Selfing in a malacostracan crustacean: why a tanaidacean but not decapods

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The crustacean class Malacostraca, with over 22,000 species, includes commercially important members such as crabs, shrimps, and lobsters. A few simultaneous hermaphrodites are known in this group, but self-fertilization was unknown. Here we show, through microscopy and breeding experiments, that the simultaneously hermaphroditic malacostracan *Apseudes* sp. (order Tanaidacea) can self-fertilize; individuals reared in isolation become hermaphroditic via a male-like phase and produce eggs that develop into fertile adults. Although selfing occurs in crustaceans like the Branchiopoda, in which simultaneous hermaphrodites have the sex ducts united, in decapods the separation of gonadal ducts and gonopores, specialized mating organs, and complex mating behaviour appear to have constrained the evolution of selfing. In contrast, in most tanaidaceans, sperm is released externally by a male and reaches the eggs in the female brood pouch, where fertilization occurs. This mode of fertilization permitted *Apseudes* sp. to achieve selfing without large modifications in morphology or behaviour.

Keywords: Crustacea, Malacostraca, Tanaidacea, *Apseudes*, self-fertilization, hermaphroditism

1. Introduction

Simultaneous hermaphroditism (functional ovaries and testes present simultaneously in a single individual), which is usually a prerequisite for self-fertilization (selfing), is widespread in animals, having been documented in roughly half of all animal phyla (Jarne and Auld 2006). Selfing has been reported at least once in each of 17 phyla known to contain simultaneously hermaphroditic (SH) species, but its frequency varies greatly, with high incidence in some groups such as platyhelminths, pulmonate gastropods, and ascidians (Jarne and Auld 2006). Selfing is theoretically advantageous when it is difficult for individuals to find a mate, and it confers a two-fold genetic transmission advantage. However, it can also lead to inbreeding depression, which may explain why most hermaphrodites avoid it altogether or limit its adverse effects through facultative outcrossing (Maynard Smith 1978; Lande and Schemske 1985; Jarne and Auld 2006).

The evolution of selfing in SH animals logically depends on the potential for eggs and sperm from the same individual to encounter one another. Simultaneous hermaphroditism occurs in several groups in the arthropod subphylum Crustacea, but selfing is more restricted, reported only in some members of the classes Branchiopoda (e.g., tadpole shrimps, clam shrimps) and Maxillopoda (e.g., some barnacles) (Weeks et al. 2008; Zierold et al. 2009; Barazandeh et al. 2013). In both of these groups, the reproductive system is permissive of selfing: SH branchiopods have the sex ducts united (Longhurst 1955), while many barnacles have a long penis with which an individual can potentially release sperm inside its own shell rather than that of a neighbor, and some species engage in spermcasting (Barazandeh et al. 2013). In contrast, there has been no report of selfing in the Malacostraca, the largest crustacean class, with over 22,000 species including decapods (e.g., crabs, shrimps) and peracaridans (e.g., isopods, amphipods, tanaidaceans). Simultaneous hermaphrodites have been reported only among decapods and tanaidaceans (Bauer 2000; Johnson et al. 2001); members of both groups have separate gonadal ducts, a condition that we speculate to be non-permissive of selfing. In the Tanaidacea, simultaneous hermaphroditism can be inferred from individuals having both a male genital cone and a female brood pouch; corresponding ovaries and testes with their respective ducts and gonopores have been confirmed internally only in *Apseudes spectabilis* (Lang 1953).

In a population of the tanaidacean *Apseudes* sp. (ESM_1), we discovered individuals having both a genital cone and a brood pouch, indicating that they were simultaneous hermaphrodites, which we confirmed through histological sections. Through breeding experiments and DAPI (4′,6-diamidino-2-phenylindole)-stained eggs, we show that *Apseudes* sp. engages in selfing, the first case known in the Malacostraca. *Apseudes* sp. appears to be the exception that proves the rule; while the sex ducts are separate as in decapods, external fertilization provides a means by which eggs and sperm from the same individual can potentially come into contact, a condition permissive for the origin of selfing.

