Reproductive effects of heavy metals on the rough periwinkle *Littorina saxatilis* (Mollusca: Prosobranchia)

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The effects of chronic mine-related heavy metal contamination on reproduction of *Littorina saxatilis* from sites around the Isle of Man were assessed. Size at birth of young, reproductive output (i.e. number of embryos in brood pouch) and frequencies of abnormal embryos in the brood pouch were examined in animals from five sites. Laxey estuary was the most contaminated compared to other sites with less contamination (Peel) and relatively uncontaminated control sites (Castletown, Ramsey estuaries and the open coast near Derbyhaven). The size at birth of young ranged from 0.365-0.876 mm and the lowest mean size at birth (0.517 ± 0.048 mm) was found in the animals from Laxey estuary. Those from Derbyhaven had the highest mean size at birth (0.632 ± 0.063 mm). ANOVA showed a significant difference in mean size at birth of young, between individuals from the five sites (p<0.0001). However, the individuals from Laxey also had the highest absolute mean count of embryos in their brood pouch implying that there might be some trade-off between reproductive output and size of young as a result of metal contamination. No heavy metal related differences in embryo abnormality were apparent. Rather, a high proportion of abnormal embryos in animals from Derbyhaven suggested that disease conditions, genetic factors or unidentified toxins might account for the differences observed.

[Key words: *Littorina saxatilis*, Isle of Man, heavy metals, fecundity, size at birth, embryo abnormality]

Sub-lethal effects of pollutants such as depression of growth and reproduction occur at concentrations where acute toxic effects may not be apparent1,2. The embryonic and larval stages of aquatic animals are generally the most sensitive stages of the life cycle to heavy metals and other toxicants3. Hunt & Anderson4 established that at concentrations above 19 μg l⁻¹ zinc, larval metamorphosis in the abalone *Haliotis rufescens* was impaired. Oshida & Word5 reported that the number of offspring produced by the polychaete *Neanthes aranaceodentata* was reduced by exposure to 39 μg l⁻¹ of dissolved chromium (VI). The dogwhelk, *Nucella lapillus* exhibits “imposex”, the imposition of male sexual characteristics on females, as a result of exposure to tributyltin (TBT), and the decline of populations of *Nucella* along much of the Channel coast of southern England was the result of TBT and virtually all populations in England showed visible effects of TBT pollution6,7.

It has been suggested that the rough periwinkle *Littorina saxatilis* Olivi (Mollusca: Prosobranchia) could be a useful organism for monitoring the reproductive effects of contaminants because embryos are found in the brood pouches all year round8,9. Embryonic development is internal and takes place in the brood pouch of the female, hence local pollution events would affect all stages of embryonic development, and in turn the number and size of juveniles may be affected. Most previous studies on the inter-site differences in numbers of embryo and the size of juveniles at birth have concentrated on the effects of natural environmental gradients such as shore height8 or, the degree of exposure or shelter of the shores10,12. All stages of development from uncleaved egg to pre-emergent fully formed juveniles can be found in the brood pouch of female *L. saxatilis* at the same time. However, in addition to normal stages of development, the brood pouch may contain a
proportion of abnormally developed embryos. Anthropogenic contamination has also been reported as increasing the frequency of abnormalities\textsuperscript{9}.

Despite being contaminated with heavy metals from previous mining activity\textsuperscript{13}, the Laxey estuary, Isle of Man supports a large population of \textit{Littorina saxatilis}; these have been shown to possess enhanced tolerance to acute levels of zinc and lead\textsuperscript{14}. Sublethal concentrations capable of inducing tolerance to metals may exact reproductive effects. Also tolerance may have energetic costs, which might indirectly affect reproductive output\textsuperscript{15}. The occurrence of such effects in \textit{L. saxatilis} from sites around the Isle of Man with different levels of heavy metal contamination was studied. Inter-site differences in the proportions of abnormal embryos were examined to assess usefulness of reproductive indices in monitoring the effects of sub-lethal concentrations of contaminants.

