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NOTE

Effect of the copepod parasite *Nicothoë astaci* on haemolymph chemistry of the European lobster *Homarus gammarus*

Charlotte E. Davies[1,*], Claire L. Vogan[2], Andrew F. Rowley[1]

[1]Department of Biosciences, College of Science, and [2]College of Medicine, Swansea University, Swansea, SA2 8PP, UK

ABSTRACT: The gills of the European lobster *Homarus gammarus* (L.) are susceptible to parasitization by the copepod *Nicothoë astaci*, the lobster louse. This copepod feeds on haemolymph of the host and can damage the gills, potentially affecting gaseous exchange capabilities. To investigate the host response to the parasite, haemolymph levels of total protein, haemocyanin, glucose and ammonia were quantified in adult lobsters carrying varying parasite loads. Parasite loads correlated positively with total haemolymph protein and haemocyanin concentrations but not with glucose or ammonia concentrations. The data suggest that lobsters with gills damaged by the feeding activities of *N. astaci* respond by producing higher levels of haemocyanin, which is both a key defence response and may compensate for their decreased respiratory functioning.

KEY WORDS: Lobster louse · Haemolymph proteins · Haemocyanin · Glucose · Ammonia · Ectoparasites · Blood chemistry

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INTRODUCTION

The lobster louse *Nicothoë astaci* is a copepod ectoparasite that inhabits the gills of some European lobsters *Homarus gammarus* and was first reported in 1826 by Audoin & Milne-Edwards (see Jones 1861). *N. astaci* has been found on lobsters inhabiting locations including Scotland (Mason 1959), Lundy Island in the Bristol Channel (Wootton et al. 2011) and as far south as France (M. Laurans pers. comm.) and Portugal (Monteiro 2008). The louse possesses a narrow suctorial mouthpart to feed on host haemolymph (Wootton et al. 2011, Davies et al. 2014). Internally, lobsters respond to the parasite by encapsulating haemocytes in an attempt to partition off damaged gill areas, and Wootton et al. (2011) hypothesized that this response may impair gill respiratory function. Such impaired respiratory function may explain why high louse loads have been associated circumstantially with elevated mortality among lobsters in holding facilities (M. Laurans pers. comm.).

Various haemolymph factors can provide a general health indicator of crustaceans (Stewart et al. 1967). For example, haemolymph levels of hyperglycaemic hormone and heat-shock proteins increase in response to environmental stressors such as temperature, salinity and hypoxia (Chang 2005), as well as to physiological stressors such as moulting (Stewart & Li 1969, Chang 2005). In response to reduced oxygen availability due to compromised gill functioning or removal from water, reduced oxygen concentrations in haemolymph can trigger increases in haemocyanin levels (Taylor & Whiteley 1989, Burnett 1992, Brouwer et al. 2004) to bolster the oxygen transport capability of haemolymph (Depledge & Bjerregaard 1989). Levels of digestion products such as glucose and nitrogenous wastes (Claybrook 1983, Truchot 1983) can also be affected by moult stage and exoge-

*Corresponding author: cedavies72@gmail.com

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nous stressors such as temperature, handling, disease and parasitization (Spindler-Barth 1976, Santos & Keller 1993, Stentiford et al. 2001). Moreover, crustaceans excrete nitrogen produced from protein catabolism across their gills, primarily as ammonia. Haemolymph levels of ammonia can also change in response to stress (Hunter & Uglow 1993) and can fall, for example, when stressors such as handling or low salinity promote elevated gill excretion (Regnault 1987).

Reduced respiratory function could have implications for lobsters parasitized with *N. astaci*, particularly when exposed to the stress of capture and live storage. Crustacean gills undertake many physiological functions including ion transport needed for haemolymph osmoregulation and ammonia excretion. They also provide a large surface area and a thin epithelium diffusion distance (ca. 1–10 µm) to exchange gases efficiently (Taylor & Taylor 1992, Hsia et al. 2013). However, their delicate nature also makes them vulnerable to damage from both waterborne debris and pathogens, including parasites. Here, we investigated the impact of *N. astaci* abundance on *H. gammarus* haemolymph levels of glucose, ammonia and proteins including haemocyanin.

