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Androgenic Gland Implantation Induces Partial Masculinization in Marmorkrebs *Procambarus fallax* f. *virginalis*

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The androgenic gland in malacostracan crustacean species produces and secretes androgenic gland hormone, which is responsible for male sexual differentiation, such as the induction and development of male sexual traits, and in turn the suppression of female sexual traits. Marmorkrebs, *Procambarus fallax* forma *virginalis*, which was identified as the first parthenogenetic species in decapod crustaceans, produces only female offspring. In this study, in order to reveal whether the Marmorkrebs crayfish is sensitive to androgenic gland hormone, we transplanted an androgenic gland from a related congener, *P. clarkii*, to *P. fallax* f. *virginalis*. In androgenic gland-implanted specimens, partial masculinization was confirmed: the masculinization of several external sexual characteristics (i.e., thickening of the first and second pleopods; formation of reverse spines on the third and fourth pereopods) was detected, whereas that of internal sexual characteristics (e.g., the formation of ovotestes and male gonoducts) was not. Our results imply that *P. fallax* f. *virginalis* still has sensitivity to the androgenic gland hormone and, at least partly, the hormone should be able to induce male characteristics, even in parthenogenetic Marmorkrebs.

Key words: androgenic gland, masculinization, parthenogenesis, implantation, Marmorkrebs

INTRODUCTION

Among decapod crustaceans including more than 14,000 species (De Grave et al., 2009), parthenogenetic reproduction has been reported/speculated in only a few crayfish species (cf. Yue et al., 2008; Buřič et al., 2011). Marmorkrebs, or the marbled crayfish, was first identified as a parthenogenetic decapod (Scholtz et al., 2003). From the analysis of mitochondrial DNA and morphological characters, Martin et al. (2010) suggested that Marmorkrebs is the parthenogenetic form of dioecious *Procambarus fallax*, and was tentatively named as '*Procambarus fallax* f. *virginalis*'. All Marmorkrebs individuals are genetically identical females (Martin et al., 2007), and there have been no reports of the birth of male offspring to date. Little is known about how the mechanism of sexual reproduction has been altered to permit parthenogenesis. Hence, Marmorkrebs is a potential model organism for studying the mechanisms underlying the evolution of parthenogenesis from sexual reproduction.

The androgenic gland (AG) is a hormone-secreting gland specific to male malacostracan crustaceans (reviewed in Charniaux-Cotton and Payen, 1985; Payen, 1990). Ever since Charniaux-Cotton (1954) found AG in the amphipod *Orchestia gammarella*, the AG has been identified in nearly all of the orders belonging to Malacostraca. The role and function of AG has been examined by its implantation into immature females and its ablation from males with various malacostracans (reviewed in Charniaux-Cotton et al., 1992; Sagi et al., 1997). In males, AG develops to produce androgenic gland hormone (AGH), promoting the masculinization of external and internal sexual characteristics. On the other hand, in females, the AG primordium is formed but does not develop, resulting in the acquisition of female sexual characteristics. Implantation of AG with subsequent reversal of functional sex has mainly been reported in the amphipod *O. gammarella* and the isopod *Armadillidium vulgare* (cf. Katakura, 1967; Charniaux-Cotton and Payen, 1985). In decapods, functional sex reversal was only detected in *Macrobrachium rosenbergii* (Sagi and Cohen, 1990; Malecha et al., 1992) although partial masculinization was reported in many species (e.g., Nagamine et al., 1980; Nagamine and Knight, 1987). In a crayfish crustacean *Procambarus clarkii*, the localization and characteristics of AG were investigated (Taketomi et al., 1996), and the AG implantation was conducted (e.g., Taketomi and Nishikawa, 1996). Then, however, Murakami et al. (2004) reported that two kinds of AG-like tissues, TIB (the gland-like tissue located inside the body cavity) and TIC (the gland-like tissue located inside the coxa of the fifth pereopod), were attached to the subterminal ejaculatory region of the vas deferens. Judging from their location and structure obtained by histological and his-

