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Note

# A first insight into stress-induced neuroendocrine and immune changes in the octopus *Eledone cirrhosa*

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**Abstract**

A number of cephalopod species present substantial ecological and economical importance; however, data on the physiology of stress and on regulatory processes linking stress to immune defence against pathogens remain extremely scarce in these organisms. The present study examined the influence of a 5 min air exposure, a common perturbation associated with handling in aquaculture settings and fisheries, on neuroendocrine and immune parameters in the octopus *Eledone cirrhosa*. Measurements of circulating concentrations of noradrenaline and dopamine, two hormones that are released in the haemolymph during stress in bivalves and gastropods, showed that the 5 min air exposure represents a real stress to octopus. Indeed, blood levels of both hormones increased by about 2–2.5-fold in stressed animals. Concomitantly, a significant decrease in the number of circulating haemocytes was observed, whereas haemocyte phagocytotic activity and superoxide anion production increased transiently between 5 and 60 min after the beginning of the stress. These results provide a first insight into the effects of stress on catecholamine levels and immune functions in cephalopods and suggest that stress and immunity may be associated in these organisms. 

*Keywords:* Haemolymph; Catecholamine; Immunity; Cephalopods

## 1. Introduction

Cephalopods are relatively advanced molluscs which inhabit various marine environments including hydrothermal vents, pelagic environments and coastal areas of polar, temperate and tropical oceans. Being efficient predators, these animals play key roles in many ecosystems and cephalopods have emerged as commercially important resources world wide (Murata, 1989; González et al., 1994; Guerra and Rocha, 1994; Guerra et al., 1994; Raya et al., 1999). Therefore, a number of cephalopod species present an important ecological and socio-economical value that appears to be threatened by disease outbreaks (Leibovitz et al., 1977; Hanlon et al., 1984; Ford et al., 1986; Bullock et al., 1987, Hanlon and Forsythe, 1990; Ford, 1992).

Previous studies have established that cephalopod defences against pathogens involve both humoral and cellular responses. Agglutinins, lectins, lysosymes and antiproteases, are important components of humoral immunity (Ford, 1992; Malham, 1996; Malham et al., 1998a) while cellular responses involve haemocytes, a class of amoeboid cells wandering in the haemolymph and tissues (Ford, 1992; Malham et al., 1995, 1998b; Malham, 1996). Previous advances in octopus immunology have shown that following immune challenge, haemocytes alter their cytoplasmic granular content and are capable of migration, phagocytosis, bactericidal and intercellular respiratory burst activities that help eliminate invading microorganisms (Malham, 1996; Malham et al., 1997, 1998b; Malham and Runham, 1998; Beuerlein and Schipp, 1998). Furthermore, octopus, squid and cuttlefish are known to harbour both endosymbiotic bacteria (Small and McFall-Ngai, 1999) and a number of prokaryotic and eukaryotic parasites, including human pathogens (Fraj Lazaro et al., 1998; Fidalgo et al., 2000; Abollo et al., 2001). Under normal conditions, both endo-

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symbiotic and pathogenic microorganism loads are maintained within ranges that are compatible with the host's survival, which suggests that complex regulatory processes contribute to the maintenance of a normal and viable immune status in cephalopods. However, both biotic and abiotic, natural or anthropogenic perturbations can threaten homeostasis in these molluscs which results in disease and mortality (Leibovitz et al., 1977; Hanlon et al., 1984; Ford et al., 1986; Bullock et al., 1987; Hanlon and Forsythe, 1990; Ford, 1992). For example, handling practices in aquaculture settings or food deprivation are highly stressful to octopus (Seibel and Childress, 2000) and variations in temperature and water quality can have detrimental effects on the survival of cephalopods in culture conditions (Reimschuessel and Stoskopf, 1990). These data raise the possibility that stress alters immune responses in cephalopods. Indeed, substantial evidence shows that environmental changes influence the immune status of invertebrates (Sindermann, 1979; Fisher, 1988; Anderson, 1990; Fisher et al., 1996; Carballal et al., 1998).

