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Sex determination in the androdioecious barnacle *Scalpellum scalpellum* (Crustacea: Cirripedia)

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How androdioecy (coexistence of hermaphrodites and males) is maintained is still poorly understood. Therefore, sex determination was studied in the androdioecious barnacle *Scalpellum scalpellum* L. First, 247 cypris larvae from seven broods were investigated for sexual dimorphism in larval morphology and found to be all identical. Second, experiments with cyprids showed that males and hermaphrodites differ distinctly in morphology as soon as 4–5 days after settlement. Third, 14 252 cyprids were allowed to settle on the bottom of their culture cages, and all surviving larvae developed into hermaphrodites and none into dwarf males. Fourth, larvae settled in hermaphrodite receptacles (i.e. future males) were removed at increasing intervals after settlement to study if the male and hermaphrodite sexual expressions are fixed or plastic. All larvae became dwarf males if allowed to stay there for more than 8 h after settlement. But if removed within 3 h after settlement, half of them developed into hermaphrodites. We conclude that an environmental sex determination mechanism operates in *S. scalpellum*. Together with a 1:1 hermaphrodite/male ratio observed in previously reported experiments offering a free choice of settlement, we suggest that all larvae are potential hermaphrodites, but only 50% can settle in hermaphrodite receptacles and yield males. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 359–368.

KEYWORDS: androdioecy – cyprid – dwarf male – larval development – settlement – sex ratio – sexual system.

INTRODUCTION

Animals exhibit diverse sexual systems and sex-determining mechanisms. Most species are dioecious (= gonochoristic, i.e. having separate sexes), but about 5% of animals show some degrees of hermaphroditism (combined sexes) (Bachtrog *et al.*, 2014). Furthermore, the coexistence of pure males or females with hermaphrodites within a population is known, and is referred to as androdioecy and gynodioecy, respectively. Among dioecious, androdioecious and gynodioecious species, the individual sex phenotypes may be determined genetically (genetic sex determination, or GSD) or environmentally (ESD). The diversity in the modes of sexual systems and sex-determining mechanisms has fuelled the development of current evolutionary biology both theoretically and empirically (Charnov, 1982; Bull, 1983; West, 2009; Beukeboom & Perrin, 2014).

Barnacles (Crustacea: Cirripedia: Thoracica) offer a great diversity of sexual systems, and thus they have furnished a particularly attracting theatre for testing evolutionary theories on sex (Darwin, 1851; Charnov, 1987; Høeg, 1995; Kelly & Sanford, 2010). Most barnacles are simultaneous hermaphrodites, but androdioecy and dioecy have evolved several times independently within the group (Yusa *et al.*, 2012; Lin *et al.*, 2015). When pure males occur, they are always tiny and live attached to the conspecific hermaphrodites or females (called dwarf males; for terminology see Høeg, 1995; Yusa *et al.*, 2012). Androdioecy attracts particular interest as an evolutionarily intermediate stage between hermaphroditism and dioecy. In addition, theory suggests that androdioecy is a very rare sexual system, because males must attain more than twice the reproductive

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success to persist than the male function of hermaphrodites, other things being equal (Charlesworth, 1984; Pannell, 2002; Weeks, Benvenuto & Reed, 2006). As many known examples of androdioecious animals are barnacles, these are especially attractive for understanding the evolutionary transitions between hermaphroditism and dioecy (Weeks *et al.*, 2006; Weeks, 2012).

Yet, the modes of sex determination in androdioecious barnacles are not well understood. Gomez (1975) suggested that the sex in the balanomorphan barnacle *Conopea galeata* L. is genetically determined, because the sex ratio of newly settled individuals is almost fixed, with the ratio of hermaphrodites to males being 3:1. In contrast, Callan (1941) suggested ESD in the pedunculated barnacle *Scallpellum scalpellum* L. mainly due to the lack of sex chromosomes. But in the same species, Svane (1986) conducted a settlement experiment and found that the proportion of males varies from 0 to 0.5 under various densities of hermaphrodites that can serve as the settlement site for dwarf males. Based on this, he suggested that the individual sex is determined by a unique combination of genetic and environmental factors, where half of the larvae are destined to become hermaphrodites and the remaining half can choose between males and hermaphrodites according to the availability of the settlement sites. No other studies have been conducted on sex determination in androdioecious barnacles. Therefore, Svane's (1986) hypothesis on the unique mixture of genetic and environmental effects on sex determination has not been independently verified. Specifically, he did not test if individual larvae settling as males could also develop into hermaphrodites. Thus, direct evidence for the environmental effects has not been available in androdioecious barnacles.