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tochemical methods, TIB appears to have a similar function as the AG. Furthermore, they indicated that the cordlike tissue that develops into AG as described in Taketomi et al. (1996) resembles TIC, suggesting that Taketomi et al. might have mistaken not only the TIB but also the TIC for the real AG. Since then, the functions of TIB and TIC have never been analyzed separately. In parallel with the AG research, the chemical nature of AGH has also been studied and insulin-like androgenic gland factors (IAGs) — such as *Cq-IAG* isolated from *Cherax quadricarinatus*, *Mr-IAG* from *M. rosenbergii*, and *Pm-IAG* from *Penaeus monodon*— have been discovered in several decapods (reviewed in Ventura et al., 2011). From the *Cq-IAG* silencing in intersex *C. quadricarinatus*, it is suggested that the IAG both regulates male sex differentiation and plays the role of a gender switcher, balancing male/female characters of intersex crayfish (Rosen et al., 2010).

It is unknown whether *P. fallax* f. *virginalis* is sensitivite to AGH; and if so, whether masculinization occurs in this crayfish. It is possible that Marmorkrebs would lose the ability to induce male characteristics with the acquisition of parthenogenesis. In this study, to begin to improve our knowledge about the sensitivity to AGH associated with masculinization in this parthenogenetic crayfish, we implanted AG from a congener (*P. clarkii*) to *P. fallax* f. *virginalis*.

MATERIALS AND METHODS

Animals

Procambarus fallax f. *virginalis* were isolated in a plastic aquarium (2.5 L; ca.14 × 20 × 12 cm) with dechlorinated tap water (hereafter referred to as 'rearing water'), which was continuously aerated. Animals were maintained at 23°C under artificial light conditions of 14 h light and 10 h dark, and fed with dry feed for crayfish (JAN code 4971618829092; Kyorin). Rearing water was changed about once every week.

As male *P. fallax* (native to Georgia and Florida, USA) could not be prepared, *Procambarus clarkii*, which is a closely related congener to *P. fallax* (Martin et al., 2010), was used as an AG donor in this study. Its rearing conditions were the same as for *P. fallax* f. *virginalis*.

The terminology used herein for morphology follows the usage of Holdich (2002); the terminology for AG-like tissue follows the usage of Murakami et al. (2004).

Extraction of vas deferens including AG

The following method for AG extraction was performed with reference to Nagamine and Knight (1987), Lee et al. (1993), and Taketomi and Nishikawa (1996). The donor animals were anesthetized with ice for 10 min, and then dissected in crayfish saline (Van Harreveld, 1936) to extract the vas deferens including AG-like tissues (Fig. 1A). Histological observation of these two tissues stained with Delafield's hematoxylin and eosin (HE) showed that the characteristics of cells in the proximal tissue were large spherical nuclei and a distinct nucleolus (Fig. 1B), whereas those in the distal tissue were oval nuclei, which were smaller and more basophilic (Fig. 1C). From these positional and histological features, the former and the latter tissues were identified as TIB and TIC, respectively according to Murakami et al. (2004). These two tissues were used distinctively in subsequent experiments. Therefore, grafts were trimmed to three types: vas deferens with 1) TIB, 2) TIC, and 3) both TIB and TIC. Moreover, vas deferens alone (without TIB and TIC) was prepared as the control.

Implantation of AG

Immature females [ca. 7–10 mm in carapace length (CL), 20–30 days after hatching] of *P. fallax* f. *virginalis* were used as recipients; the implanted area is shown in Fig. 2. Each graft was transplanted under the soft ventral exoskeleton at the base of the fifth pereopod (cephalothorax–abdominal junction) using a needle to squeeze into the soft ventral exoskeleton. Grafts 1–3 were implanted into nine, three, and 10 individuals, respectively (Table 1). As controls, three animals were implanted with only part of the vas deferens. Post-transplant individuals were reared overnight each in a 100 mm plastic dish (353003; BD Falcon) with rearing water, and then transferred separately into cylindrical cases (ca. 6 dia. × 15 depth cm) which were placed in a plastic aquarium (20 L; ca. 35 × 40 × 18 cm) filled with rearing water. The surgical mortality of the recipients was 37.5% (15/40).

