



FEATURE ARTICLE

Simultaneous activity of male and female gonads in intersex hermit crabs

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ABSTRACT: Intersexuality in hermit crabs was investigated using anatomical, histological and electron-microscopy techniques, with *Clibanarius vittatus* as a model. The specimens were collected in the intertidal zone of Pescadores Beach, state of São Paulo, Brazil, and dissected to describe the anatomy of the reproductive organs. Different portions of the reproductive system were stained with hematoxylin and eosin, and the gonopore morphology was analyzed by scanning electron microscopy. The intersex individuals showed both male and female reproductive systems, with ducts that are connected to the gonopores. The morphology of the gonopores was similar in males and females. The intersex individuals also developed functional male and female gonads in the same individual. These observations confirmed that intersex hermit crabs can reproduce as males or females, thus supporting the hypothesis that they may be part of a true sequential hermaphroditic process.

KEY WORDS: Intersexuality · Hermaphroditism · Hermit crab · Protogyny · SEM · Histology · Morphology

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INTRODUCTION

Intersexuality has been recorded in several animal groups, from invertebrates such as molluscs (Gibbs et al. 1991) and crustaceans (Bauer 2000, Turra & Leite 2000; LeBlanc 2007) to vertebrates, including humans (Walsh et al. 1974). An intersex individual or gynandromorph is defined as an individual (male or female)



Hermit crab *Clibanarius vittatus* (Bosc, 1802) at Pescadores Beach (São Vicente, São Paulo, Brazil).

Photo: B. S. Sant'Anna

that develops alterations in primary and/or secondary sexual features, so that it possesses both male and female characters (Goldschmidt 1938, Ladewig et al. 2002, Vasquez & López Greco 2007). The alterations in secondary sexual characteristics may result in non-functional hermaphroditism, characterized by oogenesis occurring in the testes, as reported in talitrid amphipods, or, conversely, by spermatogenesis taking place in the gonads of females, which has never been observed (Charniaux-Cotton 1975). In contrast, intersexuality may also be part of a functional protandric, protogynic, or simultaneous hermaphroditism (Brook et al. 1994, Fiedler 1998, Rudolph 2002).

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Different causes have been attributed to intersexuality. Studies conducted in recent decades suggest that contact with anthropogenic chemicals and mimics of estrogens and testosterone may alter sexual development in many vertebrate groups, including fishes (Williams et al. 2009), amphibians (Reeder et al. 1998), reptiles (Guillette et al. 1994) and birds (Fry & Toone 1981). In fishes and amphibians, intersexuality is closely related to their exposure to steroid estrogens (Williams et al. 2009) or chemical pollutants (Reeder et al. 1998). Among invertebrates, molluscs show a similar phenomenon termed 'imposex', which is related to tributyltin (TBT) contamination (Gibbs et al. 1991, Bettin et al. 1996, Oehlmann et al. 1996, Rank 2009). Crustacean intersexuality has been associated with natural events, such as viral infection (Juchault et al. 1991), parasites (Ginsburger-Vogel 1991), hereditary factors including genetic abnormalities (Hough et al. 1992), social (demographic) structure (Baeza & Bauer 2004), and abiotic factors (Bauer 2002).

Investigations of intersexuality in hermit crabs are recent and have focused mainly on records of gynandromorphs—with open gonopores on both coxae of the third (female) and fifth (male) pereopods—occurring in nature. This phenomenon seems to occur at all grades of terrestrialization, from typically aquatic species to fully terrestrial species such as members of the Coenobitidae (Gusev & Zabolin 2007). Currently this sexual abnormality has been recorded in 17 species around the world from different genera and families (Hilgendorf 1897, Fize & Serène 1955, Wenner 1972, McLaughlin 1974, Charniaux-Cotton 1975, Lewinsohn 1982, McLaughlin & Lemaitre 1993, Mantelatto & Sousa 2000, Turra & Leite 2000, Turra 2004, 2005, 2007, Sant'Anna et al. 2006, Fantucci et al. 2008, Gusev & Zabolin 2007).

