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Endocrine disruption in the shore crab *Carcinus maenas* — a biomarker for benthic marine invertebrates?

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ABSTRACT: A considerable amount of research has been conducted on the effects of certain contaminants that have the potential to impact the endocrine systems that regulate vital life processes in freshwater and marine fishes. There is, however, a relative paucity of information on aquatic and especially benthic marine invertebrate species, many of which could be seriously impacted by sewage effluent and industrial discharges. The present study used a combination of end-points to assess possible endocrine disruption in a marine crustacean, the shore crab *Carcinus maenas*. These included pheromonally mediated sexual behaviour, exoskeletal morphological measures, quantities of steroid moulting hormones (i.e. ecdysteroids) and the presence of the egg yolk protein, vitellin in male crabs. Crabs were collected from sites known to elicit high oestrogenic responses in vertebrates and also from coastal reference sites. The results suggest that shore crabs around the coast of the Great Britain show effects consistent with pollutant-mediated endocrine disruption. These include a reduced behavioural response to the female sex pheromone, morphometric abnormalities such as reduced pleopod-length ratios and enlarged abdomen width, enhanced steroid moulting-hormone (ecdysone equivalent) levels and the detection of vitellin-like proteins in the hepatopancreas of male crabs. This multilevel approach may have significant potential for investigating endocrine disruption in marine crustaceans.

KEY WORDS: Endocrine disruption · Shore crab · Vitellin · Ecdysteroids · Sex pheromone · Morphology · Biomarker

INTRODUCTION

A considerable amount of research has been conducted on the effects of contaminants that have the potential to impact endocrine systems, which regulate life processes in freshwater and marine fishes (Pait & Nelson 2002). In contrast, there is comparatively little information on aquatic and more especially on benthic marine invertebrate species, many of which could be seriously impacted by sewage effluent and industrial discharges. Tributyltin (TBT)-induced imposex in dogwhelks and a number of other gastropods is arguably the best known example of endocrine disruption in any aquatic organism and still constitutes the only chemical 'case study' in which ecologically significant population-level effects have been found (Matthiessen & Gibb 1998). Field studies on endocrine disruption in bivalves have been carried out using both wild and caged individuals in various locations of the Saguenay Fjord, Quèbec, Canada. Higher levels of the female-specific protein, vitellogenin (vtg) were found in both haemolymph and gonad homogenates of the clam *Mya arenaria* and the mussel *Elliptio complanata*, and related to chemical contamination of the sites (Blaise et al. 1999, 2003, Gagne et al. 2002). Other examples of effects in invertebrates include abnormal levels of intersexuality in several species of marine harpacticoid copepods along sewage-contaminated coasts of Scot-

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land (Moore & Stevenson 1991, 1994), the occurrence of intersex in a population of lobsters exposed to sewage effluent (Sangalang & Jones 1997), masculinisation of freshwater copepod species in North American lakes (Sillett & Stemberger 1998) and the presence of dual-gender imposex in freshwater crabs inhabiting Japanese TBT-uncontaminated mountain streams (Takahashi et al. 2000). Gross et al. (2001) reported that high numbers of females displayed abnormal vitellogenic oocytes and a reduced male/female size differential in amphipods (*Gammarus pulex*) found below sewage treatment works known to contain endocrine-disrupting chemicals in their effluent.

Investigations into endocrine disruption in invertebrates have mainly focused on the potential influence of vertebrate-type hormones (e.g. oestrogens) and their analogues. There is little direct evidence that natural and anthropogenic compounds, which are capable of modifying endocrine control in vertebrates, have similar effects on invertebrates such as crustaceans (Breitholtz & Bengtsson 2001). For example, the endocrine processes associated with vitellogenesis in crustaceans are not influenced by environmental contaminants in the same way as in fishes. Thus, neither vtg nor its corresponding egg yolk protein vitellin (vt) was detected in the haemolymph of male crabs, *Carcinus maenas* or in brown shrimp *Crangon crangon* collected from several UK estuaries as part of a national sampling programme (Allen et al. 2002). In addition, laboratory exposure of crabs to known oestrogens (diethylstilbestrol, nonylphenol) failed to elicit a similar response in male crabs to that previously demonstrated in fishes (Allen et al. 2002). It is also clear that oestrogens do not have any affinity for the ecdysteroid receptor, at least not *in vitro* (Ponds et al. 2002). Thus, work to date suggests that aquatic crustaceans are relatively insensitive to endocrine-disrupting effects of oestrogens. An exception is the nauplii of the barnacle *Balanus amphitrite*, which respond to low levels of xeno-oestrogen and oestrogen exposure (0.01 to 1.0 µg l^{-1} 4-nonylphenol (4-NP) and 1.0 µg l^{-1} 17β-oestradiol) by elevation of cypris major protein (Billinghurst et al. 2000), a vitellin-like protein (Shimizu et al. 1996). A range of hormones which regulate various phenomena in crustaceans have, however, been shown to be affected by environmental contaminants (see review by Fingerman et al. 1998).