Generally, the exposure of animals to biotic or abiotic perturbations induces an adaptative process termed "stress response" which includes complex behavioural and physiological changes that help the animal to maintain homeostasis in the face of environmental changes (Chrousos and Gold, 1992; Ottaviani and Franceschi, 1996). The release of neuroendocrine messengers such as catecholamines and glucocorticoids is a primary response to stress in vertebrates (Chrousos and Gold, 1992; Ottaviani and Franceschi, 1996; Wendelaar Bonga, 1997). These neuroendocrine messengers act to shut down certain processes such as growth, reproduction and immunity to redirect bioenergetic resources to specific physiological functions (e.g. increased oxygen uptake, mobilization of energy substrates) that are immediately required for the adaptation and survival of the animal under threat. Stress-induced downregulation of immune defences implies that under stress, many vertebrates are more susceptible to diseases (Chrousos and Gold, 1992; Wendelaar Bonga, 1997). Data on invertebrate stress-induced neuroendocrine responses and immune changes remain scarce and progress in this field of research is required to understand better how relationships between stress, immunity and disease may affect marine aquatic resources.

In the present study, we investigated immune changes in octopus, *Eledone cirrhosa*, subjected to handling, a common aquaculture practice which may cause substantial stress to cephalopods.

## 2. Materials and methods

### 2.1. Animals

Octopus *E. cirrhosa* (500–800 g) was collected from the coastline round the Roscoff area of Brittany (France) and maintained in polyethylene tanks (two per tank) containing 110 l of aerated and continuously flowing (50 l $h^{-1}$) natural seawater at 14–15 °C. Animals were fed ad libitum with a diet consisting of green crabs, mussels and limpets and left undisturbed for a 10 d acclimation period. All animals were carefully inspected for the presence of wounds, scars or skin and gill infections. Injured, non-feeding and/or diseased animal were excluded from experiments.

### 2.2. Stress

The stressor consisted of manually removing octopuses from the water and leaving them exposed to the air for 5 min in an empty polyethylene tank. After the stress period, the animals were allowed to recover in their original tank. Maximum care was taken to avoid skin injuries and wounds to animals as this could have resulted in immune changes and in subsequent biased results.

### 2.3. Haemolymph collection

Animals were rapidly anaesthetized using 2.5% ethanol in seawater, and haemolymph (1 ml $animal^{-1}$) was collected from the branchial blood vessel using 2 ml syringes and 26 G × 1/2 in. needles as previously described (Malham et al., 1995). Rapidity of the sampling procedure ensured that stress associated with blood sampling was kept minimal in all experiments. Since repeated blood sampling can alter immune parameters in octopus (Malham et al., 1998b), different animals were sampled at each time point so that each animal was not sampled more than once. For each time point, blood samples from 2 to 4 animals were pooled to minimize bias due to individual variations of haemocyte numbers $ml^{-1}$ and catecholamine concentrations. Furthermore, experiments were repeated three times with new animals for each experiment in order to reduce sex or maturity bias.

### 2.4. Measurement of catecholamine concentrations and immune parameters

Noradrenaline (NA) and dopamine (DA) were extracted from 0.5 ml haemolymph subsamples by absorption on alumina and hormones were quantified using HPLC with electrochemical detection as previously described (Lacoste et al., 2001c, d). For immune parameter measurement, 200 μl of haemolymph were immediately removed, cells were counted using a haemocytometer and the haemocyte concentration was rapidly adjusted to $10^6$ cell $ml^{-1}$ with Modified Hanks Balanced Salt Solution (MHBSS) (containing 3 μg 100 $ml^{-1}$ ethyleneglycol-bis (β-aminoethyl ether) tetraacetic acid (EGTA), (Sigma)) to avoid haemocyte aggregation. One million cells $ml^{-1}$ aliquots were then used in the phagocytosis and NitroBlue Tetrazolium (NBT) assays.