MATERIALS AND METHODS

Lobster collection and maintenance

European lobsters (n = 18; 3 males and 15 females; carapace length ±SD 96.1 ± 3.5 mm; weight 581.4 ± 74.4 g) above the legal minimum landing size (≥90 mm carapace length measured from eye socket to posterior end of carapace) were collected from a commercial fishing vessel. Lobsters were caught at Ilfracombe, Devon, UK, where parasitization by *Nicothoë astaci* has been found previously (Wootton et al. 2011). An endoscope (Pro Vision PV2-2in1) was used to verify the presence of parasites in lobster gills, and the 18 lobsters examined were selected at random across 9 potting locations. It was not possible to quantify parasite loads accurately by endoscope examination. After transport in iced seawater to Swansea University (6 h duration), lobsters were acclimated for 2 wk to aid recovery from handling and transportation stress. Lobsters were maintained across 3 aquarium tanks (6 lobsters $tank^{-1}$) employing recirculated sea water (pH 7.9–8.1, 18–21°C, dissolved oxygen 6–8 mg l^{-1}, as recorded before each sampling) and fed a diet of mussels *Mytilus edulis* 3 times wk^{-1}.

Haemolymph and gill sampling

Each week over a 4 wk sampling period, haemolymph (1 ml) was withdrawn from the haemal sinus at the base of the fifth pereiopod (walking leg) using a 21-gauge needle. It was left to clot on ice for ca. 2 h and then centrifuged (5000 × *g*, 5 min), and 200 µl aliquots of supernatant (serum) were stored at −80°C. Unless otherwise stated, all assays were performed in triplicate using flat-bottom 96-well microtitre plates with absorbance measured using a Tecan Sunrise Basic microplate reader. On Days 28 or 29, 1 to 2 ml of 149 mg ml^{-1} KCl was injected into the cardiac sinus to euthanize each lobster (Battison et al. 2000). Gills were excised according to Wootton et al. (2011), and parasites were counted and recorded photographically.

Protein quantification

Serum total protein concentration was determined using a bicinchoninic acid assay kit (ThermoFisher), according to the manufacturer's instructions. Briefly, defrosted serum was diluted 100-fold in marine saline (16 g NaCl, 0.88g $CaCl_2 \cdot 2H_2O$, 0.41g KCl, 1.24 g $MgCl_2$, 2.98 g HEPES, ddH_2O 500 ml) and following addition of kit reagents, absorbance at 505 nm (A_{505}) was measured. Protein levels were quantified relative to a dilution series of bovine serum albumin standards (100–1000 µg ml^{-1}) analysed in the same 96-well plate.

Haemocyanin quantification

Using a method to quantify haemocyanin adapted from Hagerman (1983), 100 µl of defrosted serum were diluted 1:10 to 1 ml with marine saline and A_{335} measured in triplicate in 10 mm quartz-microcuvettes using a Jenway 6315 spectrophotometer. Since haemocyanin is oxygenated rapidly *in vitro*, this is an effective measure of pigment concentration using an extinction coefficient $E^{mM}{}_{1cm}$ = 17.26. This value was calculated from $E^{1\%}{}_{1cm}$ = 2.83 on the basis of a minimal functional unit size of 74 kDa (Nickerson & Van Holde 1971, Antonini & Brunori 1974).

Glucose quantification

Glucose concentrations in serum were quantified using a Glucose (GO) Assay Kit (Sigma Chemical) with slightly modified manufacturer's instructions.

Briefly, defrosted serum was de-proteinised with an equal volume of 5% trichloro-acetic acid and, following the addition of kit reagents, A_{540} was measured. The glucose levels were quantified relative to a dilution series of glucose standards (920–1000 µg ml^{-1}) analysed in the same 96-well plate.