The biological meaning of intersexuality in hermit crabs has been discussed based on behavioral, population, and morphological information (Turra & Leite 2000, Turra 2004, 2007). Turra & Leite (2000) found that intersex individuals were of intermediate size in relation to males and females, and comprised 5 to 7% of the population studied. In another locality, the size of intersex individuals was more similar to the largest males, and intersex individuals comprised 2.5% of the population (Sant'Anna et al. 2009). Turra (2004) showed that intersex individuals of *Clibanarius vittatus*, *C. antillensis* and *C. sclopetarius* may act as functional males, successfully mating as males, and that the female gonopores closed in the subsequent molt. Conversely, one intersex individual was also recorded with an egg mass (Turra 2007), thus indicating that they may also act as females. The results of these studies suggest that intersexuality in hermit crabs may be part of a true sequential protogynic hermaphroditic process. A good source

of information to illuminate this discussion is a detailed morphological analysis of the reproductive system of intersex individuals in comparison with males and females. This kind of investigation is especially important since there are no records of protogyny in decapods (Brook et al. 1994, Gusev & Zabolin, 2007).

Reproductive aspects in hermit crabs are relatively well known. For many species, the gonad histology and gonopore morphology have been described (Hinsch 1980, Varadarajan & Subramoniam 1980; Manjón-Cabeza & García-Raso 2000, Hess & Bauer 2002, Tirelli et al. 2006, 2007, Amadio & Mantelatto 2009, Mantelatto et al. 2009). However, there is no such information on intersex hermit crabs. Although the gonopore morphology of males and females has been analyzed for *Clibanarius vittatus* (Hess & Bauer 2002), an ultrastructural approach may reveal important differences or similarities between the gonopores of intersex individuals and normal males and females.

In this context, the present study comprises an anatomical, histological, and ultramorphological investigation on intersexuality in hermit crabs, using *Clibanarius vittatus* as a model, to search for evidence of a functional hermaphroditic process.

MATERIALS AND METHODS

Animals and samples. Hermit crabs *Clibanarius vittatus* were collected in the intertidal zone of Pescadores Beach (23° 58' 21" S, 46° 23' 35" W), São Vicente, state of São Paulo, Brazil. To access the reproductive system, the animals were gently removed from the gastropod shells and anesthetized by thermal shock with cold seawater. During dissection, the macroscopic morphology of the reproductive system for males, females, and intersex individuals was analyzed and the organs were then transferred to various fixative vehicles. The branchial and abdominal cavities were removed and examined under a stereomicroscope, to investigate the possible presence of parasites.

Histology. The reproductive organs of *Clibanarius vittatus* were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) for 24 h, and then washed twice for 1 h with the same buffer. The material was dehydrated in an ascending ethanol series (70 to 95%) for 20 min and embedded in glycol methacrylate resin (Leica historesin kit) for 72 h at 4°C. After polymerization, the blocks were cut in a Leica RM2245 microtome. The 5 to 7 µm sections were mounted on slides and stained with hematoxylin and eosin (HE) (Junqueira & Junqueira 1983, modified), using no xylene and ethanol baths.

Electron microscopy. For the SEM procedure, the gonopores of males, females and intersexes were re-

moved and fixed in modified Karnovsky fixative (2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M phosphate buffer prepared with seawater, pH 7.3) for 24 h. The samples were washed twice with the same buffer and dehydrated in an ascending acetone series (50 to 100%). All specimens were critical-point-dried in a Balzers CPD 030. After complete dehydration, the materials were sputter-coated with gold in a Balzers SD 050 prior to examination in a Jeol P15 SEM. All images were photographed on Neopan SS 120 film.

