Note to Reader from the authors:

Please note the scale bar measures for Figures 1-3 (not given in figure captions).

Fig. 1. Scale bar in “a” represents 260 µm in “a,” 64 µm in “b,” 14 µm in “c,” 230 µm in “d,” 62 µm in “e,” and 27 µm in “f.”

Fig. 2. Scale bar in “a” represents 230 µm in “a,” 100 µm in “b,” and 20 µm in “c.”

Fig. 3. Fig. 1. Scale bar in “a” represents 93 µm in “a,” 78 µm in “b,” 18 µm in “c,” 12 µm in “d,” 81 µm in “e,” and 78 µm in “f.”

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RESEARCH ARTICLE

Gonopore sexing technique allows determination of sex ratios and helper composition in eusocial shrimps

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Abstract An evaluation of the social organization and sexual system of eusocial species of *Synalpheus* has been hindered because it has not been possible to determine the sexual composition of colony helpers (workers). The external sexual characters typically used to sex caridean shrimps are lacking in *Synalpheus*. We used SEM sexing technique to determine the sexual composition of helpers in colonies of *Synalpheus regalis*, *S. rathbunae, S. chacei, S. rathbunae A* (see Morrison et al. Mol Phylogen Evol 30:563–568, 2004), and *S. filidigitus*. Colonies consisted of both sexes and sex ratios of helpers generally conformed to 50:50 female to male. Females were characterized by gonopores with U-shaped slits on the coxae of the third pereopods (first walking legs) while males had oval gonopore openings on the coxae of the fifth pereopods (third or last walking legs). In *S. chacei, S. filidigitus*, and *S. rathbunae A*, a few helpers were found that had both male and female gonopores (intersexes). All three reproductive females (queens) of *S. filidigitus* examined were intersexes. Sexing of helpers allowed us to test some hypotheses about sexual differences in helper morphology that might indicate task specialization (division of labor). Male helpers were not different from female ones in body size (except in *S. regalis*: males somewhat larger) and in fighting chela size. The lack of sexual dimorphism in these characters suggests no male–female specialization in colony tasks such as defense. The presence of male and female helpers similar in size suggests that the sexual system of these eusocial species is gonochoristic, although protandry of some sort in *S. filidigitus* can not be ruled out. The intersexuality observed in a few individuals may be due to developmental anomalies, protandry, or even simultaneous hermaphroditism. Finally, the sexing technique allowed us to establish that new colonizers of unoccupied sponges in *S. rathbunae* are a single male and female of helper size.

Introduction

Eusocial animals live in family groups with overlapping generations in which most colony members help to rear the progeny of one or a few queens (e.g. Wilson 1971; Michener 1974; Lacey and Sherman 1991). This reproductive skew is achieved because as a rule most non-reproductive colony members (called workers or helpers) in eusocial colonies do not have functional gonads (e.g. Wilson 1971; Ratnieks 1988). Suppression of gonadal development may occur either by self restraint in which an individual chooses not to develop sexually to avoid fights with others, or through policing by fellow helpers or the queen (e.g. Reeve and Sherman 1991; Faulkes and Bennett 2001; Hart and Ratnieks 2005). Instead of reproducing, helpers in social animal societies specialize in other tasks, such as nest defense, nest construction, and brood care, while reproductives (queens and kings) usually concentrate solely on reproduction. Even though they refrain (at least temporarily)

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from direct reproduction in many species, non-breeders in eusocial societies obtain indirect fitness benefits by helping to rear relatives (Hamilton 1964).

Eusociality has evolved in several Hymenoptera (all ants, some bees and wasps), termites, some thrips, some aphids, one beetle, two species of mole rats and some snapping shrimps (Wilson 1971; Crespi 1992; Kent and Simpson 1992; Stern and Foster 1996; Jarvis and Bennett 1993; Duffy 1996a, b, 2003). In the Hymenoptera, with haplo-diploid sex determination, in which males develop from unfertilized eggs and females from fertilized eggs (Wilson 1971), only females work in the colonies. Males are less related to their siblings than are females, and thus their incentive to help with colony activities is lower (e.g. Queller and Strassmann 1998). In termites and mole rats, however, with diplo-diploid sex determination (Wilson 1971; Alexander et al. 1991), both sexes are part of the work force.

All well-studied eusocial societies are terrestrial. Snapping shrimps of the genus *Synalpheus* (Decapoda: Alpheidae) are the only known group of marine invertebrates with eusocial species. However, the sex of helpers has not previously been determined. Eusocial shrimps, like termites and mole rats, are diplo-diploid animals. Maximum colony sizes vary according to the species. *S. filidigitus* and *S. chacei* have small colonies with up to 120 individuals, while colonies of *S. regalis* and *S. rathbunae* may have more than 300 helpers (Duffy 2003; ET pers. obs). Colonies are headed by a single or a few queens, according to the species (Duffy 2003), and, as Duffy (1996a) showed for two species, queens mate with only one male (king). Colonies consist of animals with continuous range of sizes, and individuals vary in body length from a few mm (juveniles) to about one cm (developed queens). Although eusocial shrimps are small, like all snapping shrimps they are territorial and will defend their sponge from intruders (e.g. Tóth and Duffy 2005). They fight with their formidable weapon, the snapping claw (major or larger chela), which is also used in communication (Nolan and Salmon 1970; Duffy et al. 2002). This chela houses massive muscles that enable the fingers to close quickly and powerfully producing a forward-directed, high-velocity jet of water (Ritzmann 1974; Versluis et al. 2000). In crustaceans both closing force and speed increase with chela size (Taylor 2001; Levinton and Allen 2005), suggesting that the larger the claw, the better the fighting ability of the animal. Because eusocial shrimps are “fortress defenders” (Queller and Strassmann 1998), in which young are independent and food does not need to be collectively gathered nor processed (Duffy 2003), the main colony task among colony members focuses on sponge host defense (fighting). As Duffy (1996a, b) stated, these shrimps qualify as eusocial because larger colony members take indirectly care of weaker, younger ones by defending the sponge from intruders and thus help them to survive until fully grown. To understand better these under-studied societies, knowledge of the sex and sex ratios of helpers, as well as sexual dimorphism in chela size, would be desirable.