Scalpellum scalpellum is the best studied barnacle species for sex determination and is the candidate model organism for studying androdioecy (Buhl-Mortensen & Høeg, 2006; Spremberg *et al.*, 2012). We wanted to test four aspects concerning the mode of sex determination in this species. First, sexual dimorphism in terms of larval size and morphology was explored. In the sister taxon to the thoracican barnacles, the parasitic Rhizocephala, most species have GSD coupled with sexual dimorphism in larval size. Male larvae are larger, and at the settlement stage, the cypris, they also differ from female larvae in their antennular sensory apparatus. However, some rhizocephalan species (the akentrogonids) lack such dimorphism and seem to have ESD (Glenner *et al.*, 1989; Høeg & Lützen, 1995; Yamaguchi, Høeg & Iwasa, 2014). Although never studied in detail in thoracicans, we hypothesized that there is no sexual dimorphism if environmental components exist in sex determination (Yamaguchi *et al.*, 2012). Second, the morphologies of newly settled hermaphrodites and males were compared to investigate how early they are morphologically distinguishable from each other. Third, the sex of newly settled individuals was studied in conditions where no hermaphrodites were offered, to test if all the larvae have the ability to become hermaphrodites. The hypothesis here is that all the larvae can settle as hermaphrodites following Svane's (1986) suggestions. Fourth, we transplanted individuals that were newly settled in the receptacles of the adult hermaphrodites (i.e. future males) at various timings from less than 3 h to more than 5 days. We hypothesized that the future males have the ability to develop as hermaphrodites, if they are removed from the receptacles within a short period of time after settlement.

MATERIAL AND METHODS

Collection of adults

Scalpellum scalpellum were collected from the same population used by Svane (1986) at the Kristineberg Marine Laboratory, Sven Lovén Centre, located on the west coast of Sweden. In this area, *S. scalpellum* occurs almost exclusively on hydroids, principally the thecate species *Tubularia indivisa* L. Dredging of animals was as in Spremberg *et al.* (2012).

Culture techniques

Maintenance of adults and rearing of larvae were as in Høeg (1984) and Svane (1986). Adult hermaphrodites were kept in running seawater in circular, 8-cm-diameter, acrylic cages with a nylon net bottom. Larvae of *S. scalpellum* are lecithotrophic and needed no feeding at any time from their release as nauplii to settlement as cyprids. Newly released broods were immediately transferred to a similar sized but clean cage and kept until they were used in experiments. Temperature ranged from 10 to 12 °C and salinity was 33‰. Cypris larvae appeared after 10–12 days, and moulting into this stage from the last nauplius is almost synchronous in the entire brood and lasts 6 h or less.

Mortality was normally low during the naupliar phase. Occasional broods with high naupliar mortality derived mostly from a release of abnormally long duration (several days, Table 1). Cyprids resulting from such broods showed abnormal behaviour by not swimming, but lying on the bottom, beating their thoracopods or bending the antennules without any effective motion.

Scalpellum scalpellum cyprids will not attach to any substratum until at the earliest 2 days after the nauplius–cypris moult (Kaufmann, 1965; Spremberg *et al.*, 2012). For settlement experiments, such

Table 1. Sex differentiation in culture vessels

	Larval broods		Metamorphosed cyprids		
	Parentage	No. of cyprids	%	No.	% hermaphrodites
1	G	266	97.74	260	100
2	GM	282	98.23	277	100
3	G	506	94.86	480	100
4	G	630	99.05	624	100
5	GM	950	91.16	866	100
6	G	485	90.93	441	100
7	G	910	94.4	859	100
8	GM	980	97.55	956	100
9	G	800	96.62	773	100
10	GM	1085	92.81	1007	100
11	GM	240	92.5	222	100
12	GM	1130	99.03	1119	100
13	G	481	94.8	456	100
14	GM	1875	92.27	1730	100
15	G	470	87.87	413	100
16	G	238	87.82	209	100
17	G	281	86.83	244	100
18	G	203	81.28	165	100
19	G	102	79.41	81	100
20	G	222	66.67	148	100
21	GM	128	55.47	71	100
22	G	1100	73.45	808	100
23	SM	391	69.31	271	100
24	SM	368	74.73	275	100
25	G	129	52.71	68	100
Total		14,252	89.97	12,823	100

