

1 **AMPHIPOD INTERSEX, METALS AND LATITUDE: A PERSPECTIVE**

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6
7 **ABSTRACT**

8 Intersexuality has been widely reported in crustaceans with several mechanisms being
9 directly or indirectly held responsible for its occurrence, amongst which pollution. No
10 mechanistic relationship between metals and intersex has ever been established. Also the
11 incidence of intersex in populations of the same invertebrate species in a latitudinal gradient
12 has never been studied so far. Three populations (Iceland, Scotland and Portugal) of the
13 amphipod *Echinogammarus marinus* were scrutinized. Intersex females from Iceland
14 registered the highest fecundity loss. Only in Scottish samples females with two genital
15 papillae and males with only one genital papillae were observed. Nevertheless, water, biota
16 and sediment samples pointed to equivalent metal levels, and in consonance the prevalence
17 of intersex was not significantly different between locations. An unequivocal relationship

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18 between metal presence and intersex induction cannot be presented, but our results
19 advocate the potential role of metals as a direct cause of intersexuality in *E. marinus*.

20 **KEYWORDS:** *Echinogammarus marinus*; intersex; heavy metals; Iceland; Scotland; Portugal.

21 **1. INTRODUCTION**

22 Intersexuality, the condition whereby otherwise normal gonochoristic species possess both
23 male and female characteristics, has been reported throughout the animal kingdom
24 (Reinboth, 1975). This phenomenon has been widely identified in crustaceans, among which
25 a large number of amphipod species (*e.g.* Sars, 1895; Sexton and Huxley, 1921; Dunn et al.,
26 1990; Ladewig et al., 2003; Ford and Fernandes 2005).

27 Several mechanisms are directly or indirectly held responsible for its occurrence including
28 parasitism (Bulnheim, 1977); bacterial infection (Rigaud and Juchault, 1998); genetic control
29 (Lebederf, 1939); the ablation or implantation of androgenic glands, from/into males and
30 females, respectively (Charniaux-Cotton, 1958); environmental sex determination (ESD)
31 (Dunn et al., 1993) and pollution (Moore and Stevenson, 1991; Ford et al., 2006). Recently it
32 has been demonstrated to be induced chemically by juvenile hormone mimics (Olmstead and
33 LeBlanc, 2007).

34 The full significance and costs of intersexuality for amphipods are still unknown. Some
35 studies report lower fecundity and fertility, reduced pairing success and delayed maturation
36 in *Echinogammarus marinus* (Ford et al., 2003; Ford and Fernandes, 2005). Dunn et al. (1993)
37 also reported reduced pairing success in *Gammarus duebeni* and Kelly et al. (2004) found

38 lower fecundity in intersex females of the same species. An investigation by Plaistow et al.
39 (2003) on the costs of intersex upon precopulatory guarding in *Gammarus pulex*, indicated
40 that these were correlated with female size. Consequently, pair forming with larger intersex
41 females (which grow larger than the normal counterpart) will lead not only to large energy
42 costs, but also reduced reproductive success. Barbeau and Grecian (2003) noted reduced
43 fertility in intersex male *Corophium volutator*, a finding confirmed for the same species by
44 McCurdy et al. (2004) that registered smaller broods issuing from the pairing of intersex
45 males with normal females compared to matings with normal males.

46 A model simulation for an *E. marinus* population run by Ford et al. (2007) to evaluate the
47 reproductive costs associated with intersexuality revealed that intersexes can have dramatic
48 effects: population extinction within approximately 6 years if 5% of normal females are
49 replaced by intersex females. Nevertheless, in behavioral terms no differences appear to
50 occur with intersex females and intersex males acting as do normal females and males,
51 respectively (Ford et al., 2007).

52 Besides the classical case study of organotin-induced imposex and intersex in gastropods
53 (Matthiessen and Gibbs, 1998), continued studies of metals endocrine disrupting effects in
54 invertebrates are scarce, leading to inconsistent knowledge on the subject. More recently a
55 growing body of work has been trying to bridge this gap of knowledge (e.g. Medesani et al.,
56 2004; Bondgaard and Bjerregaard, 2005; Corrêa et al., 2005; Ford et al., 2006), as the study
57 of metals as endocrine disruptors can be considered highly relevant bearing in mind their

58 ubiquitous presence in the environment (from both natural and anthropogenic sources) and
59 in metabolic processes.