Consistent and accurate identification of the sexes, and thus the sexual composition of helpers, is not possible in *Synalpheus* using standard techniques of examination of abdominal appendages with light microscopy. In members of the same family, e.g. *Alpheus* species, males can be distinguished from females by the appendices masculinae on the second pleopods. These appendices have never been observed in *Synalpheus* spp. (Banner and Banner 1975; Felder 1982; Dardeau 1984). Other possible characteristics for sexing *Synalpheus* specimens, such as the shape of abdominal pleurae and the relative position of other pleopodal appendices, have been suggested (e.g., Banner and Banner 1975) but have not proved reliable. Although some species of *Synalpheus* are eusocial, most sponge dwelling species live in heterosexual pairs (Duffy 2003). Mature reproductive females of such pairs are characterized by features such as developing ovaries with eggs (visible through the carapace), the modification of their pleopods and pleural plates to form an open abdominal brood pouch, the presence of embryos carried in that space, and by their larger body size. Identification of males in these pairs has usually been done by default: non-ovigerous individuals of adult size are usually considered males (e.g. Banner and Banner 1975; Dardeau 1984). This assumption may be valid when the shrimps live in pairs but not in eusocial shrimps in which several to many non-breeding individuals live with a few or a single breeding female (queen). Ovarian development, incubated embryos, and modified pleopods are features found in breeding females of *Synalpheus*, and thus an absence of these characters might occur both in males and in non-reproductive females.

In eusocial shrimps, as in the pair-forming species, the reproductive females (queens) based on the above mentioned characters are easily recognized. Other colony members have generally been assumed to be males and subadults (Chace 1972; Banner and Banner 1981a, b; Duffy 1996b, 1998, 2003; Duffy and Macdonald 1999; Duffy et al. 2002; Tóth and Duffy 2005; Didderen et al. 2006), resulting in a 100% male sex ratio among helpers. After eusociality in these shrimp was recognized, the excess number of males was suggested to occur because of a protandric sexual system in which the queen was derived by sex change from one of these males. The sex change would be environmentally (socially) mediated, which has been proposed in some of the approximately 40 species of protandric decapods (Bauer 2000), e.g. the alpheid genus *Athanas* (Suzuki 1970; Nakashima 1987; Gherardi and Calloni 1993) and *Pandalus* spp. (Pandalidae) (Charnov 1982; Bregström 2000), as well as in the protandric simultaneous hermaphroditic genus *Lysmata* (Baeza and Bauer 2004). In protandric species, the population may consist of some mixture of individuals that do not change sex (primary males or females) and protandric sex changers (Bauer 2000). In eusocial *Synalpheus* spp., helpers might thus also be a mixture of males, sex changers and females with undeveloped gonads. Repressed ovarian development, however, is a very common helper feature in most eusocial taxa (e.g. Hart and Ratnieks 2005). Assuming repressed ovarian development but no sex change, helpers in eusocial shrimp colonies could also consist of primary males and females in a 50:50 ratio.

As noted above, identification of males in *Synalpheus* by the external morphological characters normally used for sexing shrimps is not reliable (Felder 1982; Dardeau 1984). The presence or absence of male gonadal characters, such as ejaculatory ducts with sperm (e.g. Bauer 1986), might be used to distinguish male and female individuals. However, because the ejaculatory ducts of *Synalpheus* males are not as large or well developed as those of other male caridean shrimps (see "Results"), routine observation of ejaculatory ducts by dissection is fairly difficult (pers. obs.), especially in the eusocial species, in which helpers are quite small in body size. The position of gonopores on the third (female) or fifth (male) pereopods are a reliable indicator of sex in caridean shrimps and all malacostracan crustaceans (Bauer 2004). Male shrimps might be distinguished from undeveloped females by the presence of gonopores, through which sperms are ejaculated, on the coxae of their fifth pair of legs (pereopods). In female shrimps, eggs are spawned through gonopores located on the third pair of legs. In *Synalpheus* spp., however, male and female gonopores are very difficult to observe with light microscopy using dissecting microscopes (Dardeau 1984), the traditional and most convenient method for examination of external morphology in crustaceans. These gonopores, on the other hand, are readily apparent and observable using the scanning electron microscopy (SEM), as reported herein. Accurate sexing of colony members of known body size would allow evaluation of hypotheses about size, age, and sex-related polyethism ("division of labor") in eusocial colonies of *Synalpheus*, as well as the sexual system (gonochory, sequential or simultaneous hermaphroditism) of these species.

In this study, we tested the null hypothesis that helpers are undifferentiated males (male-phase hermaphrodites and/or primary males) in five eusocial species of *Synalpheus*, as well as the alternative hypothesis that the helper population also includes non-reproductive females. We tested these hypotheses by sexing helpers and queens of measured size using scanning electron observations on the location of gonopores (third pereopods, female; fifth pereopods, male). Moreover, we were able to determine the sex of dispersing individuals colonizing unoccupied sponges and thus to test hypotheses about sexual composition in colony founders. In addition, accurate sexing of individuals also allowed us to compare possible differences in defensive roles between the sexes, as measured by the relative size of cheliped weapons used in agonistic interactions.

