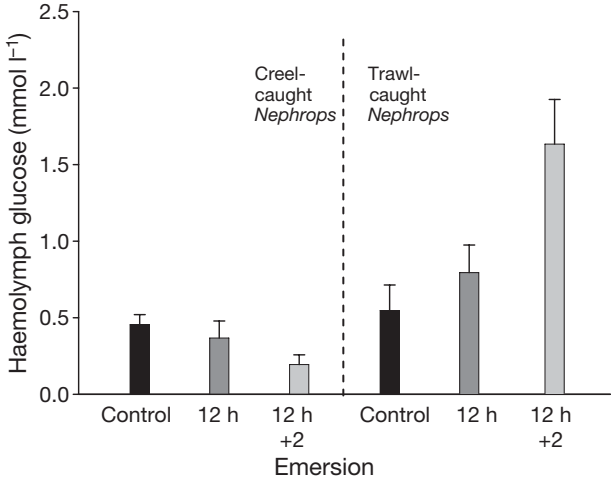

Fig. 3. *Nephrops norvegicus*. Haemolymph glucose concentrations (mean + SE) in creeled and trawled specimens after 0 h (control), 12 h emersion, and 12 h emersion and 2 h re-immersion (n = 6)

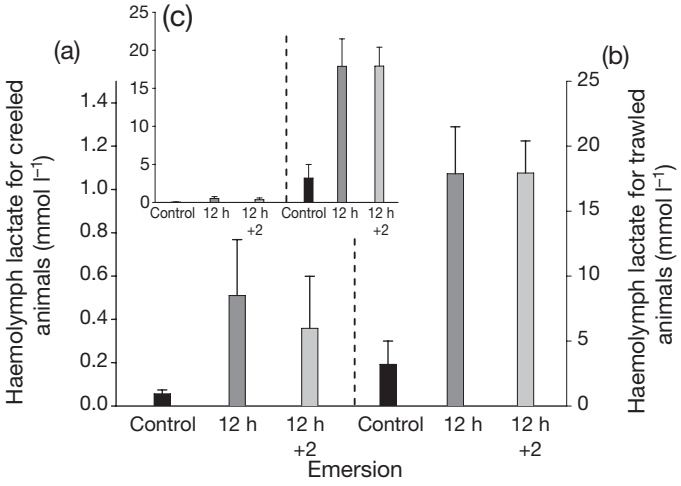

Fig. 4. *Nephrops norvegicus*. Haemolymph L-lactate concentrations (mean + SE) in (a) creeled, and (b) trawled specimens after 0 h (control), 12 h emersion, and 12 h emersion and 2 h re-immersion. (c) Combined plot of creeled and trawled L-lactate concentrations (n = 6)

by haemocyte counts, ProPO activation, phagocytic indices, and release of free oxygen radicals.

The release of mature haemocytes from the haematopoetic tissue (HPT) is induced when haemocytes are degranulated in response to 'non-self' molecules, e.g. β-1,3-glucans and lipopolysaccharides (LPS) (Söderhäll et al. 2003). Degranulation capacity in *Nephrops norvegicus* decreased by 75% in the presence of Mn (Hernroth et al. 2004) and Mn is also suggested to inhibit the induction of proliferation of the stem cells. A compensatory proliferation is otherwise a normal reaction to decreased THC. Here, it appears that a decrease in degranulation occurred in trawled animals exposed to 12 h of emersion. This suggests that the animals are attempting to compensate for increased stress brought about by the capture method. In contrast, the number of circulating haemocytes in the creeled animals increased during the 12 h emersion period in the creeled lobsters but decreased during the same emersion period in trawled individuals.

A low circulating haemocyte number in crustaceans is strongly correlated with long clotting times (Sindermann 1971) and a greater sensitivity to pathogens (Persson et al. 1987, Le Moullac et al. 1998). Hence, an attrition-induced, low THC indicates a higher susceptibility to infectious diseases (Newman & Feng 1982, Field & Appleton 1995, Jussila et al. 1997, Lorenzon et al. 2001). The finding here of a significant THC decrease in trawled *Nephrops norvegicus* (cf. the increase in the creel-caught lobsters) suggests that trawling may impose irreversible stress. Smith et al. (1995) found that *Crangon crangon* exposed to dredge spoils showed an elevation in recoverable haemolymph volume and a reduction in their THC; thus, such environmental stresses may produce a similar response in other species.

In this study, THC and PO activity were always negatively related in the trawled *Nephrops norvegicus* after emersion and this contrasts with the increases in both parameters (cf. control lobsters) in the creeled lobsters. Lowered THC and increased PO activity that followed a 24 h exposure of *Litopenaeus stylirostris* to hypoxia was attributed to lower amounts of plasma serine proteinase inhibitors regulating the ProPO system (Le Moullac et al. 1998). Negative correlations between PO activity and THC have also been found in the shore crab *Carcinus maenas* (Hauton et al. 1995) and in the common shrimp *Crangon crangon* (Smith & Johnston 1992).

