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1   Inter-population variability in the reproductive morphology of the shore crab (*Carcinus*

2   *maenas*): evidence of endocrine disruption in a marine crustacean?

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1   Abstract

2   Environmental contaminants that are capable of causing endocrine disrupting effects are

3   currently a major cause for concern. These chemicals are known to influence the

4   reproductive development of vertebrates by mimicking or antagonising the actions of

5   endogenous hormones. However, little is known regarding their potential effects on

6   invertebrates. Here we examine variations in the reproductive morphology of the shore

7   crab (*Carcinus maenas*) for evidence of endocrine disruption. Crabs were collected from

8   a number of sites comprising a putative gradient of exposure to endocrine disrupting

9   chemicals. Patterns of inter-population variability in the expression of sexually

10   dimorphic traits were then examined for evidence of hormone disruption. Extensive

11   variability was detected and patterns of chelal morphology were consistent with the

12   gradient of endocrine disruption. However, overall, the patterns of morphological

13   variability were not consistent with hormonally-mediated effects. This suggests that

14   shore crabs are not susceptible to the same type of endocrine disrupting effects that have

15   been detected in vertebrates, which are most commonly mediated via the oestrogen

16   receptor. However, the potential for androgenic effects on crustacean morphology are

17   discussed.

18

19   Key words: shore crab; *Carcinus maenas*; endocrine disruption; oestrogen; reproductive

20   development; morphology.

1. Introduction

There is now unequivocal evidence that a wide variety of chemicals that enter the aquatic environment are capable of disrupting endocrine function in wildlife and humans (IEH, 1999). Endocrine disrupting chemicals (EDCs) that interfere with the actions of the sex hormones are of particular concern, having been associated with reproductive dysfunction in all classes of vertebrate (Ashby et al., 1997; Tyler et al., 1998). The mechanisms responsible for these effects are very similar across this group of organisms as the vertebrate hormone-receptor system is highly conserved. Consequently, this phenomenon is relatively well understood. In contrast, little is known regarding the potential implications of these chemicals for invertebrates, largely due to our rudimentary understanding of invertebrate hormone-receptor systems. This paucity of knowledge is dangerous, given that invertebrates comprise 95% of all animal species and play a pivotal role in ecosystem dynamics (Defur et al., 1999; Depledge & Billinghurst, 1999).

The phenomenon known as "imposex" remains one of the few clear examples of endocrine disruption in invertebrates in the field. This morphological abnormality occurs in gastropod molluscs exposed to organotin compounds and it is characterised by the superimposition of male reproductive characteristics, including a penis and vas deferense, on the female genitalia (Bryan et al., 1986). The mechanism by which this occurs is still under investigation. However, interference with the aromatase enzymes, which are normally responsible for the conversion of testosterone to 17β estradiol, is the most widely accepted explanation. The resulting accumulation of testosterone is thought to be responsible for these masculinising effects (Matthiessen & Gibbs, 1998).

3

1 There is putative evidence that the reproductive morphology of crustaceans may also be

2 affected by EDCs. For example, increased rates of intersex and female biased sex ratios

3 have been reported in harpacticoid copepods from sewage polluted locations along the

4 East coast of Scotland, although a direct correlation between the frequency of intersex

5 and distance from the discharge locations was not observed (Moore & Stevenson, 1991;

6 1994). Ovotestes formation has also been reported in lobsters (*Homarus americanus*)

7 around the coast of Nova Scotia (Sangalang & Jones, 1997) and duel-gender intersex,

8 characterised by the presence of penis-like appendages on females and gonopore-like

9 openings and ovotestes in males, has been observed in Japanese freshwater crabs

10 (*Geothelphus dehaani*) from contaminated sites (Takahashi et al., 2000). Recent data

11 have revealed increased rates of intersex and female biased sex ratios in the marine

12 amphipod, *Echinogammarus marinus*, from the Scottish coast (Ford et al., 2004).

13 Furthermore, discriminant analysis of sexual dimorphisms, such as gnathopod length,

14 revealed that "normal" males from polluted sites closely resembled intersex specimens.