Materials and methods

Taxon sampling

We collected in 2005 four eusocial species in Belize close to the Carrie Bow Cay marine station of the Smithsonian Institution (N16°46.46′, W88°04.46′), *Synalpheus "rathbunae* A", *S. filidigitus, S. regalis*, and *S. chacei*, and another, *S. rathbunae*, in Panama at Bocas del Toro marine station (N09°21.10′, W82°15.54′). We collected whole sponges either by SCUBA or snorkeling. Each sponge was put in a very fine-meshed bag and closed immediately. At the surface sponges were broken into small pieces and all animals collected from them. Colonies were preserved in formalin or ethanol. *Synalpheus filidigitus, S. regalis, S. rathbunae and S. "rathbunae* A" are in one clade with a single origin of eusociality, whereas *S. chacei* has a separate, independent origin of eusociality (Morrison et al. 2004).

Initially we sexed (using SEM, see later in this section) 7 and 80 helpers of various sizes (randomly selected) from two colonies, respectively, of *S. rathbunae*, along with six queens (of which four came from different colonies) with results clearly showing separate sexes. Based on these results, we decided to subsample and thus to select, measure, and sex 15 large, 15 mid-sized, and 15 small individuals all coming from the same colony of each species, or more when we needed clarification of the observed sex ratio. Because eusocial colonies have often only one sole queen and because we wanted to analyze three queens per species, we added two extra queens originating from different colonies for *S. rathbunae, S. "rathbunae* A", and *S. regalis*. The *S. filidigitus* colony analyzed had one large and two smaller queens, and we examined those. For the examination of additional queens of the other species originating from different colonies we chose when possible the smaller-sized individuals, reasoning that if there is sex change, the smaller queens might be still showing male gonopores. We thus sampled 45 helpers originating from one colony and three queens from *S. filidigitus, S. rathbunae, and S. "rathbunae* A". From *S. regalis* we sampled 15 large, 25 midsized and 25 small non-reproductive individuals. For *S. chacei*, with a different origin of eusociality, which species is atypical in that there are often helpers with very slight ovarian development, we sampled two colonies. Because there is usually only one, or a few females with fully developed ovaries and embryos in *S. chacei* colonies, we assumed that individuals with slight ovarian development (but otherwise with characters of helpers) either leave their colonies to start new colonies themselves, or remain in their colonies without further gonadal development. In these two *S. chacei* colonies we measured all individuals (13 and 9, respectively) that showed some ovarian development as well as, from each colony, 10 large individuals without any indications of ovarian development, 10 mid-sized and 10 small individuals, plus the single queen from each colony. Additionally, we included another queen from a different colony (Fig. 4b smaller queen).

We also determined the sex of 34 dispersing individuals of *S. rathbunae* that colonized 17 unoccupied experimental sponges (Tóth, submitted). We expelled all shrimp from small pieces of sponges by dipping them into fresh water and relocated them into the sea for 45 days. After this period we collected these experimental sponges by SCUBA, put the sponges individually in a very fine-meshed bag and closed the bags. In the laboratory the sponges were destroyed and all colonizers collected. In some of these colonizers ovarian development was observable, but none of these individuals were developed enough to carry embryos.

Size measurements

Most morphometric data were obtained using a dissecting microscope, digital camera, and the program Image Pro Plus 4.1 for windows (Media Cybernetics L.P. 1999). Magnified images of animals were projected onto the computer screen, allowing us to draw straight lines between two points. For *S. filidigitus* queens, which change their large chela for a small one upon obtaining queen status (Duffy and Macdonald 1999), the largest of the two small chelae was measured. A number of specimens of *S. chacei* (30) and *S. regalis* (20) were measured with a dissecting microscope and an optical micrometer in the laboratory of the second author.

We measured chela length and carapace length for each shrimp. We separated the major (fighting) chela of each individual shrimp and placed the shrimp on its left side for measurements. We recorded the commonly used metric of body size in crustaceans, carapace length, as a straight line from the tip of rostrum to the posterior margin of the carapace. Chela length was measured as a straight line from the tip of the fixed (immovable) finger to the proximal edge of the chela. In individuals that were missing the fighting chela, we measured only the carapace length and plotted chela length as 0 (Fig. 4).

Sexing of individuals

For SEM, specimens of *S. chacei* that were initially preserved in seawater formalin and later changed to 70% ethanol (ETOH) for storage, or 70–95% ETOH and were later taken through the solution series: 95% ETOH to 100% ETOH, then 50:50 ETOH: hexamethyldisilazane (HMDS), and finally 100% HMDS, with at least 1 h in each solution. Other specimens preserved in 95% ETOH were taken through the solution series: 95% ETOH to 100% ETOH, then 50:50 ETOH: HMDS, and finally 100% HMDS, specimens were drained of HMDS and air dried. Several dried specimens (up to 26, depending on size) were attached with adhesive to round stubs of 25 mm in diameter for sputter-coating with 40–80 nm gold prior to observation with a Hitachi S-3000-N scanning electron microscope. The presence or absence of gonopores on the coxae of the third and fifth pereopods was recorded for each specimen. Reproductive females in colonies (queens) were identified as individuals carrying embryos and/or with many eggs clearly visible from dorsal view through the translucent exoskeleton of the cephalothorax and abdomen.