A lowered THC may be a consequence of haemocyte immobilisation in the gills, as shown in mercury-exposed prawns (Victor et al. 1990), and is similar to the inflammatory reaction observed in *Carcinus maenas* after an infection (Smith & Ratcliffe 1980). The 12 h emersion-induced THC increase in the trawled group and decrease in the creeled group ($p < 0.05$ in each case) suggests that a lowered haemocyte count may have little to do with altered gill function in *Nephrops*. Jussila et al. (1997) suggested that a THC $<4 \times 10^6$ cells ml^{-1} was indicative of poor condition or health in lobsters. The trawled *Nephrops norvegicus* in this study had mean THC values of $1.1 \pm 0.20 \times 10^6$ cells ml^{-1} and $0.7 \pm 0.07 \times 10^6$ cell ml^{-1} for the 12 h-emersed and re-immersed groups, respectively, suggesting that their condition had deteriorated (cf. the creel-caught lobsters which maintained a THC

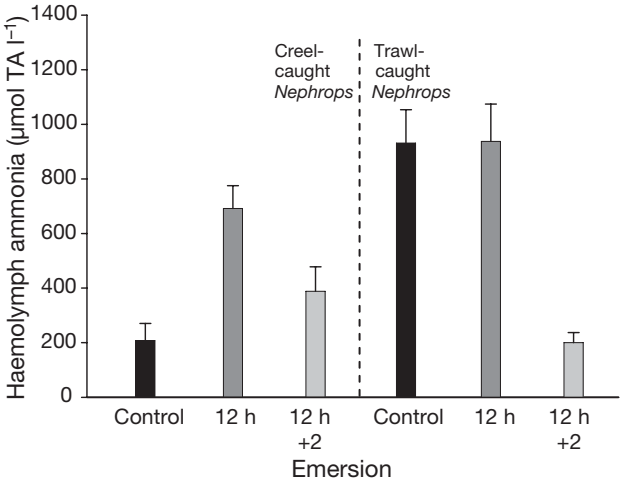

Fig. 5. *Nephrops norvegicus*. Haemolymph ammonia concentrations (mean + SE) in creeled and trawled specimens after 0 h (control), 12 h emersion, and 12 h emersion and 2 h re-immersion (n = 6). TA: total ammonia

which was probably more representative of the lobsters in the wild: 11.0 ± 2.07 × 10^6 cells ml^{-1} and 10.0 ± 1.37 × 10^6 cells ml^{-1}).

The initial haemolymph TA difference between the control groups probably reflects the different stress intensities imposed by the 2 capture methods. During trawling, animals not only have to endure the stress of being dragged behind the boat in a net for hours but, once hauled, the animals are released into a shoot and may be left there for up to an hour prior to being sorted by the fishermen.

Emersion-induced increases in haemolymph TA have been described for *Cancer productus* (DeFur & McMahon 1984), *C. pagurus* (Regnault 1992), *Nephrops norvegicus* (Schmitt & Uglow 1997), and *Necora puber* (Durand & Regnault 1998). Here, despite the differences in capture method, most of the accumulated ammonia was excreted from both groups within 2 h of re-immersion.

Under severe hypoxia, it is important for active crustacean species that use normal glycolysis with L-lactate (lactic acid) as their anaerobic end-product, to minimise activity and retard the depletion of glycogen reserves (Hagerman 1998). The present findings suggest that the extent of glycogen reserve depletion when captured varies with the capture method used as substantiated by the significant difference in the L-lactate concentrations between the creeled and trawled groups (0.06 ± 0.02 and 3.20 ± 1.80 mmol l^{-1}, respectively). This difference widened with the continued lactate increase in the trawled group during the 12 h emersion period and, again, probably reflects the severity and duration of the stresses associated with trawling, which was observed to induce extensive tail-flipping escape behaviour when the nets were being emptied).

Experimental emersion induced a pronounced haemolymph hyperglycaemia in the trawled group and this persisted into the subsequent re-immersion. On the other hand, the creeled group which had significantly lower mean haemolymph glucose levels before emersion, became hypoglycaemic when emersed and their haemolymph glucose concentration continued to decrease during re-immersion. These findings further highlight the greater stress intensity that trawling (vs. creeling) has on *Nephrops norvegicus* and implies that trawling and emersion induce a more severe depletion of the glycogen reserves than creeling does.

CONCLUSION

This study has demonstrated that the capture method and subsequent emersion of *Nephrops norvegicus* result in stress responses that are manifested by changes in their physiology and immunology. As a benthic burrowing species, *N. norvegicus* can be and has been affected by eutrophication-induced prolonged periods of severe hypoxia and anoxia. Hagerman & Baden (1988) indicate that the species is very hypoxia-tolerant, but this relates to relatively immobile individuals. The combined stresses of lengthy capture procedures, physical injury and emersion that occur when *N. norvegicus* is fished suggest that their emersion tolerance will be compromised unless positive steps are taken to avoid emersion (e.g. keeping lobsters immersed in good quality seawater whenever possible, especially soon after capture, while awaiting first sale, and certainly before packing and consigning). Large temperature variations should also be avoided (e.g. between ambient temperature at place of capture and at place of despatch) in existing protocols so that these better meet the needs of the animals. As *N. norvegicus* currently comprises the second most valuable UK fishery (7% of the 2005 landings but 18% of total value; DEFRA July 2006), and as customer requirement is increasingly for live, good quality product delivered to a distant point of sale, then pragmatic commercial (as well as humane) considerations indicate that protocol changes based on the physiological tolerances of the animals are needed to meet customer requirements.

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