Ammonia quantification

Ammonia concentrations in serum were quantified using the indophenol assay method (Bolz & Howel 1978). Defrosted serum was de-proteinised in an equal volume of 5% trichloro-acetic acid. After centrifugation, 100 µl of supernatant were diluted 5-fold in marine saline. To 100 µl of diluted serum, we added 50 µl of reagent 1 (10 g phenol, 50 mg sodium nitroferricyanide [III] dehydrate, 50 ml ddH_2O), and the sample was shaken for 30 s. Reagent 2 (50 µl; 0.5 g NaOH, 1 ml NaClO, 50 ml ddH_2O) was then added and the plate incubated at 37°C for 30 min before being measured at A_{635}. The serum ammonia content was quantified relative to a dilution series of NH_4Cl standards (0–1 µg ml^{-1}) analysed in the same 96-well plate.

Statistics

Statistical analyses employed Microsoft Excel and GraphPad Prism 6.0 for Mac (OSX Version 10.9.1). All data were first tested for a Gaussian distribution using a Kolmogorov–Smirnov test (K–S test). Spearman's rank correlation coefficient was then used to correlate parasite load (parasite count per lobster) against protein, haemocyanin, ammonia and glucose concentration, as well as lobster carapace length.

RESULTS

All 18 lobsters examined were found to be parasitized by *Nicothoë astaci*. Total numbers of parasites determined from gill photographs varied from 27 to 130 lobster^{-1} (n = 18, mean ± SD = 91 ± 63; male n = 3, 55 ± 19; female n = 15, 98 ± 67; Table 1, Fig. 1). During the 28 d study, no lobsters moulted, but 2 females became berried and as their haemolymph protein levels increased during this time, they were excluded from the analyses (data not shown). As all other lobsters showed only minor changes in haemolymph total protein, haemocyanin, glucose or ammonia levels at each of the 4 wk sampling points (data not shown), only data obtained on Day 28 are reported here.

Haemolymph total protein levels correlated significantly with parasite load ($p = 0.02$; Fig. 2A). Haemolymph haemocyanin levels also correlated significantly with parasite load, but only when 1 pre-moult lobster (R in Table 1) with a high protein concentration throughout the 28 d study was omitted from the dataset ($p = 0.0065$; Fig. 2B). In contrast, no

Table 1. Summary of European lobsters *Homarus gammarus* sampled and levels of parasitization by the copepod *Nicothoë astaci*. F: female; M: male

Lobster	Sex	Size (mm)	Weight (g)	Parasite number			Additional observations
				Left gills	Right gills	Total	
A	F	100	622	51	56	107	Epibiont (suspected monogenean) also found on gills
B	F	92	529	91	110	201	Deformed carapace
C	F	94	577	16	20	36	
D	F	97	581	12	15	27	
E	F	92	489	62	45	107	
F	F	98	689	48	28	76	Became berried
G	M	99	648	30	25	55	
H	F	92	531	135	95	230	
I	F	91	470	20	18	38	
J	F	95	573	14	17	31	
K	F	95	530	17	30	47	
L	F	94	602	83	104	187	
M	F	97	581	74	65	139	Became berried
N	M	105	776	14	22	36	
O	F	96	536	75	54	129	
P	F	97	566	26	41	67	
Q	F	97	524	19	24	43	Right chela missing
R	M	99	641	42	31	73	Pre-moult upon dissection