Materials and Methods

The sites from which samples were collected around the Isle of Man (Castletown, Derbyhaven, Laxey, Peel and Ramsey) are shown in Fig. 1. The catchments leading into the estuaries at Laxey and Peel are still contaminated by past mining activities\textsuperscript{13,16}. Details of heavy metal concentrations in the environment and bioindicators are given elsewhere\textsuperscript{14}. Metal burdens in the soft tissue of \textit{Littorina saxatilis} from the five sites are presented in Table 1. The animals from Laxey are exposed to highest concentrations of heavy metals, especially zinc but high levels are also present at Peel. In contrast the estuaries at Castletown and Ramsey have no mining contamination, but there is little industrial contamination of Castletown from boating and an industrial estate and Ramsey has been shown to be affected by TBT contamination\textsuperscript{17}. The Castletown Bay side of Langness near Derbyhaven was presumed to be a clean coastal reference site.

\textit{Littorina saxatilis} of reproductive size (>6 mm shell height) were collected from Castletown, Derbyhaven, Laxey, Peel and Ramsey (from 8-10 May 1995; 1 July 1996), relaxed in 7.5% magnesium chloride and fixed in 10% formaldehyde-seawater solution\textsuperscript{18}. Animals were then preserved in 70% ethanol until dissected. The shell height of each individual was measured before cracking on a bench vice and separating the tissue from the shell. The presence of a

Table 1 — Concentrations (mean ± s.d., n=5) of zinc, lead, copper and cadmium (µg g\textsuperscript{-1} dry tissue) in \textit{Litorina saxatilis} from five sites around the Isle of Man.

<table>
<thead>
<tr>
<th></th>
<th>Zinc</th>
<th>Lead</th>
<th>Copper</th>
<th>Cadmium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castletown</td>
<td>121 ± 5</td>
<td>102 ± 3</td>
<td>11.5 ± 2.5</td>
<td>11.5 ± 2.5</td>
</tr>
<tr>
<td>Derbyhaven</td>
<td>89 ± 3</td>
<td>103 ± 7</td>
<td>2.3 ± 0.9</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>Laxey</td>
<td>438 ± 42</td>
<td>504 ± 28</td>
<td>16.6 ± 0.7</td>
<td>16.6 ± 0.7</td>
</tr>
<tr>
<td>Peel</td>
<td>157 ± 6</td>
<td>128 ± 9</td>
<td>16.6 ± 2.2</td>
<td>16.6 ± 2.2</td>
</tr>
<tr>
<td>Ramsey</td>
<td>148 ± 7</td>
<td>135 ± 4</td>
<td>7.7 ± 1.7</td>
<td>7.7 ± 1.7</td>
</tr>
</tbody>
</table>

Fig. 1 — Locations of sampling sites around the Isle of Man and past mining activities. Filled triangles and circles represent major and minor producing mines respectively with an indication of the order of importance of the ores produced (Cu=copper, Fe=iron, Pb=lead, Zn=zinc).
brood pouch and penis was used to identify females and males, respectively. The brood pouch of each female was removed into a gridded Petri dish, teased open and the contents examined under a stereomicroscope and counted.

About 50 animals of reproductive size (> 6 mm shell height) from each site were placed in white plastic containers after acclimatisation to 16°C for 6 to 7 days in other tanks. The individuals transferred were thoroughly cleaned to avoid transfer of juveniles on the shells. The warm temperature was a means of inducing the free young to emerge from the brood pouch of the parents, which is entirely a function of the juvenile activity. Juveniles that emerged were removed from the containers every 24 hours for five days and preserved in 70% ethanol prior to measurement. The measurements (accurate to ±0.001 mm) were made on a stereomicroscope (Wild Heerbrugg, Finlay Microvision Ltd.) equipped with a video camera. Images were captured and calibrated to a set magnification using a NIH Image Analysis software (1.58 VDM) on a Macintosh Computer.