The present study uses a combination of end-points to assess possible endocrine disruption in a marine crustacean, the shore crab *Carcinus maenas*. These included (1) pheromone-mediated sexual behaviour, (2) exoskeletal morphological measures, (3) quantities of steroid moulting hormones (i.e. ecdysteroids) and (4) the presence of vt in the hepatopancreas of male crabs. The shore crab was chosen as it inhabits both benthic communities of estuaries, a habitat often susceptible to contamination from anthropogenic sources, and open coastlines. Amongst a number of invertebrate species representative of different trophic levels, the shore crab has also been identified as the organism most sensitive to anthropogenic contamination (Galloway et al. 2004). In addition, crustaceans have been shown to be particularly susceptible to insecticides that target arthropod endocrine systems (Oberdörster & Cheek 2000).

MATERIALS AND METHODS

Collection and maintenance of crabs. Intermoult *Carcinus maenas* (n = 250) were collected by trawl from May to July 2003 at 2 sites where effects of exposure to oestrogenic contamination have been observed in fishes, the Tyne estuary downstream of Howdon sewage treatment works and the Tees estuary, Dabholm Gut (Lye et al. 1997, Allen et al. 1999), and 2 'non-contaminated' coastal sites, Newton-by-the-Sea and Ross Sand, Lindisfarne, in NE England. The Tamar estuary, SW England, was sampled as an additional contaminated site. The sexes were separated and held in tanks with a seawater flow-through system. The crabs were allowed an acclimatisation period of 7 d and separated for 3 h prior to any behavioural experiment. The muscle tissue of white fishes was fed to the crabs twice a week.

Bioassay. Behavioural assays (n = 1024) were carried out on 128 intermoult crabs as described by Hardege et al. (2002). All assays were carried out in triplicate except for the negative controls, which were carried out in duplicate. Briefly, assays were undertaken in glass tanks holding 25 l of filtered seawater at ambient temperature. The bioassay procedure involved a stone being dipped into pre-copula female-conditioned seawater (harvested from pre-copula females separated from their guardian male) and introduced into a tank containing an intermoult male. The response of male crabs to female sex-pheromone exposure varied and was graded according to its intensity (Grades 1 to 5: Table 1). A positive bioassay result required a specific stereotyped behavioural response in which the assay male grasped the pheromone-treated stone (the so-called pseudofemale) and tested the hardness of the cuticle with its chelipeds (Grades 4 and 5). An intermediate bioassay result required the selected assay male to rise to tiptoe posture and move towards the pheromone-treated stone (Grades 2 and 3). No behavioural change was consequently required for a negative response (i.e. Grade 1). Negative (stones dipped into filtered seawater) and positive (pre-moulted females) controls were always used. Male crabs were used only once a day for behavioural assays.

Morphometric analyses. Morphometric analyses were performed with an image-analysis system (Matrox Imaging Codec for Windows 2000), and a digital image was recorded for each crab. In *Carcinus maenas* the parasitic barnacle *Sacculina carcini* induces broadening of the abdomen, a typical secondary sex characteristic of the female, most probably via an effect on the endogenous endocrine system of the crab (Høeg 1995). These characteristics have recently been indicated in non-parasitised *C. maenas*, both female and male, from oestrogen-contaminated waters (Bentley pers. comm.). Morphometric analyses therefore focused on secondary sexual characteristics including claw depth, pleopod height, abdomen dimensions and carapace width. As it was difficult to obtain crabs of a standard size from each site, morphological data were compared after generating a ratio for each measurement to carapace width. Approximately 250 shore crabs, (carapace width >30 mm, collected from the various sites) were examined.

Identification and quantification of ecdysteroids. Samples of haemolymph taken with a 1 ml syringe from intermoult males (n = 40, from both contaminated and reference sites), were prepared by adding 100% ethanol to precipitate protein. Samples were incubated at 4°C for 18 h and then centrifuged at 2000 × *g* for 20 min at 4°C. The supernatant was removed and evaporated to dryness. Samples were purified by solid-phase extraction using Varian-Bond-Elut LRC-C18, 500 mg C_{18}Sep-Pak cartridges. The crude extract residue was resuspended in 4 ml 10% (v/v) methanol, and applied to the cartridge. The C_{18}Sep-Pak cartridges were sequentially eluted with 6 ml of 10% methanol, 6 ml of 25% methanol, 10 ml of 60% methanol and 6 ml of 100% methanol. The free ecdysteroids were eluted in the 60% (v/v) methanol fraction and collected in silanised glass vials.

Table 1. *Carcinus maenas*. Grading of behavioural responses of intermoult male shore crabs upon exposure to female pheromone samples (Hardege et al. 2002)

Grade	Response
1	No response: no behavioural change observed
2	Rise to 'tip toe' posture: random-assay male raises its body on extended pereiopods 2 to 4, and may raise pereiopod 5 above and behind posterior of its carapace
3	Searching behaviour: assay male engages in slow forward-searching motion towards source of cue
4	Tactile investigation: on locating 'female-like object', male engages in tactile examination using chelipeds to inspect source of stimulus
5	Adopting pairing stance: assay male covers pseudo female, his ventral surface overlying dorsal surface of the pseudo female. Assay male uses his ptereopods to clasp and support the pseudo female from below

All radioimmunoassays (RIA) were carried out in triplicate and used ecdysone as standard, with bound and unbound [23,24-^{3}H] ecdysone (NEN Ltd) being separated by ammonium sulphate precipitation (Mendis et al. 1983). The antiserum employed was H-22 which was produced by immunisation with ecdysone 22-succinylthyroglobulin amide and shows greatest specificity towards the ecdysone nucleus (Warren & Gilbert 1986). A typical 50% binding occurred at about 125 pg of 25 deoxyecdysone, 150 pg of 3-dehydroecdysone, 175 pg of ecdysone, and 600 pg of 20-hydroxyecdysone (Chang & O'Connor 1979, E. S. Chang pers. comm.).

