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First record of intersexuality in the Amazon River shrimp *Macrobrachium amazonicum* (Heller, 1862) (Caridea: Palaemonidae)

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ABSTRACT

Intersexuality in a gonochoric palaemonid shrimp is reported for the first time in a wild population of *Macrobrachium amazonicum* (Heller, 1862). This phenomenon was confirmed based on an investigation of anatomic and morphometric traits and the ultrastructure of gonopores. The intersex had both reproductive organs despite the absence of vitellogenic oocytes and spermatozoa. This individual had a vestigial male gonopore complex and female gonopores. The intersexuality was related to a genetic mutation and/or hormonal disruption because there was no detection of chemicals in the water or parasites in the samples. Non-functional hermaphroditism is a very rare event (0.06%) in *M. amazonicum*, indicating that this species is gonochoric, and intersex individuals are not reproductively viable.

Key Words: gonopores, morphology, non-functional hermaphroditism, ultrastructure

Intersexuality in carideans is associated with the development of male and female gonopores and other non-functional secondary sexual characters, and/or abnormal reproductive organs of both sexes in the same individual (Nagamine *et al.* 1980a, b; Tóth & Bauer, 2007, 2008). In crustaceans, this phenomenon is caused by hormonal disruptions and sexual differentiation during late embryonic development (Ford, 2012). Among palaemonids, only Rao (1967) has so far reported a possible sexual reversal in individuals in a wild population of *Macrobrachium rosenbergii* (De Man, 1879). Nagamine *et al.* (1980a, b), however, questioned his study due to the short and subjective description and the lack of anatomical or histological details. These authors pointed out that *M. rosenbergii* is not hermaphroditic, and masculinized females induced by laboratory procedures retain female gonopores even after induction and complete sex change. The anomalous pattern detected by Rao (1967) in *M. rosenbergii* might be associated with a non-functional hermaphroditism. The Amazon River shrimp *Macrobrachium amazonicum* (Heller, 1862), like *M. rosenbergii*, exhibits strong sexual dimorphism, with dominant male morphotypes green claw 1 (GC1) and green claw 2 (GC2), and submissive translucent claw (TC) and cinnamon claw (CC) (Pantaleão *et al.*, 2014). Populations of this species have significant ecological plasticity, high morphological and reproductive variability, and can inhabit different aquatic environments (Vergamini *et al.*, 2011). We present the first confirmed record of intersexuality in native populations of a gonochoric palaemonid.

Monthly samples of water, sediment, and individuals of *M. amazonicum* were collected from October 2014 to December 2015 in Grande River (20° 30′ 53″S, 46° 50′ 16″W), Marechal Mascarenhas de Morais reservoir, Cássia municipality, Minas Gerais state, southeastern Brazil. Supplementary material Table S1 summarizes the analyzed physical and chemical parameters. Shrimp were collected by active sampling (use of nets) and passive collection (six baited-traps). All individuals were anesthetized by cooling and fixed in 70% ethanol. Individuals were sexed in the laboratory by verifying the secondary sexual characters, determining the position of the gonopores, and dissecting the reproductive organs. Measurements of structures (following Pantaleão *et al.*, 2014) were made using a caliper with a precision of 0.02 mm. The height of the propodus was also measured. The wet weight (W) was obtained with an analytical scale to 0.0001 g. The color of the chelipeds, angles of the spines on the carpus and propodus, and the pubescence on dactyls were verified and used to determine morphotypes in males (see Pantaleão *et al.*, 2014). The ovaries were classified into five developmental stages based on morphometrical and histochemical characteristics (Paschoal & Zara, unpublished data). Principal component analysis (PCA) was conducted using all the body dimensions examined ($\log_{10}$) in females (Supplementary material Table S2). A linear regression was carried out on the variable with the highest contribution in the PCA. The coefficient of determination (r^2) and degree of allometry (*b*, verified with Student's *t* test) of the function were also calculated. The

reproductive organs were dissected, followed by a smear of the ejaculatory ducts of males, ovary of females, and both organs of the intersex individuals. The abdominal sterna with pereiopods of six females (two ovigerous), two males, and the intersex were fixed in 4% paraformaldehyde in sodium phosphate buffer (pH 7.2) and examined using scanning electron microscopy (SEM).