60 *Echinogammarus marinus* (Leach, 1815) (= *Chaetogammarus*; = *Marinogammarus*) is a highly
61 abundant amphipod species of the marine and estuarine intertidal north-eastern Atlantic
62 (Lincoln, 1979), with documented intersex prevalence (Ford et al., 2003). This species occurs
63 in close association with *Fucus spp.* (Phaeophyta, Fucales), which is used by the amphipod as
64 source of food and shelter to live and breed (Schreider et al., 2003).

65 To the author's best knowledge, no mechanistic relationship between endocrine disruptive
66 capabilities of metals and the occurrence of intersex in crustaceans has ever been
67 established. The incidence of intersex in populations of an invertebrate species in a
68 latitudinal gradient is also unknown. In this context, this study aimed to ascertain the
69 incidence of intersexuality in independent populations of *E. marinus* (encompassing the
70 entire breadth of its distribution), while at the same time attempted to unveil possible
71 correlations with the presence of heavy metals.

72 **2. MATERIALS and METHODS**

73 **2.1. Study sites**

74 The organisms used in this study to evaluate intersex variation in latitudinal terms were
75 collected in Iceland, Scotland and Portugal (Figure 1). A literature survey was performed to
76 choose specific sites where *Echinogammarus marinus* occurs and metal levels were of
77 approximately similar magnitude.

78 Icelandic individuals were captured in Reikjanes Peninsula (64°02'N, 22°42'W). Despite these
79 shores being considered pristine environments (Sarà et al., 2007), there is indication of heavy
80 metal background values being higher than in equally remote areas. Volcanic activity is
81 regarded as the probable cause (IME, 2001), mainly because eruptions substantially increase
82 metals concentration in Icelandic rivers. The redistribution of these metals is efficiently
83 performed by the unique hydrography of the Icelandic coastal current, which begins in the
84 northeast, moves clockwise and ends at the mid-north coastal areas (Egilsson et al., 1999).
85 The closest human settling from the collection site is the small town of Sandgerði (1700
86 inhabitants), which is one of the most important fishing harbours in Iceland, where fish
87 processing facilities are installed. Olafsson (1986) used *Mytilus edulis* to run a “mussel
88 watch”-type survey on the south-western coast to identify anthropogenically enhanced
89 metal concentrations. The conclusion was that localized spots existed, being the Sandgerði
90 area one of them. This verdict was confirmed by Leung et al. (2005) who allude to Sandgerði
91 as a “polluted site”.

92 Loch Fyne is the longest, deepest fiordic sea loch of Scotland, being part of the Firth of Clyde
93 watershed (one of the country’s most contaminated). In spite of this, it was expected to be
94 relatively free from metal contamination due to serving mainly forestry, extensive grazing,
95 aquaculture and recreation purposes (SEPA, 2005). Leung et al. (2001) detected higher metal
96 contents in tissues of *Nucella lapillus* from this area than in individuals collected in areas
97 traditionally described as metal polluted. The organisms for the present study were collected

98 in the vicinity of Strachur (56°10'N, 5°05'W), located a few km to the south of a "Marine
99 Consultation Area" (NCC, 1986).

100 The Mondego Estuary is located in the Portuguese Atlantic coast. It comprises two arms
101 (north and south) with very different characteristics. North arm is highly hydrodynamic,
102 possesses navigational capabilities and it is pointed as having pristine conditions in terms of
103 heavy metals (Vale et al., 2002). South arm dynamics are much weaker, depending partly of
104 the tidal excursion and of the artificially controlled discharges (according to the necessities of
105 the extensive lower valley rice crops) of a small river – the Pranto. This leads to extended
106 residence times and fine particles deposition. The Mondego possesses a sizeable watershed
107 (6670 km²) upon which the impact of urban areas summing up to 695.000 inhabitants is felt
108 (INAG, 2004). Few industries are to be noted, but deactivated uranium mines are located
109 approximately 100km from the estuary and increases of metals, particularly Zn, Mn, Fe, U, Pb
110 and Ra (Pinto et al., 2004) are registered in the areas directly receiving the mine effluents.
111 The organisms were collected in the south arm (40°07'N, 8°49'W) in the confluence with
112 Pranto river. Castro et al. (2007) noted an increase of Pb in sediment profiles from this area
113 since the 1960's, but emphasized that the Mondego was not heavily contaminated compared
114 to other European estuaries. Pereira et al. (2005) indicated enrichment in Hg, Cu, Cd, Cr, Mg,
115 Zn and Fe of the fine fraction of the south arm, compared to the remainder of the estuary,
116 pointing the Pranto river as the presumable source.