Determination of vitellin by western blotting. Tissue samples of *Carcinus maenas* extracts, including gonads, hepatopancreas and muscle tissue (n = 40), were homogenised in Lysis buffer (see Chausson et al. 2004), sonicated and centrifuged at 15 000 rpm, for 15 min at 4°C. The supernatant was analysed by sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE). SDS-PAGE was generally performed using 7.5% polyacrylamide gels in an ATTO mini-gel electrophoresis system under reducing conditions (Laemmli 1970). After separation by SDS-PAGE, the proteins were electrotransferred to a flurotrans nitrocellulose membrane by soaking the gel in 10 mM 3-(cyclohexamino)-1-propanesulfonic acid transfer buffer using a Trans-Blot SD apparatus (Bio-Rad) (15 V, 60 min). For western blotting, the membranes were incubated in blocking buffer (5% skimmed milk in a Tris-buffered saline, TBS) for 1 h. After blocking, they were rinsed with TBS and incubated with a rabbit anti-(*Carcinus*) vt primary antibody isolated in our laboratory (Sanders 2004) (dilution 1:500) for 2 h. After extensive washing with TBS-Tween (TBS containing 0.05% Tween 20), the membranes were incubated with a horse radish peroxidase-conjugated goat anti-(rabbit IgG) secondary antibody for 1 h. The vt bands on the membrane were visualised by electrochemiluminescent immunoassay (Pollini et al. 1993).

Statistical analyses. The data were checked for normality and variance homogeneity. The mean behavioural response grade was compared between sites using Kruskal-Wallis tests ($p < 0.05$) and Mann-Whitney ANOVA. All other parameters were tested by ANOVA using the general linear model procedure in the Minitab Statistical Package (Zar 1984).

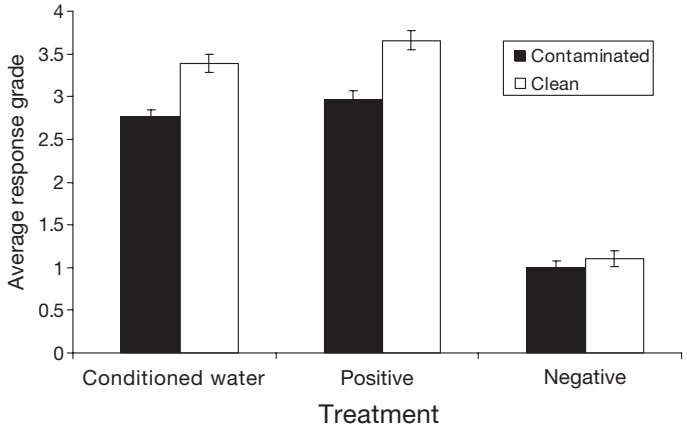

Fig. 1. *Carcinus maenas*. Pheromone-mediated behavioural response (mean ± SE) of selected assay males to stones dipped into pre-copula female-conditioned seawater, to pre-copula females (positive control), and to stones dipped into fresh seawater (negative control)

RESULTS

Sex pheromone communication

A total of 1024 bioassays were carried out (Table 2). As no significant difference could be detected between the 2 contaminated sites or between the 2 reference sites (Mann-Whitney ANOVA, $p > 0.05$), the data were pooled. Fig. 1 compares the mean behavioural response grade of males from the contaminated sites to that of males from the reference sites. The results reveal a significant difference between the mean response grade of male crabs from the contaminated sites and that of males from the coastal reference sites (Mann-Whitney ANOVA, $p < 0.05$), with male crabs from the Tyne and Tees showing a significantly reduced response compared to crabs from the coastal reference sites. The same pattern was prevalent in the response of male crabs to pre-copula females (positive control) (Mann-Whitney ANOVA, $p < 0.05$). None of the sites showed significant difference between the response of males exposed to female-conditioned water and to pre-copula females (Mann-Whitney ANOVA, $p < 0.05$), whereas male crabs from both sites differed significantly in their response to female-conditioned water and to fresh seawater (negative control) (Mann-Whitney ANOVA, $p < 0.05$).

Exoskeletal morphological measures

Crabs from the 2 contaminated sites had significantly smaller (ANOVA, $p < 0.001$) right and left pleopod-length ratios than those from the 2 reference (Fig. 2). Ross Sand crabs also had smaller (ANOVA, $p < 0.05$) pleopod-length ratios than Newton-by-the-Sea crabs. The Tees crabs had a significantly larger abdomen-width ratio than crabs from all other sites (ANOVA, $p < 0.05$), whereas Ross Sand crabs had a significantly smaller abdominal area than crabs from all other sites (ANOVA, $p < 0.001$). No obvious pattern was discernible from the morphometric measurements of claw depth.

