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The cypris larvae of the parasitic barnacle *Heterosaccus lunatus* (Crustacea, Cirripedia, Rhizocephala): some laboratory observations

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Abstract

Heterosaccus lunatus parasitizes the portunid crab, *Charybdis callianassa* in Moreton Bay, Australia. With the host crabs maintained at 22.5°C this sacculinid rhizocephalan released larval broods every 6–7 days. During July–August 1996 and particularly August 1999 such broods showed the change-over from male only larvae in the early broods to females only in the later broods. As the host crabs were maintained under similar aquarium conditions in both years it is concluded that the light/dark cycle is the principal cue triggering this larval sex reversal. Oogenesis in the parasite externa is somehow controlled to produce two different sized ova — male larvae develop from large ova and females from small ova. A working hypothesis outlining how sex is probably determined for the larvae of sacculinids is erected. *H. lunatus* is considered the ideal sacculinid for the further experimental work necessary to verify the proposed sex-determining mechanism and its control processes. Measurements of the maximum swimming speeds of *H. lunatus* male and female cyprids showed the larger males to be the faster in absolute terms (27.95 compared with 17.60 mm s^{-1}, respectively), however, the calculated relative speeds were almost identical at ≈ 90 body lengths s^{-1}. Settlement experiments confirmed that female *H. lunatus* cyprids settle only on the gills of *C. callianassa*; these cyprids needed to be at least 2 days old before they were able to settle.

Keywords: Rhizocephalan barnacle; Cyprids; Larval sex determination; Swimming speeds; Settlement

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1. Introduction

Heterosaccus lunatus Phillips is a rhizocephalan barnacle which parasitizes the portunid crab, *Charybdis callianassa* (Herbst) in Moreton Bay, Queensland, Australia (Phillips, 1978). *H. lunatus* is classified as a member of the Sacculinidae (Phillips, 1978) and its life history follows that of the generalized kentrogonid rhizocephalan (see Høeg and Lützen, 1995), involving lecithotrophic nauplii and cyprids of both sexes (Walker, 1999). Female cyprids settle on the gills of the host crab and their subsequent kentrogon stage probably infects the crab by injecting the invasive vermigon stage through a stylet (Glenner et al., 2000). Within the crab this stage develops parasitically to become the interna with proliferating rootlets that take up nutriment from the haemolymph of the host as they grow out into its haemocoelic spaces; in time a virgin externa erupts out onto the crab's ventral abdomen. This virgin externa houses the primordial ovary. A mantle aperture is acquired at the second moult of the externa and male cyprids are attracted to settle in or around this aperture. A trichogon is released from each male cyprid to pass through the aperture into the mantle cavity of the externa, where a maximum of only two implant, one into each of two preformed receptacles, to become the primordial male germ cells (see Høeg, 1987, 1992; Høeg and Lützen, 1995). Each externa, once fecund, must therefore be comprised of two or three individuals — a female and one or two males, the latter parasitic on the former, which is itself endoparasitic within the host crab.

Such a bizarre male/female relationship, termed 'cryptogonochorism' by Bresciani and Lützen (1972) is diandrous (or monogamous if only one male implants) as it exists for the life of the externa, which may extend over several years.

The observations reported here on *H. lunatus* cyprids were made whilst one of us (G.W.) was undertaking study visits to The University of Queensland, Brisbane, Australia in 1996 and 1999.

2. Materials and methods

Parasitized *Charybdis callianassa* were collected and maintained as described previously (Walker and Lester, 1998). A single *H. lunatus* externa on the abdomen of *C. callianassa* was the most common relationship found. The pertinent conditions in the aerated holding aquaria were salinity 31±1‰, temperature 22.5±0.5°C and a 12-h light/dark cycle.

During July and August 1996 and August 1999 each released brood of *H. lunatus* nauplii was attracted to a point-light source and pipetted into 500-ml glass beakers, two-thirds filled with 31‰ 'storage water' (see Walker and Lester, 1998). The openings of the beakers were covered over with cling-film to minimize dust contamination and evaporation and the nauplii allowed to develop uninterrupted to cyprids over 3 days at room temperature (22°C) under ambient light conditions.