117 **2.2. Sampling**

118 *E. marinus* specimens were collected by gently scraping the surface of intertidal rocks with
119 attached algae and were readily preserved in 75% ethanol. Simultaneously, samples of brown
120 algae (*Fucus spp.*), water and sediment from the collection sites were collected for the
121 analysis of metal contents. Sediment was collected with a Van Veen grab, and each sample
122 consisted of the top 2 cm of three grabs. No sediment was collected in Iceland due to the
123 rocky nature of the substrate at the sampling site.

124 **2.3. Intersex analysis**

125 Seven hundred and five adult specimens were collected (Iceland = 230, Scotland = 254, and
126 Portugal = 221), sexed (females: presence of four pairs of brood plates – oostegites – in the
127 thoracic region; males: presence of two genital papillae (GP) between the last pair of
128 pleopods), observed for external intersex characteristics (presence of both structures) and
129 measured (distance between the base of the first antenna to the base of the telson) (Ford et
130 al., 2003). In ovigerous females, eggs were removed from the brood pouch (formed by the
131 oostegites) and counted.

132 **2.4. Metal analysis**

133 **2.4.1. Sediment**

134 Extraction of metals from sediments was carried out using a nitric acid–hydrogen peroxide
135 digestion. Approximately 2g wet weight (ww) of sediment per sample site was dried at 110°C
136 for 24 hours and ground to a fine powder. Three 500mg sub-samples were added to 20ml

137 Teflon screw top digestion vessels. 5ml of concentrated nitric acid (69%, Aristar, BDH, 106
138 U.K.) was added and the sample was heated to 110°C for 24-hours. Once cooled, 3ml of
139 hydrogen peroxide (Aristar, BDH, U.K.) was added in 1 ml steps until the sample became
140 totally clear and ceased effervescing. Samples were re-heated at 110°C for a further 2 hours,
141 allowed to cool, made up to 15ml with distilled water and centrifuged at 2000 rpm for 15
142 minutes. Quantification (cadmium, copper, nickel and zinc) was performed using a THERMO™
143 ICP - Mass Spectrophotometer.

144 **2.4.2 *Fucus spp* and *E. marinus* tissues**

145 The algae were washed with deionised water to remove adhering sediment and the bladders
146 and tips were removed by tearing (Dobson, 2000). Tissues of amphipods and algae were
147 digested and subsequently analyzed using the methods for metals analysis previously
148 described (sub-section 2.4.1).

149 **2.5. Statistical analysis**

150 One-way Analysis of Variance (ANOVA) was used to evaluate the existence of significant
151 differences between groups of the different stations and between sampled compartments
152 among stations. When data proved to have a non-normal distribution (by application of the
153 Kolmogorov-Smirnov test) the Kruskal-Wallis test (ANOVA on ranks) was used. Tukey Test or
154 Dunn's Method multiple comparison procedures (normal or non-normal distributed data,
155 respectively) helped elucidate further the differences within groups of the same station. All
156 statistical analysis was performed using SigmaStat (Version 3.1) statistical software.

157 **3. RESULTS**

158 **3.1. Organism size**

159 The size of the organisms was variable in all three stations (Figure 1) and within each station
160 decreased in the following order: intersex males, normal males, intersex females and normal
161 females. All groups were significantly different ($p < 0.001$), except normal males and intersex
162 females ($p > 0.05$). Intersex specimens were consistently bigger than the normal counterparts
163 (Figure 2).

164 **3.2. Intersex incidence**

165 In general terms, intersex incidence was higher in Scotland (18.5%), followed by Portugal
166 (14.3%) and Iceland (10.0%). Females registered higher intersex rates, once again with
167 Scotland leading with 13.0%, followed by Portugal (9.5%) and Iceland (6.7%) (Table 1). Only in
168 Scottish samples was possible to find females with two genital papillae (3.7% of the
169 individuals analysed, Table 1).

170 Regarding males, the general pattern was once again repeated, though with more discreet
171 differences, namely with 5.6%, 4.8% and 3.3% for Scotland, Portugal and Iceland, respectively
172 (Table 1). Males with only one genital papillae were only observed in samples from Scotland
173 (1.9%, Table 1).