Cyprids from 25 separate broods of *Scalpelllum scalpellum* were kept incubated and allowed to settle in naked culture trays without any other objects present (see Fig. 3B). Parentage: G, gregarious hermaphrodite without dwarf male; GM, gregarious hermaphrodite with dwarf male; SM, solitary hermaphrodite with dwarf male. All specimens that settled and survived until examined microscopically had developed into hermaphrodites; none developed into males. Broods 20, 21, 23 and 25 suffered high mortality during the naupliar phase. Broods 22 and 24 resulted from abnormally long duration brood release (days instead of normally a few hours). All broods with high mortality (> 20%) during the settlement experiments contained many cyprids that immediately after nauplius–cyprid moult would lie on the bottom without ever swimming or exploring.

2-day-old cyprids were transferred to clean cages to avoid any contamination. The cyprids will readily settle in the laboratory on their natural substrata (Svane, 1986; Spremberg *et al.*, 2012). Cyprids that attach in the receptacle area of adult hermaphrodites (alongside the edges of the scutal plates) develop into dwarf males (Svane, 1986; Spremberg *et al.*, 2012), and those that attach to hydroids or other substrata develop into hermaphrodites. Metamorphosis never occurs in cyprids that have not cemented themselves to a substratum (Kaufmann, 1965; Svane, 1986; Spremberg *et al.*, 2012).

In the field, *S. scalpellum* normally occurs as an epibiont, but in the laboratory cyprids would eventually settle and metamorphose if just left in the empty culture vessels without any other substratum present. Such settlement to naked physical substrata also occurs naturally, as we have, in a separate study, dredged many adult *S. scalpellum* attached to nylon line from lost fishing gear at Roscoff, France.

Cypris morphology

To investigate if *S. scalpellum* has sexually dimorphic cyprids (indicating a GSD component as in several rhizocephalans, Glenner *et al.*, 1989), we sampled cyprids from seven separate broods of larvae. They were fixed in 2.5% glutaraldehyde and their carapace length was measured from photographs taken while mounted on a slide. Subsamples of these (always more than ten per brood) were then prepared for scanning electron microscopy (SEM) as described by Jensen *et al.* (1994) in search of possible sexual dimorphism in the antennules or elsewhere in the body.

Male and hermaphrodite morphology

This experiment explored at what time after settlement male and hermaphrodite type development can be morphologically separated. We used the natural substrata (hydroids or adult hermaphrodites) to yield settled cyprids. Both types of settlement could be achieved within less than 3 h after placing the substrata in the cypris holding cages. After 12–24 h of exposure the hydroids or adult hermaphrodites were removed and flushed with seawater to remove any still unattached cyprids. The settled cyprids were then left in place and incubated for up to 10 days. Each day, randomly selected individuals were removed and fixed in 2% glutaraldehyde. These settlers of known age were mounted on slides in glycerin and examined and photographed in a Leica DM RXA microscope at 10–40× magnification.

Sex differentiation in culture vessels

Two-day-old cyprids (N = 14 252) from 25 broods were pipetted into clean cages and left to themselves. The broods derived from known parentage (solitary or gregarious hermaphrodites; with or without dwarf males). After 7–16 days, which suffices to separate male from hermaphrodite development, all contents of the vessels were fixed in glutaraldehyde and the

specimens were studied microscopically. All larvae that remained loose (not settled) or had died while attached (disintegrating or showing growth of mould) were counted as mortality. All remaining larvae were classified as males or hermaphrodites according to the criteria deriving from the study of male and hermaphrodite morphology (Table 1).

Transplanting of settled larvae from the receptacle

In *S. scalpellum*, cyprids settled in the receptacle of a hermaphrodite were detached by cutting though the anchoring cement and then transferred with no accompanying hermaphrodite tissue to a small 20-mm-diameter Petri dish containing micropore filtered seawater. The settled but detached specimens could be maintained *in vitro* and proved to metamorphose normally for at least another 6–7 days, which sufficed to estimate the type of development they followed. Comparison with settled larvae that remained attached on their natural substratum showed that the transplanted and *in vitro* cultured specimens continued with a completely normal development into either males or hermaphrodites.