RESULTS

A total of 72 adult hermit crabs *Clibanarius vittatus* were analyzed, including 27 males, 27 females, and 18 intersex individuals. In the anatomical observations (Fig. 1), 15 intersex individuals showed only male gonads (testis and vas deferens), while 3 specimens

showed both male and female gonads that were not macroscopically distinct (Fig. 1c,d), similar to those recorded in normal males (Fig. 1a) and females (Fig. 1b). The intersex female gonads showed light-orange pigmentation, slightly different from normal females that have reddish-orange gonads when fully developed. Both oviducts were found during the dissections; however only one is shown in the image because the second one broke during dissection. No crabs contained parasites in the branchial or abdominal cavities.

The distal ends of the vas deferens, the ejaculatory ducts, are located within the coxae of the fifth pereopods of the normal male, and open to the exterior via the gonopores, which are protected by a flap-like operculum surrounded ventrally by stout setae (Fig. 2a). The normal female gonopores open in the coxae of the third pereopods and have slender setae (Fig. 2b). The male and female gonopores of an intersex individual are shown in Fig. 2c,d. The male intersex gonopore

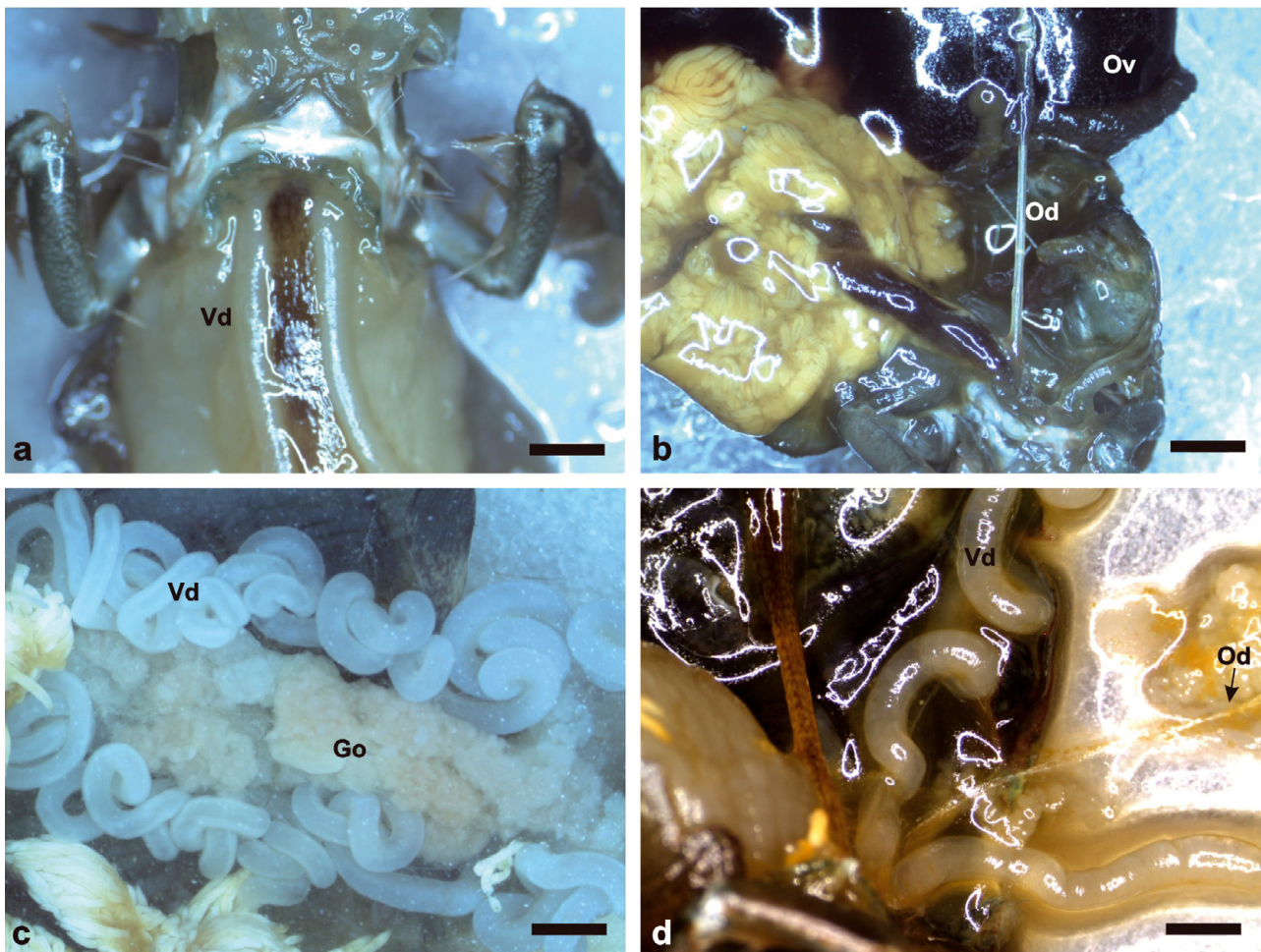


Fig. 1. *Clibanarius vittatus*. (a) Normal male abdominal cavity showing the vas deferens (Vd) leading to the gonopore; (b) detail of normal female anatomy with ovary (Ov) and oviduct (Od); (c) intersex individual with both gonads (Go) and vas deferens; (d) detail of vas deferens and oviduct from an intersex individual, leading to the gonopores. Scale bar = 2 mm