174 **3.3. Female fecundity**

175 Normal ovigerous female's average fecundity (assessed by the number of eggs present in the
176 brood cavity) showed little variation between the three locations (15.0, 16.7 and 15.3 for
177 Scotland, Portugal and Iceland, respectively) (Table 1). Intersex specimens registered a
178 generalized decline in these values: 13.2 for Scotland, 13.0 for Portugal and a pronounced
179 decrease for Icelandic organisms, with 7.9 eggs/female (Table 1).

180 **3.4. Metal Analysis**

181 Figure 3 presents data on the analysis of water, sediment (except Iceland), *Fucus spp.* tissue
182 and *E. Marinus* tissue of samples collected at the three sites, respectively. Significant
183 differences (evaluated by pairwise comparison methods – Tukey Test for water, *Fucus spp.*
184 tissue and *E. marinus* tissue; Dunn's Method for sediment) showed no consistent patterns
185 among the three sites for any of the analysed compartments. Nevertheless, Scotland
186 systematically presented the most elevated metal concentrations for all compartments (with
187 the exception of water samples from Iceland).

188 **4. DISCUSSION**

189 The relationship between pollution and the occurrence of intersex in crustaceans was never
190 irrefutably established. The available literature provides several examples of studies that
191 failed to prove this connection (despite some of them produced evidence that other
192 endocrine disruptive effects were occurring). Marine copepod communities (*Paramphiascella*
193 *hyperborean*, *Stenhelia gibba* and *Halectinosoma sp.*) associated with sewage outfalls

194 registered elevated numbers of intersex individuals (Moore and Stevenson, 1991). The
195 simultaneous occurrence of this phenomenon in several species indicated the involvement of
196 a common environmental factor. However, no clear relationship could be established
197 between exposure to sewage effluent and incidence of intersex (Moore and Stevenson,
198 1991). Gross et al. (2001) surveyed *Gammarus pulex* populations from an area where sewage
199 effluent was being discharged, but failed to reveal any relationship between intersex
200 incidence and the exposure, in spite of a highly significant number of females having oocytes
201 containing a reduced number of yolk bodies and lipid globules (suggesting reduced
202 vitellogenesis). In a study using populations of *Gammarus fossarum* (Jungmann et al., 2004),
203 the incidence of intersex increased among gammarids transplanted from a location with a
204 low incidence of intersex to a location having a high incidence of intersex, suggesting that
205 some environmental factor was responsible for the high incidence of intersex at some
206 localities. Sites considered unaffected by this factor had incidences of intersex lower than
207 1%. Yet, no discernible relationship between intersex incidence and pollution could be
208 established (Jungmann et al., 2004).

209 The interpretation of the results from the present study could point in a different direction.
210 The differences obtained among locations for all the four sampled compartments (water,
211 sediment, *Fucus spp.* tissues and *E. marinus* tissues) were in its majority non-significant. This
212 means that, from a metal contamination point of view, the initial literature-based selection
213 of sampling sites, which aimed to select locations with a profile of limited disturbance and a
214 moderate presence of metals above natural backgrounds, was proper. A similar setting for all

215 three sampled populations was thus confirmed. Coincidentally, the incidence of intersex in the
216 three sampling sites was also not significantly different (one way ANOVA, $p>0.05$). Seemingly,
217 the statistics-based conclusion from this study is that when exposed to approximately the
218 same environmental metal concentrations, populations of *E. marinus* show similar incidences
219 of intersex. However, we are aware that despite being statistic inference a fundamental tool,
220 it should not be the sole contributor to the interpretation of a data set. Discreet differences
221 can be otherwise detected, allowing the observer to retain palpable information regarding
222 the capability of a certain location to exert a more marked influence upon organisms than
223 others judged (e.g. by statistical analysis). Nevertheless, an “influence ranking” of the three
224 sites is hard to establish. Chemical stressors, like heavy metals, tend to be discreetly present
225 in the water column both in time and space (Hall et al., 1998). Periodical disturbances may
226 occur and these pulse events (of natural and anthropogenic origin) when ended can leave the
227 physical and chemical environment similar to pre-disturbance conditions (Bender et al.,
228 1984). If sampling occurs in these intermediate periods, erroneous conclusions could be
229 drawn in terms of frequency and levels of perturbations.