15 Similar abnormalities have been reported in amphipods (*Hyalella azteca*) exposed to

16 ethinylestradiol (Vandenbergh et al., 2003), which provides further evidence that these

17 effects may be endocrine-mediated. These findings are consistent with the hypothesis

18 that, like vertebrates, crustaceans are susceptible to the effects of EDCs.

19 In this study, we examine patterns of variability in the reproductive morphology of the

20 shore crab, *Carcinus maenas* L., for evidence of endocrine disruption. The shore crab

21 provides an ideal focus for this type of study as it has a particular affinity to estuarine

22 habitats, which are particularly susceptible to pollution from anthropogenic sources.

23 Recent evidence indicates that this species is sensitive to contaminant-induced effects

4

1 (Galloway et al., 2004). The shore crab also fulfils many of the criteria for the selection

2 of sentinel species outlined at the Institute for Environmental Health workshop on "The

3 Ecological Significance of Endocrine Disruption" (Leicester, 1997) in that it is common

4 and widespread in Northern Europe, it reproduces sexually and is sexually dimorphic.

5 Unlike a number of decapods that are naturally hermaphroditic, the shore crab is single

6 sexed throughout life and adult males and females are readily identifiable.

7 However, male shore crabs are known to be capable of exhibiting an intersex condition,

8 which is induced by parasitic castration by the thoracican barnacle, *Sacculina carcini*

9 (Charniaux-Cotton, 1960). This leads to the development of more feminine features,

10 such as a broadened abdomen and a reduction in dominant claw size, through changes in

11 their endogenous endocrine regime. Preliminary data indicate that these feminised

12 features are also exhibited by male shore crabs from polluted environments, with males

13 from the Tyne and Tees estuaries, which are impacted by EDCs (Allen et al., 1999a; b;

14 Lye et al., 1999; Matthiessen et al., 1998), appearing to be less male than those from a

15 reference population (unpublished data). This indicates that the reproductive

16 development of this species may be susceptible to endocrine disruption by exogenous

17 agents such as EDCs. Here we present the findings of an extensive field survey that

18 aimed to investigate these patterns of inter-population variability in shore crab

19 morphology and assess their potential use as a biomarker of endocrine disrupting effects

20 on crustaceans in the field.

21 2. Materials and Methods

22 Approximately one hundred shore crabs were collected from each of eight sites around

1 northern Britain. Sampling site locations are shown in Figure 1. These locations were

2 selected on the basis of previous reports of endocrine disruption, including the induction

3 of vitellogenin and intersexuality in wild flounder (Allen et al., 1999a; b). In increasing

4 order of impact, these were located in the estuaries of the rivers Dee, Clyde, Tyne,

5 Mersey and Tees. Three reference sites were also identified, two of which were located

6 on the west coast of Scotland at Arisaig and Appin, and one of which was located on the

7 east coast at Belhaven Bay. No evidence of endocrine disrupting inputs was available for

8 these sites, but given their remoteness from centres of population and industry, the risk of

9 endocrine disrupting effects is likely to be low.

10 Samples were collected during a six-week period in the summer of 2001. Adult crabs

11 (>30mm carapace width) were collected by hand from the intertidal zone. Individuals

12 that exhibited signs of infection by *S. carcinii* were discarded. The remaining crabs were

13 returned to the laboratory for morphological examination (see Figure 2). This focused

14 on the analysis of sexually dimorphic traits. Carapace length and width and

15 cephalothorax depth were measured at the widest, longest and deepest dimensions,

16 respectively. The depth of the chelae was also measured between the maximum points.

17 The degree of heterochely was taken to be the difference in depth between the left and

18 right claws. Individuals that had lost chelae or that were suspected to have regenerated

19 one or more cheliped were omitted from the analysis of claw morphology. Periopod and

20 propodus lengths were measured by taking an average from the fourth and fifth pairs of

21 limbs, respectively. Again, data from crabs with missing or regenerating limbs were

22 ignored. These dimensions were measured using digital callipers (Browne & Sharpe).