Phenotype analysis

Implanted females were maintained for roughly one year. During the experiment, external evidence of masculinization was scrutinized with every molt. External morphologies were observed under a stereomicroscope (MZ16; Leica) and recorded with a digital camera (DS-Fil-L2; Nikon and/or CX-3; RICOH). At the end of the experiment, animals were examined for external and internal morphology. They were anesthetized with ice for 5 min, and then dissected in crayfish saline to extract the ovaries. After observing the external morphology of the ovaries in the same manner as described above, they were fixed in Bouin's fluid. The fixed ovaries were embedded in paraffin, serially sectioned at 5 μm thickness, stained with HE, and observed under a light microscope (BX50; OLYMPUS).

RESULTS

Morphological changes of external sexual characteristics

Table 1 summarizes the results of the implantation experiment based on the type of grafts used and masculin-

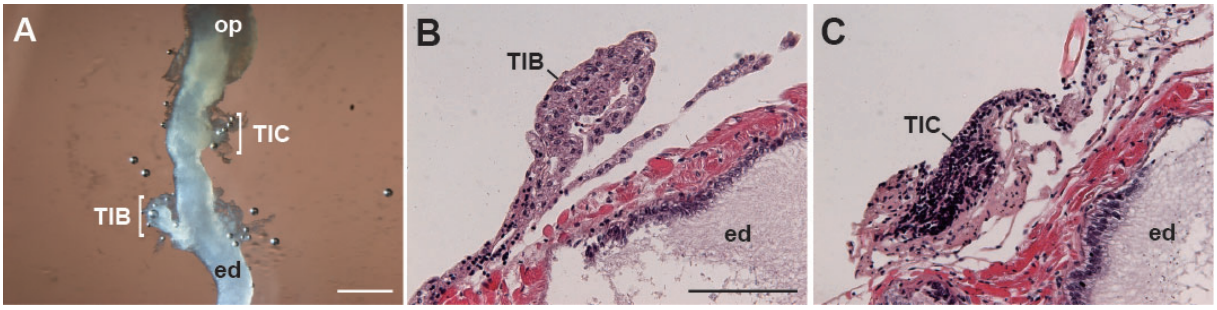


Fig. 1. The subterminal ejaculatory region of the vas deferens in *P. clarkii*. **(A)** Light micrograph. **(B, C)** HE-stained sections of TIB and TIC, respectively. ed, ejaculatory duct; op, opening of vas deferens. Scale bars = 1 mm **(A)**, 0.1 mm **(B, C)**.

ized phenotypes. Although the degree of masculinization varied among individuals, all females implanted with TIB only (graft 1) or both TIB and TIC (graft 3) became masculinized, as judged by the external sexual characteristics (Figs. 3–5). In all cases, first evidence of masculinization was seen on the first pleopod (abdominal appendages), which began appearing 34–83 days (mean 52 days, n = 12) after AG implantation. The shape of the first pleopod began to change with the onset of thickening at the base, and then thickening and sclerotizing in every molt, resulting in a tendency to develop a male-like morphology. At the end of the experiment, individual variability was observed in terms of the degree of changes in the first pleopod such as total size, or thickness of its basal portion (Fig. 3A–C). After the appearance of masculinization in the first pleopod, the formation of a reversed spine on the fourth pereopod was detected in one individual (Fig. 4B), and both third and fourth pereopods in two individuals (Fig. 4A). One of the latter was also masculinized in the endopod of the second pleopod which displayed thickening and calcification (Fig. 5A). These masculinized phenotypes were initially observed 118 days after the AG implantation. The degree of masculinization between left and right pleopods or pereopods was usually unequal, with one pleopod/pereopod being more masculinized than the other (Figs. 3 and 4, respectively). There was no evidence of masculinization in other male-specific characteristics such as the shape of chelipeds and the male gonopore. The female genital openings on the coxae of the third pereopods were unaffected and retained their morphology in all recipients.