230 Icelandic organisms are likely to be more frequently influenced by natural pulse-like
231 perturbation, whilst Scottish and Portuguese organisms more liable to frequently receive
232 anthropogenic influences. These, due to their diverse and complex nature (e.g. accidental
233 spills, urban runoff, licensed periodic discharges) represent added stress upon these
234 organisms (atop natural occurring events). In the present study, the differences in intersex
235 female fecundity between sites could represent a sign of this influence. Charnov (1982)

236 predicted that intermediates (=intersexes) should incur in reduced fitness. Our data
237 confirmed that overall prediction, but Icelandic intersex females had a two-fold decrease in
238 fecundity when compared to Portuguese individuals (48% versus 22%) and four-fold when
239 compared to Scottish individuals (48% versus 12%). *E. marinus* eggs are relatively large for
240 amphipods (Clarke et al., 1985) and as expected in the arcto-boreal zone they become even
241 larger (Skadsheim, 1984). This means that intersex females from Iceland will have more
242 difficulties in maintaining their brood inside the marsupium, especially if we consider that
243 one of the effects of intersex is the parcial or total loss of the setae closing the brood pouch
244 (Ladewig et al., 2003).

245 In a scenario of pollution-caused intersex, intermediates from populations that have been
246 inhabiting for numerous generations environments where recurrent episodes of stress occur,
247 hence subjected to selection mechanisms (Luoma, 1977), would be more prone to cope with
248 that fecundity loss, compared to individuals living in areas were those episodes are more
249 sparse. This idea is reinforced by given proof of a genetic basis for metal tolerance in
250 crustaceans (Barata et al., 1998; Ross et al., 2002). Besides this gradation in fecundity,
251 another fact reinforcing a discrepancy between locations is that females with two genital
252 papillae and males with only one genital papillae were only present in Scottish samples.
253 Despite a certain similarity in the observed conditions, this occurrence allows a
254 differentiation between the intensity of the intersex mechanisms affecting the Scottish
255 population and the ones affecting the Portuguese population.

256 Recent work by Ford et al. (2006) confirmed a consistently higher level of intersexuality in *E.*
257 *marinus* throughout the year at sites receiving industrial contaminants (metal rich pulp mill
258 effluent) compared to reference sites. In this context, one can envisage environmental
259 constraints modulating pollution-derived intersex (possibly metal pollution), keeping the
260 former an open door to make intermediates viable. Furthermore, one should expect that this
261 situation would only occur in sites with marginal and not heavy pollution. A plausible
262 scenario for *E. marinus* intersex, as it was observed in the present study.

263 In our study, specific focus was put upon the environmental occurrence of metals and their
264 possible relationship with intersexuality. An unequivocal relationship between the two
265 factors, such as for instance the case of organotin-caused intersex in gastropods
266 (Matthiessen and Gibbs, 1998) cannot be presented, but our results advocate the potential
267 role of metals as a direct cause of intersexuality in *E. marinus*.

268

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277

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411

412

413 **FIGURE CAPTIONS**

414 **Figure 1** - Geographic location of the three sampling sites.

415 **Figure 2** - Organism length in the three locations. Error bars denote standard deviations.

416 **Figure 3** - Metal concentrations in samples of water [A], sediments [B], *Fucus spp.* tissue [C]
417 and *E. marinus* tissue [D] collected from the sites in Iceland, Scotland and Portugal (in ug/l).
418 Error bars denote standard deviations. Letters (a, b) denote statistically significant
419 differences ($p < 0.05$) between locations for each metal analysed. Nickel results were not
420 obtained for Portuguese sediments.

421

422 **TABLE CAPTIONS**

423 **Table 1-** Incidence of intersex (%) for males and females in the three stations and average
424 number of eggs inside females brood pouch. GP- genital papillae.