23 The length of the first pair of pleopods was measured under a dissecting microscope.

1   These structures exhibit clear sexual dimorphism, with the pleopods of males being

2   modified for copulation and those of females being used to hold eggs. Pleopod structure

3   was therefore used to confirm the sex of each crab. The size and shape of the abdomen

4   also exhibits sexual dimorphism. Hence, the area of the abdomen was recorded using the

5   image analysis package, Image Tool.

6   Each of the characters measured was plotted against carapace width in order to

7   investigate their relationship with body size. All characters increased with body size,

8   although the nature of these allometric relationships varied between traits. It was

9   necessary to remove the effects of size dependence to allow the morphological

10   comparison of crabs of varying size. This was achieved by calculating the residuals of

11   the line of best fit between each trait versus carapace width, which was used as a

12   reference dimension. These residuals were then used as adjusted trait values (Reist,

13   1985; Debuse et al., 2001). For some traits, the residuals required log transformation in

14   order to fulfil the assumptions of normality. In other cases, the adjusted trait values

15   increased with body size. This required that they were divided by carapace width in

16   order to achieve homogeneity of variance. These transformations enabled the statistical

17   analysis of inter-population variations in morphology, which was carried out using

18   ANOVA and Tukey's pairwise comparisons. The correlations between each trait and the

19   gradient of exposure to EDCs was explored by ranking the data and calculating Pearson's

20   product moment correlation coefficient. Traits were ranked from 1-8 according to their

21   mean adjusted values. Sites were ranked according to their pollution status: the three

22   reference sites were given a mean rank of 2 and the remaining five contaminated sites

23   were ranked 4-8 according to the extent of the effects reported by Allen et al. (1999a; b).

1 3. Results

2 The mean body size of crabs collected varied extensively between sites. In general, crabs

3 were smallest at the Dee estuary, where the majority of individuals were of between 35

4 and 40mm carapace width, and were largest at Arisaig, where the majority of crabs fell

5 within the 45 and 50mm carapace width range. For this reason, the mean trait values

6 presented in Tables 1 (a and b) have been calculated for a crab of average size.

7 Each of the traits analysed exhibited some degree of sexual dimorphism. This required

8 that male and female crabs were considered independently for the analysis of inter-

9 population variability. These analyses revealed that males exhibited significant

10 variability in nine out of the ten traits measured and that females exhibited significant

11 inter-site variability in all ten traits (See Table 2). However, no consistent pattern in the

12 distribution of this variability among populations from reference sites and those that had

13 evidence of endocrine disruption was apparent from the results of the post-hoc tests.

14 The pattern of variability expressed by each trait was then considered in terms of

15 exposure to EDCs. In male crabs, a correlation between depth of the right chelae and the

16 putative gradient of endocrine disrupting effects was observed (r=-0.81, p<0.05). This

17 pattern was not evident from the analysis of the left chelae (r=0.27, p=0.52). This meant

18 that the degree of heterochely also correlated with the pollution gradient, with male crabs

19 from sites with deeper right chelae exhibiting greater differences between the size of the

20 left and right claw (r=-0.88, p<0.01). Although this characteristic is generally more

21 pronounced in male crabs, the degree of heterochely expressed by female crabs also

22 correlated with the pollution gradient (r=-0.78, p<0.05). These patterns are highlighted

1   on Table 1. Tukey's tests revealed that there was a significant difference between the

2   degree of heterochely expressed by male and female crabs at the reference sites compared

3   with those at the Mersey and Tees, and to a lesser extent, the Clyde and Dee. The only

4   anomaly to this pattern was at the Tyne, where the chelal morphology of male and female

5   crabs differed little from that at the reference sites.

6   4. Discussion

7   This study has revealed that there is extensive inter-population variability in the

8   morphology of male and female shore crabs around the coast of Northern Britain.