In contrast, none of the females implanted TIC only

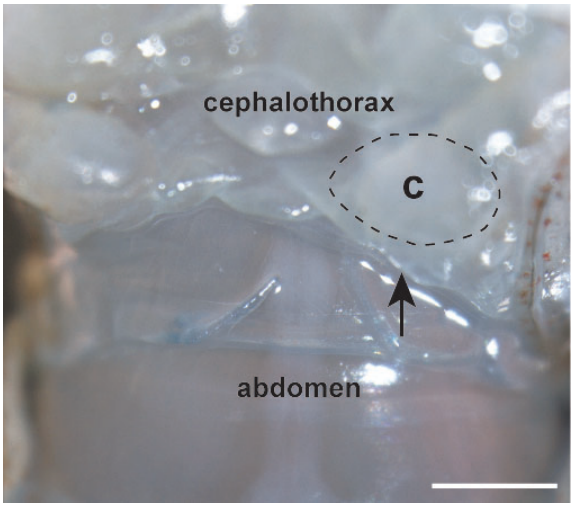


Fig. 2. The site of implantation (dashed line) in *P. fallax* f. *virginalis*. A graft was inserted under the ventral exoskeleton at the coxa of the fifth pereopod as shown by the direction of the arrow. c, coxa of fifth pereopod. Scale bar = 1 mm.

Table 1. Summary of AG implantation. The first row shows the type of grafts implanted into *P. fallax* f. *virginalis*. The first column represents the combination of body parts in which masculinized phenotypes were observed. The figures indicate the number of individuals observed. Pl, pleopod; P, pereopod; vd, vas deferens; –, null.

Masculinized phenotype	Type of grafts			
	1)	2)	3)	control
	TIB	TIC	TIB + TIC	vd
No change	–	3	–	3
Pl1 (strong)	5	–	5	–
Pl1 (weak)	3	–	3	–
Pl1 + P4	–	–	1	–
Pl1 + P3 + P4	1	–	–	–
Pl1 + Pl2 + P3 + P4	–	–	1	–
Total (n)	9	3	10	3