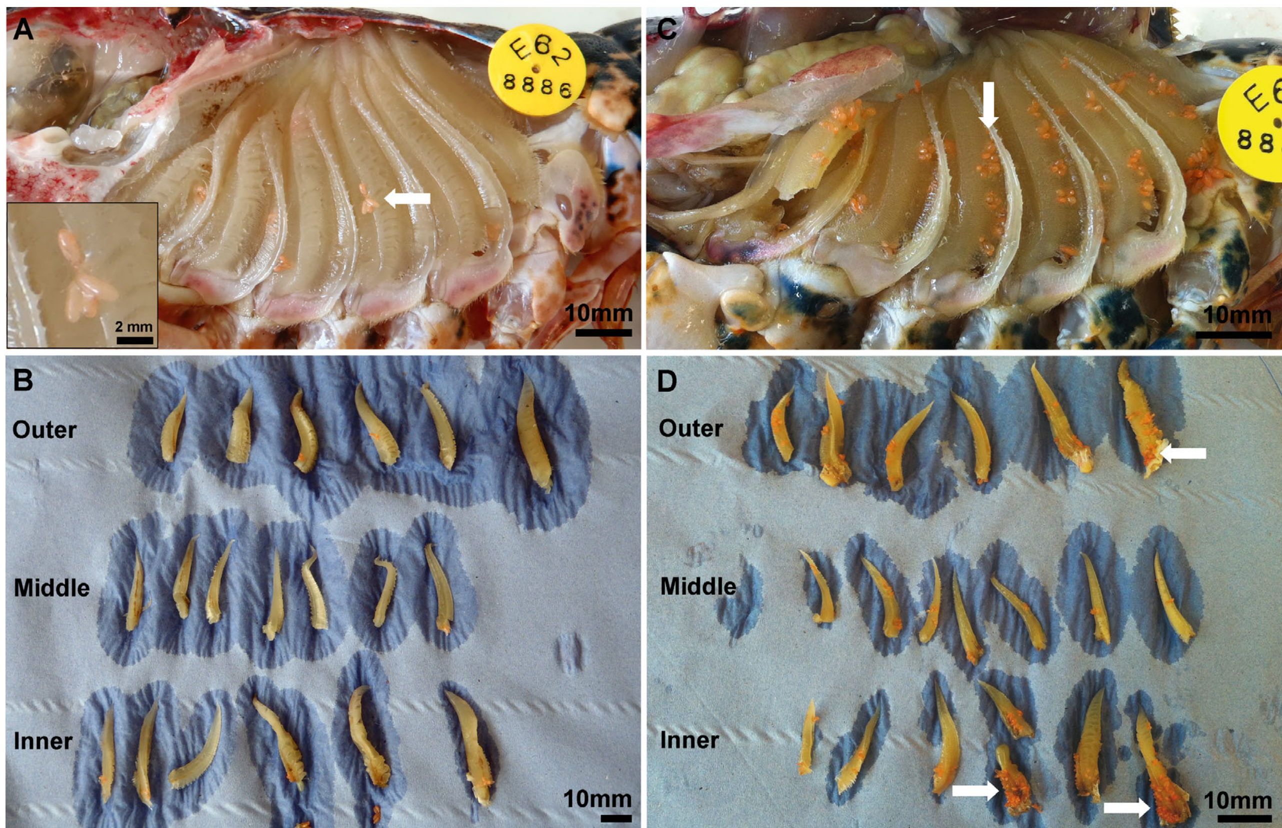

Fig. 1. Photographs showing examples of (A,B) low and (C,D) high levels of *Nicothoë astaci* (arrows) in the gills of European lobster *Homarus gammarus* before (A,C) and after (B,D) excision. Inset shows the structure of the parasites. Note the high numbers of parasites at the base of the gills in the lobster with high parasite load (arrows). The excised gills show the arrangement of gills into outer, middle and inner sets

correlations were apparent between gill parasite load and haemolymph glucose levels ($p = 0.2112$; Fig. 2C), ammonia levels ($p = 0.1290$; Fig. 2D) or lobster size ($p = 0.1269$, $R^2 = 0.1699$). Haemocyanin accounted for 84.2 ± 19.1% (mean ± SD, n = 15) of the total haemolymph protein.

DISCUSSION

Higher loads of *Nicothoë astaci* copepods in the gills of *Homarus gammarus* lobsters were here associated with elevated haemolymph total protein and haemocyanin levels. As haemocyanin constitutes ca. 90% of total protein in crustacean haemolymph (Uglow 1969, Ghidalia 1985), increased haemocyanin levels appear the most likely contributor to elevated total protein levels. However, increases in other less abundant proteins cannot be discounted. Increased circulating haemocyanin levels may result in lobsters as a compensatory mechanism for reduced respiratory function due to parasite-induced gill damage as reported by Wootton et al. (2011). Indeed, hypoxia can trigger elevated haemocyanin levels in several crustacean species (Hagerman & Pihl Baden 1988, Hagerman et al. 1990). Moreover, in other crustacean species such as the blue crab *Callinectes sapidus*, injection of the pathogenic bacterium *Vibrio campbellii* results in haemocytes aggregating in the gill lamellae, thus restricting haemolymph circulation and oxygen uptake through these narrow channels (Burnett et al. 2006). Hence the cellular defence response (aggregation of haemocytes) caused by parasites and pathogens may cause temporary or permanent damage to the capacity of gaseous exchange in the gills, which the lobster attempts to compensate for by producing increased amounts of haemocyanin.