2.1. Determination of sex of larvae

A sub-sample of the cyprids was pipetted into a small glass vial (3 ml capacity) to be

relaxed in 0.1% MS-222 (Sandoz) in seawater for 20 min. The carapace lengths of the relaxed cyprids ($n = 50$) were then measured, using a microscope with a calibrated eye-piece graticule; from these carapace lengths the percentage sex figures were determined (see Walker and Lester, 1998). The terminal region of the protruding antennules was also examined under the light microscope to confirm the sex (Walker, 1999).

2.2. Measurement of swimming velocity

On 28th August 1999, two single-sex batches of cyprids, one male and one female, were available for experimentation. The male cyprids were 7 days old and the female cyprids 2 days old and most were active, strongly photopositive swimmers. The batches were in their separate respective culture beakers of internal diameter 9 cm at room temperature (22°C). Using fibre-optic point-light sources as directional stimuli, individual cyprids of each batch were timed using a stopwatch (0.01 s accuracy) swimming the 9 cm distance from one side of the beaker to the other. The two light sources were positioned diametrically opposite each other at the same height up the beakers, so that when one was turned off and the other on the cyprids swam in the horizontal plane towards the light. It is assumed that maximum speeds were being observed.

2.3. Female cyprid settlement

A single brood of *H. lunatus* female cyprids was used to examine settlement onto the host crab, *C. callianassa*, at 22°C. Unparasitized crabs (32–44 mm carapace width, CW) were used as the settlement targets. For all three experiments a single crab was introduced into each of two of the beakers in which the larval brood had developed to female cyprids. Each beaker was then placed inside a black plastic bag, which was sealed, and left for 2 h, the time arbitrarily chosen for cyprid settlement to occur, after which each crab was removed and placed into a container filled with fresh seawater for 30 min. All external surfaces of the crab were then carefully examined under a stereomicroscope for any settled cyprids before the crab was killed and its eight pairs of gills dissected out. Each gill was carefully examined under the microscope; settled cyprids were found only on the gills. When present, the number and position (epibranchial/hypobranchial side) on each gill were noted.

3. Results

3.1. Sex of larvae

Fig. 1 shows the *H. lunatus* larval batch release dates and the proportions of the sexes of the respective cyprids that developed during July–August 1996 and August 1999. Collection of the parasitized crabs took place in 1996 on two occasions — 1st and 18th July, but only on a single occasion in 1999 — 4th August.

In 1996 the first larval brood released was 100% male and there then followed broods