Ecdysteroids levels

The amounts of free ecdysteroids detected in the haemolymph samples by RIA with the H-22 antiserum are shown in Fig. 3. It was decided to analyse the free ecdysteroids collectively, as separating the different ecdysone compounds would have required all samples to be subjected to high-performance liquid chromatography (HPLC) followed by RIA of multiple fractions. This would have significantly restricted the number of crabs that could efficiently be analysed. The standard

Table 2. *Carcinus maenas.* Graded response (mean ± SE) of intermoult males exposed to stones dipped into female-conditioned seawater to pre-copula females (positive control) and to stones dipped into fresh seawater (negative control). Grades are as in Table 1

Sampling site	No. crabs	No. bioassays	Avg. response grade
Female-conditioned water			
Newton	20	60	3.5 ± 0.18
Ross Sand	26	78	3.4 ± 0.13
Tyne	42	126	2.6 ± 0.12
Tees	40	120	2.8 ± 0.13
Positive control			
Newton	20	60	3.9 ± 0.16
Ross Sand	26	78	3.5 ± 0.15
Tyne	42	126	2.9 ± 0.13
Tees	40	120	2.9 ± 0.13
Negative control			
Newton	20	40	1.4 ± 0.13
Ross Sand	26	52	1.3 ± 0.1
Tyne	42	84	1.3 ± 0.08
Tees	40	80	1.4 ± 0.09

Table 3. *Carcinus maenas.* Average amount of ecdysone titres (ng ml^{-1} haemolymph) in intermoult-stage males collected from different sites. Values (mean ± SE) ecdysone equivalents

Site	Avg. ecdysone titre
Lindisfarne	4.1 ± 2.4
Tyne	9.9 ± 5.1
Tamar	10.5 ± 3.8

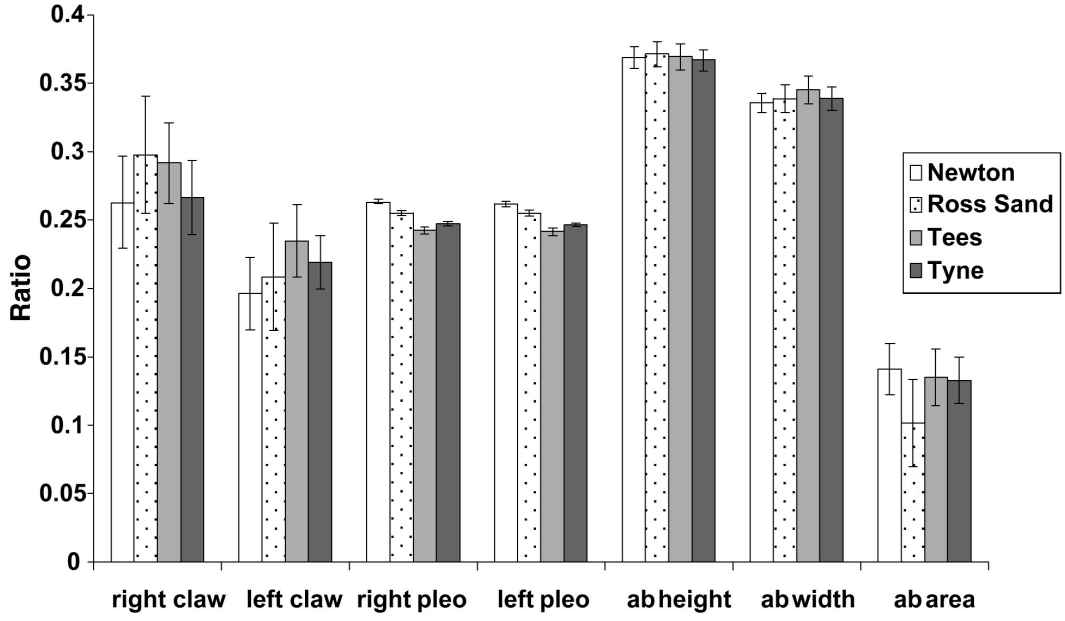

Fig. 2. *Carcinus maenas*. Comparison of morphometric data (mean ± SE) in male shore crabs sampled from contaminated and references sites. ab: abdomen; pleo: pleopod

Fig. 3. *Carcinus maenas*. Quantification of free ecdysteroids extracted from haemolymph samples of males from different sites, determined by radioimmunoassay of extracts with H-22 antiserum. Values (mean ± SE) are ecdysone equivalents (ng ml^{-1} haemolymph)

unlabelled ligand was ecdysone. Thus, RIA activity was expressed in ecdysone equivalents (ng ml^{-1} haemolymph). In reality the change in ecdysone equivalents between contaminated and reference sites probably represents a change in a mixture of ecdysteroids including ecdysone and 20-hydroxy-ecdysone.

As no significant difference could be detected between the contaminated sites (Tyne and Tamar: Table 3) (Mann-Whitney ANOVA, $p > 0.05$), the data for these sites were pooled. The results in Fig. 3 indicate that levels of ecdysone equivalents are significantly higher in intermoult stage crabs from contaminated sites than in those from the reference site (ANOVA, $p < 0.05$).

Vitellin analyses

A limited number of *Carcinus maenas* extracts (n = 40), including hepatopancreas, gonads and purified vt standard, were subjected to western blotting, following SDS-PAGE with antibody raised against vt purified from *C. maenas* ovaries (Sanders 2004). As shown in Fig. 4A, female-specific protein was detectable by western blotting using antibodies to *Carcinus maenas* vt. There was no discernible cross-reactivity between the polyclonal antisera against vt used in this study and the proteins present in the testis extract and juvenile ovarian extract (Fig. 4A, Lanes 2 to 5). This feature was used to validate the suitability of the present antiserum for investigating the presence of vt-like proteins in the hepatopancreas of male shore crab.