9   However, examination of the patterns of spatial variability expressed by each trait

10 revealed that only the size of the dominant claw, and hence the degree of heterochely,

11 exhibited any correlation with previous reports of endocrine disrupting effects in fish

12 (Allen et al., 1999a; b). In male shore crabs, the dominant claw is a secondary sexual

13 characteristic that is used in aggressive and sexual interactions. As its development is

14 determined by sex hormones (Charnioux-Cotton, 1960), variations in the size or shape of

15 this structure could be indicative of endocrine disruption. However, if this were the case,

16 we would also expect there to be effects on the expression of other sexually dimorphic

17 traits, such as abdominal area, that are known to be affected by endogenous endocrine

18 disruption. We might also expect to see an increase in the degree of sexual dimorphism

19 expressed by crabs at the affected sites. The lack of further evidence of feminisation,

20 combined with the fact that similar patterns of variability in claw morphology were

21 evident in female crabs, indicates that endocrine disruption is an unlikely explanation for

22 the patterns observed.

1   This finding was not consistent with that of a previous, smaller scale survey of crab

2   morphology, although the same patterns of variability were expressed by each of the

3   traits analysed at each of the sites in question (Brian, unpublished data). This should

4   serve to caution against claims of endocrine disruption when small datasets, containing

5   fewer sites and/or lower sample sizes, are used to investigate such effects. In contrast,

6   the results of this study were in close agreement with those of a similar survey of shore

7   crab morphology, which was carried out under the EDMAR programme (Allen et al.,

8   2002). This revealed patterns of inter-population variability in the morphology of the

9   right chelae that were analogous to those presented in this study, but overall, it was

10   concluded that the data was equivocal and the mechanism responsible for variations in

11   claw size was unknown.

12   Potential explanations for these patterns of morphological variability are wide ranging.

13   For example, it is possible that differences in the chelal morphology of crabs from

14   reference and contaminated sites reflect a more general effect of pollution on crustacean

15   health. Alternatively it may be that the patterns observed have arisen as an indirect

16   consequence of contamination on ecological parameters. For example, changes in

17   community composition may alter the availability of different prey types. Diet has been

18   found to play an important role in the chelal development of the blue crab, *Callinectes*

19   *sapidus* (Smith & Palmer, 1994). Variations in morphology may have also arisen in

20   response to natural environmental differences between the sites, such as the structure of

21   the substrate. Further research is required to establish the potential influence of the

22   habitat characteristics on morphological variability.

1   However, the lack of evidence of a relationship between variations in shore crab

2   morphology and the putative gradient of endocrine disruption does not rule out the

3   possibility that shore crabs may be susceptible to the same type of effects reported in

4   crustaceans in the literature (e.g. Ford et al., 2004; Moore & Stevenson, 1991; Sangalang

5   & Jones, 1997; Takahashi et al., 2000). It is possible that a correlation was not detected

6   because the levels of EDCs encountered by the crabs from these populations did not

7   exceed the threshold required to elicit an affect. Alternatively, it may be that the methods

8   employed in this study were not sufficiently sensitive to reflect the effects of exposure to

9   environmentally relevant concentrations of EDCs or that the high levels of background

10   variability have obscured any contaminant-induced patterns in morphology. However, it

11   is also possible that these results reflect the fact that, unlike vertebrates, crustaceans such

12   as the shore crab are not susceptible to developmental effects exerted by EDCs.

13   The reproductive development of crustacea is similar to that of vertebrates in that sex is

14   genetically determined, but the expression of secondary sexual characteristics is largely

15   under hormonal control (Highnam & Hill, 1976). However, the structures and functions

16   of crustacean hormones are very different to those found in vertebrates. Although

17   vertebrate-like oestrogens and androgens have been identified in some invertebrate phyla,

18   such as cephalopods, bivalves and gastropods (DeLoof & DeClerk, 1986; Joosse, 1982),

19   the principal sex steroids in the crustacea are the ecdysteriods. Non-steroidal compounds

20   such as methyl-farnesoate have been associated with the reproductive control of some

21   species (Baldwin et al., 1995). This indicates that crustaceans are unlikely to be affected

22   by the same type of chemicals that are capable of interfering with the reproductive

23   development of vertebrates.