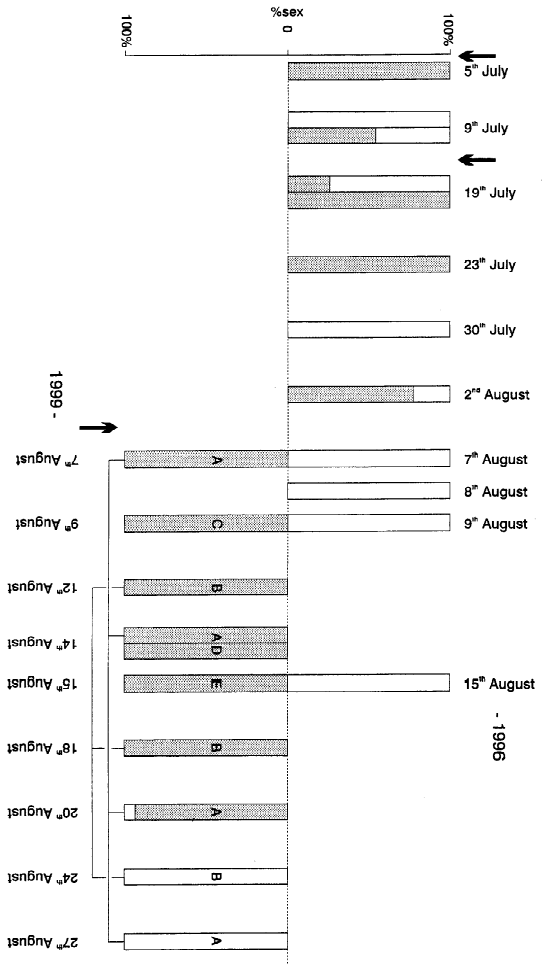

Fig. 1. Release dates of *Heterosaccus lunatus* larval broods in 1996 and 1999. Each brood is presented as a box in which the shaded (male) and unshaded (female) areas represent the percentage proportion of the respective sexes. Individual parasites (A–E) were monitored in 1999 with parasites A and B releasing multiple broods as indicated. The bold arrows show when the host crabs were collected — 1996 (1st and 18th July), 1999 (4th August).

of single and mixed sexes, until during most of August only 100% female broods were released. Because there were two crab collections and the fact that all the parasitized crabs were maintained together it is not possible to know after 18th July whether released broods emanated from parasites collected on the first or second occasion. However, from Fig. 1 it is highly relevant that from 7th–15th August the four released broods were all 100% female.

In 1999 only six parasitized crabs were collected and their parasites were monitored individually in terms of the proportions of the sexes of their released broods. One host crab died without any parasite larval release, but for each of the remaining five at least

one parasite larval brood was released; two of the parasites (A and B) gave multiple broods. Between 7th and 18th August male only broods were released, whilst from 24th to 27th August the released broods were 100% female (see Fig. 1).

Parasite A gave four broods with an interbrood interval of 6–7 days and parasite B gave three broods with a 6-day interbrood interval. Parasite A released 100% males in the first two broods, then a low proportion of females (5%) in the third brood with the fourth brood 100% female. Although some female larvae first appeared 16 days following crab collection, 100% females occurred in the brood released 23 days post-host collection. Parasite B gave 100% males in the first and second broods and 100% female in the third brood. This change-over to femaleness occurred in the brood released 20 days following host crab collection. Parasites C, D and E (Fig. 1) each gave only a single larval brood (100% males) before their respective host crabs died.

It is clear from the 1999 data that males only made up the early broods with a change-over to female and continuation as females in the later broods. The same trend occurred for the 1996 data, but the earlier broods were more sexually mixed. However, femaleness was dominant in broods released during the first half of August 1996, whilst in 1999 maleness was dominant during this same calendar period.

3.2. Cyprid swimming velocity

The calculated maximum swimming velocities for male and female *H. lunatus* cyprids are given in Table 1; the smaller female cyprids were significantly slower than the larger male cyprids by a factor of 0.63. The swimming velocities expressed in relative terms of body lengths s^{-1}, however, are remarkably similar (Table 1).

3.3. Female cyprid settlement

Female cyprids, <24 h old, were used in the first settlement experiment. The two target crabs, both males (40 mm CW; 37 mm CW) had no settled cyprids on them after the exposure period.

In the second experiment using the same batch of cyprids, but now 2 days old, three settled cyprids were found in the right branchial chamber of a crab (34 mm CW), two on gill 2 and one on gill 6. No cyprids settled on the gills in the left branchial chamber. The second target crab (32 mm CW) had only two settled cyprids, both on gill 2 from the right branchial chamber. All five settled cyprids in this experiment were found on the hypobranchial (inside) side of the gills.

Table 1
Comparison of the swimming velocities of male and female *Heterosaccus lunatus* cyprids

	Carapace length (μm) (mean±S.D.)	Mean time (s) to swim the 9 cm	Mean maximum swimming velocity (mm s^{-1})	Corresponding relative swimming velocity (body lengths s^{-1})
Male cyprids	305.25±4.87 ($n=50$)	3.22±0.32 ($n=10$)	27.95	91.56
Female cyprids	201.98±4.46 ($n=50$)	5.11±0.49 ($n=10$)	17.60	87.14

Table 2
Settlement experiment 3: number and position of settled female *Heterosaccus lunatus* cyprids on each gill (1–8) of the two target *Charybdis callianassa* (A,B) after the 2 h exposure[a]