Data analysis

In most individuals, we observed only male or female gonopores, but both types of gonopores were observed in some individuals ("intersexes"). Data from these latter were grouped with that of males or females or treated apart in different tests. We used X^2 to test the null hypotheses of no difference in observed versus expected sex ratios. Expected sex ratios proposed in different species were 50:50 females: males. We used log transformed data and used *t*-tests and ANCOVA to

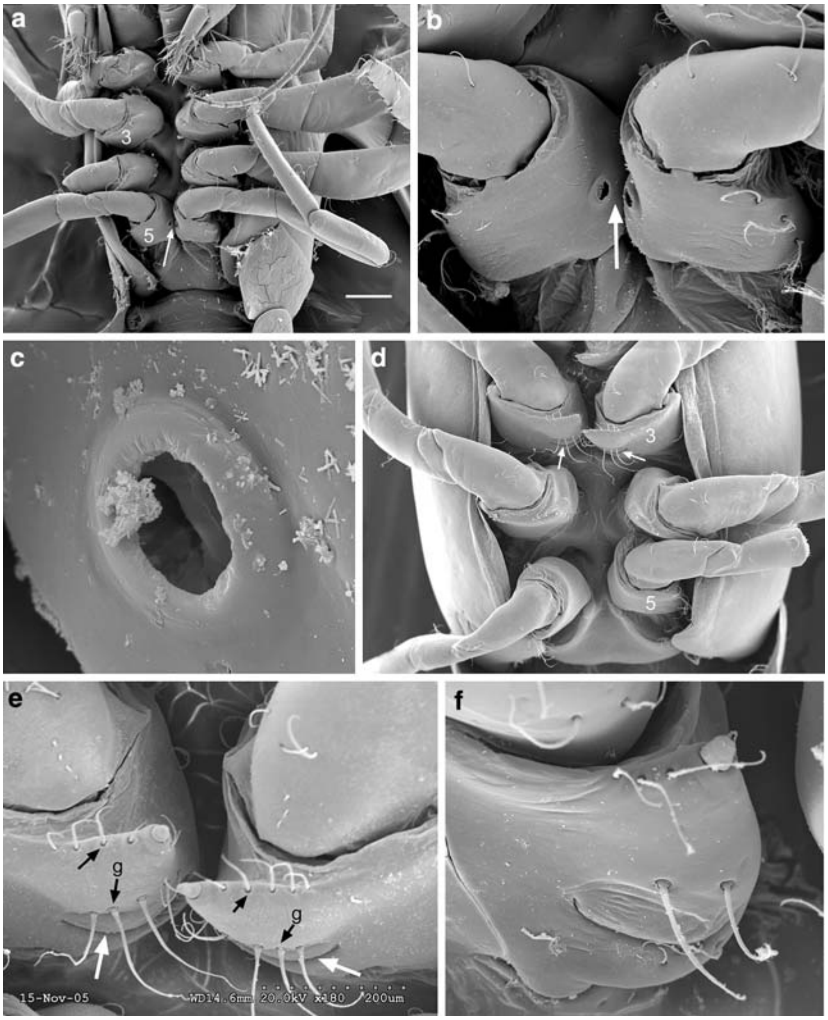

Fig. 1 Male and female gonopores in *Synalpheus* spp. **a** Ventral view of posterior thorax and pereopods of a male *S. r*athbunae helper, showing gonopores (*arrow*) on the coxae of pereopods 5 and their absence from the coxae of the third pereopods; **b** higher magnification of male P5 coxae and gonopores (*arrow*) from (**a**); **c** male gonopore; **d** ventral view of posterior thorax of a female *S. rathbunae A* helper, showing gonopore slits (*arrows*) on P3 coxae and an absence of gonopores on P5 coxae; **e** higher magnification of female gonopores in (**d**) showing gonopore slits (*white arrows*), presumed egg guiding setae (*black arrows* marked "g"), and setae on the distal spur of coxae (*unmarked black arrows*); **f** female gonopore from the P3 coxa of a *S. chacei* female helper. 3, coxa of pereopod 3; 5, coxa of pereopod 5; g, egg-guiding setae

test the null hypothesis of no differences in carapace and chela length between male and female helpers.

Results

Sexing

Helpers and queens of all *Synalpheus* species examined by SEM had gonopores typical of decapod crustaceans in appearance and position. Males were identified by gonopores on the basal segments (coxae) of the fifth pereopods (last pair of walking legs) and females by gonopores on the coxae of the third pereopods (=first pair of walking legs). Well developed male gonopores appear as protuberances with a conspicuous opening on the medial sides of the peropod 5 coxae (Fig. 1a–c). Female gonopores are broadly U-shaped slits on the proximomedial sides of the third pereopod coxae (Fig. 1d–f). Just distal to the female gonopore are several posteriorly directed setae (Fig. 1d–f) unique to both helper and reproductive females. This somewhat fan-shaped row of presumptive egg-guiding setae is not to be confused with other setae, which may be found on the coxae of both males and females, especially a line of setae along a distal medially pointing spur (Fig. 1e, f), which may also include short stout spines. All reproductive females (queens) examined had female gonopores.

Observation of ejaculatory ducts by dissection, although not consistent enough for reliable sexing, did reveal information about these structures (Fig. 2). This structure in *Synalpheus* spp. widens from a narrow juncture with the distal vas deferens into a thin sac that extends into the coxa of the last walking leg (Fig. 2a–c). Sperm cells appear as discoid refractile structures within the ejaculatory duct (Fig. 2c).