1 Previous research has revealed that changes in the reproductive morphology of the shore

2 crab can be induced by the manipulation of the androgenic gland, which is the site of

3 male sex hormone synthesis in crustacea (Barki et al., 2003). The removal of this gland

4 from juvenile male shore crabs has been found to inhibit the growth of male secondary

5 sexual characteristics and result in the development of the female form. Conversely, the

6 implantation of an androgenic gland into immature females stimulates the development of

7 male characteristics and inhibits female reproductive processes, such as vitellogenesis

8 (Charnioux-Cotton & Payen, 1988). This indicates that the expression of male and

9 female morphological characteristics is largely determined by the presence or absence of

10 androgenic hormones. This would explain the absence of a correlation between

11 variations in shore crab morphology and the gradient of endocrine disrupting effects in

12 flounder, which reflect the levels of estrogenic, as opposed to androgenic, activity.

13 The significance of androgenic hormones in determining the reproductive development of

14 the shore crab highlights the potential for androgenically-mediated effects on crustacean

15 morphology. It is unlikely that androgenic endocrine disruptors are responsible for the

16 patterns of inter-population variability detected in this study, which were not consistent

17 with the effects of hormone disruption. However, the de-masculinising actions of anti-

18 androgenic chemicals provide a plausible explanation for previous reports of feminisation

19 and intersexuality in copepods, decapods and amphipods *in situ*. Further research is

20 clearly required to elucidate the potential for androgenically-mediated effects on the

21 reproductive development of crustacea and to establish the implications for invertebrate

22 populations in the field.

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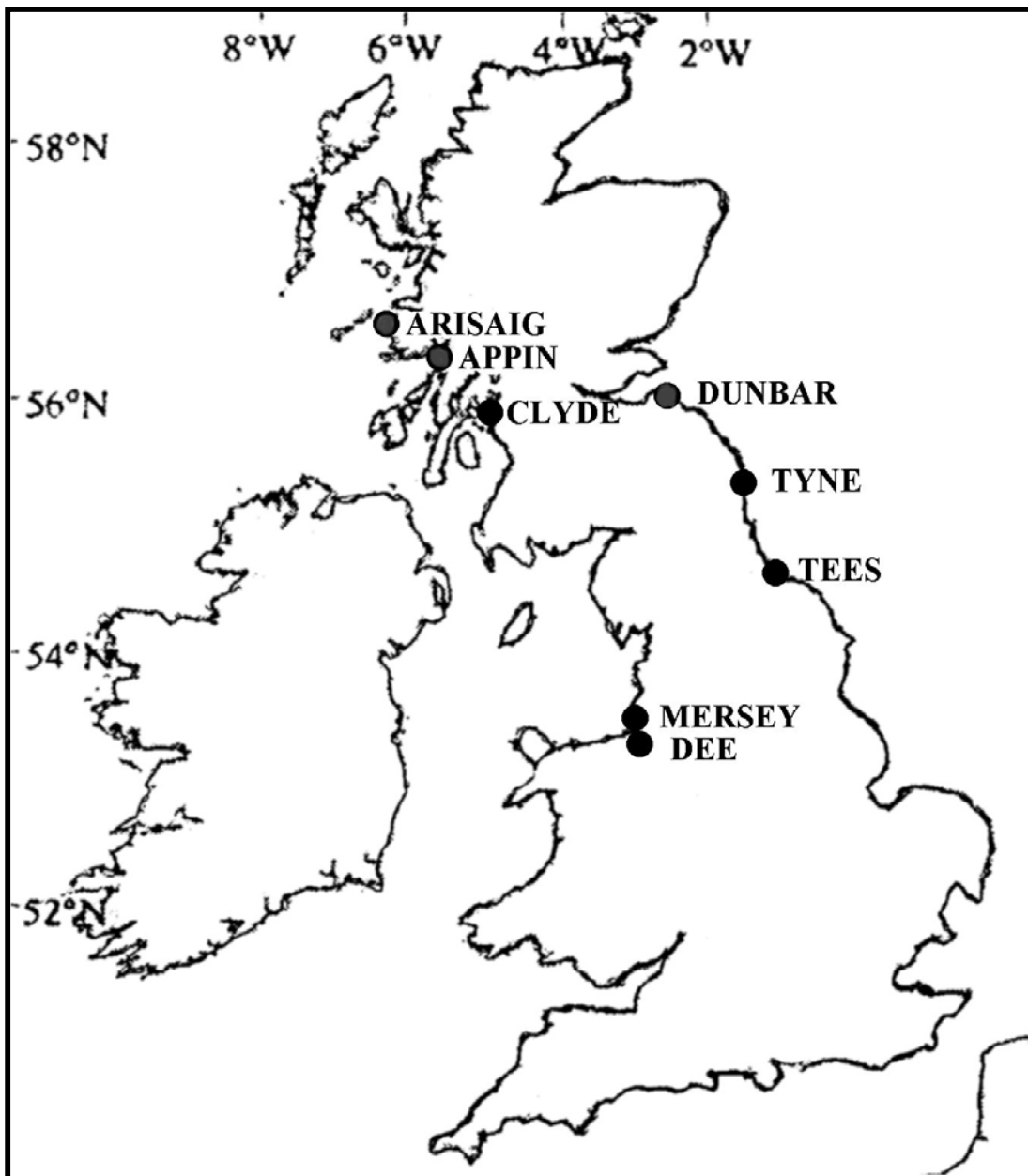6   Tyler, C.R., Jobling, S. & Sumpter, J.P. 1998. Endocrine disruption in wildlife: A