depicted has almost the same morphology as a normal male, i.e. with stout setae above a flap-like operculum. The main difference is the exoskeleton evagination, which is convex in the male intersex individual (Fig. 2c). The female gonopore of the intersex individual has a small evagination of the exoskeleton, without slender setae (Fig. 2d), which may indicate gonopore closure. In 12 of the 18 intersex individuals, 3 gonopores were identified (Fig. 3a), and 6 individuals showed 4 gonopores (Fig. 3b). In 7 of the 18 individuals, the exoskeleton evagination around the female gonopores was observed.

Normal males and females were used as a histological control to check the functionality of the reproductive system (Fig. 4a,b). The intersex individual that showed only male gonads macroscopically proved, on

histological examination, to have a functional male reproductive system. The tests showed the same germinal cells in different stages of spermatogenesis, and mature sperm in the lumen of the seminiferous duct, as in normal males (Fig. 4c).

The histology of the 3 intersex individuals that showed both male and female gonads, upon dissection (Fig. 1), also confirmed that the testis and ovary were functional (Fig. 4d,e). Macroscopically and histologically, the gonads are a paired organ with functional ovary and testis (Fig. 4f,g), characterized by normal oocytes filled with yolk granules, indicating the vitellogenesis or exogenous phase (Fig. 4f), and normal spermatogenesis showing mature sperm (Fig. 4g). Each ovary and testis is clearly surrounded by its own thin layer of connective tissue (Fig. 4d–f), i.e. they are not arranged as an ootest.