Adult hermaphrodites were selected that had no previously settled males (to avoid male specimen confusion). They were offered to cyprids for intervals of 3, 8 and 24 h. Thereafter, the adults were flushed with seawater to remove cyprids that had not settled. Cyprids settled in the receptacles were carefully detached and incubated as described above without any hermaphrodite tissue present. The time these specimens had spent attached in the receptacle could therefore be in the ranges of 0–3, 0–8 and 0–24 h, respectively. Development of the incubated specimens was checked every day. After 4–5 days they were fixed as above, and by microscopy classified to the type of metamorphosis they had followed (see Table 2). Some specimens were allowed to remain settled in the receptacle for at least 4–5 days and thus under constant influence of the hermaphrodite until they were fixed and examined as above.

Table 2. Results from the male settlement and transplant experiment

Time in receptacle before transplant (h)	Type of development: Dwarf males No.	Dwarf males %	Hermaphrodites No.	Hermaphrodites %	Total No.
0–3	8	53.3	7	46.6	15
0–8	5	100	0	0	5
0–24	10	100	0	0	10
24>	64*	100	0	0	64

Cyprids were allowed to settle in the receptacles of adult hermaphrodites and after increasing time intervals removed and incubated *in vitro* (see Fig. 3D). After 4–5 days they were examined microscopically to determine their type of development.

*These larvae were not transplanted, but remained in the receptacle for at least 5 days until examined by microscopy.

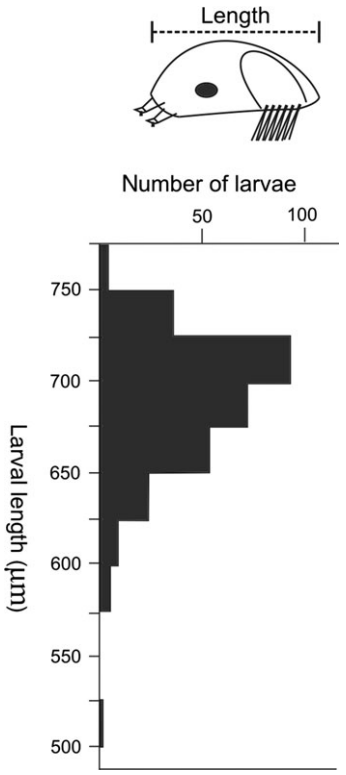

Figure 1. Size distribution in laboratory reared cypris larvae of *Scalpellum scalpellum*. Larval length was measured in 243 cyprids from seven different broods.

RESULTS

Cypris morphology

Carapace length and height were measured in 22–52 cyprids from each of seven broods of larvae ($N = 243$). Although the size distribution (Fig. 1) showed that they were not normally distributed ($P < 0.001$, Shapiro–Wilk test), body length did not indicate any dimorphism in size among the larvae, indicating the lack of two morphologically distinct types of larvae as observed in rhizocephalans (Fig. 1). There were significant differences among broods in length of the larvae ($P < 0.001$,

Kruskal–Wallis test), but bimodality did not appear, even when each brood was separately analysed. SEM investigation also failed to detect any morphological dissimilarity among the larvae.

Male and hermaphrodite morphology

All cyprids could unproblematically be separated into dwarf males and hermaphrodites as early as 4–5 days after settlement (Fig. 2). In external features, the dwarf males have already completed their development 7 days after settlement, and their terminal morphology does not resemble similarly aged hermaphrodites or any other stage in hermaphrodite development. Hermaphrodites develop more slowly and start cirral feeding after *c.* 3 weeks. At 4–5 days: (1) males have a round-ovoid shape, while hermaphrodites have a more elongated shape with a clearly developing peduncle; (2) hermaphrodites have developed the carinal plate, while this plate never develops in males; and (3) the paired terga and scuta are small, and circularly shaped in males, when they first become visible – in hermaphrodites these plates have a distinct angular shape from their first appearance. Further structural details on male and hermaphrodite development will be given elsewhere.