Western blotting revealed 3 dark bands of approximately 180, 100 (arrowed in Fig. 4A) and 90 kDa molecular mass (Lane 1). These bands coincided with similar bands in the female hepatopancreas sample (Lane 6). Other bands visible in the female hepatopancreas had molecular masses of 66, 80 and 130 kDa. These could be additional subunits; 5 polypeptide subunits of approximately 170, 102, 88, 76.7 and 72 kDa

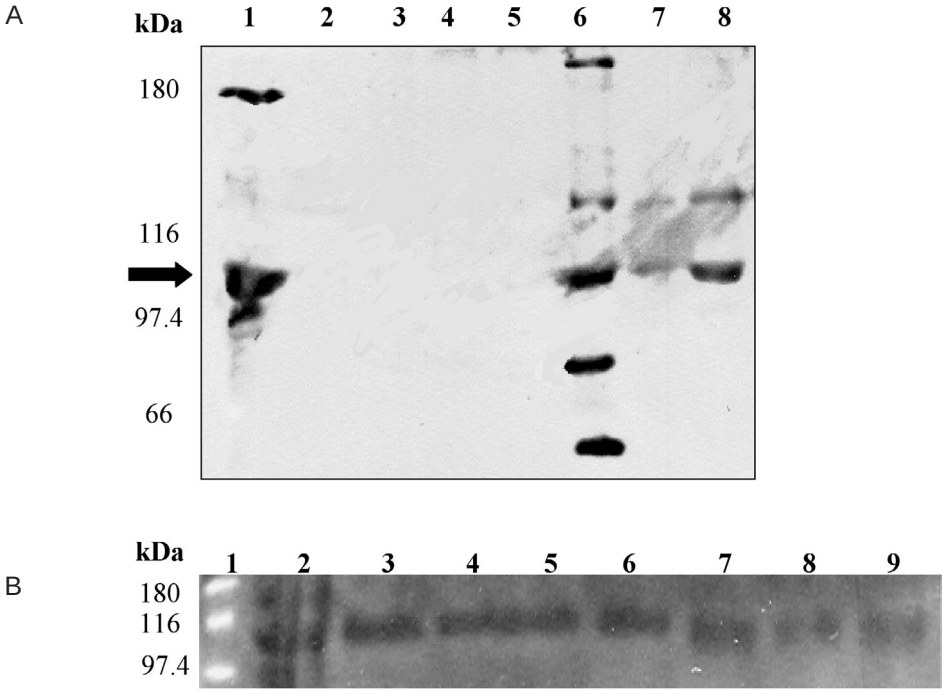

Fig. 4. *Carcinus maenas*. Western blot showing cross-reactivity between antibodies raised against *C. maenas* vitellin and *C. maenas* tissue samples (7.5% acrylamide). (A) Lane 1: purified vitellin standard; Lanes 2 and 3: testes extract; Lanes 4 and 5: juvenile ovarian extract; Lane 6: female hepatopancreas; Lanes 7 and 8; male hepatopancreas. (B) Lane 1: molecular weight standards; Lane 2: vitellogenic female ovarian extract; Lanes 3 to 9: male hepatopancreas

molecular mass have previously been identified in our laboratory in the haemolymph of vitellogenic female shore crabs (Sanders 2004) using the same antiserum as that used in this study. The high molecular weight band of approximately 170 was vtg. Lanes 7 and 8, which contained male hepatopancreas extract, displayed a strong polypeptide band of approximately 100 kDa molecular mass like the vt standard (Lane 1). An additional band of around 130 kDa molecular mass was also seen in the female hepatopancreas sample. A faint band of 170 kDa, visible on the original blot, but not evident in Fig. 4A, is presumed to be vtg.

Similar results were obtained with western blots of additional samples of male hepatopancreas. Fig. 4B shows the areas of vt immunoreactivity on the resulting developed membranes (Lanes 3 to 9 male hepatopancreas). These areas are coincident with the subunits obtained from ovarian extract of a vitellogenic female crab (Lane 1), suggesting the presence of vt-immunoreactive material in the hepatopancreas of male shore crabs from both contaminated and reference sites.

DISCUSSION

As in most crustaceans, mating in *Carcinus maenas* is coincident with the female moult (Hartnoll 1969) and copulation occurs shortly after this event. *C. maenas* males and females produce sex pheromones, which attract the opposite sex prior to the female moult. This enables pre-copula pairs to be established so that the female can be inseminated after she has moulted and before the genital pore becomes blocked (Bamber & Naylor 1996). Although given little consideration, pheromonal communication is likely to be susceptible to interference by compounds capable of mimicking pheromones, as has been found for hormones (Pinder et al. 1999, Mueller et al. 2003).

The results of this study suggest that male crabs from sites contaminated with endocrine-disrupting substances, (the Tyne and Tees estuaries) showed evidence of reduced behavioural pheromone-mediated response towards females compared to crabs from Lindisfarne, a relatively clean coastal site. In addition, abnormalities in secondary sexual characteristics, such as reduced pleopod-length ratios and an enlarged abdomen width, were noted in crabs from contaminated sites.