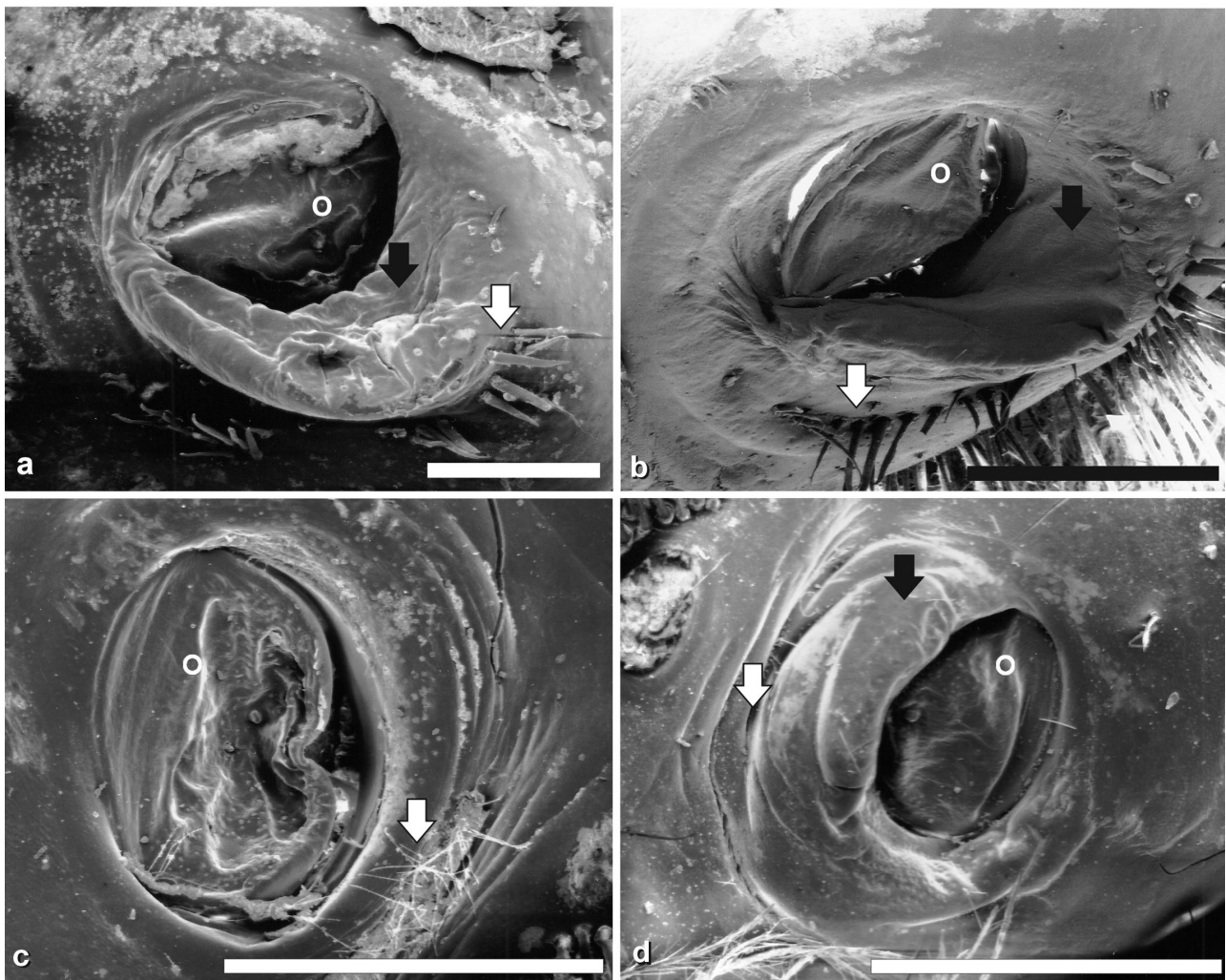


Fig. 2. *Clibanarius vittatus*. (a) Male gonopore and operculum (o) showing concave evagination of the exoskeleton (black arrow) surrounded by stout setae above them (white arrow); (b) male gonopore of intersex individual displaying convex evagination of the exoskeleton (black arrow) and stout setae (white arrow); (c) female gonopore, marked ventrally by slender setae (white arrow); (d) female gonopore of intersex individual, operculum, and evagination of exoskeleton (black arrow) lacking slender setae (white arrow). Scale bar = 0.5 mm