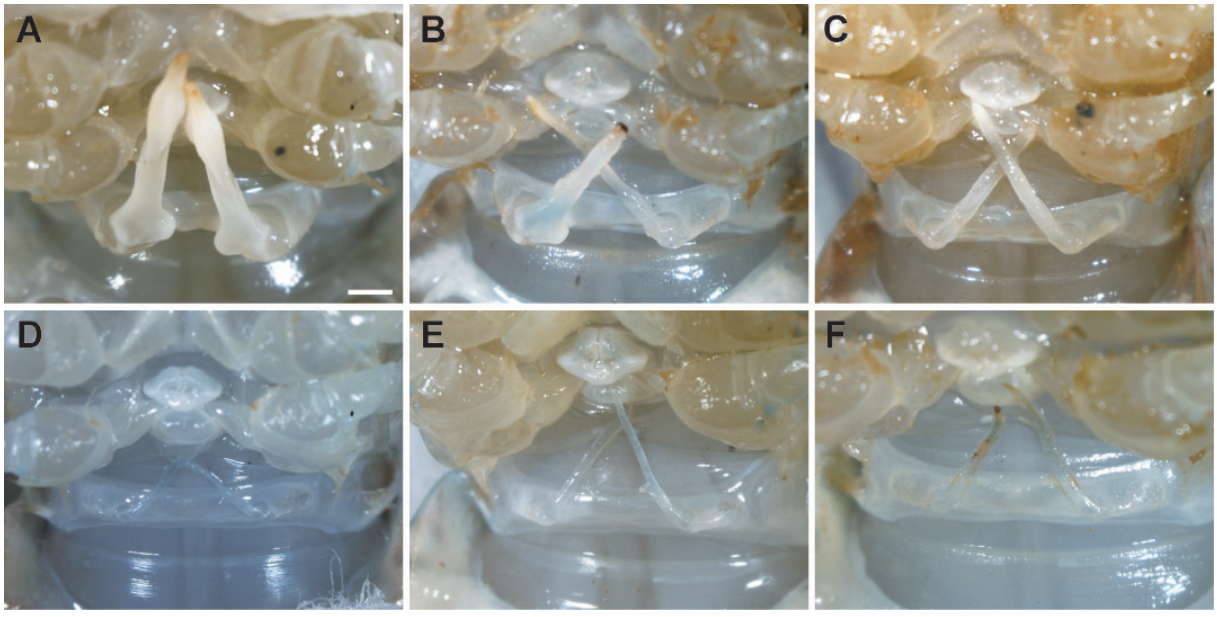


Fig. 3. Morphological changes of the first pleopod of *P. fallax* f. *virginalis* by implantation. **(A–C)** TIB-implanted individuals. The first pleopods were masculinized. **(D)** TIC-implanted individual. The first pleopods were not masculinized. **(E)** Vas deferens-implanted individual (control). The first pleopods were not masculinized. **(F)** Normal individual without an implant. Scale bar = 1 mm.

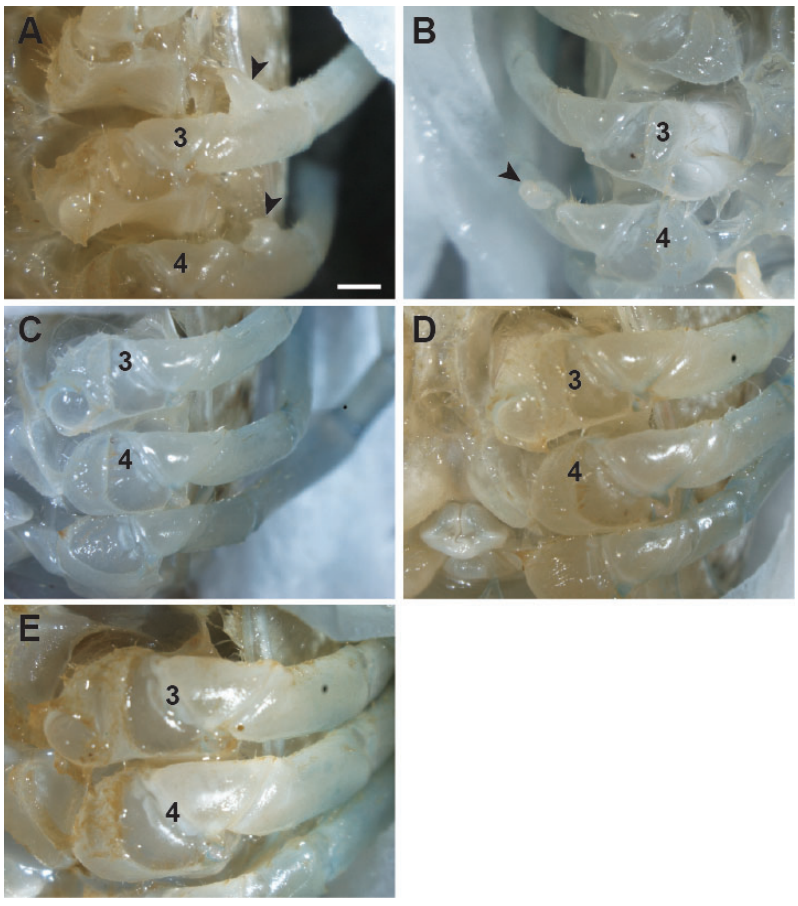
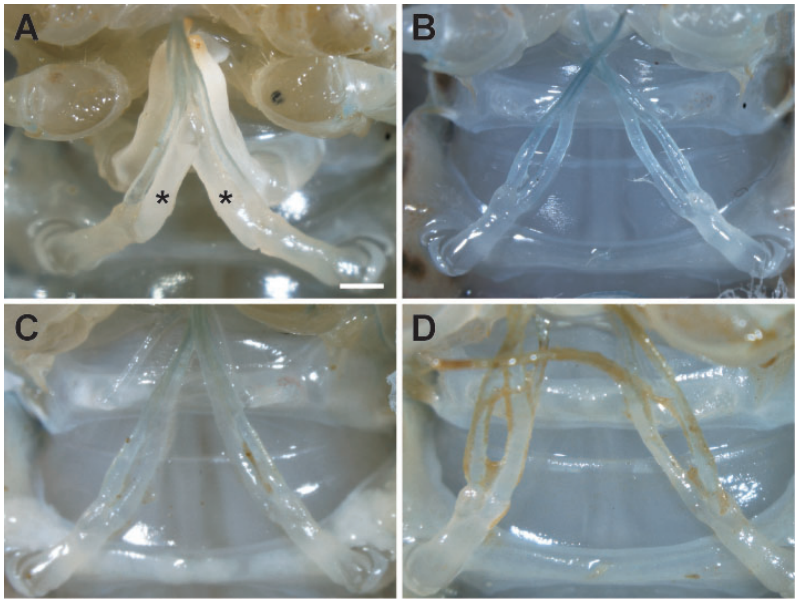


