AMPHIPOD INTERSEX, METALS AND LATITUDE: A PERSPECTIVE

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ABSTRACT

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

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

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

1. INTRODUCTION

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

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

The full significance and costs of intersexuality for amphipods are still unknown. Some studies report lower fecundity and fertility, reduced pairing success and delayed maturation in *Echinogammarus marinus* (Ford et al., 2003; Ford and Fernandes, 2005). Dunn et al. (1993) also reported reduced pairing success in *Gammarus duebeni* and Kelly et al. (2004) found lower fecundity in intersex females of the same species. An investigation by Plaistow et al. (2003) on the costs of intersex upon precopulatory guarding in *Gammarus pulex*, indicated that these were correlated with female size. Consequently, pair forming with larger intersex females (which grow larger than the normal counterpart) will lead not only to large energy costs, but also reduced reproductive success. Barbeau and Grecian (2003) noted reduced fertility in intersex male *Corophium volutator*, a finding confirmed for the same species by McCurdy et al. (2004) that registered smaller broods issuing from the pairing of intersex males with normal females compared to matings with normal males.

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

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

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

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

2. MATERIALS and METHODS

2.1. Study sites

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

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

Loch Fyne is the longest, deepest fiordic sea loch of Scotland, being part of the Firth of Clyde watershed (one of the country’s most contaminated). In spite of this, it was expected to be relatively free from metal contamination due to serving mainly forestry, extensive grazing, aquaculture and recreation purposes (SEPA, 2005). Leung et al. (2001) detected higher metal contents in tissues of *Nucella lapillus* from this area than in individuals collected in areas traditionally described as metal polluted. The organisms for the present study were collected in the vicinity of Strachur (56°10'N, 5°05'W), located a few km to the south of a "Marine Consultation Area" (NCC, 1986).

The Mondego Estuary is located in the Portuguese Atlantic coast. It comprises two arms (north and south) with very different characteristics. North arm is highly hydrodynamic, possesses navigational capabilities and it is pointed as having pristine conditions in terms of heavy metals (Vale et al., 2002). South arm dynamics are much weaker, depending partly of the tidal excursion and of the artificially controlled discharges (according to the necessities of the extensive lower valley rice crops) of a small river – the Pranto. This leads to extended residence times and fine particles deposition. The Mondego possesses a sizeable watershed (6670 km^2) upon which the impact of urban areas summing up to 695.000 inhabitants is felt (INAG, 2004). Few industries are to be noted, but deactivated uranium mines are located approximately 100km from the estuary and increases of metals, particularly Zn, Mn, Fe, U, Pb and Ra (Pinto et al., 2004) are registered in the areas directly receiving the mine effluents. The organisms were collected in the south arm (40°07'N, 8°49'W) in the confluence with Pranto river. Castro et al. (2007) noted an increase of Pb in sediment profiles from this area since the 1960's, but emphasized that the Mondego was not heavily contaminated compared to other European estuaries. Pereira et al. (2005) indicated enrichment in Hg, Cu, Cd, Cr, Mg, Zn and Fe of the fine fraction of the south arm, compared to the remainder of the estuary, pointing the Pranto river as the presumable source.

2.2. Sampling

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

2.3. Intersex analysis

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

2.4. Metal analysis

2.4.1. Sediment

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

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

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

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

2.5. Statistical analysis

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

3. RESULTS

3.1. Organism size

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

3.2. Intersex incidence

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

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

3.3. Female fecundity

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

3.4. Metal Analysis

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

4. DISCUSSION

The relationship between pollution and the occurrence of intersex in crustaceans was never irrefutably established. The available literature provides several examples of studies that failed to prove this connection (despite some of them produced evidence that other endocrine disruptive effects were occurring). Marine copepod communities (*Paramphiascella hyperborean*, *Stenhelia gibba* and *Halectinosoma sp.)* associated with sewage outfalls registered elevated numbers of intersex individuals (Moore and Stevenson, 1991). The simultaneous occurrence of this phenomenon in several species indicated the involvement of a common environmental factor. However, no clear relationship could be established between exposure to sewage effluent and incidence of intersex (Moore and Stevenson, 1991). Gross et al. (2001) surveyed *Gammarus pulex* populations from an area where sewage effluent was being discharged, but failed to reveal any relationship between intersex incidence and the exposure, in spite of a highly significant number of females having oocytes containing a reduced number of yolk bodies and lipid globules (suggesting reduced vitellogenesis). In a study using populations of *Gammarus fossarum* (Jungmann et al., 2004), the incidence of intersex increased among gammarids transplanted from a location with a low incidence of intersex to a location having a high incidence of intersex, suggesting that some environmental factor was responsible for the high incidence of intersex at some localities. Sites considered unaffected by this factor had incidences of intersex lower than 1%. Yet, no discernible relationship between intersex incidence and pollution could be established (Jungmann et al., 2004).