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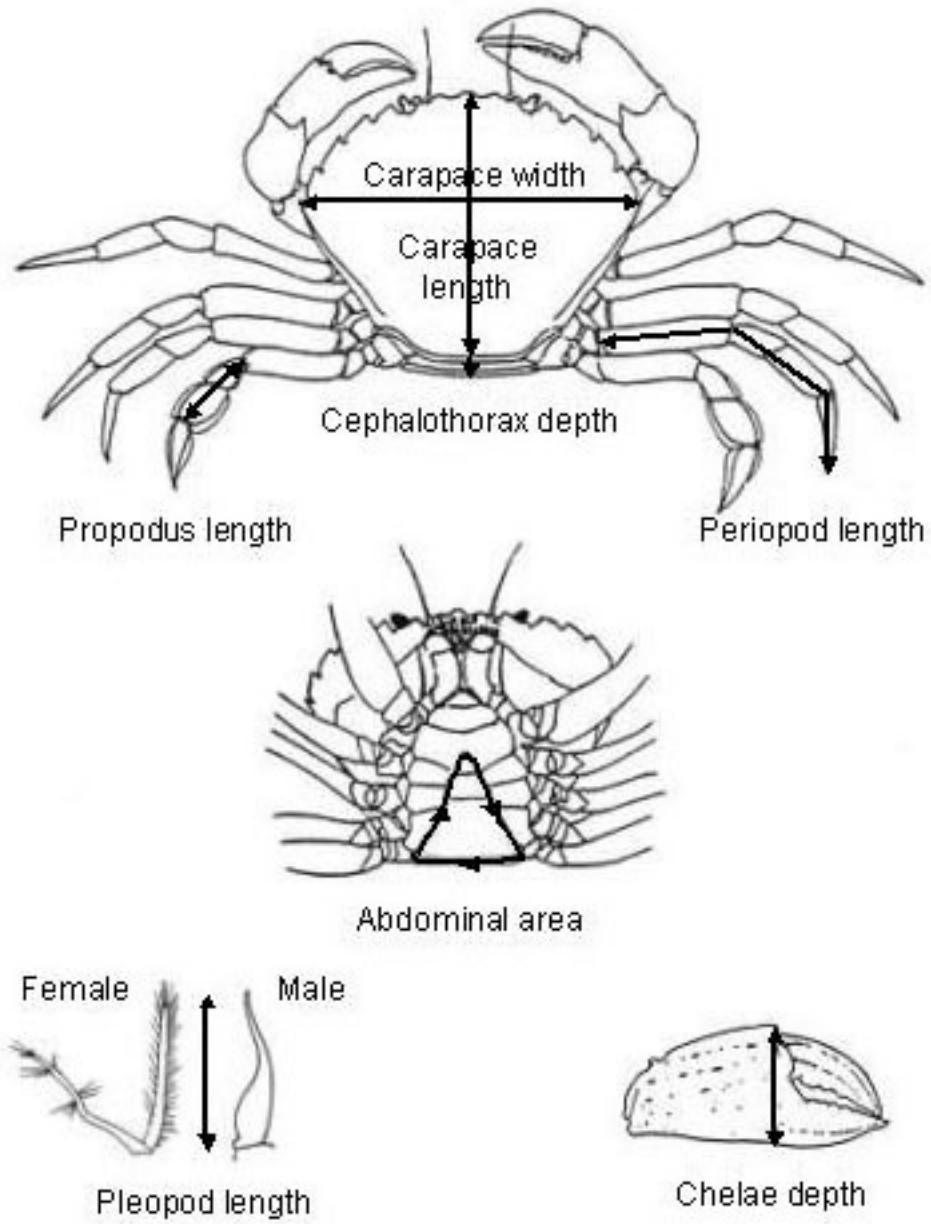11 Ecotoxicology and Environmental Safety 54, 216-222.