Fig. 4. Morphological changes of the third and fourth pereopods in *P. fallax* f. *virginalis* by implantation. **(A, B)** TIB-implanted individuals. Reversed spines formed on the third and fourth pereopods (arrowheads). **(C)** TIC-implanted individual. Reversed spines on the third and fourth pereopods did not form. **(D)** Vas deferens-implanted individual (control). Reversed spines on the third and fourth pereopods did not form. **(E)** Normal individual without an implant. 3, 4, third and fourth pereopods, respectively. Scale bar = 1 mm.



(graft 2) and control animals exhibited external masculinization (Figs. 3D, E, 4C, D, 5B, C), showing no difference from normal females (Figs. 3F, 4E, 5D).

Morphological changes of internal characteristics

At a gross anatomical level, all females implanted TIC only (graft 2) and control females did not show any prominent change in their ovaries (Fig. 6D and E) compared to normal females (Fig. 6F). On the other hand, the females with an implanted TIB (grafts 1 and 3) had both yellow and gray eggs (Fig. 6A, B). There was no association between the ratio of yellow and gray eggs with the degree of masculinization in external sexual characters. In the females implanted with TIB, the shape of the ovary varied from a Y-shape to an awkward shape, and also showed no association with the degree of masculinization in external features.

From the observation of HE-stained ovary sections, all females implanted TIC only (graft 2) and control females did not show any prominent difference in the accumulation of yolk granules in their ovaries compared to normal females. Although the females with an implanted TIB (grafts 1 and 3) also showed the accumulation of yolk granules, the observed ovarian eggs can be divided into two types: one with fine yolk granules (Fig. 7, asterisks) and the other with fine and larger granules as normal eggs. In all of the masculinized females, there was no evidence of ovotestis or male gonoducts. Moreover, implanted tissues could not be found in either individual at dissection for fixation.

DISCUSSION

Our results present the first evidence of experimentally induced masculinization of Marmorkrebs by AG implantation of another related species. In other words, *P. fallax* f. *virginalis* still has sensitivity to AGH, although the implanted specimens were not completely, rather only partially, masculinized. All females implanted with a graft including TIB were masculinized in external sexual characters to various degrees, whereas all females implanted with only TIC were not. These results strongly

Fig. 5. Morphological changes of the second pleopod of *P. fallax* f. *virginalis* by implantation. **(A)** TIB-implanted individual. The endopods of second pleopods showed partial thickening and calcification (asterisks). **(B)** TIC-implanted individual. The second pleopods were not masculinized. **(C)** Vas deferens-implanted individual (control). The second pleopods were not masculinized. **(D)** Normal individual without an implant. Scale bar = 1 mm.