Embryo abnormalities were determined in samples collected on 1 July 1996 during the middle of the reproductive season. After collection, animals were stored at ambient temperature in the laboratory for a few days before dissection. The shell height of each animal was measured as above before cracking on a bench vice. The brood pouch content of females were placed in a Petri dish and viewed under a stereomicroscope for counting and examination of embryo abnormality. Total number of embryos in each pouch was counted and each embryo was examined for abnormality. Abnormalities involving unshelled embryos (mainly characterised by the physical disruption of soft tissues, resulting in the presence of a number of separate, floating, cell masses within the egg capsule; double embryos) and shelled embryos (multiple embryos, mostly twins; abnormal shells, mostly uncoiled shells) were counted separately.

Differences in the absolute number of embryos between sites were compared by means of Kruskal-Wallis one-way analysis of variance using MINITAB for Windows. Where significant differences were obtained with the Kruskal-Wallis test, the Dunn non-parametric multiple comparison test was used to make pairwise comparisons between average ranks. Significant differences in size at birth between animals from the five populations were tested by means of one-way analysis of variance (ANOVA) and Tukey HSD multiple comparisons. The proportions of abnormal embryos in each female were calculated for each of the two categories of abnormality (i.e. unshelled and shelled) and for total abnormalities. Significant inter-site differences in the proportions of unshelled, shelled and total abnormality were tested using the Kruskal-Wallis test and the Dunn test where applicable.

Results

Fecundity and size of juveniles at birth

The absolute numbers of embryos in the brood pouch L. saxatilis from the five sites were variable with total counts ranging overall from 24 at Castletown to 1117 at Laxey (Table 2). Animals from Laxey, the contaminated site, had the highest mean number of embryos in both the May 1995 and July 1996 samples. The mean absolute brood pouch content recorded in July 1996 was generally similar to values for May 1995 except for numbers in samples from Peel and Castletown. In animals from these two sites marked increases in mean number of embryos were recorded, which were coincident with the relative increase in the shell height of females from which the embryo were obtained. Kruskal-Wallis tests on median total brood pouch content showed significant differences for both sets of samples, but at different levels (df=4, H=89.27, P < 0.0001 for May 1995 and df=4, H=9.86, p = 0.043 for July 1996). Dunn multiple comparisons on the May data showed that pairwise significant differences in numbers of embryo followed the form: Laxey > Ramsey = Derbyhaven = Castletown > Peel. However, the Dunn test could not detect any significant differences between any pair of sites for the July data in spite of the significant differences shown by the Kruskal-Wallis test.

The relationships between shell height and number of embryos in brood pouch were very weak. When regressions were drawn (log. number of embryos against shell height), the coefficients of determination ranged from 0.050 (Derbyhaven) to 0.471 (Laxey) for May, 1995 and 0.072 (Derbyhaven) to 0.484 (Peel) for July, 1996. This precluded the application of Analysis of Covariance to test for differences in regression coefficients as way of determining significant differences in size-specific number of embryos.

The range and mean sizes of newly emerged juveniles (0-24 hrs old) for the five study sites are given in Table 3. Animals from Laxey (with the highest absolute pouch content) had the smallest juveniles while those from Derbyhaven were the largest. One-way
ANOVA showed a significant difference in the mean length at birth (F<sub>4,1412</sub> = 123.0, p < 0.001). Tukey multiple comparisons indicated that significant differences conformed to the following order: Derbyhaven > Castletown > Ramsey > Peel = Laxey.

**Embryonic abnormality**

The percentage of females with abnormal embryos was very high, ranging from 92% in samples from Castletown to 100% in those from Peel and Ramsey. The percentages of females with abnormal embryos from Derbyhaven and Laxey were 98% and 94% respectively.