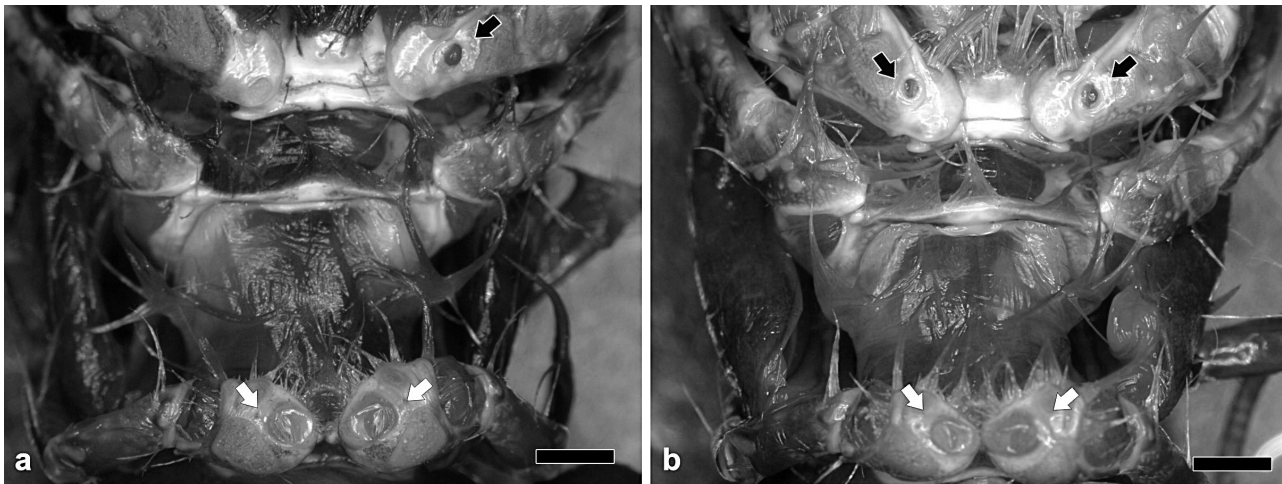


Fig. 3. *Clibanarius vittatus*. Ventral view of coxae and sternites of the pereopods from intersex individuals with (a) 3, and (b) 4 gonopores. Black arrow: female gonopores; white arrow: male gonopores. Scale bar = 2 mm

DISCUSSION

The present study is a step forward in the investigation of hermit crab intersexuality, extending the findings on morphology, behavior, and reproductive activity reported by Turra (2004, 2007). Intersex individuals of *Clibanarius vittatus* may copulate as males that close the female gonopores after molting (Turra 2004), which indicates a protogynic process. Intersex individuals of this species have also been reported as ovigerous females (Turra 2007), reinforcing the hypothesis presented above about the existence of occasional sex reversal in this group, which was first proposed by Wenner (1972), Turra & Leite (2000) and Sant'Anna et al. (2009), based on analyses of population data.

In *Clibanarius vittatus*, both Turra & Leite (2000) and Sant'Anna et al. (2009) obtained anomalous sex-ratio curves. As pointed out by Wenner (1972), such anomalous curves might be explained by differences between the sexes in longevity and time for growth, differential migration, different mortality or growth rates between the sexes, and sex reversal. Turra & Leite (2000) and Sant'Anna et al. (2009) explained this observed sex ratio by differences in the growth rate, longevity, or habitat partitioning between the sexes. Although the percentage of intersex individuals (the hypothetical transitional form) was very low (2 to 7%), the sex-reversal theory was also used to explain the anomalous curve in these 2 studies.

However, since a true protogynic hermaphroditic system requires that both sexes be expressed in a single individual, a very important piece of information in this discussion was lacking. The morphological analysis presented here shows that male and female

gonads can be present and active in the same intersex individual. Most of the intersex individuals contained male gonads, but some of them contained both male and female gonads. In addition, electron microscopy revealed almost the same morphology for male, female, and intersex gonopores. If intersex individuals can act as males (Turra 2004) or reproductive females (Turra 2007), and develop both functional gonads, it may be possible that in a certain stage of female masculinization an individual could reproduce as a male and/or a female. This would indicate a simultaneous hermaphroditism, before female gonopore closure, as observed by Turra (2004), and possible ovary reabsorption if a protogynic process is occurring.

Although protogyny is commonly recorded among crustaceans (Brook et al. 1994), mainly in peracarids, there is no conclusive evidence of this phenomenon in decapods. Although definitive evidence is still lacking, i.e. a record of sex change from female to male in the same individual during its growth, the results of the present study—which may be the first record of protogyny in decapod crustaceans—support this hypothesis.