2. Material and Methods

(a) Animals

Apseudes sp. were collected from the bottom sand in a tank housing giant clams (24–26°C) in the Port of Nagoya Public Aquarium (PNPA), Japan, on 14 June 2009 and 12 April 2010. The animals were maintained at 25°C and fed every three days with porphyrized dry feed for crayfish (JAN code 4971618829092; Kyorin). Body length was measured from the tip of the eye to the tip of the pleotelson.

(b) External and internal morphology

Animals were examined for presence of a genital cone and brood pouch under a stereoscopic microscope. For scanning electron microscopy, specimens were fixed in 70% ethanol, dehydrated in an ethanol series, treated with hexamethyldisilazane, sputter-coated with gold, and observed at 15 kV accelerating voltage. For histology, specimens were fixed in Bouin's fluid; paraffin sections 5 μm thick were prepared and stained with Delafield's hematoxylin and eosin using standard techniques.

(c) Reproduction

Three adults were isolated, each in a small aquarium with coral sand covering the bottom (average temperature 23.6°C; light cycle, 14 h light : 10 h dark). After each adult had released juveniles, it was transferred by pipette to another aquarium; the juveniles were maintained in a group for ca. 3 weeks, after which they were separated, and each was reared singly in a small aquarium. These juveniles then matured in isolation and themselves produced eggs and juveniles. Two subsequent generations of juveniles after the first were handled in the same manner as described for the first. Isolated individuals were observed once a day when they lacked a brood pouch; every two hours after the brood pouch began to develop; and every 30 minutes from their molt until they laid eggs.

(d) DAPI staining of eggs

Independently propagating simultaneous hermaphrodites can produce offspring by either a sexual (self-fertilization) or a parthenogenetic pathway, and the temporal pattern in the number of separate chromosome sets in eggs stained by DAPI (which binds to DNA and fluoresces strongly when bound) can distinguish between these alternatives (see ESM_2). Individuals with a developing brood pouch were isolated and fixed in 99% glacial ethanol at intervals from the release of eggs into the brood pouch. Eggs were washed three times in PBST (0.05% Tween 20 in PBS, phosphate buffered saline) for 10 min each; stained with 0.5 μg/ml DAPI (MP Biomedicals) in PBS for 1 h at 4°C; washed twice for 1 h and then overnight in PBS at 4°C; mounted in Vectashield (H-1000; Vector Laboratories); and observed under a fluorescence microscope. Images taken at different focal depths were combined by using Adobe Photoshop CS5. In total, 590 eggs from 27 adults were examined.

3. Results

(a) External and internal morphology

Adults (greater than 4.35 mm long) all had a complete or developing brood pouch, a pair of ovarian gonopores on the pereonite-4 sternite, and a genital cone with paired testicular gonopores (Fig. 1a–c). Internally, adults had paired ovaries and testes (Fig. 1d), each organ with a duct leading independently to a gonopore. The male component of the reproductive system developed first. Juveniles 1.93–2.47 mm long at 7 dpr (days post-release from the brood pouch) had only a spine where the genital cone would develop and lacked testes and ovaries; individuals at 18–23 dpr (2.69–3.08 mm long) had testes with ducts and a genital cone with gonopores, but the ovaries were lacking or only developing (“male-like phase” individuals).

(b) Reproduction

We isolated juvenile individuals at 20–21 dpr, before their ovaries had appeared or were fully developed. Approximately 5 weeks later, these juveniles had matured and themselves laid eggs, which developed normally to the adult stage. We observed individuals to produce at least three clutches of eggs in isolation, laying the first clutch into the brood pouch at ca. 57 dpr, releasing the resulting larvae from the brood pouch ca. 15 days later, and laying the second clutch ca. 13 days after that. Between the release of larvae and laying the next clutch, adults lost the brood pouch and formed a new one in the course of two molts. We also observed maturation and egg laying by three successive generations of juveniles reared singly, beginning from one of the three adults initially isolated.