Other members of the haemocyanin protein family include cryptocyanin and phenoloxidase (Terwilliger et al. 2006). While cryptocyanin does not bind oxygen like haemocyanin or phenoloxidase due to critical copper-binding histidine residues being reduced, levels of these proteins become elevated immediately prior to ecdysis before returning to nor-

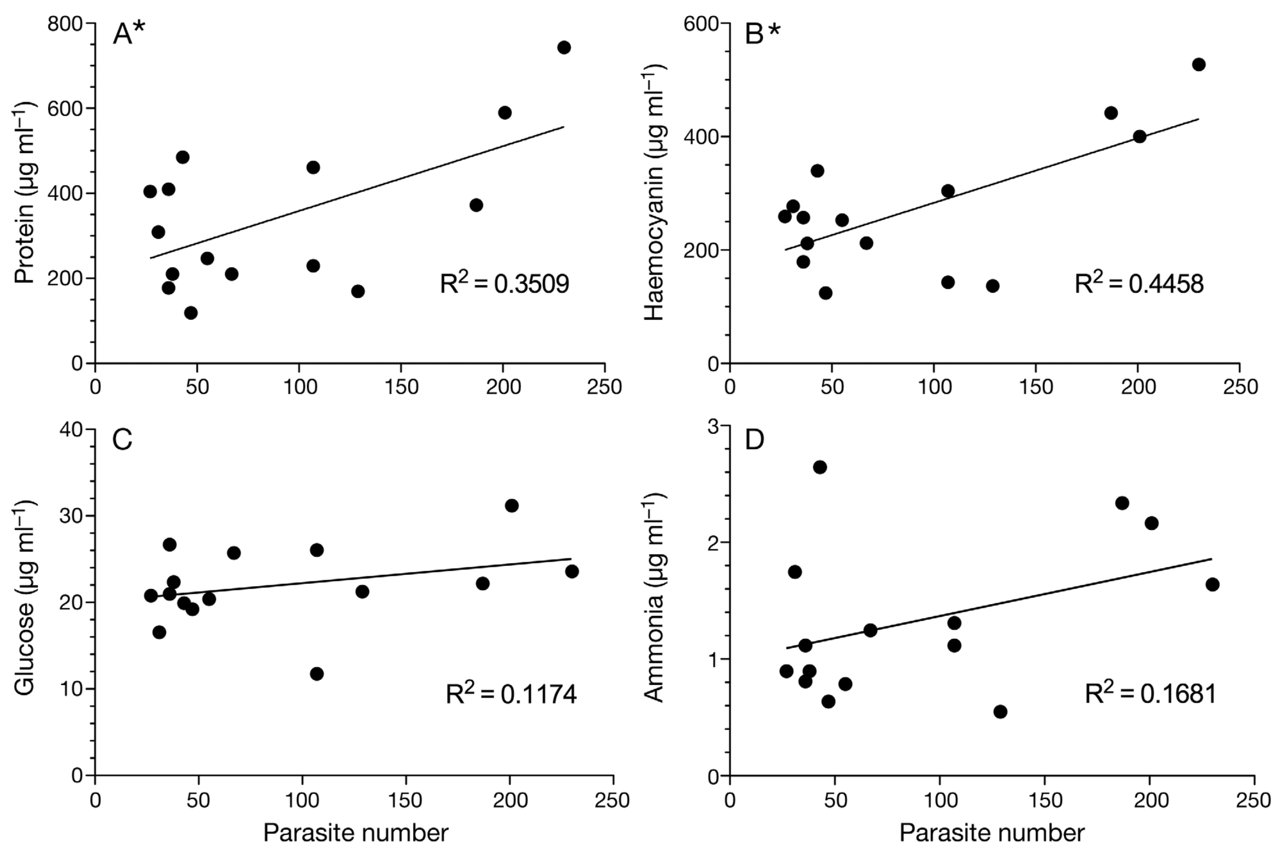

Fig. 2. Spearman's correlation coefficient analysis showing correlations between parasite *Nicothoë astaci* numbers and haemolymph concentrations of (A) total protein (p = 0.02), (B) haemocyanin (p = 0.0065), (C) glucose (p = 0.2112) and (D) ammonia (p = 0.1290) in European lobster *Homarus gammarus*. Asterisks denote significance

mal post-moult (Terwilliger et al. 1999, 2006). One lobster examined here (R in Table 1) was found to be pre-moult when dissected at the final sampling point. Since cryptocyanin and haemocyanin also have an absorbance peak at A_{340}, and both increase markedly in pre-moult crustaceans (Terwilliger et al. 1999, Kuballa et al. 2011), haemocyanin levels quantified for this individual might have been anomalous and were thus omitted from the dataset.

In invertebrates, the enzyme phenoloxidase catalyses melanin formation via the prophenoloxidase cascade (Cerenius et al. 2008). This cascade also results in the formation of key immune modulators; for example, gene knockout of this enzyme leaves shrimp immune deficient (Liu et al. 2007). In some crustaceans, haemocyanin cleavage results in the formation of an enzyme with catalytic activities similar to that of phenoloxidase (Adachi et al. 2003, Decker et al. 2007, Terwilliger 2007). In addition, some peptide fragments of haemocyanin have antifungal (Destoumieux-Garzón et al. 2001), antibacterial (Lee et al. 2003) and antiviral (Lei et al. 2008) activities. It is possible, therefore, that haemocyanin levels in lobsters parasitized by *N. astaci* might also become elevated as part of the defence response to this parasite.