In all, 5,088 individuals were collected. In the subsample of 1,540 individuals, 1,244 were females and 295 were males (male:female ratio = 1:4.2; $\chi^2 = 293.02$, $df = 1$, $P < 0.001$). External and internal parasites were absent in the studied population. We observed one individual with external and internal characteristics of both sexes (Fig. 1), representing 0.06% of the sampled population. This intersex individual was collected in October 2014 and had the typical external features of a dominant GC2 male (Fig. 1A, Supplementary material Table S3).

The sexual characters of the intersex differed from females of a similar size (Fig. 1B, Supplementary material Table S3). The intersex had no appendix masculina on either of the two endopodites of the second pleopods (Fig. 1C). The abdominal sternum of the intersex had female gonopores in the third pair of pereiopods and two whitish cuticular scars on the fifth pair of pereiopods (Fig. 1G). The ovary was in a normal position, located over the hepatopancreas, and connected to a pair of normal oviducts ending in the gonopores on the coxopodites of the third pereiopods

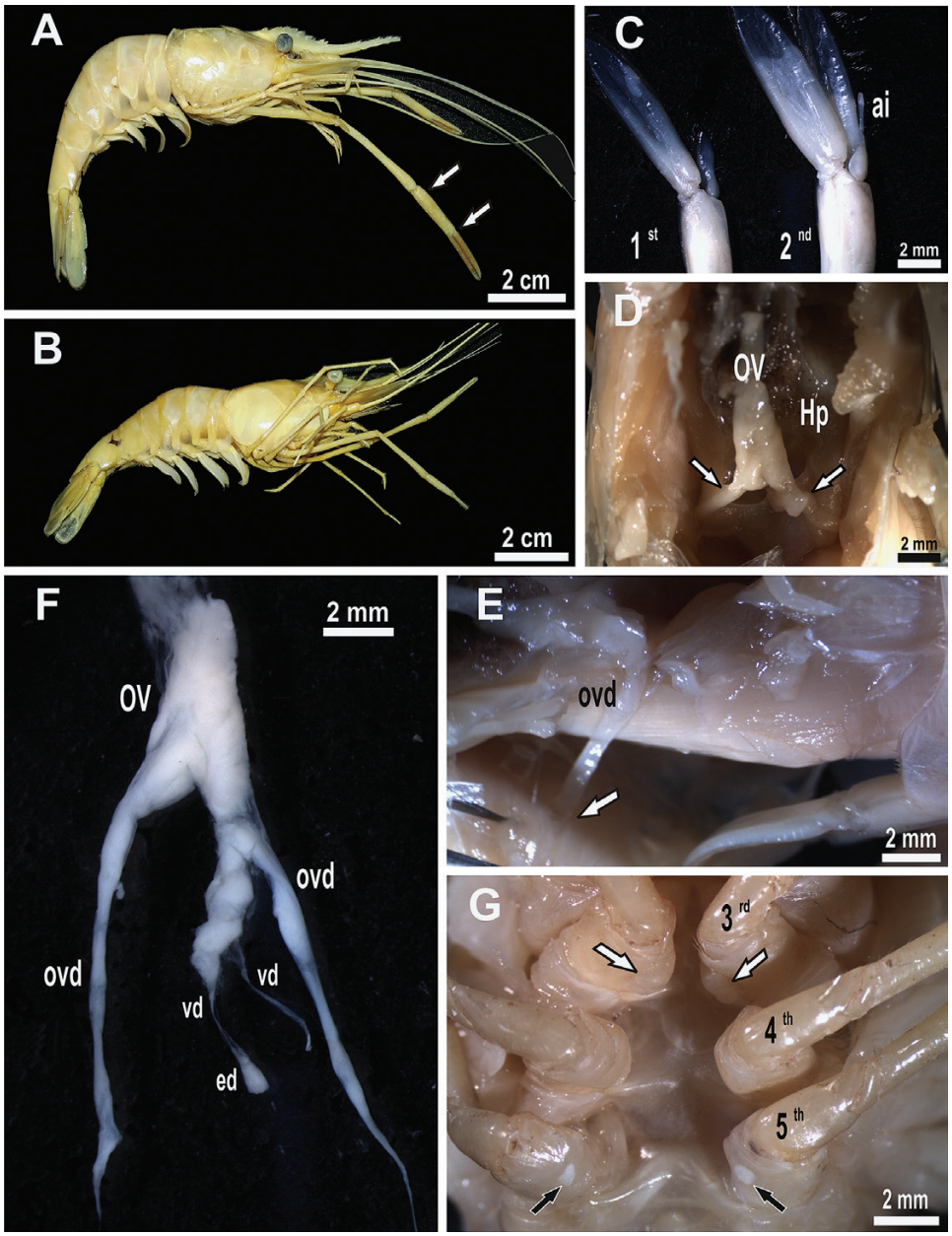

Figure 1. *Macrobrachium amazonicum*, Cássia, Minas Gerais, southeastern Brazil. **A**, Lateral view of the intersex. The shrimp had only one well-developed cheliped (white arrows). **B,** Lateral view of a female (for details, see Female 1 in Supplementary material Table S3). **C,** First and second pleopods of the intersex. **D,** Ovary of the intersex located on the hepatopancreas, with oviducts arranged on the sides of the carapace (white arrows). **E**, Oviducts of the intersex connected to the gonopores of the third pereiopod (white arrow). **F**, Overview of ovary from the intersex, with one extremity showing vasa deferentia. The right ejaculatory duct was removed. **G**, Ventral view of the sternum of the intersex, with openings of the female gonopores in the third pair of pereiopods (white arrows) and cuticular scars on the fifth pair of pereiopods (black arrows). ai, appendix interna; OV, ovary; hp, hepatopancreas; ovd, oviduct; vd, vas deferens; ed, ejaculatory duct. This figure is available in color at *Journal of Crustacean Biology* online.