In the male shore crab, secondary sex characteristics of the female, including broadening of the abdominal area, are induced by the parasite barnacle *Sacculina carcini*, and are thought to be linked to degeneration of the androgen gland (Høeg 1995). These feminising characteristics, i.e. reduction in abdomen area and

claw depth, can also be induced in crabs by removing the androgen gland experimentally (Charniaux-Cotton 1960). Furthermore, environmental oestrogens such as Bisphenol-A and 4-tert-octylphenol are known to induce a complex syndrome of alteration in the female prosobranch snail *Marisa cornuarietis*, with affected individuals forming additional female organs and gross abnormalities of sex glands, resulting in an increased mortality and impaired egg production (Oehlmann et al. 2000). In addition, significantly wider abdomens, compared to normal females, were induced in female blue crabs *Callinectes sapidus* parasitised by *Loxothylacus texanus* (Hochberg et al. 1992). The abdomen flap of the mud crab *Scylla serrata* has shown to be relatively larger in those female crabs parasitised by the Rhizocephalan parasite *Loxothlacus ihlei* compared to non-parasitised crabs and the mechanism is presumed to be due to hormonal changes (Knuckey et al. 1995). This suggests that endocrine-mediated changes in dimensions of exoskeletal morphology can be induced in crustaceans.

It is thought that the effect of many endocrine disrupters is due to; (1) mimicking or antagonising the effects of hormones, (2) altering the pattern of synthesis and metabolism of hormones, and (3) modifying receptor levels (Soto et al. 1995). This implies that if anti-androgenic substances bind to male hormone receptors and subsequently block the action of naturally produced hormones in the organism, then a demasculinising effect could result. Thus, changes in the abdominal area of the male crabs from the Tyne and Tees estuaries areas could be indicative of contaminant exposure and disruption of endocrine processes.

The behavioural and morphological findings of this study are consistent with recent work on aquatic invertebrates. First, Clare et al. (2003) investigated the relationship between pheromonally mediated behaviour and external morphology of shore crabs collected from 'contaminated sites' around the UK (the Tyne, Tees and Tamar estuaries) and 'non-contaminated' sites (Oban, Arisaig and Lindisfarne). Using the same bioassay as in the present study, Clare et al. (2003) found that for all sites there was a strong relationship between male crabs showing female-type dimensions (i.e. larger abdomen) and a lack of response to pheromone-dipped stones. In addition, significantly more crabs showing smaller claw depth were also found at the contaminated sites compared to the relatively non-contaminated sites. Second, differences in morphological characteristics indicative of endocrine disruption (i.e. smaller pleopod lengths and claw depth and larger abdomens) have also been reported by other workers for shore crabs collected from oestrogen-contaminated estuaries (i.e. Tees, Tyne, Clyde at Bowling) compared to reference sites (the Alde) (Allen et al. 2002, Bentley pers. comm.). A third study by Straw & Rittschof (2004) investigated the relationship between pheromonally mediated behaviour in female mud snails and the extent and degree of imposex (i.e. the presence of male secondary sexual characteristics in female snails) as a morphological indicator of sub lethal exposure to organotin compounds. Straw & Rittschof (2004) found that the responses of molluscs from sites where a high degree of imposex was present (e.g. Morehead City, North Carolina), were significantly lower than responses of snails from low-imposex sites (e.g. Carrot Island, Beaufort, North Carolina) suggesting that most snails from the high-imposex sites do not respond to gender-specific pheromones.

Thus, the present results suggest that male crabs (showing intermediate morphology at contaminated sites) do not display the stereotypical behaviour of males to females that is induced by female sex pheromone in crabs from cleaner sites. Furthermore, the incidence of crabs showing abnormal exoskeleton morphology is higher at sites contaminated with oestrogens compared to reference sites. Although this data set is too small to draw any firm conclusions about induced abnormalities in the reproductive morphology of the shore crab, it adds to the growing body of circumstantial evidence that feminisation of shore crab exoskeleton from locations contaminated with endocrine disrupting substances could be linked to exposure to these compounds (Allen et al. 2002, Clare et al. 2003, Bentley pers. comm.).

The ecological significance of the present finding has yet to be determined. As there was no breeding or capsule deposition at the high-imposex site in the study of Straw & Rittschof (2004), the authors concluded that impacted molluscs (whether or not they showed morphological effects) which had lost their ability to respond to pheromones, were behaviourally and reproductively compromised. Considering that a pheromonally mediated sexual response that synchronises gamete release is required for initiating successful pairing in shore crabs (Bamber & Naylor 1996), disruption in the control of normal sequence and duration of pairing behaviour could ultimately affect the reproductive success of these crabs.

The present findings support the hypothesis that endocrine systems, other than those controlling sexual processes, may be affected in crustaceans. This study provides limited evidence that the steroid moulting hormones in crabs from 'contaminated' sites were significantly higher (ecdysone equivalents) than from 'non-contaminated' sites. Ecdysteroids are involved not only in the moulting process but have also been shown to be involved in reproduction and vitellogenesis and may have a role in behaviour and rhythmic locomotory processes (Watson et al. 2003). Interference

with ecdysone could therefore have profound ecological significance. Ecdysones, produced endogenously in the Y-organ exert their effect by binding to an intracellular receptor protein within the target tissue (i.e. epidermis and hepatopancreas) of crustaceans (Chang & O'Connor 1988). Synthesis of ecdysone is under hormonal control from moulting inhibitory hormone and methyl farnesoate (Tamone & Chang 1993) and could therefore be potentially sensitive to environmental steroids and their mimics.