Sexual composition of eusocial colonies

In all species, the helper population had a mixed sexual composition. We observed helpers that had

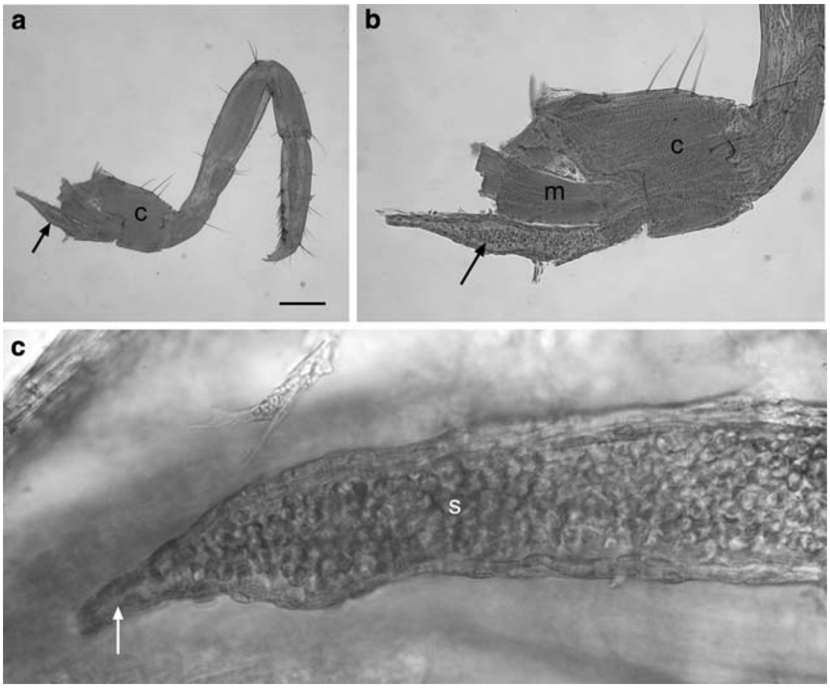

Fig. 2 *Synalpheus* spp. ejaculatory ducts. **a** Entire pereopod 5 of *S. regalis* with ejaculatory duct (*arrow*) attached to limb coxa; **b** higher magnification of (**a**) showing proximal articles of pereopod 5 with ejaculatory duct (*arrow*) attached just inside limb coxa; **c** ejaculatory duct of *S. rathbunae* filled with sperm cells; *arrow* shows broken distal end of vas deferens. *c* coxa, *m* muscle tissue, *s* mass of sperm cells

gonopores either on the fifth pair of pereopods (males) or the third pair of pereopods (females). Additionally, there were a small number of individuals that had both sets of gonopores (termed here "intersexes"). We observed no gonopores in a few of the smallest individuals, which we term "juveniles." We found such individuals of small size in *S. chacei*, (one in colony 1, and two in colony 2), *S. "rathbunae* A" (1), S. *filidigitus* (6) and *S. rathbuanae* (2) without gonopores. In *S. rathbunae* and *S. regalis*, and *S. chacei* colony 1, helpers were males, females, and juveniles, whereas one helper in *S. chacei* colony 2, four in *S." rathbunae* A", and nine individuals in *S. filidigitus* appeared to be intersexes (Fig. 3a, b). The male gonopores of intersexes (Fig. 3c) appeared relatively smaller and rudimentary compared to male gonopores of purely male individuals, or only one of the male gonopores was present while the other was not (Fig. 3a). The reproductive females (queens) in all species had only female gonopores except in *S. filidigitus*, in which all three queens examined, also had male gonopores (Fig. 3d), i.e., were intersexes.

The 34 individuals of *S. rathbunae* colonizing unoccupied sponges consisted of 17 male–female pairs. The females of such pairs had female gonopores and egg-guiding setae like those of other female helpers in this species (Fig. 3e, f).

Sex ratios of helpers were calculated for all species. Exclusion of intersex individuals from the calculations results in a significant deviation from a 50:50 sex ratio in colony 2 in *S. chacei*, resulting in a female biased sex ratio, and in *S. filidigitus*, providing a male biased sex ratio (Table 1).

Table 1 Results of X^2 (1 *df*) for five social *Synalpheus* species testing the null hypothesis of a 50:50 female–male sex ratio in colony helpers (juveniles are excluded from the analysis)

Species	Total	X^2 value	*P*
S. chacei colony 1	39	0.421	0.516
S. chacei colony 2	40	4.333	0.037*
S. filidigitus	30	6.533	0.011*
S. rathbunae A	40	1.600	0.206
S. rathbunae	43	0.023	0.879
S. regalis	65	3.461	0.063

* Represents significant *P* values

Body and fighting-chela size of helpers

Body size (carapace length) did not differ significantly between males and females in *S. chacei* col 2, *S. "rathbunae* A", *S. filidigitus*, and *S. rathbunae* (Fig. 4; Table 2). Among *S. chacei* colony 1, helper females were slightly larger than males, and among *S. regalis* helpers, males were larger than females (Table 2). Intersex individuals were scattered among males and females in *S. "rathbunae* A" and *S. filidigitus* (Fig. 4).

The size of the major (fighting) chela did not differ among the sexual types of helpers (males, females) in any of the species examined (Fig. 4; Tables 3, 4).