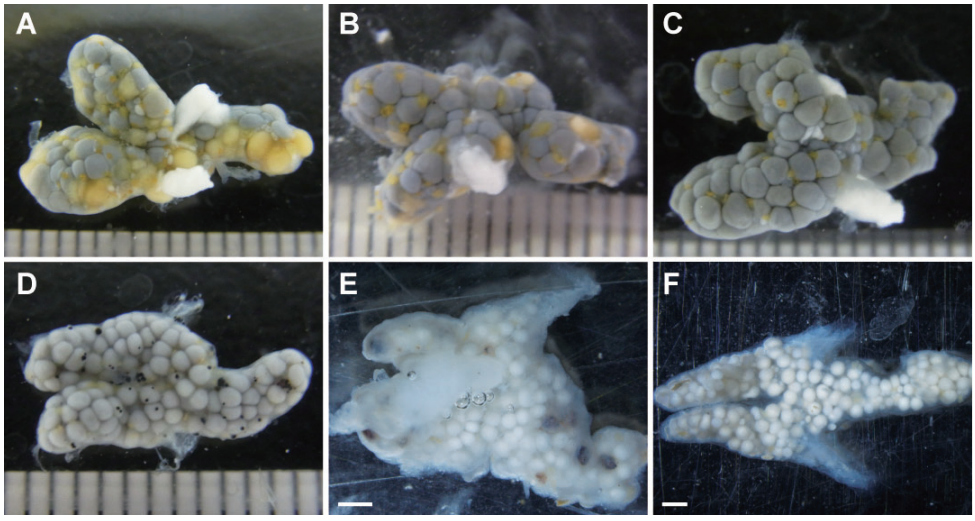


Fig. 6. Morphological changes of ovaries and ovarian eggs in *P. fallax* f. *virginalis* by implantation. **(A–C)** TIB-implanted individuals. Yellow eggs as well as gray eggs formed. **(D–F)** TIC-implanted, vas deferens implanted, and normal (no implant) individuals, respectively. Scale bars (one graduation in A–D) = 1 mm.