Sex differentiation in culture vessels

Table 1 provides all details on this experiment including: number of broods, parentage, brood size, type of development and mortality during the trials. A total of 14 252 cyprids from 25 separate broods were used in this experiment. Of these, 12 823 cyprids (90.0%) developed into hermaphrodites according to the morphological criteria described above (Fig. 3B). The remaining 1429 cyprids (10.0%) perished during the experiment, either as unsettled cyprids or as settled specimens before their developmental fate could be assessed. There was no difference in sex differentiation between broods with different parentage, although we cannot entirely exclude that larger numbers of broods with a more equal representation of parentage (G, GM and SM see Table 1) could yield a difference.

Within the individual brood mortality (from settlement experiment start to termination) varied from 1 to 47% for all broods, but was generally low (Table 1). High mortality (20–47%) occurred mostly in six broods that had either suffered high mortality during the nauplius phase or resulted from an abnormally prolonged (for days) release period of the nauplii, both being signs of suboptimal health. If these are excluded, only two broods had rather high mortalities (18.1 and 20.1%). There was a significant difference among broods in mortality ($P < 0.001$, likelihood $\chi^2 = 1212.3$, d.f. = 23), but overall mortality was significantly lower than 50%, even if we included broods with high mortalities ($P < 0.001$, likelihood $\chi^2 = 4670.0$, d.f. = 1). This latter result indicates that a 1:1 sex ratio was rejected even if all dead larvae had been males.

Male settlement and transplant

Table 2 shows the result from the transplant experiments, where cyprids settled in the receptacles were

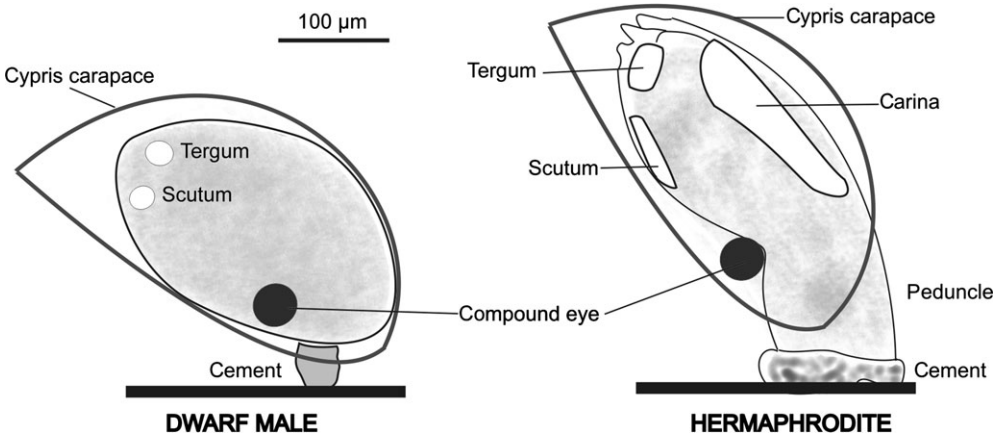

Figure 2. Male and hermaphrodite metamorphosis in the pedunculated barnacle *Scalpellum scalpellum*. Dwarf males and hermaphrodites can be clearly separated just 4–5 days after settlement. Hermaphrodites are distinctly elongated with an incipient peduncle, have angularly shaped scuta and terga and a dorsal carinal plate. Males are ovoid without a peduncle, their scuta–terga are ovoid and they always lack the carina. At this 4–5-day stage, the compound eyes are in the process of being shed from both males and hermaphrodites. The carapace is sometimes shed but may remain for some additional time. Drawn by camera lucida from live specimens 3–5 days after settlement.

removed after increasing time intervals and incubated *in vitro* until their type of development was determined. All specimens that were allowed to stay settled in the receptacle for 8 h or longer developed into dwarf males. This included all those that were transplanted after 8 h (five specimens), after 24 h (ten specimens) and 64 specimens that remained in place for longer than 24 h in the receptacle without transplantation (Fig. 3C). Hermaphrodites appeared only among the 15 specimens that were transplanted within 3 h after settlement in the receptacle and amounted to half (53.3%) of these (Fig. 3D). A logistic regression showed that the difference in sex differentiation between < 3 and > 3 h (even excluding the not transplanted > 27 h class) was significant ($P < 0.001$, likelihood $\chi^2 = 11.87$, d.f. = 1).