(Fig. 1D, E). Two conspicuously thin vasa diferentia were observed running from the ovarian caudal extremity and ending in a terminal expansion, the bulb of the ejaculatory duct, inserted into the fifth coxopodite (Fig. 1F, G). No spermatozoa were found in the right ejaculatory duct (Fig. 1F). Smears with fragments of the ovary revealed only oogonia and pre-vitellogenic oocytes. Yolk granules were not observed in any of the preparations of different portions of the ovary. A comparison of the morphometric variables of individuals of similar body size with the variables of the intersex revealed a shrimp with hybrid traits (Table S3). The abdomen and the second pleura of the intersex were larger than those observed in the males. The size of the major cheliped, propodus, and dactyl, however, were larger than those of the females. The length, angle of spines, and pubescence of the chelipeds were similar to those recorded for CG2 morphotype males (Supplementary material Table S3). The morphometry of body traits did not separate females into morphotypes, despite visual differences in the cheliped patterns in 94 females. These females exhibited hypertrophied chelipeds, whereas the remaining females ($N = 1{,}153$) had normal chelipeds (Figs. 2, 3). The first two PCA axes explained most of the variability of the data (95.45%) (Fig. 2). Although morphotypes were not separated, the variable with the greatest contribution to axis 1 (90.12%) was the length of the major cheliped (MCL) (Supplementary material Table S2). This morphometric variable increases with female growth, showing a positive allometric growth (t: 133.08, $P < 0.001$) (Fig. 3). The intersex was clearly different from the rest of the females, appearing as an outlier in the analyses (Figs. 2, 3).

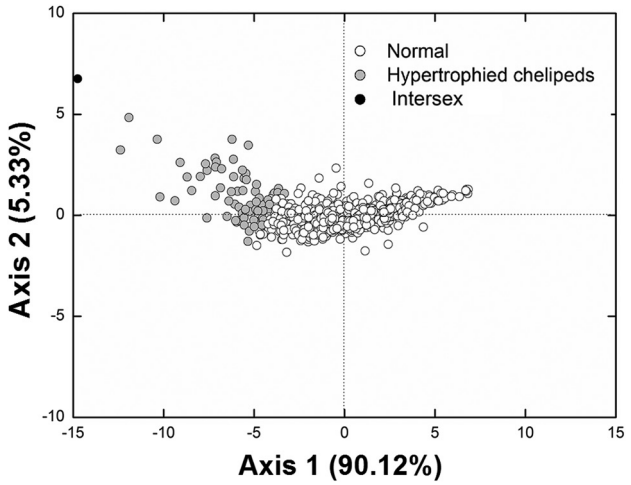

Figure 2. Ordination diagram of the morphometric variables for normal females, females with hypertrophied chelipeds, and the intersex in the population of *Macrobrachium amazonicum* based on the values obtained in the first two axes of the principal component analysis.