### 2.5. Phagocytosis assay

The phagocytosis assay was performed as previously described (Mortensen and Glette, 1996; Lacoste et al., 2002) with some modifications. Briefly, *Vibrio anguillarum* was grown at 18 °C for 24 h on Zobell liquid (Gibco) before being killed in 10% formalin, washed, and labelled with fluorescein 5-isothiocyanate, Isomer 1, (FITC, Sigma) as described in Mortensen and Glette (1996). After further centrifugation ($13\,000 \times g$) and resuspension in phosphate buffered saline (PBS), the bacteria were diluted to $1 \times 10^8$ bacteria ml$^{-1}$ and stored at –20 °C.

One hundred microlitres of haemolymph containing haemocytes at $10^6$ haemocytes ml$^{-1}$ in MHBSS were placed on a glass slide and allowed to adhere for 10 min in a moist incubation chamber before rinsing in MHBSS and adding 100 µl of FITC-labelled *V. anguillarum* (giving a ratio of 100 bacteria haemocyte$^{-1}$). Glass slides were incubated for a further 30 min after which they were rinsed and unphagocytosed bacteria counterstained for 2 min with 100 µl of an ethidium bromide (Sigma) solution (50 µg ml$^{-1}$ in PBS). Three counts of 200 cells were immediately carried out from each of the duplicate slides using a 488 nm emission filter on a Zeiss microscope. Phagocytic cells were easily distinguishable from non-phagocytic ones as they contained green fluorescent bacteria. Percentage of phagocytic cells was then calculated for each slide and the mean and standard error recorded for each time point.

### 2.6. Measurement of intracellular superoxide anion production by nitroblue tetrazolium reduction assay

The nitroblue tetrazolium assay was performed as previously described (Pipe, 1992). Briefly, 200 µl of haemocytes at $10^6$ cells ml$^{-1}$ in MHBSS were added to 1.5 ml centrifuge tubes in triplicate for each sample. Two hundred microlitres of a nitroblue tetrazolium (NBT, Sigma) solution (2 mg ml$^{-1}$ in Tris/HCl buffer containing 2% NaCl, pH 7.6) were added to each tube. NBT solution without cells or tubes containing either cells, NBT and superoxide dismutase (SOD, Sigma, 300 Units ml$^{-1}$) in MHBSS or cells in MHBSS only were used as negative controls. Tubes were incubated for 1 h (in the dark), centrifuged ($120 \times g$ for 10 min) and the supernatant removed. The cells were resuspended in MHBSS, and washed twice before the addition of 200 µl of methanol (100%) to each tube for 10 min to fix the cells. The tubes were centrifuged ($300 \times g$), the supernatant removed and the cells air dried. The cells were then rinsed three times with 200 µl of methanol (50%) followed by the addition of 240 µl of potassium hydroxide (2 M) and 280 µl of dimethylsulphoxide. After vortexing, the supernatant was removed and placed in 500 µl cuvettes. The optical density value at 620 nm was measured on a spectrophotometer (Uvikon 943, Biotek-Kontron Instruments) and the results expressed as OD values $\times 10^5$ cells$^{-1}$ ml$^{-1}$.

### 2.7. Statistical analyses

All data are presented as means and standard errors of at least three experiments. For comparison of two means, paired or unpaired Student's *t*-tests were used where appropriate. For multiple comparisons, the data were analysed by one-way analysis of variance or Dunnett's multiple comparisons test where appropriate. $P < 0.01$ was considered as the limit of significance.

## 3. Results

### 3.1. Catecholaminergic response to stress

Measurements of NA and DA concentrations indicate that a 5 min exposure to air induced a transient state of stress in octopus (Fig. 1). Noradrenaline and dopamine concentrations (Fig. 1a,b) increased ($P < 0.01$) to 2–2.5-fold higher levels 5 min after the beginning of the disturbance. Following stressor application, NA and DA concentrations decreased to basal values 30–60 min after the beginning of the experiment.