Much of the abnormalities in animals from all sites were found in the unshelled embryonic stages (Table 4). Individuals from Derbyhaven had by far the highest proportion of unshelled abnormalities; those from Peel had the least with Laxey site showing intermediate values. The mean proportions of shelled abnormalities were similar in all sites. There was very high intra-site variability in the proportion of abnormalities. Coefficients of variation ranged between nearly 100% to well over 200%. The Kruskal-Wallis test showed a significant difference in median unshelled abnormalities between sites (df = 4, H = 33.87, p < 0.001); with individuals from Derbyhaven showing significantly higher abnormalities than those from all other sites (Dunn tests, Table 4). No significant differences were found for shelled abnormalities between sites. The animals from Derbyhaven also had the highest proportions of total abnormality. The Kruskal-Wallis test showed a significant difference in median total

### Table 2

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>Min.</th>
<th>Max.</th>
<th>Median</th>
<th>Mean</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castletown</td>
<td>45</td>
<td>24</td>
<td>652</td>
<td>256</td>
<td>225</td>
<td>58.3</td>
</tr>
<tr>
<td>Derbyhaven</td>
<td>84</td>
<td>54</td>
<td>731</td>
<td>262</td>
<td>277</td>
<td>49.5</td>
</tr>
<tr>
<td>Laxey</td>
<td>45</td>
<td>52</td>
<td>955</td>
<td>398</td>
<td>435</td>
<td>44.7</td>
</tr>
<tr>
<td>Peel</td>
<td>73</td>
<td>27</td>
<td>396</td>
<td>131</td>
<td>145</td>
<td>46.6</td>
</tr>
<tr>
<td>Ramsey</td>
<td>44</td>
<td>39</td>
<td>1018</td>
<td>269</td>
<td>316</td>
<td>58.1</td>
</tr>
</tbody>
</table>

**May, 1995:**

- Laxey ≠ Ramsey = Derbyhaven = Castletown ≠ Peel

**July, 1996:**

- Laxey = Castletown = Peel = Ramsey = Derbyhaven

Kruskal-Wallis One-way Analysis of Variance (H) and Dunn Tests (p < 0.05):
- May 1995: H = 89.27, p < 0.001
- Laxey ≠ Ramsey = Derbyhaven = Castletown ≠ Peel
- July 1996: H = 9.86, p = 0.043
- Laxey = Castletown = Peel = Ramsey = Derbyhaven

### Table 3

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castletown</td>
<td>186</td>
<td>0.405</td>
<td>0.876</td>
<td>0.574</td>
<td>0.071</td>
</tr>
<tr>
<td>Derbyhaven</td>
<td>103</td>
<td>0.457</td>
<td>0.776</td>
<td>0.632</td>
<td>0.063</td>
</tr>
<tr>
<td>Laxey</td>
<td>419</td>
<td>0.356</td>
<td>0.676</td>
<td>0.518</td>
<td>0.048</td>
</tr>
<tr>
<td>Peel</td>
<td>417</td>
<td>0.375</td>
<td>0.700</td>
<td>0.525</td>
<td>0.053</td>
</tr>
<tr>
<td>Ramsey</td>
<td>292</td>
<td>0.394</td>
<td>0.732</td>
<td>0.547</td>
<td>0.047</td>
</tr>
</tbody>
</table>

Analysis of Variance F<sub>4,1412</sub> = 123.0, p < 0.001.
Multiple Comparisons (Tukey Tests, p < 0.05):
Derbyhaven ≠ Castletown ≠ Ramsey ≠ Peel = Laxey
abnormalities between sites (df=4, H = 25.02, p < 0.001) and Dunn tests found a significantly higher abnormality in animals from Derbyhaven in comparison with those from Ramsey, Peel and Laxey. However, no significant difference in total abnormality was found between animals from Derbyhaven and those from Castletown. Spearman’s rank correlation coefficients indicated that no significant correlation existed between shell height and the proportion of abnormal embryos in the brood pouches of female winkles at any of the sites studied [Castletown: \( r_s (39) = 0.046, \) Derbyhaven: \( r_s (63) = -0.002, \) Laxey: \( r_s (52) = -0.121, \) Peel: \( r_s (46) = -0.206, \) Ramsey: \( r_s (50) = 0.044, \) in all cases p > 0.05].