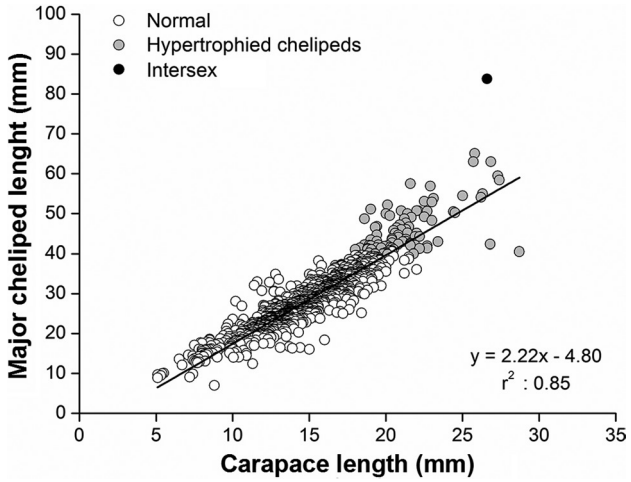

Figure 3. Dispersion of points for the morphometric relationship major cheliped length (MCL) *versus* carapace length (CL) for normal females, females with hypertrophied chelipeds, and the intersex in the population of *Macrobrachium amazonicum*.

The gonopores of non-ovigerous and ovigerous females were located on the medial surface of the coxopodite of the third pereiopod with the opening facing the base of the sternum (Fig. 4A, B). Setae were absent on the edges of the gonopore of non-ovigerous females (Fig. 4D), whereas many temporary egg-guiding setae were observed on this structure and on the sternum in ovigerous females (Fig. 4E). The medial/basal surface of the coxopodite of the fifth pereiopod of females showed a slight depression toward the center of the coxopodite, extending to its base, and devoid of gonopores (Fig. 4G, H). The gonopores of male GC morphotypes were located on the medial surface of the arthrodial membrane between the coxopodite of the fifth pereiopod and the abdominal sternum. The gonopore was covered with flap-like extensions and the opening was horizontally oriented toward the opposite opening (Fig. 4C, I). The surface of the coxopodites of the third pereiopod of males was smooth and regular, and devoid of gonopores and setae (Fig. 4F). A projection with a prominent edge was observed in the same region of the coxopodite of the fifth pereiopod under which we observed the flap of the gonopore on the medial surface of the arthrodial membrane of the coxopodite (Fig. 4I). Female gonopores of the intersex were located on the medial surface of the coxopodite of the third pereiopod, whereas gonopore complexes with underdeveloped gonopods, were observed on the medial surface of the arthrodial membrane between the coxopodite of the fifth pereiopod and the abdominal sternum (Fig. 4J, N). The opening of the female gonopore faced the base of the sternum and was devoid of setae (Fig. 4K) as in the non-ovigerous females. Gonopore complexes were present underneath a projection with a prominent border (Fig. 4L, N) in the same location where a cuticular scar was located on the central portion of each coxopodite of the fifth pereiopod (Fig. 1G). This region was characterized by a central groove interrupted near the scar of each coxopodite (Fig. 4L). Unlike the males, the gonopore complexes of the intersex were not covered by flaps, and had a rudimentary appearance (Fig. 4M, N).

This study confirms for the first time a rare case of intersexuality in a wild population of *M. amazonicum*. Sex determination in decapods is primarily regulated by the androgenic gland (Nagamine *et al.*, 1980a, b). Ventura *et al.* (2011) reported that the androgenic gland of *Macrobrachium* is perpendicular to the ejaculatory ducts and is responsible for secreting an insulin-like peptide (IAG), which modulates primary (spermatogenesis) and secondary sexual characters (e.g. hypertrophy of chelipeds and formation of gonopores) as well as sexual behavior.