Gill	Crab A (44 mm CW, female)		Crab B (40 mm CW, berried female)	
	RBC	LBC	RBC	LBC
1	0	0	0	0
2	0	0	2 hypo.	0
3	1 epi.	0	3 hypo.	3 hypo.
4	0	0	8 − 1 epi., 7 hypo.	3 hypo.
5	1 hypo.	0	4 hypo.	0
6	0	0	3 hypo.	3 hypo.
7	0	0	9 hypo.	6 hypo.
8	1 hypo.	2 hypo.	8 hypo.	5 hypo.
No. cyprids	3	2	37	20

[a] CW, carapace width; epi., epibranchial side of gill; hypo., hypobranchial side of gill; LBC, left branchial chamber; RBC, right branchial chamber.

For the third settlement experiment with the same cyprid batch, but now 4 days old, increased settlement occurred. The numbers of settled cyprids on each of the gills of the two target crabs are given in Table 2. Crab A had a total of five settled cyprids, whereas crab B had 57 settled cyprids, highlighting the variability in cyprid settlement between host crabs. In this experiment all but two of the settled cyprids were found on the hypobranchial side of the gills.

It was noted that at the start of all three settlement experiments the *H. lunatus* cyprids were actively swimming in their culture beakers, but at the end of the 2-h exposure period with a crab they had become noticeably inactive, and indeed, most were moribund as they did not recover when placed in fresh seawater.

4. Discussion

For those sacculinids so far studied in both northern and southern hemispheres, males dominate larval broods in winter and females dominate in summer, respectively. In comparing the *H. lunatus* larval broods during the Australian winter periods in 1996 and 1999, with a lag of about a month between the years when released larval broods were first monitored, it is unequivocal that femaleness had total dominance during the first half of August 1996, but during the same period in 1999 maleness dominated (Table 1). In both years the initial broods were males, then broods were female and remained female with time. Maintenance conditions of the parasitized crabs in the aquarium room were essentially the same in both years, but because of the lag time between years when the crabs were collected it is now apparent that either temperature or the light/dark cycle (or both) is the likely environmental trigger helping to determine larval sex in *H. lunatus* broods. In mid-winter (June 21st) in Brisbane natural daylength lasts 10 h 24 min and seawater temperature in Moreton Bay is 17–18°C, whereas in mid-summer (December 22nd) daylength lasts 13 h 52 min and seawater temperature is 27–28°C (temperatures

taken from Shields and Wood, 1993). Although the aquarium lights were on a timer (lights on 06:00–18:00 h) there were times after 18:00 h when the aquarium room lights were switched on by researchers at a wall switch. Such 'out of hours' lighting effectively extends the light period on those days, even beyond the natural maximum summer daylight time. The constant 22.5°C in the aquarium room is roughly mid-way between the natural summer and winter seawater temperatures. Walker (1987) using continuous lighting and constant temperatures was unable to alter the natural northern hemisphere seasonal sex reversal for the larval broods of *Sacculina carcini*. He concluded that light could be ignored as an environmental factor influencing larval sex, which in view of the present findings for *Heterosaccus lunatus* may be an unfortunate and inappropriate conclusion. Walker (1987) was also convinced that temperature was not involved, the evidence being that at a constant 18°C (summer seawater temperature) the change-over to males (the winter sex) still occurred. By deduction, therefore, it is now proposed that the varying seasonal photoperiod is the sole environmental trigger modulating larval sex in *H. lunatus*.

Oogenesis is the process which actually determines egg size and hence larval sex in sacculinids — large eggs develop into male larvae and smaller eggs into female larvae; these eggs may also have a different chromosome complement (see Yanagimachi, 1961). How oogenesis, essentially a cellular process, is switched to produce different sized ova must remain mere conjecture at present. Sacculinid externae do not have a recognizable sense organ for light reception, although there is a well developed single nerve ganglion with radiating nerves (see Delage, 1884). If light can somehow be perceived in the externa then there would be no need to invoke any intermediary involvement of the host crab in the control of oogenesis in the parasite. *Heterosaccus lunatus*, with its short interbrood times, would seem to be the ideal parasite for the critical experimentation now needed to gain the precise understanding of how rhizocephalan larval sex is determined.