12

1   Figure 1

2

1   Figure 2

Carapace width

Carapace length

Cephalothorax depth

Propodus length

Periopod length

Abdominal area

Female   Male

Pleopod length

Chelae depth

2

3

Table 1 (a and b)

	Reference Sites			Increasing degree of contamination				
(a) *Trait Value (mm)*	Arisaig	Appin	Dunbar	Dee	Clyde	Tyne	Mersey	Tees
Carapace width	50.4 ± 8.66	47.7 ± 8.35	48.1 ± 10.46	41.5 ± 6.34	44.1 ± 8.85	45.3 ± 9.78	43.5 ± 7.56	43.2 ± 9.65
Carapace length	34.5 ± 0.47	34.5 ± 0.09	34.7 ± 0.12	34.9 ± 0.09	34.8 ± 0.09	34.6 ± 0.76	34.5 ± 0.84	34.5 ± 0.41
Cephalothorax depth	18.9 ± 0.45	19.0 ± 0.64	19.1 ± 0.10	18.9 ± 0.58	18.6 ± 0.59	18.7 ± 0.60	18.8 ± 0.06	19.0 ± 0.39
Left chelae depth	9.59 ± 0.55	9.62 ± 0.39	10.3 ± 0.57	9.81 ± 0.48	9.54 ± 0.47	9.92 ± 0.29	9.89 ± 0.33	9.92 ± 0.46
Right chelae depth	**13.4 ± 1.12**	**13.1 ± 0.93**	**13.6 ± 1.02**	**12.4 ± 0.06**	**11.8 ± 0.68**	**12.9 ± 0.96**	**11.6 ± 0.94**	**12.1 ± 0.77**
Heterochely	**3.84 ± 0.90**	**3.45 ± 0.81**	**3.37 ± 0.59**	**2.61 ± 0.62**	**2.39 ± 0.46**	**3.17 ± 0.65**	**1.83 ± 0.82**	**2.23 ± 0.63**
Pleopod length	11.5 ± 0.48	12.8 ± 0.49	13.3 ± 0.43	13.2 ± 0.38	12.6 ± 0.61	13.1 ± 0.44	13.1 ± 0.41	12.9 ± 0.41
Abdominal area	145 ± 8.20	149 ± 9.16	155 ± 11.15	145 ± 8.46	145 ± 7.81	149 ± 8.03	148 ± 6.49	153 ± 8.98
Periopod length	48.8 ± 1.74	50.0 ± 1.88	48.9 ± 1.46	48.9 ± 1.73	48.4 ± 1.79	49.6 ± 2.02	48.7 ± 1.42	49.8 ± 1.7
Propodus length	16.7 ± 0.47	16.8 ± 0.47	16.7 ± 0.42	16.5 ± 0.54	16.5 ± 0.43	16.7 ± 0.46	16.5 ± 0.30	16.8 ± 0.46