In the anomuran *Galathea intermedia*, an intersex individual in transitional form can indicate a sex change, as discussed by Kronenberger et al. (2004). These authors suggested that activation of the androgenic gland (AG) and synthesis of the androgenic hormone (AGH) in females are responsible for the intersex morphology observed. This is also a plausible explanation for intersexuality in *Clibanarius vittatus*, since the hormonal action is directly related to male and female differentiation (Charniaux-Cotton & Payen 1985).

However, one question remains unanswered: What could cause this hormonal disruption and what may be

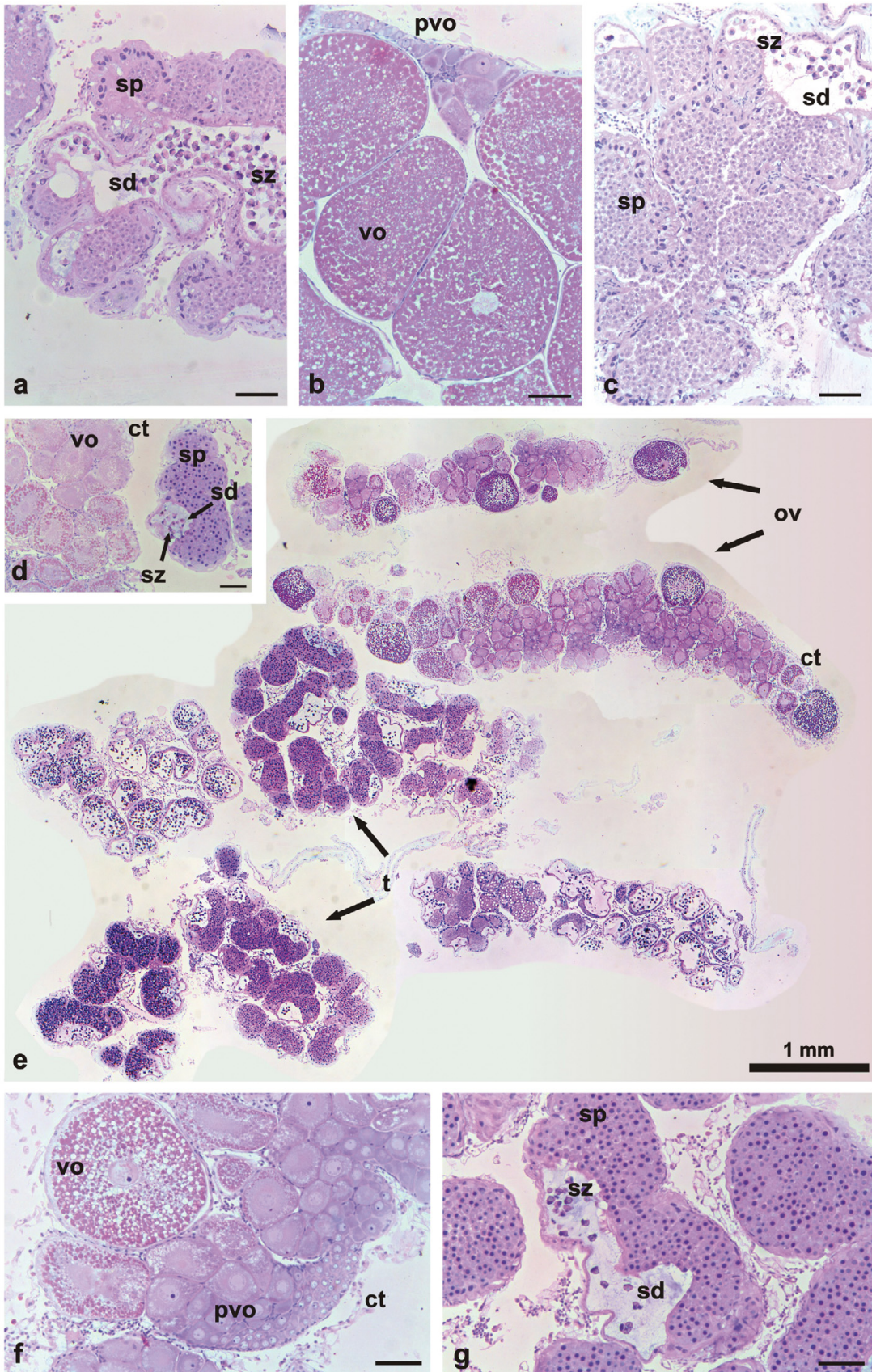


Fig. 4. *Clibanarius vittatus*. Histological sections. (a) Functional male testis with normal characteristics, showing the seminiferous duct (sd), male germinative cells (sp), and mature sperm (sz). (b) Female ovary surrounded by a thin capsule of connective tissue (ct) displaying normal vitellogenesis, depicting vitellogenic oocytes (vo) and pre-vitellogenic oocytes (pvo). (c–g) Histological sections from intersex individuals. (c) The most usual intersex condition observed, showing the testis filled by germinative cells and seminiferous ducts filled with mature sperm. (d) Another intersex condition that occurs in low frequency, showing details of testis, with germinative cells, seminiferous duct and mature sperm, and the ovary, with vitellogenic oocytes bounded by their own connective tissue. (e) Montage pictures from intersex individual observed in Fig. 4d, forming the total histological section of paired gonads, testes (t) and ovaries (ov). (f) Detail of vitellogenic and previtellogenic oocytes bounded by their own connective tissue in an intersex individual with both gonads. (g) Detail of seminiferous duct, male germinative cells, and mature sperm in an intersex individual with both gonads. Scale bar = 50 μ m; except for (e)

occurring in these hermit crabs to result in intersex individuals and, at least hypothetically, in a protogynic process? The answer to this question may be twofold, based on the sex-determination system of hermit crabs and also on external causes such as parasites and pollution.