DISCUSSION

Larval morphology and sex

We found no evidence for any sexual dimorphism among the larvae of *S. scalpellum*. Within cirripedes, such a dimorphism is well established for the majority of the parasitic barnacles (Rhizocephala) where it is linked to a GSD mechanism (Glenner *et al.*, 1989; Høeg & Lützen, 1995; Walker, 2001). It would be interesting to search for sexual dimorphism in cyprids of other scalpellid barnacles. Many of these are dioecious (Yusa *et al.*, 2012; Lin *et al.*, 2015), and sexual dimorphism could be indicative of their having a GSD (Yamaguchi, Høeg & Iwasa, 2014). However, the lack of dimorphism does not mean the lack of genetic components in sex determination.

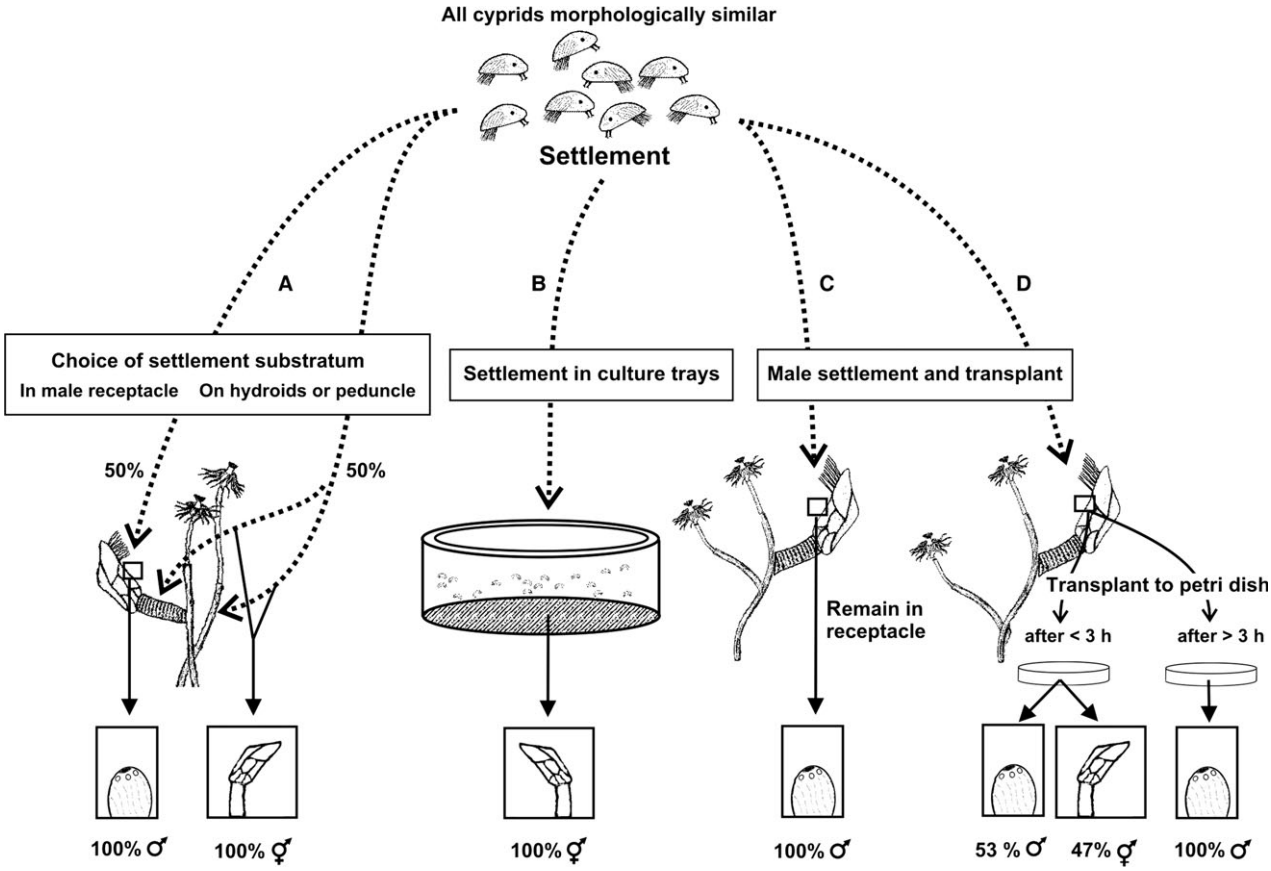

Figure 3. Experiments (A–D) on settlement and sex determination in the pedunculated barnacle *Scalpellum scalpellum*. A, choice experiment, where cyprids were offered settlement as both hermaphrodites and males; this yielded a maximum of 50% cyprids settling as males in the receptacles of adult hermaphrodites, while the remaining larvae settled as hermaphrodites on the hydroids or on the peduncle of their adult conspecifics. B, experiment in which numerous broods of cyprids were kept until they settled in naked culture cages; this resulted in all surviving cyprids developing into hermaphrodites (see Table 1). C, experiment in which cyprids settled in a receptacle and remained there; all these larvae developed into dwarf males. D, experiment in which cyprids were transplanted at increasing time intervals after their settlement into a receptacle and then incubated *in vitro*; all cyprids allowed to stay settled in the receptacle for more than 3 h before transplant became males; hermaphrodites appeared among cyprids removed and incubated prior to 3 h after settlement (see Table 2). Results in A are from Spremberg *et al.* (2012) and in B–D from the present paper.

Settlement choice and sex determination

Our experiment on sex differentiation in culture vessels demonstrated very clearly that all larvae can develop into hermaphrodites irrespective of their parentage (Table 1). We therefore conclude that in *S. scalpellum* all larvae are potential hermaphrodites, and they will always follow this type of development if no receptacles in adult hermaphrodites are available for them to settle as males (Fig. 3B). By contrast, the studies of Svane (1986) and Spremberg *et al.* (2012) both indicate that only a maximum 50% of *S. scalpellum* larvae will settle as dwarf males, while the rest settle as hermaphrodites (Fig. 3A). Thus, while our study merely indicates the presence of the environmental component of sex determination, these results in common support the hypothesis by Svane (1986), where half of the larvae are genetically destined (GSD) to be hermaphrodites and the remaining half can choose between males and hermaphrodites according to the environment (ESD).