No significant correlation was detected between *N. astaci* load and *H. gammarus* haemolymph glucose levels. However, stress or pathology resulting from disease infection has previously been associated with either increases (Telford 1968, Spindler-Barth 1976, Santos & Keller 1993, Powell & Rowley 2008) or decreases (Stentiford et al. 2001) in glucose levels. Parasites may absorb glucose from haemolymph, thereby forcing the host to resupply tissues with this sugar from glycogen reserves in the hepatopancreas in order to maintain carbohydrate homeostasis (Stentiford et al. 2001). It is possible that elevated glucose was not identified here due to *N. astaci* either being less invasive than other pathogens previously studied, or potentially not feeding constantly on haemolymph.

No significant correlation was detected between *N. astaci* load and *H. gammarus* haemolymph ammonia

levels. Stressors including pathogens can lead to increased respiration in crustaceans, resulting in elevated ammonia excretion due to levels becoming elevated in haemolymph (Yoganandhan et al. 2003). However, since ammonia is excreted from gills (Taylor & Taylor 1992), significant damage to this tissue may be expected to result in ammonium and other cellular metabolites becoming accumulated and therefore elevated in the haemolymph. Thus either the gill damage caused by *N. astaci* was not sufficient to impair ammonia excretion, or there is a switch in nitrogenous wastes to products such as urate or urea (Dykens 1991, Chen et al. 1994, Vogan & Rowley 2002) which were not examined. Another explanation may be that since ammonia is a minor component of haemolymph, and as all lobsters examined were parasitized by *N. astaci* to some extent, any increase in levels was not able to be resolved as easily as other constituents assayed for.

In summary, increased loads of *N. astaci* on the gills of European lobsters were found to correlate with increased haemocyanin and thus total protein concentrations in haemolymph. This most likely compensates for reduced respiratory function due to gill damage reported previously (Wootton et al. 2011). Increased haemocyanin, which may be a generalized host response as well as compensatory, may well be advantageous for infected lobsters. It would now be of great interest to investigate a wider host immune response on a larger scale.

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