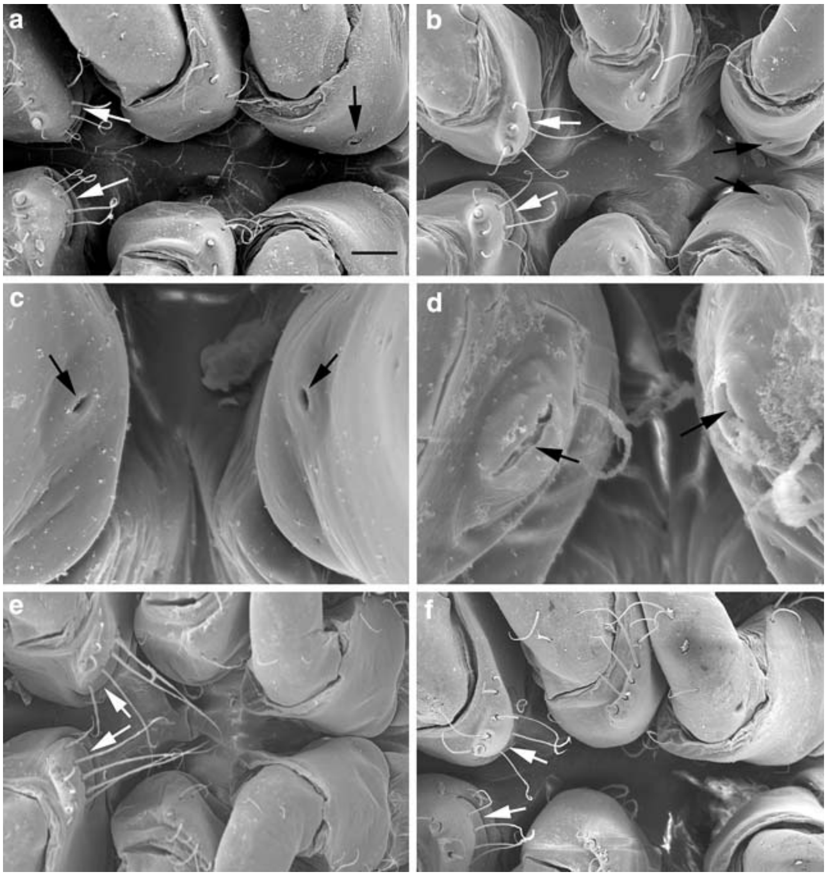

Fig. 3 Gonopores of intersexes and colonizing individuals in *Synalpheus* spp. **a** *S. rathbunae A* helper, **b** *S. filidigitus* helper, **c** rudimentary male gonopores of *S. filidigitus* helper, **d** male pores of *S. filidigitus* queen, **e** *S. rathbunae* colonizing female, **f** *S. rathbunae* helper from established colony, for comparison with (**e**). *Black arrows* indicate male gonopores on pereopod 5 coxae, *white arrows* show female gonopores on pereopod 3 coxae

Discussion

Our results show that SEM observation of gonopores is a reliable method, the only one to date, for sexing many helpers of social *Synalpheus* that can be done relatively quickly, and likely will prove essential for sexing males and non-reproductive females in other *Synalpheus* species. The small size of helpers in eusocial species makes this practical, i.e., several to numerous specimens can be mounted and examined on a single SEM observation stub. In all five species studied in this report, females show a fan-shaped row of setae just below (distal to) the female gonopores. Höglund (1943) hypothesized that in another caridean shrimp, *Palaemon squilla* (Palaemonidae), these "egg-guiding" setae (his "Group I" setae) direct the eggs posteriorly towards the abdomen, under which they will be attached during spawning. Because these setae show up consistently, they might be useful for sexing individuals of *Synalpheus* spp. of larger body size using light microscopy to save money and time or when sex determination of living animals is needed. Presence (female) or absence (male) of these egg-guiding setae, which are present in non-reproductive females observed in this study, could be determined using light microscopic observation of the coxa of the third pereopod (first walking leg) dissected from a specimen. However, for each species, the combination of (1) the presence of these setae above female gonopores on pereopods 3 plus the absence of gonopores on pereopods 5 (=female) and (2) the absence of these setae and pereopod 3 gonopores plus the presence of gonopores on pereopods 5 (=male) should be verified initially on some ovigerous and non-ovigerous specimens using SEM.

The currently accepted assumption that in eusocial shrimps helpers are males or juvenile males is not supported by our study. Our results show that the helper populations mainly consist of males, females, and a few smaller individuals which have not yet developed gonopores (juveniles). In some species, there were individuals with both male and female gonopores (intersexes). Our results generally confirm a 50:50 male: female sex ratio (except for *S. chacei* colony 2 and *S. filidigitus*), suggesting that the basic sexual system for *S. chacei, S. rathbunae* and *S. regalis*, is gonochory (separate sexes) rather than protandric hermaphroditism. Observed deviations may be due to non-random sampling in *S. chacei* (see "Materials and methods") and thus except for *S. filidigitus*, the 50:50 sex ratio among helpers, with males and non-reproductive females of similar size, is not indicative of protandry (Bauer 2004). Moreover, in *S filidigitus*, if intersex