During the moult cycle, circulating ecdysteroids vary considerably. At postmoult, haemolymph titres of ecdysteroids are negligible and remain so throughout the intermoult. Ecdysteroids levels increase dramatically in the premoult, and then drop steeply prior to ecdysis (Chang 1989). In this study, HPLC was not performed prior to RIA, and so it is not possible to determine the relative amounts of ecdysone, 20-hydroxyecdysone and ponasterone-A recently described in the male shore crab *Carcinus maenas* by Styrishave et al. (2004). However, it is clear that the levels detected in our study were similar to those determined during intermoult, and much lower than those found during moulting by Styrishave et al. (2004).

The significance of elevated ecdysone levels, as found in this study, remains an open question. There is evidence to suggest that exposure to 20-hydroxyecdysone (20-HE, or chemicals mimicking 20-HE) results in hyperecdysonism (persistently high 20-HE titres) and accelerated moulting (Freeman & Costlow 1984, deFur et al. 1999). Such a disturbance could potentially have a range of effects on the rate and timing of development and/or moulting success (Fingerman et al. 1998). Still other more recent studies have shown that exposure of *Carcinus maenas* to 20-HE has limited impact on processes such as vitellogenesis, moulting and locomotor activity rhythms, although potential disruption due to moulting-hormone mimics could not be ruled out (Watson et al. 2003). Work will continue in this area to investigate the full implications of different steroid mimics on the processes regulated by ecdysteroids.

It has been suggested that ecdysteroids may stimulate vitellogenesis (Fingerman 1997) and ovarian maturation and protein synthesis (Chan 1995, Oberdörster & Cheek 2000) although the mechanism is still poorly understood. Indeed, conflicting results have been reported in studies where ecdysteroids were administered *in vivo* or *in vitro*, with the effect on vitellogenesis being either inhibitory or stimulatory (Chang 1989, 1993, Cheng & Chang 1991). There is also some evidence that the ability of ecdysteroids to promote vitellogenesis is species-dependent (Loeb 1993), as is the case with some crustaceans, including *Carcinus maenas* (Lachaise et al. 1981). Correlations between haemolymph ecdysteroid levels and vtg levels have been reported (Young et al. 1993), although it is still unclear whether ecdysteroids directly influence vitellogenesis or are simply indicative of the corresponding stage of the moult cycle (Watson et al. 2003). In this study, the presence of vt and elevated ecdysone levels in male crabs suggests that these processes may possibly be interlinked. A more thorough understanding of the biology of vitellogenesis is however needed to conclude an interrelationship between ecdysone levels and vitellogenesis.

It has been suggested that vtg is synthesised in the ovary and also in extra-ovarian tissues like the hepatopancreas in crustaceans. The extent of extra-ovarian vitellogenesis is species-dependent however, with some species (e.g. *Callinectes sapidus*) exhibiting synthesis of vtg exclusively in the ovary (Lee et al. 1996), whereas other species (e.g. *Carcinus maenas*) synthesise vtg or its vt in both the hepatopancreas and the ovary, depending on the stage of vitellogenesis (Paulus & Laufer 1982). Thus, in order to identify any hormonal disturbances in the process of vtg in male crustaceans, the approach of the present study was to analyse extracts of hepatopancreas for the presence of vtg.

This study has detected the presence of vt in the hepatopancreas of male shore crabs. Thus, it confirms that induction of female-specific proteins is taking place in male crabs collected from sites in NE England. A band corresponding to approximately 100 kDa similar to that in the vt standard was present in both the female and male hepatopancreas. The high molecular mass band of approximately 180 kDa (present in the standard and female hepatopancreas) but not in the male hepatopancreas, suggested that the immunoreactive profiles were vtg. In addition, other bands of roughly 80 and 60 kDa molecular mass were also visible in the female hepatopancreas, as was a band of about 130 kDa visible in both female and male samples. These were considered to be additional subunits. Indeed, the number of vt subunits reported in crustaceans has been shown to vary from 2 to 11 (reviewed in Kawazoe et al. 2000). A similar vt sub-unit of approximately 130 kDa has also been detected in the prawn *Penaeus japoncius* (Kawazoe et al. 2000).

In *Carcinus maenas* vtg is immunologically identical to vt, with 2 subunits of approximately 100 and 85 kDa and usually with an additional subunit of 170 kDa (Sanders 2004). These units are remarkably similar among crabs, shrimps and brine shrimps (i.e. 182 ± 13, 100 ± 7 and 79 ± 6 kDa respectively: Spaziani et al. 1995). Thus, although the molecular weights were not accurately defined in the present study, but calculated on the electrophoretic mobility of markers on the gels, they still corresponded approximately to the molecular

masses of vt/vtg reported in other studies on crustaceans.