The male transplant experiment showed that larvae that attach and remain in hermaphrodite receptacles will invariably develop into dwarf males (Fig. 3C, Table 2). Moreover, transplanting of such receptacle-settled cyprids demonstrated for the first time in barnacles that an irreversible commitment to male development is not present prior to their choosing this attachment site. Many hermaphrodites appeared from transplanted specimens that had been in the receptacle only for 3 h or less, and development into a male required that the settled larva remained in contact with the receptacle for a minimum of 3 h (Fig. 3D, Table 2). This agrees with data from the field, where juvenile *S. scalpellum* hermaphrodites have never been observed in the receptacle area of adult specimens (Spremberg *et al.*, 2012). We therefore conclude that development into a male requires a time-dependent stimulus, probably chemical, from the restricted receptacle area. Cyprids that settle on the peduncle of adult conspecifics, or even externally on their capitulum but away from the receptacle, develop into hermaphrodites (Fig. 3A; Svane, 1986). This further supports that male development requires a stimulus originating specifically from the receptacle. In other words, some environmental factor is needed for a larva to develop as a male (an ESD component).

The reverse experiment, i.e. transplanting cyprids settled on hydroids to the receptacle, was tried a few times. But we did not obtain yield sufficient data for analysis, due to the difficulty of securely fixing the small cyprids (0.7 mm) to the restricted area of receptacle in a hermaphrodite without causing harm to either organism. For the same reasons, we could not have a control treatment, in which the larvae settled on the receptacle were first removed, incubated and later replaced into a receptacle. Therefore, the effect of the transplanting procedure itself (cutting and reattaching the newly settled individuals) could not be evaluated. But the effect is not responsible for the different responses among individuals that had been in the receptacle for different durations, because all the individuals experienced this procedure.

Structural details of the confined receptacle area await close study, but from Spremberg *et al.* (2012) it does not appear to have any striking characteristics in external features. The stimulus to become male is therefore most likely to be chemical in nature. Settlement near or onto conspecifics is common in barnacles and generally held to be due to SipC proteins, formerly called 'arthropodins' (Aldred & Clare, 2009). A localized, high concentration of SipC is therefore one probable candidate for the male determining factor in *S. scalpellum*.