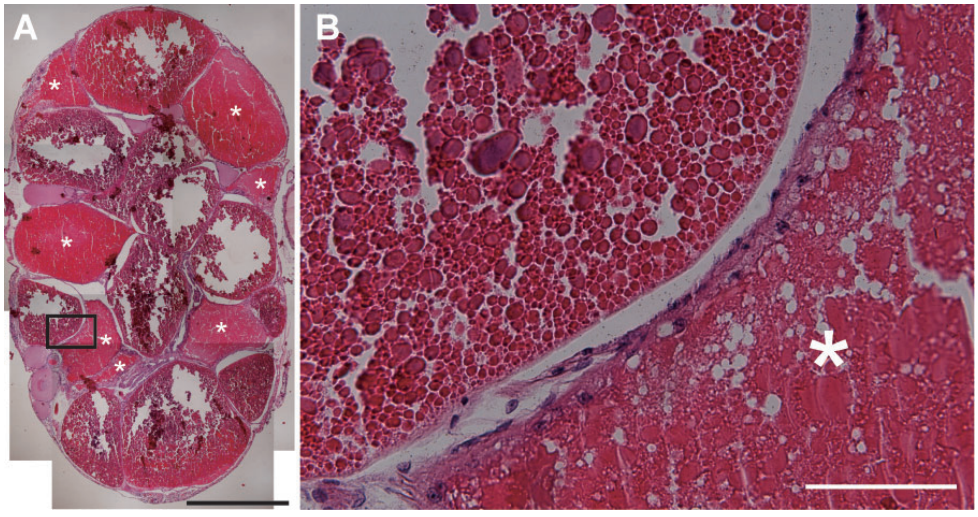


Fig. 7. Changes in internal morphology of *P. fallax* f. *virginalis* ovaries by implantation. **(A)** HE-stained section of ovary in TIB-implanted individual of Fig. 6A. There were two types of ovarian eggs: one with fine yolk granules (asterisks) and another with fine and larger granules. **(B)** Magnified view of the boxed area in **(A)**. Scale bars = 1 mm **(A)**, 100 μm **(B)**.

support the hypothesis that TIB is the AG of *P. clarkii*, as advocated by Murakami et al. (2004). All females implanted with a graft including TIB in this study were masculinized in the first pleopod, which is similar to previous reports in AG-implanted *P. clarkii* (Nagamine and Knight, 1987; Taketomi and Nishikawa, 1996). Furthermore, the magnitude of change in the first pleopod varied among different individuals, which is in accordance with an AG implantation experiment in *P. clarkii* (Taketomi and Nishikawa, 1996). In addition to the first pleopod, masculinization was also seen in the second pleopod, and the third and fourth pereopods in several specimens (Table 1). Because *P. fallax* f. *virginalis* reproduces by apomixis, resulting in the production of genetically identical individuals (Martin et al., 2007), the cause of the differing degrees of masculinization can be explained by differences in the developmental stages among studied individuals at the time of implantation rather than the individual variability caused by genetic differences.

In the AG implantation study of *P. clarkii*, vitellogenesis in ovarian eggs was inhibited in masculinized females (Taketomi and Nishikawa, 1996). In the present study, however, vitellogenesis occurred in both types of ovarian eggs observed in masculinized females; one type with only fine granules may be the degenerating oocyte, as suggested by Nagamine and Knight (1987). In the AG removal experiment of *M. rosenbergii*, the abnormal multi-lobulated ovaries were observed in incomplete feminized males, whereas the two ovarian lobes (normal female phenotype) were manifested in completely feminized males (Aflalo et al., 2006). Even though their experiment was the opposite to our study, both experiments resulted in the formation of abnormal-shaped ovaries in incomplete feminized or masculinized individuals, suggesting the intervention by removed or implanted AG in the sex differentiation process. Nagamine et al. (1980) showed, in AG-implanted *M. rosenbergii*, that the spermatogenesis initiated in the ovaries was observed in two masculinized females, which were small at the time of implantation (8.6 and 11.8 mm in CL), but not in other masculinized females, which were larger at the time of implantation (12.7–32.8 mm in CL). On the other hand, our results detected no evidence of spermatogenesis in the ovaries of masculinized females. These differences in the degree of masculinization may be due to differences in species and/or size of the females when the AG was implanted.

In *P. clarkii* (Nagamine and Knight, 1987; Taketomi and Nishikawa, 1996) and *M. rosenbergii* (Nagamine et al., 1980), implanted AG tissues remained and some of them enlarged. However, in this study, we could not recover the grafts from either individual at the end of the experiments. Even though immature females raised for 20–30 days after hatching to a CL of 7–10 mm were used as recipients, the masculinization of internal sexual characteristics was not observed, and functional males could not be induced. This

failure of complete masculinization in *P. fallax* f. *virginalis* may be attributable to the destruction or degeneration of AG following masculinization. More or complete masculinization may have occurred if we had been able to obtain smaller surviving recipients after implantation. It is also possible that cross-species implantation of AG caused incomplete masculinization.

In this study, we found that even an all-female species has a genetic disposition to be able to respond to masculinizing hormone. Therefore, Marmorkrebs may serve as a useful model for studying the physiological effects of AGH in decapod crustacean. It will be necessary to investigate IAG in Marmorkrebs and conduct subsequent functional analyses by overexpression/silencing.

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