The interpretation of the results from the present study could point in a different direction. The differences obtained among locations for all the four sampled compartments (water, sediment, *Fucus spp.* tissues and *E. marinus* tissues) were in its majority non-significant. This means that, from a metal contamination point of view, the initial literature-based selection of sampling sites, which aimed to select locations with a profile of limited disturbance and a moderate presence of metals above natural backgrounds, was proper. A similar setting for all three sampled populations was thus confirmed. Coincidently, the incidence of intersex in the three sampling sites was also not significantly different (one way ANOVA, $p>0.05$). Seemingly, the statistics-based conclusion from this study is that when exposed to approximately the same environmental metal concentrations, populations of *E. marinus* show similar incidences of intersex. However, we are aware that despite being statistic inference a fundamental tool, it should not be the sole contributor to the interpretation of a data set. Discreet differences can be otherwise detected, allowing the observer to retain palpable information regarding the capability of a certain location to exert a more marked influence upon organisms than others judged (e.g. by statistical analysis). Nevertheless, an "influence ranking" of the three sites is hard to establish. Chemical stressors, like heavy metals, tend to be discreetly present in the water column both in time and space (Hall et al., 1998). Periodical disturbances may occur and these pulse events (of natural and anthropogenic origin) when ended can leave the physical and chemical environment similar to pre-disturbance conditions (Bender et al., 1984). If sampling occurs in these intermediate periods, erroneous conclusions could be drawn in terms of frequency and levels of perturbations.

Icelandic organisms are likely to be more frequently influenced by natural pulse-like perturbation, whilst Scottish and Portuguese organisms more liable to frequently receive anthropogenic influences. These, due to their diverse and complex nature (e.g. accidental spills, urban runoff, licensed periodic discharges) represent added stress upon these organisms (atop natural occurring events). In the present study, the differences in intersex female fecundity between sites could represent a sign of this influence. Charnov (1982) predicted that intermediates (=intersexes) should incur in reduced fitness. Our data confirmed that overall prediction, but Icelandic intersex females had a two-fold decrease in fecundity when compared to Portuguese individuals (48% versus 22%) and four-fold when compared to Scottish individuals (48% versus 12%). *E. marinus* eggs are relatively large for amphipods (Clarke et al., 1985) and as expected in the arcto-boreal zone they become even larger (Skadsheim, 1984). This means that intersex females from Iceland will have more difficulties in maintaining their brood inside the marsupium, especially if we consider that one of the effects of intersex is the parcial or total loss of the setae closing the brood pouch (Ladewig et al., 2003).

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

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

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

Acknowledgements

We are very grateful to Dr. Gudmundur V. Helgason and Dr. Halldór P. Halldórson for arrangements and hospitality at the Sandgerði Marine Centre, Iceland (SMC). Work at the SMC was supported by the programme ‘‘Improving Human Potential: Access to Research Infrastructures’’ (IHP/ARI) provided by the European Community under Marie Curie Actions - EC-IHP Transnational Access Grant. This research was funded through a PhD Grant by FCT –

Fundação para a Ciência e Tecnologia (SFRH/BD/4778/2001). We are also extremely grateful to the two anonymous reviewers whose comments helped improve this manuscript.

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FIGURE CAPTIONS

Figure 1 - Geographic location of the three sampling sites.