(c) DAPI staining of eggs

We designated the time at which the first eggs were released into the brood chamber (here termed ovulation) as 0 h. Eggs showed 3 DAPI spots at 1–2 h post-ovulation (hpo); 4 spots at 3–4 hpo, and 3 spots at 5–6 hpo (Fig. 2). Although eggs contained a variable number of DAPI spots in some time intervals, this was because ovulation extended over a period of 1–2 h, and thus embryos within a clutch were not developing completely synchronously. The progression of spots observed at 1.5, 3.5, and 5.5 hpo but prior to the first cleavage was 3-4-3, the transition pattern characteristic of sexual reproduction rather than any of the modes of parthenogenesis (compare Fig. 2 with Fig. S2 in ESM_2).

4. Discussion

While *Apseudes* sp. can self-fertilize, it remains unknown whether cross-fertilization also occurs, with male-like phase and/or SH individuals functioning as males. If outcrossing occurs, *Apseudes* sp. could resemble protandric SH species among carideans (Bauer and Holt

1998; Baeza 2009; Onaga et al. 2012). Another analogue might be the pulmonate snail *Biomphalaria glabrata* (Trigwell et al. 1997), in which individuals self-fertilize in isolation but preferentially outcross when paired. This mode potentially avoids the disadvantage of inbreeding depression while obviating the absolute necessity of encountering a mate in order to reproduce.

Among the Malacostraca, simultaneous hermaphroditism is known only in decapods and tanaidaceans, which engage in different forms of mating. In decapods, males use special copulatory organs (gonopods) during a mating ritual to inseminate a female internally or to attach spermatophores/spermatophoric-mass to the ventral side of the female (Bauer 1976; Thiel and Duffy 2007). In contrast, male tanaidaceans lack special copulatory structures, but simply align themselves near a female during mating and release sperm, which the female sweeps into the brood pouch with water currents (Bückle Ramírez 1965; Johnson and Attramadal 1982). The evolution of selfing in decapods requires evolutionary changes in the complex behaviours and morphological structures involved in copulation, whereas in tanaidaceans few evolutionary changes are required: a simultaneous hermaphrodite need only release eggs to the brood pouch and, in the absence of another individual, release sperm externally and sweep the sperm to its own eggs. Other SH animals such as some ascidians, corals, bryozoans, and polychaetes that have evolved selfing likewise release sperm externally and required few or no behavioural or morphological modifications, but only a breakdown in gamete self-incompatibility systems, which are only now beginning to be understood in animals (e.g., Harada et al. 2008). Like all other tanaidaceans, *Apseudes* sp. releases non-planktonic larvae with low dispersal ability, increasing the chance of encountering kin during mating, which may have led to reduced inbreeding avoidance. By the time selfing evolved, *Apseudes* sp. might already have survived the initial, deleterious phase of inbreeding depression.

Several "likely" modes of reproduction have been discovered recently in familiar animals, including parthenogenesis in a crayfish (Scholtz et al. 2003), facultative parthenogenesis in non-captive pit-vipers (Booth et al. 2012), spermcast mating in a gooseneck barnacle (Barazandeh et al. 2013), and now selfing in a malacostracan. Each of these discoveries constitutes an independent test of life-history theory, which will remain incomplete without fuller knowledge of the phenomenology and taxonomic distribution of reproductive modes. While it remains unclear why hermaphroditism has a limited distribution among crustacean higher taxa, our study helps explain why, among SH malacostracans, selfing evolved in tanaidaceans but not in decapods.