Sex determination in Cirripedia and other animals

The Cirripedia are becoming a choice group for studying sexual evolution, because they comprise hermaphroditic, androdioecious and dioecious species (Kelly & Sanford, 2010; Yusa *et al.*, 2012; Lin *et al.*, 2015). All species of the parasitic barnacles (Rhizocephala) are dioecious and the majority have GSD, but some appear to sport ESD (Yanagimachi, 1961; Høeg & Lützen, 1995; Yamaguchi *et al.*, 2014). The small group of burrowing barnacles (Acrothoracica) are similarly dioecious, but the mechanism of sex determination is unknown (Kühnert, 1934; Turquier, 1972; Larsen, Høeg & Yusa, 2016). The pedunculated and balanomorphan barnacles (Thoracica) comprise hermaphroditic, androdioecious and dioecious species, but only few experiments exist on sex determination. In *Conopea galeata*, a commensal and androdioecious balanomorphan barnacle, Gomez (1975) used an artificial hormone to induce settlement and metamorphosis on cyprids without their natural substratum being present. The male to hermaphrodite ratio obtained was *c.* 1:3 and thus far from 1:1. Although unpublished, he reports that a largely similar ratio was obtained in choice experiments with natural substrata, just as carried out by Spremberg *et al.* (2012) for *S. scalpellum*. This notwithstanding, the details and ecological significance of sex determination in this species remain unclear, and it cannot be completely ruled out that an ESD component is also at play.

It seems that within cirripedes variation in sex determination matches the well-documented variation

in sexual systems. On the one hand, we have a pure GSD system in dioecious, kentrogonid rhizocephalan barnacles and possibly in the androdioecious balanomorph *Conopea galeata*. On the other hand, in several pedunculated and balanomorphan barnacles, the dwarf males are virtually small hermaphrodites that attach to the conspecifics and mature much earlier as males and retard (or in most cases cease) the development of the female sexual organs (Crisp, 1983; Yusa *et al.*, 2010, 2015; Sawada *et al.*, 2015). Here, environmental effects appear to operate in sex determination although a GSD component has not been tested (Yusa *et al.*, 2013). In between, we suggest that both ESD and GSD are active in *S. scalpellum*. Evolutionary addition of GSD or ESD components to purely ESD or GSD systems, respectively, will give rise to the interactive sex determination as suggested in *S. scalpellum*. Such interactive effects of genetic and environments are not rare in sex determination of other animals (fish in Conover & Heins, 1987; oysters in Guo *et al.*, 1998; lizards in Holleley *et al.*, 2015; reviewed by Bull, 1983; Beukeboom & Perrin, 2014). However, known examples of androdioecy in non-cirripede animals have pure GSD systems (nematodes in Hodgkin, 1986; clam shrimps in Sassaman & Weeks, 1993), and hence they are not compatible with the supposedly genetic and environmental effects on sex determination in *S. scalpellum*. The detailed mechanism of sex determination in this species remains unresolved. Further experiments are required, such as studying the proportion of male and hermaphrodite offspring under various densities of hermaphrodites and using broods of known parentage (i.e. mating between hermaphrodites or between males and hermaphrodites). Settlement behaviour should also be observed, because one type of larvae (future hermaphrodites in Svane's hypothesis) might refuse to explore the hermaphrodite receptacle before settlement, whereas the other type (larvae that can choose to become males or hermaphrodites) might explore both hermaphrodites and the normal substrata. Finally, the putatively chemical factor emanating from the receptacle and inducing male development should be explored.

The sex-determining system in *S. scalpellum* may be adaptive. The environmental effects mean that at least some of the larvae can choose their own sex according to the degree of encounters with possible mating partners, which is rather difficult to predict for the parents that release the larvae from distant places. Moreover, the supposed genetic component limits the maximum proportion of males, which agrees with a version of local mate competition theory applied for barnacles, where the male to hermaphrodite ratio varies from 0:1 (in large mating groups) to 1:1 (in very small groups) (Charnov, 1987; Urano *et al.*, 2009; Yamaguchi *et al.*, 2012, 2013). Under a simple ESD system, where cues from hermaphrodites induce larvae to become males, the proportion of males may become higher than being optimal for parents under the local mate competition. Perhaps possessing both ESD and GSD components is adaptive under environments such as variable and unpredictable mating group sizes, although local mate competition theory applies. In fact, mating group size differs greatly in this species. Darwin (1851) commented on variations in the frequency of males in *S. scalpellum* between various geographical areas, and our unpublished investigations indicate that mating group size can vary significantly, both between populations and through time within a population. More details of sex determination should be studied in this species to fully understand the mechanisms that maintain androdioecy, a rare biological phenomenon.

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