Sex determination in crustaceans can also be altered by external factors such as pollution and parasitism (Sant'anna *et al.*, 2010; Ford, 2012). These external factors could not explain intersexuality in *M. amazonicum* because internal and external parasites were not observed in the intersex or other gonochoric individuals in the population, and heavy metals and other contaminants were not found among the more than 30 physical and chemical variables analyzed in the water and sediment (Table S1). The presence of pesticides, however, was not analyzed in the water and sediment, but the water in the sites is used for human consumption, so these contaminants are expected to be absent or at low levels. A higher frequency of intersex individuals would be expected if this hormonal dysfunction were caused by pollution, as in the 2% to 7% reported in three species of hermit crabs in Brazil (Turra & Leite, 2000). Intrinsic factors seem to be the most likely explanation in *M. amazonicum*, unlike that proposed for hermit crabs by Sant'anna *et al.* (2010, 2012). Rumbold *et al.* (2015) hypothesized

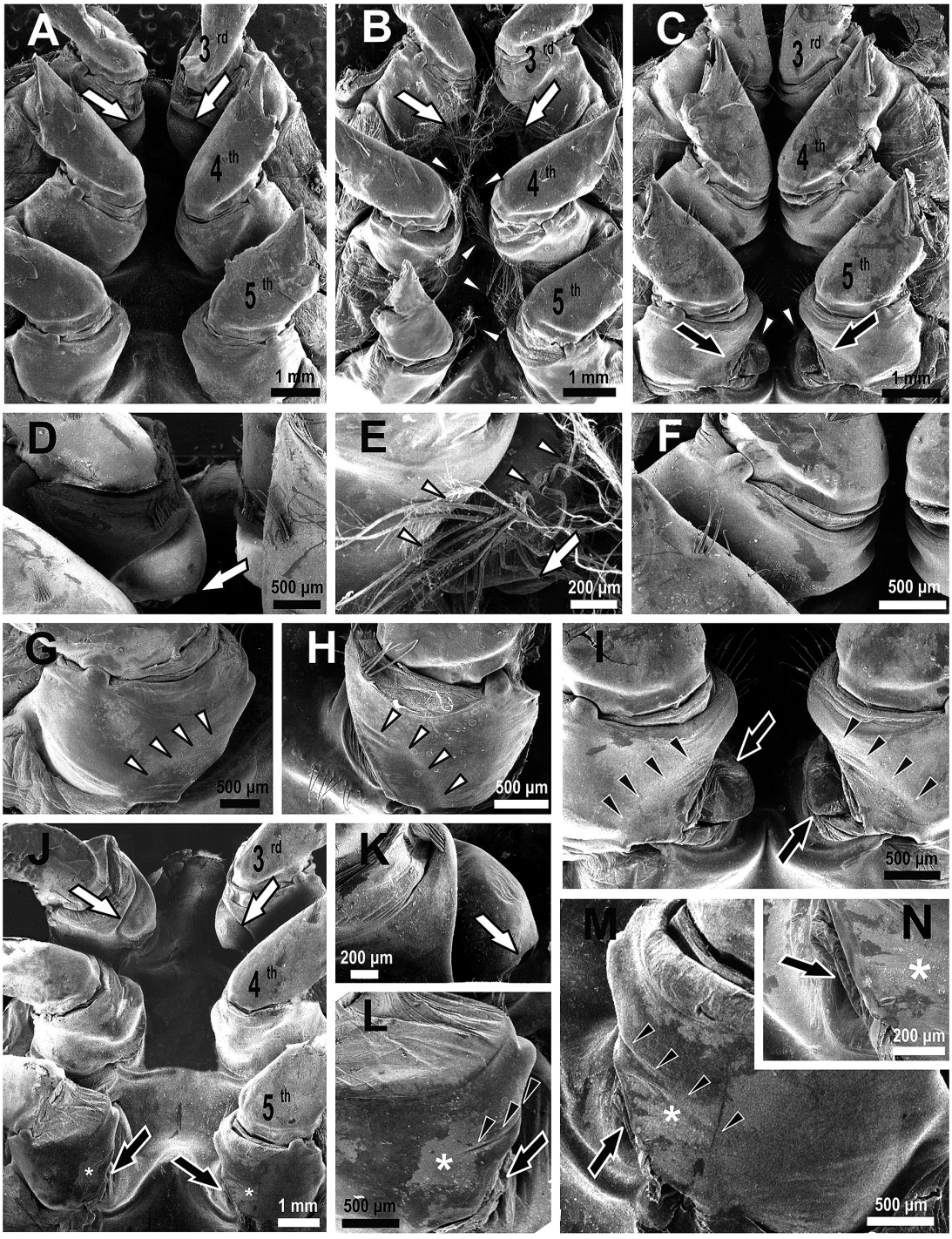

Figure 4. *Macrobrachium amazonicum*. **A**, **B**, Ventral view of the sternum of a non-ovigerous and ovigerous female, respectively, showing gonopores in the third pereiopod (white arrows). Note the egg-guiding setae (white arrowheads) in the ovigerous female. **C**, Ventral view of the sternum of a GC1 male showing gonopores in the fifth pereiopod (black arrows) covered by flaps (white arrowheads); **D**, **E**, Gonopore of a non-ovigerous and ovigerous female, respectively, with opening facing the base of the sternum (white arrow). The structure is surrounded by setae (white arrowheads) in the ovigerous female. **F**, Absence of gonopore in the third pereiopod of a GC1 male. **G**, **H**, Depression toward the central portion of the coxopodite (white arrowheads) in the fifth pereiopod of a non-ovigerous and ovigerous female, respectively. **I**, Projections with prominent edges of the fifth pereiopod (black arrowhead), located above the flaps of gonopores (black arrows) of a GC1 male. **J**, Ventral view of the sternum of the intersex showing female gonopores (white arrows) and male gonopore complexes (black arrows) with scars in the central portion of coxopodites (asterisks). **K**, Opening of the female gonopore of the intersex (white arrow). **L**, Groove in the central portion of the fifth pereiopod (black arrowheads) where the gonopore complex is located (black arrow), interrupted near the scar (asterisk). **M**, Gonopore complex (black arrow) underneath the projection of the fifth pereiopod (black arrowheads). **N**, Gonopore complex (black arrow) located between the coxopodite of the fifth pereiopod (asterisk) and the abdominal sternum.