In fishes, induction of vtg in males has been used extensively to signal exposure to xeno-oestrogens (Sumpter & Jobling 1995). A vt-like cyprid major protein has also been induced in barnacle cypris larvae of *Balanus amphitrite* (Billinghurst et al. 1998) upon exposure to the model xeno-oestrogen 4-NP and the natural oestrogen 17β-oestradiol. Recent studies concerning endocrine disruption in both marine and freshwater bivalves have confirmed the induction of vtg after injection of various concentrations of oestrogenic compounds such as nonylphenols (NPs), 17β-oestradiol, pentachlorophenol and coprostanol into the adductor muscle of the clam *Mya arenaria* (Blaise et al. 1999) and the mussel *Elliptio complanata* (Gagné et al. 2001). In males of the Manila clam *Tapes philippinarium* exposed to 4-NP (100 and 200 µg l^{-1}), vtg levels have been shown to increase significantly in both the haemolymph and digestive gland, whereas no changes were observed in those of females (Matozzo & Marin 2005). However, vtg was not detected in the haemolymph of *Carcinus maenes*, following exposure (100 µg l^{-1}, 21 d) to the oestrogens 4-NP and diethylstilbestrol and an androgen (testosterone) (Allen et al. 2002).

Since the process of vitellogenesis in crustaceans is indeed very similar to that in fishes, with vtg sequestered from the haemolymph by developing oocytes, the development of a biomarker based on the detection of vtg in male crustaceans has consequently been investigated (Allen et al. 2002). The results showed that vtg was undetectable in the haemolymph of male shore crabs *Carcinus maenas* and shrimps *Crangon crangon* from oestrogen-contaminated sites, and further in male crabs which had been feminised through parasitism by *Sacculina carcini.* On the basis of these results Allen et al. (2002) concluded that vt expression does not provide evidence of endocrine disruption in crustaceans in response to oestrogens and androgens, although this does not necessarily mean that endocrine disruption is not occurring in crustaceans. As Allen et al. (2002) did not measure the levels of vt in the hepatopancreas of examined individuals, and vt levels were not measured in haemolymph samples collected in the present study, it is not possible to comment on the potential production of vt in both tissues of an individual. If, however, vt is present in the hepatopancreas but not in the haemolymph of an individual animal, it could be speculated that since no active uptake by the ovary tissue exists in male shore crabs, there is little transportation of the vt into the haemolymph. It should also be noted that in some invertebrate species (e.g. prosobranch molluscs), vt does not enter the haemolymph but, rather, is produced locally for uptake into the gonad (Jobling et al. 2004). Comparison of the vt levels of crab haemolymph to those of hepatopancreatic tissue of individual crabs should clarify the situation.

For a comparison to be made it is also essential to consider the specificity of the antibodies used in the different studies. In our study, vt was detected by western blotting using a polyclonal antiserum raised specifically against the delipidated form of lipovitellin which had been extracted from the ovaries of shore crabs by ion-exchange chromatography (Sanders 2004). In Allen et al. (2002), the protein was detected by ELISA using polyclonal antibodies raised against the native lipoprotein of crab ovaries. Thus, as both antibodies used were polyclonals and therefore produced in different hosts, and the sensitivity and specificity of delipidated antisera and native antisera may differ, differences may exist in response (Spaziani et al. 1995).

In crustaceans, the gene(s) necessary for the synthesis of vtg appears to be present in both sexes. In males, the gene(s) seems to be directly or indirectly repressed by action of the androgenic hormone (Adiyodi 1985). It is generally accepted that the androgenic gland (AG), which synthesises the androgenic hormone, regulates the development of male characteristics. Its absence results in feminisation, often including the induction of vtg in the hepatopancreas of AG-ablated individuals (Sagi et al. 2002). Thus, although there is no conclusive evidence that the current finding of vt in male hepatopancreas is caused by anti-androgens, the possible role of this type of steroid mimic in the onset of vitellogenesis in male shore crabs must not be overlooked. Of particular relevance in this context is a recent national survey of effluent from over 40 selected sewage-treatment works distributed throughout England and Wales. This study clearly confirmed not only the presence of significant oestrogenic activity, but also revealed a widespread occurrence of anti-androgenic activity discharged into these waters (Environment Agency UK 2004, M. Gross-Sorokin pers. comm.). The possible association of male crabs exhibiting vt induction in their hepatopancreas with exposure to these kind of substances warrants investigation. Clearly, much is still to be learnt about vitellogenesis in marine crustaceans and further work is needed to understand adequately the factors that influence this process. This will also include determining whether the incidence of the induction of vt expression is higher at polluted sites than at cleaner sites. Nevertheless, in the light of the present findings of vt present in the hepatopancreas of male crabs, it can be suggested that vt induction should be reassessed as an indicator of endocrine disruption in marine crustaceans.

While the detection of a vt-like protein in the hepatopancreas of male shore crabs is of significance, it is difficult to speculate further on the physiological

effects of this phenomenon at present. In this study, differences in secondary sexual characteristics, possibly indicative of feminisation, were detected in male crabs from contaminated sites compared to reference sites. In fishes, it has been shown that vtg induction following oestrogenic exposure in most instances is accompanied by the presence of inter-sex conditions (Jobling et al. 1998), suggesting that similar scenarios may occur in other animals.

In conclusion, the present findings are consistent with the potential for endocrine disruption by environmental contaminants. The results suggest that the multilevel approach, as proposed in this study and described elsewhere (Clare et al. 2003), may have significant potential as a measure of endocrine disruption. Particularly promising biomarkers for further investigation are induction of vt/vtg in the male hepatopancreas, abnormal levels of ecdysteroids (and the processes regulated by ecdysteroids), and pheromonally mediated sexual behaviour.

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