### 3.2. Number of circulating haemocytes

The number of circulating haemocytes ml$^{-1}$ significantly decreased ($P < 0.01$) during the application of the stress from $11 \times 10^6$ to $5 \times 10^6$ cells ml$^{-1}$ (Fig. 2a). The haemocyte count ml$^{-1}$ significantly increased ($P < 0.01$) from the 5 min sample point to a maximum of $61 \times 10^6$ cells ml$^{-1}$ at 60 min. One hour later (120 min) haemocyte counts ml$^{-1}$ had returned to roughly the same value ($12 \times 10^6$ cells ml$^{-1}$) as that obtained at the beginning of the experiment.

### 3.3. Haemocyte phagocytosis assay

Percentage of phagocytosis significantly increased ($P < 0.01$) during the 5 min stress compared to the initial value of 16.75% (Fig. 2b). The increase in phagocytosis rates continued until the 30 min sample (27.62%). From 60 to 120 min, haemocyte phagocytic rates significantly decreased ($P < 0.01$) below the initial value.

### 3.4. Measurement of intracellular superoxide anion production by nitroblue tetrazolium reduction assay

Intracellular reactive oxygen intermediate production significantly increased ($P < 0.01$) from the initial value of 0.098 OD value $\times 10^5$ cells$^{-1}$ ml$^{-1}$ to 0.641 OD value $\times 10^5$ cells$^{-1}$ ml at the 5 min sample (Fig. 2c). Reactive oxygen production decreased significantly ($P < 0.01$) from the 5 min to the 30 min sample after which it significantly increased ($P < 0.01$) to the 60 min sample. At the end of the experiment (120 min) (0.258 OD

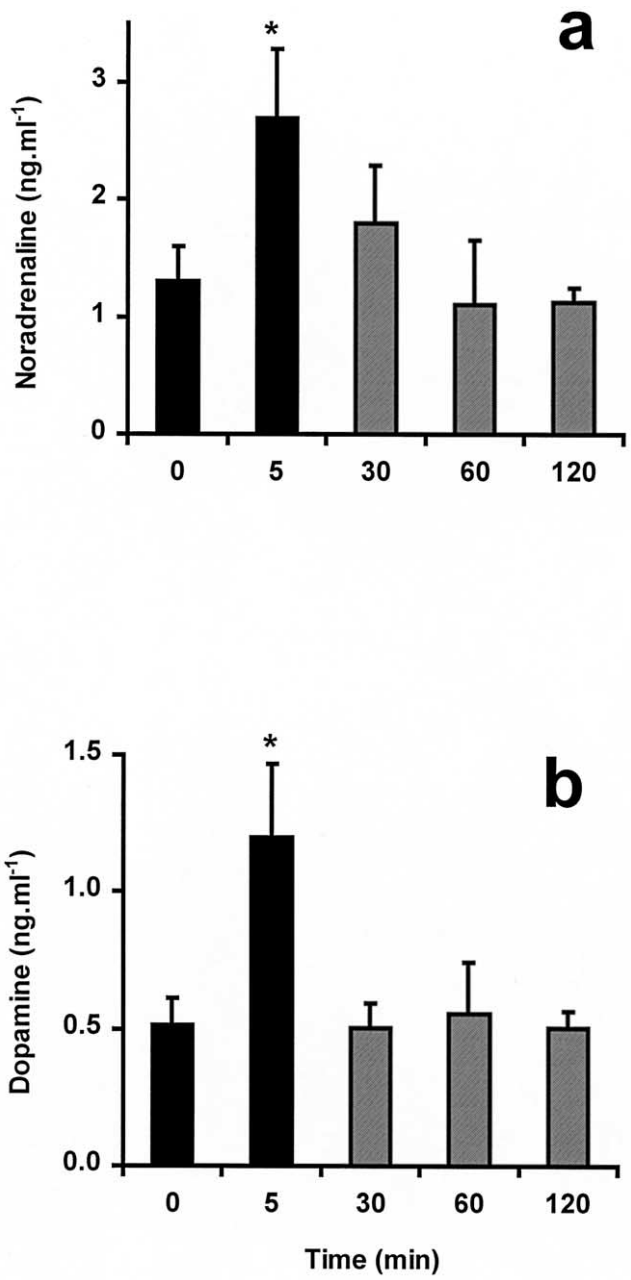


Fig. 1. Effect of a 5 min exposure to air on haemolymph (a) noradrenaline and (b) dopamine concentrations in the octopus *E. cirrhosa*. Black bars indicate values recorded during the stress period and hatched bars indicate values recorded after the disturbance. All data are presented as mean and standard errors of a minimum of three experiments. Asterisks denote significant ($P < 0.01$) differences from initial values (sample time 0).