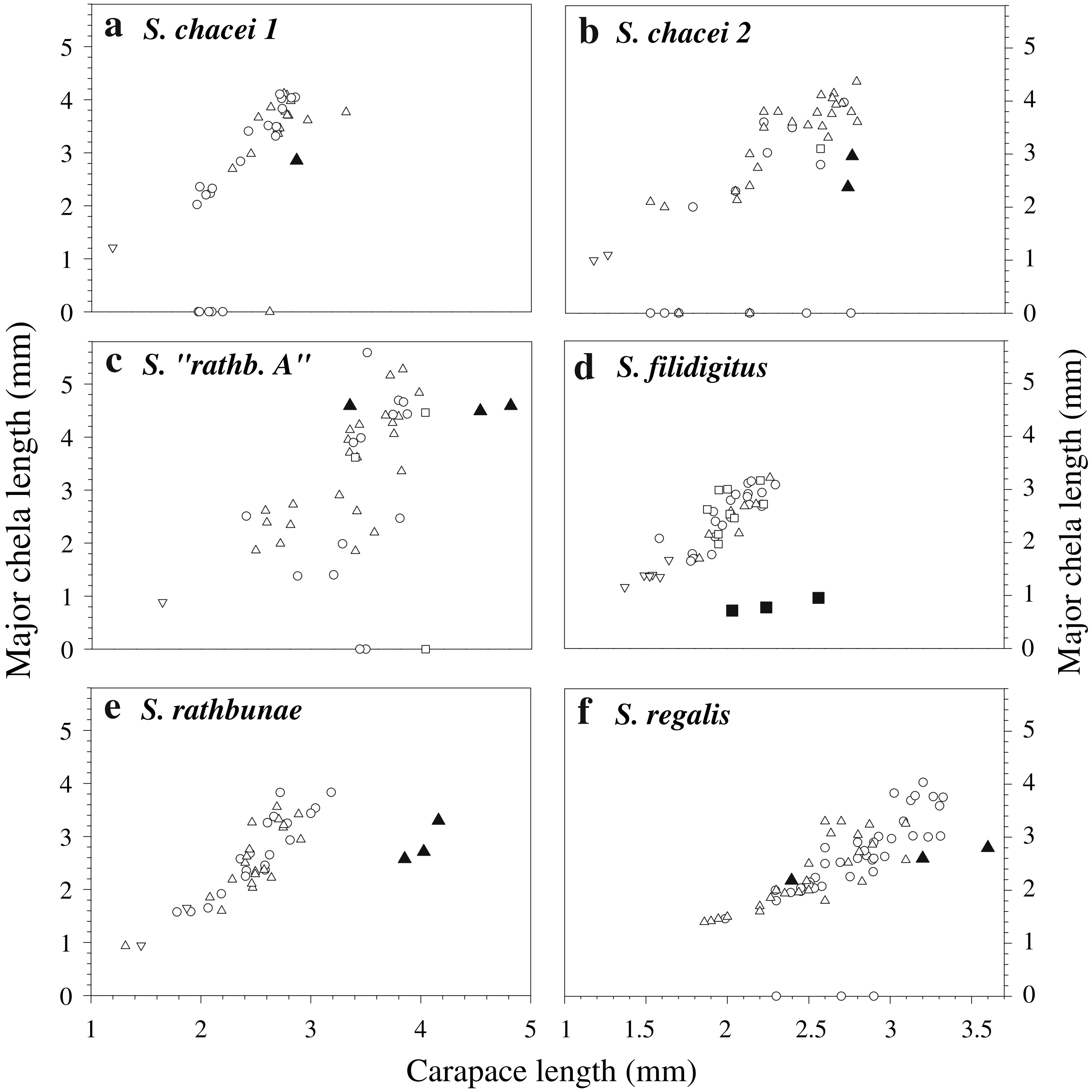

Fig. 4 *Synalpheus* spp. Relationship between carapace length and major chela length in helpers (males *open circles*, females *open triangles*, intersexes *open squares*, and juveniles *upside down triangles*) and queens (female *filled triangles*, intersexes *filled squares*) in **a** *S. chacei* colony 1, **b** *S. chacei* colony 2, **c** *S. rathbunae A*, **d** *S. filidigitus*, **e** *S. rathbunae*, **f** *S. regalis*. Note that the dimensions on the *x*-axis are different for the left and right column

helpers are considered as females along with the queens, there is no deviation from a 50:50 sex ratio ($N = 38$, $X^2 = 0.641$, $P = 0.423$). It seems more likely, given the mixed sexual composition of the helper population, that queens are derived by reproductive maturation of a female helper (except in *S. filidigitus*, see below). Supporting this assumption are also the results from *S. chacei* helpers with slight ovarian development having only female gonopores, as well as from the female dispersers of *S. rathbunae* (future queens) colonizing empty sponges showing only female gonopores.

Lastly, female and male helpers were similar in body size except in *S. regalis*, in which male helpers were slightly but significantly larger than female helpers, and not as one would expect in protandric sex change, in which females are the larger sex. The hypothesis that some individuals may mature sequentially from male to female (protandry), suggested as a possibility in Duffy (2003), is not supported by our study. Instead, a gonochoristic sexual system is indicated for these species in which reproductive males are derived from male helpers and queens from female helpers.

Table 2 Mean carapace length (CL) of male and female helpers and the results of *t*-tests of the null hypothesis: no difference in log carapace length between male and female helpers of 5 social *Synalpheus* species (juveniles and intersexes are excluded from the analysis)

Species	Mean CL females	Mean CL males	*df*	*T*-value	*P*
S. chacei colony 1	2.87	2.46	38	3.781	0.001*
S. chacei colony 2	2.63	2.39	39	1.760	0.085
S. filidigitus	2.1	2.0	29	0.652	0.520
S. rathbunae A	3.34	3.43	39	0.633	0.531
S. rathbunae	2.44	2.56	42	0.720	0.473
S. regalis	2.5	2.8	64	3.483	0.0001*

* Represents significant *P* values

In *S. chacei* colony 2, *S. rathbunae A* and *S. filidigitus* (Fig. 4), we also found intersex individuals having both types of gonopores. Intersexes have been found in a variety of crustacean species, with different causes proposed, both non-functional (developmental anomalies; e.g. Sagi et al. 1997; Ford et al. 2004) and functional (protandric hermaphroditism, Rudolph 1995). In parastacid crayfishes, in which the presence of a double set of gonopores is common, intersexes have been shown by histological studies to be male to female sex changers, i.e. protandric hermaphrodites (Rudolph 1995; Almeida and Buckup 2000). In the caridean genus *Lysmata*, individuals have both pairs of gonopores but the sexual system is protandric simultaneous hermaphroditism, in which individuals mature first in a male phase and later acquire female characters, and thereafter are functional simultaneous hermaphrodites (Bauer 2000).