that a genetic mutation and/or hormonal disruption leads to the rare occurrence (0.1%) of intersexes in tanaids. Laboratory experiments involving andrectomy, the ablation of the androgenic gland, showed that males of *M. rosenbergii* underwent feminization after this procedure (Nagamine *et al.*, 1980a). The same authors reported the masculinization of *M. rosenbergii* females after the

implantation of the androgenic gland (Nagamine *et al.*, 1980b). The anatomical and histological pattern found in *M. rosenbergii* after the induction of sex change was most probably that observed in the intersex of *M. amazonicum*.

The positions of gonopores on coxopodites are reliable traits for sex recognition in carideans (Bauer, 2004). The presence of gonopores of both sexes in the same individual has been reported is some alpheid shrimps (Tóth & Bauer, 2007, 2008). The gonopore characteristics observed in males and females agree with the common pattern described for gonochoric carideans; however, the male gonopore of the intersex, the gonopore complex, appeared undeveloped or vestigial. Piyaviriyakul & Darawiroj (2014) demonstrated the process of sex differentiation in *M. rosenbergii* and found the gonopore complexes only in juvenile males. These complexes had a similar structure and were arranged in the same position as the complexes observed in the intersex of *M. amazonicum*.

Studies involving the gene structure and IAG expression can provide further insight into the occurrence of intersexes and even hermaphrodites in this species and other palaemonids. Despite the instance of intersexuality found in *M. amazonicum*, this phenomenon is rare, indicating that this species is gonochoric and that intersexes are not reproductively viable.

SUPPLEMENTARY MATERIAL

Supplementary material is available in *Journal of Crustacean Biology* online.

S1 Table. Mean, minimum, and maximum values for the limnological variables analyzed in water and sediment between October 2014 and December 2015 in Cássia (Minas Gerais, southeastern Brazil).

S2 Table. Correlation and contribution of morphometric variables in the principal component analysis used in Figure 2.

S3 Table. Comparison of morphometric variables and other variables analyzed in female and male shrimp with body proportions similar to those of the intersex found in a similar population of *Macrobrachium amazonicum* in Cássia (Minas Gerais, southeastern Brazil).

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