Figure 1

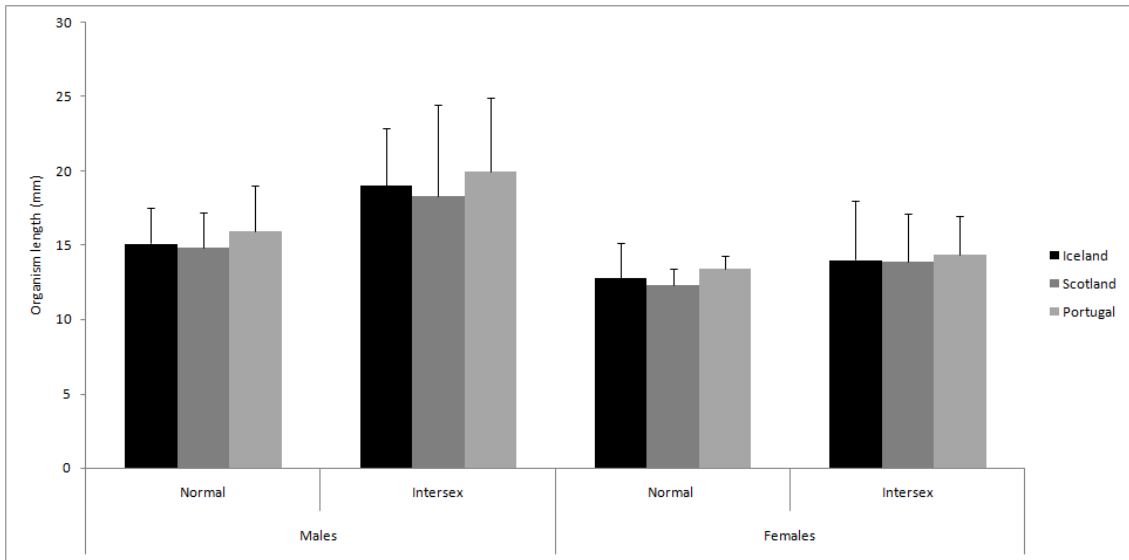


Figure 2

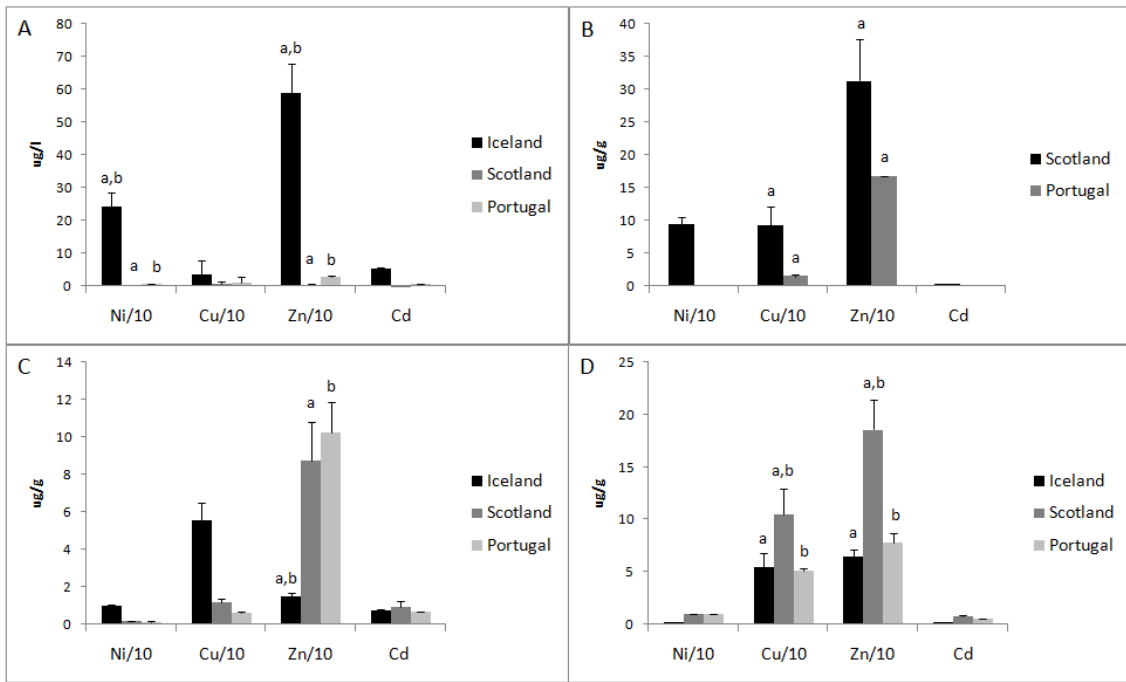


Figure 3

	Females			Males		
	Iceland	Scotland	Portugal	Iceland	Scotland	Portugal
Normal	33.3	29.6	33.3	56.7	51.9	52.4
Avg egg	15.3	15.0	16.7	-	-	-
Intersex	6.7	13.0	9.5	3.3	5.6	4.8
Avg egg	7.9	13.2	13.0	-	-	-
1 GP	6.7	9.3	9.5	0	1.9	0
2 GP	0	3.7	0	-	-	-

Table 1