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

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

TABLE CAPTIONS

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


Sandgerði,
Iceland
Loch Fyne,
Scotland
Mondego estuary,
Portugal

Figure 1

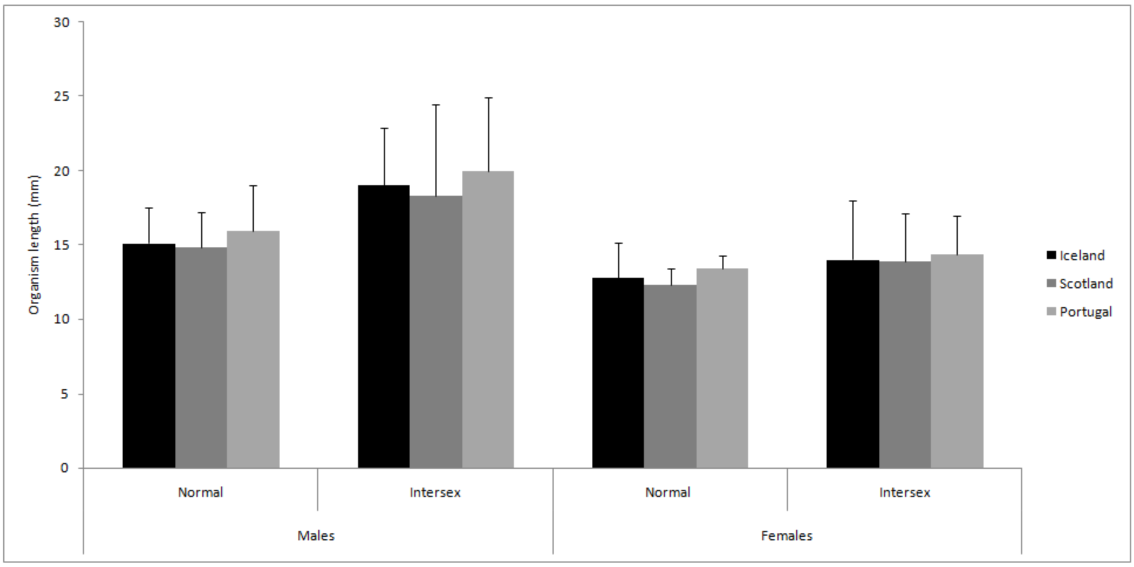
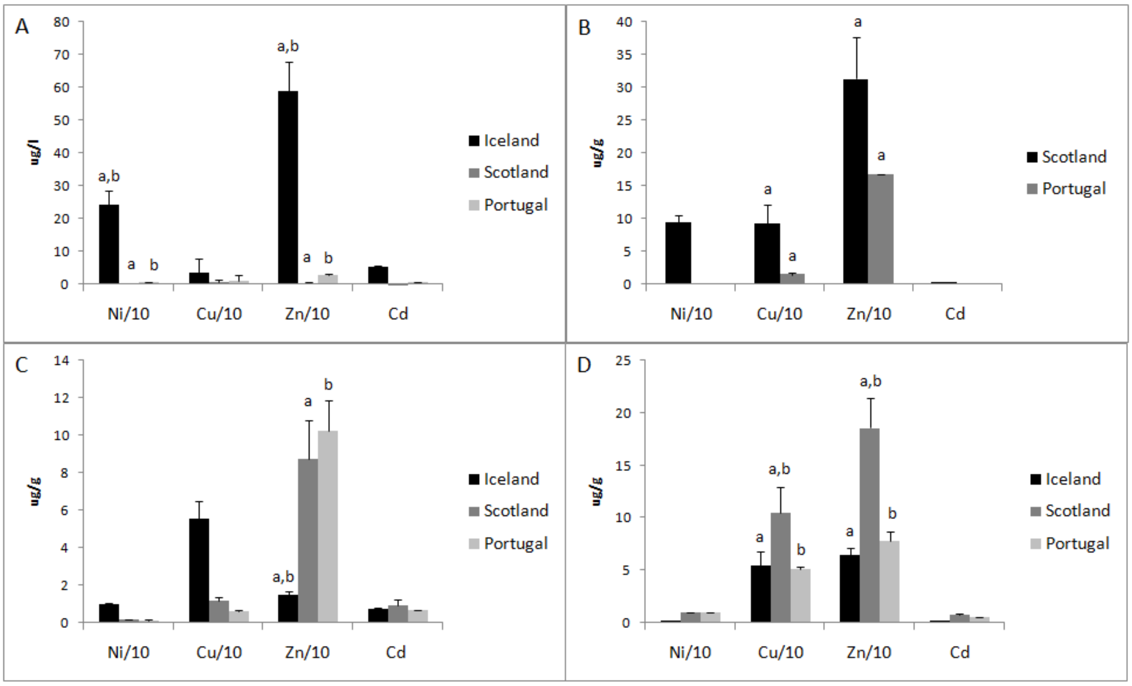

30
25
20
15
10
5
0
Organism length (mm)
Normal
Intersex
Normal
Intersex
Males
Females
Iceland
Scotland
Portugal

Figure 2



A
ug/l
Iceland
Scotland
Portugal
Ni/10
Cu/10
Zn/10
Cd
B
ug/g
Scotland
Portugal
C
ug/g
D
ug/g

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