value $\times 10^5$ cells$^{-1}$ ml$^{-1}$), reactive oxygen production was significantly higher ($P < 0.01$) compared to the initial sample. Optical density values of negative controls were <0.01 OD value $\times 10^5$ cells$^{-1}$ ml$^{-1}$.

## 4. Discussion

Recent progress has established that the application of a mechanical stressor can alter immune responses in bivalves (Lacoste et al., 2002) and gastropods (Malham et al., in press) which can cause increased susceptibility to disease (Lacoste et al., 2001a, b). Research has also demonstrated that bivalves possess a primitive form of catecholaminergic stress-response system involving noradrenaline and dopamine (Lacoste et al., 2001c, d) and further studies in the gastropod *Haliotis tuberculata*, demonstrated that gastropods also secrete these catecholamines in the haemolymph when they experience stressful situations (Malham et al., in press). Catecholamines are known to be present in cephalopods where they act as neurotransmitters and neurohormones (Budelmann and Bonn, 1982; Williamson, 1989; Kime and Messenger, 1990) controlling various processes such as digestion, heart function and activity of the retina (Andrews et al., 1983; Silver et al., 1983; Versen et al., 1999). The present results provide evidence that, as in bivalves and gastropods, catecholamines are also involved in the neuroendocrine response system to stress in the octopus *E. cirrhosa*. Upon exposure to air, both NA and DA concentrations increased significantly in the haemolymph of this cephalopod. These data suggest that stress-induced catecholamine release in the haemolymph is a conserved process among molluscan taxa.

Stress caused significant alterations in all immune parameters studied, the number of haemocytes and their ability to engulf, phagocytose and kill, via reactive oxygen intermediate production, invading microorganisms. The number of haemocytes circulating in the haemolymph decreased rapidly after the start of the disturbance. A similar phenomenon is observed in bivalves and gastropods subjected to a mechanical stressor resembling those imposed by grading and sorting practices in aquaculture (Lacoste et al., 2002; Malham et al., in press). It is possible that during stress, octopus haemocytes leave the haemolymph and migrate to tissues that are prone to injury or infection. Although work with other molluscs such as the oyster *Crassostrea gigas*, showed that stress augments the susceptibility of the animal to infection (Lacoste et al., 2001a), this may be because pathogen multiplication is greater than the haemocyte defence function. An alternative suggestion is that during stress, haemocytes may not leave the haemolymph specifically for immune functions. It is known that bivalve and gastropod haemocytes are also involved in nutrient transport (Cheng, 1996). It is, therefore, possible that these cells may play a role in stress management by the redirection of bioenergetic resources and leave the main haemolymph vessels in order to convoy nutrients to certain tissues involved in adaptation and survival. However, knowledge on the role of cephalopod haemocytes in nutrient transport is currently too limited to determine whether these hypotheses are plausible or not.

Interestingly, phagocytosis and intracellular superoxide production by haemocytes remaining in the haemolymph increased upon exposure of *E. cirrhosa* to stress, whereas the same immune parameters significantly decreased in stressed *C. gigas* (Lacoste et al., 2002) or *H. tuberculata*