**Discussion**

**Fecundity and size at birth of juveniles**

Intraspecific differences in numbers of embryo an size of juveniles at birth in *Littorina saxatilis* have been reported to vary with the level of exposure of the shore from which samples are collected\(^{10,11,20}\). Such variation should be taken into account when interpreting the likely effects of pollution on these reproductive parameters. Conflicting reports have been found in the literature with respect to how exposed/sheltered shore types affect these reproductive parameters. For example, Janson\(^{20}\) compared the sizes of newly born juveniles of two morphs of *L. saxatilis*: exposed (E-morph) and sheltered (S-morph) from Swedish coast populations and found that despite being smaller, E-morph females on the average gave birth to larger juveniles than did S-morph females; and concluded that this difference in hatching size is likely to be of genetic origin and an aspect of the adaptation to the environment. On the contrary, Faller-Fritsch\(^{10}\) reported that investigations of *Littorina rudis* (=*L. saxatilis*) in many parts of Britain support the view that differences in reproductive energy expenditure were general in occurrence; such that fewer, larger embryos were characteristic of sheltered populations while a large number of smaller offspring are favoured in exposed shores. Similarly, Roberts & Hughes\(^{11}\) found from studies of three Welsh sites (a sheltered saltmarsh, a semi-sheltered boulder shore and an exposed cliff) that the largest juveniles were produced by the population from the most sheltered site. However, the smallest juveniles were not obtained from the exposed shore but rather from the semi-sheltered boulder shore and they did not find any significant differences in the size-specific number of embryo in the brood pouch. They concluded that there was no significant trade-off between the size of offspring at birth and parental fecundity in *L. rudis* (=*L. saxatilis*).

The locations from which samples were collected for this work were essentially sheltered, efforts having been made to control for the effect of exposure. However, some differences were apparent in the degree of exposure of the locations from which samples were taken with Peel and Ramsey being slightly more exposed, the former being more so than the latter. Overall, Castletown and Derbyhaven were the most sheltered and Laxey was intermediate between the above four sites. The size structure of adults at these sites reflects the above pattern, the animals from Peel being the smallest and those from Castletown the largest\(^{14}\). The degree of exposure of shore may therefore not be responsible for the variations in the size of juveniles.

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**Table 4—Proportions of abnormal embryos (unshelled, shelled and total abnormals) in *Littorina saxatilis* from five sites in the Isle of Man.**

<table>
<thead>
<tr>
<th>Site</th>
<th>Unshelled abnormals</th>
<th>Shelled abnormals</th>
<th>Total abnormals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Median</td>
<td>Mean</td>
</tr>
<tr>
<td>Castletown</td>
<td>39</td>
<td>2.6</td>
<td>3.3</td>
</tr>
<tr>
<td>Derbyhaven</td>
<td>63</td>
<td>3.6</td>
<td>14.3</td>
</tr>
<tr>
<td>Laxey</td>
<td>52</td>
<td>1.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Peel</td>
<td>46</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Ramsey</td>
<td>50</td>
<td>1.9</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Analysis of Variance: H=33.87, p < 0.001

Dunn Tests (p<0.05): D C R L P

Underlined=no difference

C=Castletown, D=Derbyhaven, L=Laxey, P=Peel, R=Ramsey

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[ N.B. Large coefficients of variation. N=number of animals. Results of Kruskal-Wallis One-way Analysis of Variance and Dunn tests are shown ]
observed as *L. saxatilis* from Laxey had the smallest mean embryo size and the highest mean absolute number of embryos both in May and July. The situation of the animals from Laxey does not also conform to the contention by Roberts & Hughes\(^1\)\(^1\) that there is no trade-off between size at birth and fecundity, since, the lowest size at birth coincided with the highest fecundity in the samples from the Laxey.