	Reference Sites			Increasing degree of contamination				

(b) *Trait Value (mm)*	Arisaig	Appin	Dunbar	Dee	Clyde	Tyne	Mersey	Tees
Carapace width	46.5 ± 6.72	46.5 ± 6.71	40.3 ± 6.93	33.9 ± 4.09	49.4 ± 5.76	43.2 ± 10.7	38.1 ± 5.72	37.4 ± 5.48
Carapace length	34.7 ± 0.88	34.7 ± 0.46	34.8 ± 0.59	35.2 ± 0.07	34.8 ± 0.07	35.2 ± 0.57	34.9 ± 0.12	34.5 ± 0.05
Cephalothorax depth	19.3 ± 0.74	19.4 ± 0.46	19.5 ± 0.66	19.4 ± 0.36	19.2 ± 0.52	19.1 ± 0.63	19.0 ± 0.37	19.4 ± 0.31
Left chelae depth	9.02 ± 0.31	9.62 ± 0.28	9.15 ± 0.26	8.66 ± 0.25	8.98 ± 0.26	9.26 ± 0.36	8.66 ± 0.13	8.79 ± 0.26
Right chelae depth	11.6 ± 1.22	10.6 ± 0.82	11.1 ± 0.72	10.2 ± 0.36	10.0 ± 0.51	11.2 ± 0.95	9.10 ± 0.22	9.64 ± 0.43
Heterochely	**2.66 ± 1.04**	**1.74 ± 0.85**	**2.11 ± 0.65**	**1.73 ± 0.40**	**1.06 ± 0.46**	**2.04 ± 0.85**	**0.43 ± 0.23**	**0.84 ± 0.33**
Pleopod length	20.2 ± 1.06	19.1 ± 0.87	20.1 ± 1.14	20.7 ± 1.29	19.0 ± 1.38	19.8 ± 1.05	19.2 ± 0.75	20.1 ± 0.80
Abdominal area	255 ± 22.7	262 ± 10.7	277 ± 19.4	257 ± 11.9	294 ± 19.9	277 ± 26.3	234 ± 10.3	258 ± 13.9
Periopod length	46.2 ± 1.90	47.8 ± 1.34	44.4 ± 1.63	44.0 ± 0.74	46.5 ± 1.02	47.8 ± 1.83	45.9 ± 0.77	46.5 ± 1.19
Propodus length	16.3 ± 0.65	16.2 ± 0.48	15.7 ± 0.46	14.8 ± 0.31	16.0 ± 0.38	15.8 ± 0.52	15.7 ± 0.37	15.9 ± 0.36

Table 2

Trait	Males			Females		
	df	F	p	Df	F	p
Carapace Width	340	5.31	<0.01	305	17.56	<0.01
Carapace Length	338	4.26	<0.01	299	3.99	<0.01
Cephalothorax depth	253	1.83	0.08	223	2.76	<0.05
Left chelae depth	185	6.60	<0.01	153	3.98	<0.01
Right chelae depth	187	1.59	<0.01	157	17.33	<0.01
Heterochely	176	18.14	<0.01	151	19.40	<0.01
Pleopod length	216	6.22	<0.01	171	4.16	<0.01
Abdominal area	211	5.48	<0.01	179	6.83	<0.01
Periopod length	209	5.09	<0.01	165	15.68	<0.01
Propodus length	215	5.49	<0.01	192	13.98	<0.01

Legends

Figure 1. Locations of the sampling sites.

Figure 2. Illustration of morphological dimensions measured. (Reproduced and modified from Crothers (1967) with the kind permission of J.H. Crothers).

Table 1 (a) and (b). Relative mean trait values for male (a) and female (b) shore crabs at each site. With the exception of carapace width, the mean trait values have been calculated for a crab of average size (45.6mm carapace width) to enable the comparison of populations of varying size. This was achieved using the regression equation of the relationship between each trait and carapace width. The error values represent the mean of the residuals of this relationship for each population. Traits that correlated with the gradient of endocrine disrupting effects are highlighted.

Table 2. Inter-population differences in the morphological characteristics of male and female crabs.