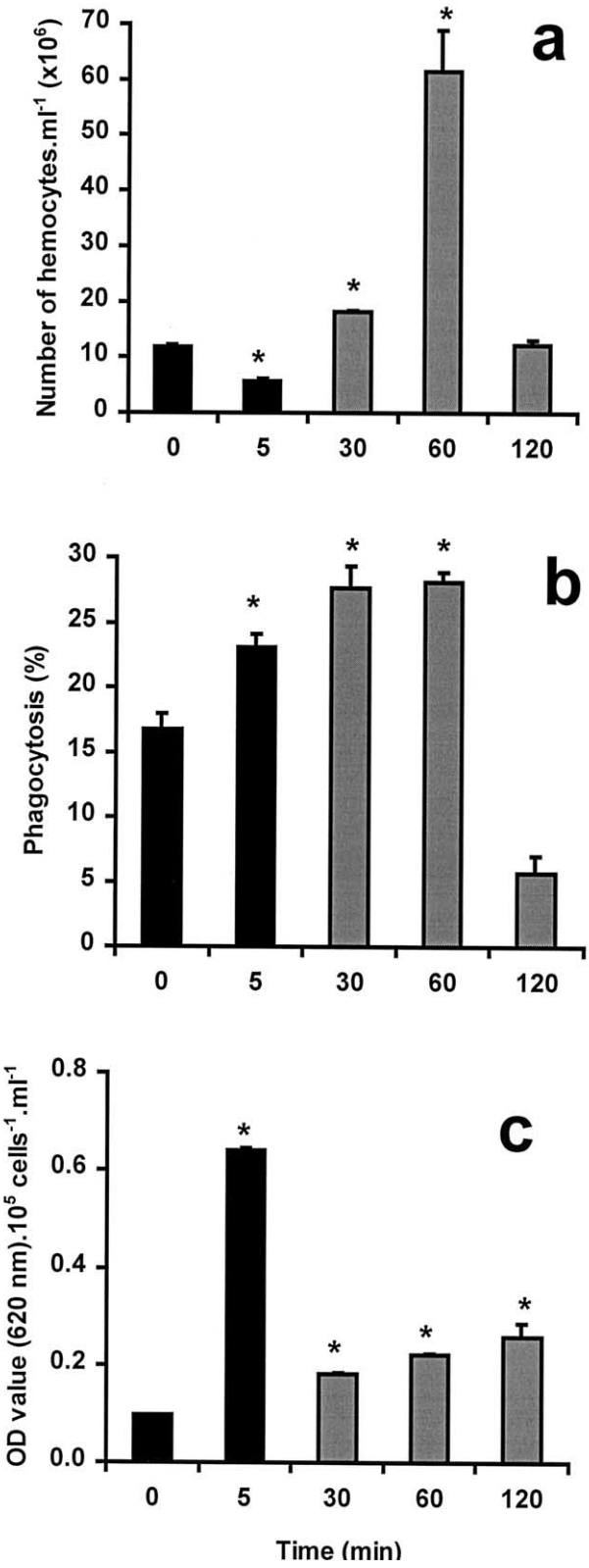


Fig. 2. Effect of a 5 min exposure to air on (a) the number of circulating haemocytes $ml^{-1}$, (b) the percentage of haemocyte phagocytosis and (c) the production of intracellular superoxide anion (nitroblue tetrazolium reduction reaction) by haemocytes. Black bars indicate values recorded during the stress period and hatched bars indicate values recorded after the disturbance. Each point is the mean (±S.E.) of triplicate counts performed in duplicate. Asterisks denote significant ($P < 0.01$) differences from initial values (sample time 0).

(Malham et al., in press). The results for both the oyster and the abalone were in line with in vitro experiments which show that the principal catecholamine, noradrenaline, exerts a dose dependent inhibitory effect on phagocytosis and respiratory burst activity (Lacoste et al., 2001d, e). The reasons for the difference in the results between the molluscs are not presently known. However, such a discrepancy in the results may be due to differences in stressors applied to the animals. Indeed, in previous experiments, *C. gigas* (Lacoste et al., 2002) or *H. tuberculata* (Malham et al., in press) was subjected to 15 min shaking whereas in the present study, octopuses were exposed to air for 5 min. Alternatively, cephalopod immune functions may react differently to stress compared to bivalves or gastropods. Further studies are required to clarify these points but the present results suggest that in molluscs, relationships between stress and immunity exist and are much more complex than expected.

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