In our study we found that in intersexes, the male gonopores of these helpers were smaller than those of pure males or, in a few individuals, only one male gonopore was present (in addition to the female ones). In *S. filidigitus* 20% of the helpers had rudimentary male gonopores as well as female gonopores. In this species only, all three queens examined (coming from the same colony), which clearly reproduce as females, were also intersexes. Although helpers do not function sexually, it seems likely that the sexually reproductive males and queens must first pass through a helper (non-reproductive) phase when smaller (younger). In this case, intersexes may have the potential to become a reproductive male or female. It is possible that in some species (*S. filidigitus*) individuals change sex from male to female (Fig. 4) as a sex change hypothesis would predict. However, intersexes in this species were not smaller than females and larger than males, on the contrary, the largest and oldest individual (queen) in the colony was an intersex. It is also difficult to explain why not all animals in the colony with female gonopores possessed also male gonopores. What would be the function of pure female helpers if the queens (breeding females) were intersex? Because our species results are based on individuals coming from single colonies, and because it is not known how many genes or chromosomes are required to determine their sex, or whether factors such as sponge secondary metabolites can affect sexual development, we need to be careful with drawing conclusions. Developmental anomalies occur quite frequently in social shrimps, such as major chela missing the pollex, or animals missing their rostrum etc. (Didderen et al. 2006, pers obs). Such developmental anomalies, possibly including double sets of gonopores (intersexes) might be due to genetic events such as population bottleneck events or inbreeding. Inbreeding could occur in some colonies if the original queen dies and she is replaced by a daughter. In *S. filidigitus* all three queens analyzed came from the same colony and this makes generalizations invalid. Even if the underlying mechanism for intersexuality were known, interpretation of this phenomenon would be difficult without further behavioral observations and experiments. Unfortunately at this stage experimental studies can not be carried out because individuals need to be sacrificed to sex them by gonopore examination with SEM. In summary thus, the intersex characteristics of *S. filidigitus* queens may be due to (1) a developmental

Table 3 Mean chela length (ChL) of male and female helpers, the slopes of the regression lines of log transformed carapace versus chela length, and the results of ANCOVA of the null hypothesis: no difference in log chela length between male and female helpers of 5 social *Synalpheus* species (juveniles and intersexes are excluded from the analysis). Parasitized individuals were excluded from the analysis

Species	Mean ChL females	Mean ChL males	Slope females (R^2)	Slope males (R^2)	SS	*N*	*F*-value	*P*
S. chacei colony 1	3.58	3.10	1.45 (0.71)	1.54 (0.95)	0.000	30	0.225	0.639
S. chacei colony 2	3.47	3.09	1.32 (0.61)	1.27 (0.61)	0.004	34	1.298	0.262
S. filidigitus	2.50	2.50	2.53 (0.83)	2.50 (0.80)	0.050	30	1.012	0.324
S. rathbunae A	3.43	3.45	1.71 (0.53)	1.80 (0.80)	0.003	38	2.065	0.160
S. rathbunae	2.53	2.77	1.90 (0.59)	1.77 (0.87)	0.002	38	0.628	0.434
S. regalis	2.29	2.72	1.68 (0.77)	1.84 (0.84)	0.006	63	1.007	0.320

Table 4 Summary of characteristics observed in the five investigated *Synalpheus* species. Median colony size for *S. chacei* and *S. regalis* come from Duffy (2003); for *S. filidigitus* the modes are given (Duffy and Macdonald 1999). Original colony size indicates the size of the colonies that were sub-sampled to sex their helpers

Species	Range colony size	Median colony size	# queens / species	Ovary development helpers	Original colony size	# Analyzed	Females	Males	Intersex	Juveniles	Sex of queens	Size helpers male = female	Equal sex ratio helpers
S. chacei 1	2–238	23	1 to few	few	69	41	17	21	0	1	1 Female	F > M	–
S. chacei 2	2–238	23	1 to few	few	103	44	26	13	1	2	2 Female	yes	–
S. filidigitus	1–121	10–30	1 to few	no	72	48	8	22	9	6	3 Intersex	yes	no
S. "*rathbunae* A"	3–189	67	1 to several	no	161	48	24	16	4	1	3 Female	yes	yes
S. rathbunae	32–380	138	1 to several	no	222	48	22	21	0	2	3 Female	yes	yes
S. regalis	1–356	135.5	1	no	271	68	25	40	0	0	3 Female	M > F	yes

anomaly, (2) a protandric origin from a male or intersex helper, or (3) simultaneous hermaphroditism. Acceptance or rejection of these or other sexual scenarios is not possible at the moment. Future studies need to involve more colonies to obtain additional data including larger sample sizes, behavioral experiments, genetic methods, and histological studies to help to elucidate the sexual functionality in these animals.

Colony founders, i.e. dispersing individuals colonizing unoccupied sponges, were examined in *S. rathbunae*, the only species in which the sponge hosts survive experimental manipulation. Colonizing individuals occurred in heterosexual pairs, suggesting that dispersing males and females similar in size to helpers give rise to the reproductive males and queens in a new colony (Tóth, submitted). It is thus possible that colony founders originate from male or female helpers that develop into reproductives by release from the inhibition of gonadal development that occurs within their mother colony.

One characteristic of eusocial animals is specialization in tasks among helpers. In eusocial shrimps the most important task is colony defense (Duffy 2003). In social Hymenoptera, thrips, and beetles, mostly females perform defensive tasks, while in termites and mole rats both sexes help equally (Wilson 1971; Lacey and Sherman 1991; Choe and Crespi 1997). We investigated the possibility that helper females and males differ in defensive roles by measuring of the size of the major (fighting) chela, which is used in colony defense in snapping shrimps. However, we found no significant differences among males and female helpers in major chela size, suggesting no such specialization among the sexes.

The reliable method for sexing *Synalpheus* individuals reported herein has allowed us to investigate some important characteristics of the sexual composition and social organization of these eusocial shrimps, and the results can be used as a stepping stone for further investigations. Our results show that the sexual organization of these diploid shrimps resemble that of other diplo-diploid animals societies, such as mole-rats (Mammalia) and termites (Isoptera), in which workers are composed of both males and females and not just a single sex. Most other aspects of social organization and sexual biology of eusocial shrimps, e.g. the developmental origin of colony queens and reproductive males, and the mode of helper gonad repression remain still unknown and open to further study.

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