A likely explanation for the size at birth and fecundity of *L. saxatilis* at Laxey relative to the snails from other sites (Tables 2 and 3), is exposure to high levels of heavy metals (mainly zinc but also cadmium and lead). Bodar *et al.*\(^2\)\(^1\) observed from laboratory experiments with *Daphnia magna* exposed to cadmium that concentrations of 0.5, 1.0 and 5.0 ppb caused a significant increase in the average number of neonates per female, but the size of the neonates were smaller. They found, however, that at higher concentrations both brood size and body size declined. The lower size at birth and higher absolute number of embryos in *L. saxatilis* may be similar to the above finding. It is possible that the growth rate of embryo in the brood pouch is lowered by metal pollution such that it takes a longer time for the embryo to develop to the emergent stage at which it crawls out of the brood pouch. The longer gestation time may have led to the presence of large number of embryos in the brood pouch at any given time since the release of eggs is continuous. The smaller size of the embryo may then be the result of either a packed brood pouch with limited space for growth or the direct effect of the reduced growth rate of embryos. Notably, the size of young at birth followed the reverse order of metal contamination such that the cleanest (Derbyhaven) had the largest and Laxey the smallest with Castletown > Ramsy > Peel.

Other factors which may, however, affect the differences in embryo numbers of *L. saxatilis* include the availability of food and the relative period of tidal exposure which could affect foraging time.\(^1\)\(^0\) Berry\(^8\) observed that fecundity in snails was higher for individuals collected from the upper shore than those from the lower shore. Spatial differences in such factors from our study sites may have contributed to the differences observed.

**Embryo abnormality**

The results of this study did not show any correlation in the proportion of abnormal embryos (Table 4) in the Isle of Man populations of *Littorina saxatilis* with metal pollution (Table 1). In contrast, Dixon & Pollard\(^9\) working on the southern coast of Wales found that individuals from a site which was furthest away from the known sources of pollution had the lowest frequency of abnormal embryos in comparison with those from other sites, but there was no correlation between the gradient of contamination and frequency of abnormality and they suggesting that a complex aetiology might be involved. It is possible that the animals from Derbyhaven (highest embryo abnormality but least metal contamination) experience some form of contamination that has not been detected in this work, but this is unlikely. Derbyhaven has full strength seawater so it is not the cause of the high frequency of abnormalities at this site.

Dixon & Pollard\(^9\) suggested that abnormalities may be caused by a latent pathogen which is activated to disease condition by environmental stress. This assertion was based on the physical features of the most dominant type of abnormality they observed were reminiscent of the kind of uncontrolled breakdown that might be expected from a neoplastic disorder, in this case confined within the egg capsule. However, they did not find any pathological condition to be associated with the somatic tissue of adult snails containing high incidence of abnormality. The predominant type of embryo abnormality observed in *L. saxatilis* from Derbyhaven bear the above features described by Dixon & Pollard\(^9\) and it is possible that some disease condition may be responsible for the high incidence of abnormalities at that site.

Janson\(^1\)\(^2\) argues that genetic factors may play an important role since environmental stress factors do not unquestionably influence the degree of embryo abnormality. Large within-site variations in embryo abnormality were suggested to be due to genetic rather than environmental factors. Presumably, in-breeding in small random mating groups was the primary genetic factor involved. The possibility of such an effect occurring in the Isle of Man populations of *L. saxatilis* cannot be ruled out as very high coefficients of variation were found in the proportions of abnormalities in these sites.

Contamination by heavy metals may be exerting direct or indirect effects leading to a reduction in the size of embryo at birth in *L. saxatilis* from Laxey estuary. No evidence for induction of embryo abnormalities by heavy metals was apparent in this study. The high proportions of abnormal embryos in animals from Derbyhaven which are not known to be exposed to high levels of heavy metal pollution may be due to disease and/or genetic factors. It is concluded that...
embryo abnormality in *L. saxatilis* may not be a good indicator of sub-lethal effects of heavy metals in the field.

**Acknowledgement**

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**References**