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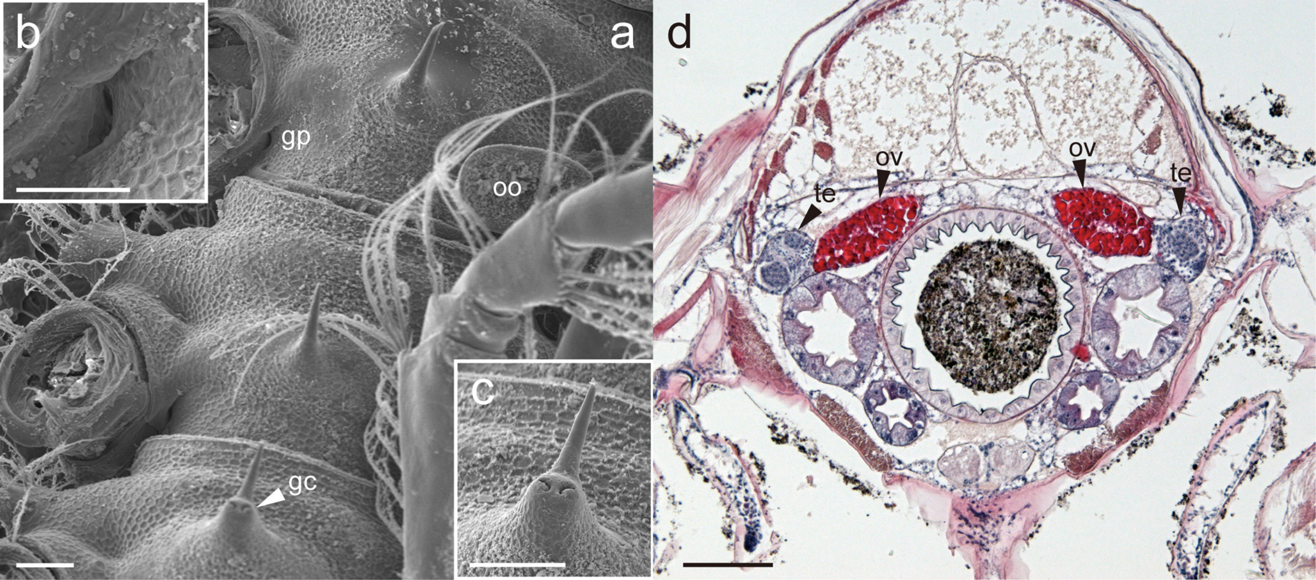
Figure legends

Fig. 1 External and internal reproductive organs in *Apseudes* sp. **a** Ventral view of an adult showing the ovarian gonopore (gp), genital cone (gc*)* with paired testicular gonopores, and oostegite (oo), part of the developing brood pouch (right pereopods and oostegite removed). **b, c** Enlargements of the ovarian gonopore and genital cone, respectively. **d** Transverse section of the adult body showing ovaries (ov) and testes (te). HE-stained. Scale bars: a, c, d, 0.1 mm; b, 0.05 mm

Fig. 2 Number of DAPI-staining spots (*arrowheads*) at intervals through oogenesis and early development in *Apseudes* sp. **a** Two half-bivalent sets and one sperm chromosome set. **b** First and second polar bodies, and male and female pronuclei. **c** Fused pronuclei and the two polar bodies. **d** First cleavage, with two nuclei and two polar bodies. **e** Second cleavage, with four nuclei and one polar body. Scale bars: 0.1 mm

Short title: Selfing in a malacostracan crustacean



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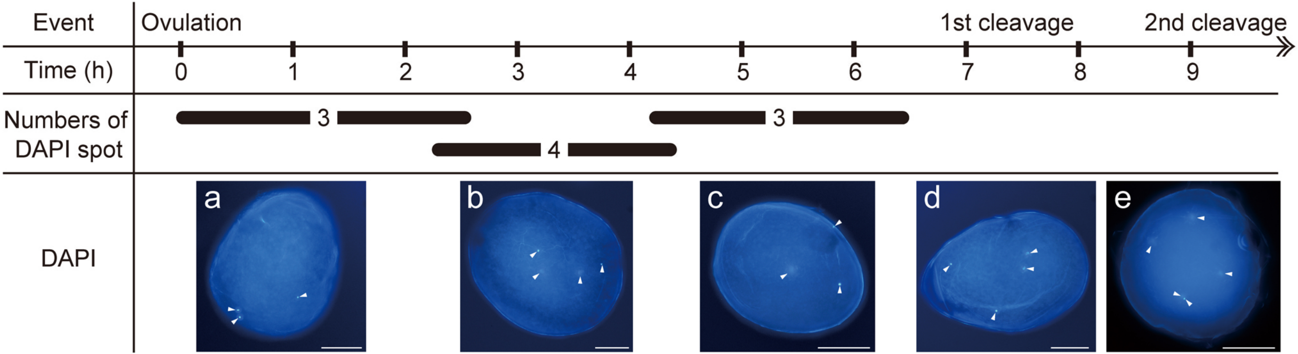

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