In crustaceans, protogynic hermaphroditism is often socially mediated (Brook et al. 1994). For example, in the tanaid *Leptochelia dubia*, the presence of a male influences the sex-reversal rate (Highsmith 1983), probably because males produce a pheromone that inhibits sex reversal in potentially protogynous individuals (Buckle-Ramirez 1965). Since the sex ratio in *Clibanarius vittatus* is significantly skewed toward females (Turra & Leite 2000, Sant'Anna et al. 2009), the lack of males may trigger this process.

According to Potts (1906) and Nielsen (1970), parasites may modify sexual characteristics in some hermit crab species. However, in the detailed anatomical analysis, no parasites were found in the individuals of *Clibanarius vittatus* analyzed here.

Endocrine disruption caused by pollutants may be another possible explanation. In molluscs, the antifouling agent TBT used in marine paints causes the phenomenon called imposex, in which female individuals develop male characteristics due to endocrine disruption (Limaverde et al. 2007). Worldwide, the imposex state has been recorded in about 120 gastropod species (Garaventa et al. 2006), and in Brazil this phenomenon was documented in the gastropod *Stramonita haemastoma* (Fernandez et al. 2002). This species lives in coastal regions where there are large concentrations of boats and consequently of TBT (Godoi et al. 2003). Because *Clibanarius vittatus* inhabits estuaries on the Brazilian coast, is commonly recorded using shells of *S. haemastoma* (Turra & Leite 2002, Sant'Anna et al. 2006), and has been found to be contaminated by TBT in nature (B. S. Sant'Anna unpubl.), this pollutant may also be affecting these organisms. Therefore, a careful investigation of the possible effects of TBT on the reproductive development of *C. vittatus* is needed.

Finally, one may also argue that intersexuality in hermit crabs may be a consequence of the synergism of different causes. Therefore, an experimental ap-

proach that considers the effects of pollutants as well as the intrinsic reproductive determination system and behavior would be appropriate to shed more light on this intriguing phenomenon in hermit crabs.

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