It is perhaps pertinent to integrate the facts and suppositions into a working hypothesis — the sex-determining mechanism is a function of the production of different sized eggs (maternal oogenesis/chromosomes?) which is likely to be modulated by hormone(s) generated within externa tissues or by the host crab. Production of the hormone concerned will be environmentally controlled by the varying seasonal photoperiod (see also Yanagimachi, 1990).

The similar relative speeds of both male and female *H. lunatus* cyprids (≈ 90 body lengths s^{-1}) can be compared with that of *Semibalanus balanoides* cyprids at 95 body length s^{-1}, as measured by Yule (1982, unpublished PhD thesis). Although the cyprids of this thoracican barnacle are much larger, being 1 mm carapace length, the surprising parity of the speeds probably reflects the highly efficient hydrodynamic body shape, coupled with relative thoracic limb beat rate and combined limb thrust of this instantly recognizable larval stage conserved throughout the Cirripedia. These cyprid swimming velocities are exceptionally high when compared with other non-ciliated marine invertebrate larvae (Chia et al., 1984).

Only female *H. lunatus* cyprid settlement was examined in the present study with the eight pairs of host crab gills being the settlement targets for these larvae. These cyprids need time to attain competence to settle, which is clear from the results of the settlement

experiments. It is now generally acknowledged for rhizocephalans that 2 days is the minimum time, as cyprids, before they are competent to settle (see Høeg and Ritchie, 1987).

Settlement of female *Sacculina carcini* cyprids occurs on external surfaces of the host crab with the actual fixation taking place at the base of a seta in different regions (Delage, 1884; see Walker, 1985). This sacculinid may yet prove to be the exception, as host gills are the settlement site for female cyprids of other sacculinids (Glenner and Høeg, 1995; Walker, 1999) and other kentrogonids (Høeg, 1985; Glenner and Høeg, 1994). Crab gills are vulnerable not only because of their thin cuticle, easily pierced by ketrogon stylets, but also because female cyprids can be taken passively into branchial chambers in the respiratory current after being attracted to the vicinity of the crab. Once in the confined space of a branchial chamber, the cyprids can explore the gills in earnest, needing only to avoid the wiping action of the maxilliped mastigobranchs (Bauer, 1989). *H. lunatus* female cyprids fit easily into the spaces between gill lamellae, so by moving quickly into these spaces mastigobranch action can be avoided (see Walker, 1999). Although the gill lamellae of *Charybdis callianassa* have distinct morphological protrusions (knobs), including setae (Walker, 1999), such protrusions are not selected by settling *H. lunatus* female cyprids (Walker, unpublished data).

Water in the normal respiratory current through a crab's branchial chamber is first drawn in at the entry points at the bases of the chelae (Milne-Edwards openings) and pereopods. Once inside the chamber the water impinges first onto the hypobranchial side of the gills, the side settled on most in the settlement experiments. Water then passes between the lamellae and into the epibranchial space to exit past the baling appendage, the scaphognathite. In the holding aquaria, as probably in nature, *C. callianassa* buries itself into sand. When buried, the crab's respiratory current must reverse to minimize sediment entry into the branchial chambers. Even for unburied crabs there are periods of current reversal allowing water flow to the hind region of the branchial chamber and at the same time helping to keep the gills clean. When the current is reversed, settlement of *H. lunatus* female cyprids will be favoured on the epibranchial side of the gills.

The short 2-h interaction time of female *H. lunatus* cyprids with a single *C. callianassa* in the confined seawater volume was still sufficient to cause a major change to the cyprids from being highly active at the start to moribund at the end. Although the larvae had already developed in the same volume of seawater for at least 5 days before the introduction of the crab, it is the increasing levels of nitrogenous excretion (NH_3) and/or reduced oxygen tension in the water, both created by the crab, that are the likely cause of such a dramatic and irreversible effect on the larvae. Alternatives to this 'closed' condition should be considered in any future laboratory experimentation on rhizocephalan female cyprid settlement, particularly if protracted larval–host interaction periods are deemed necessary.

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References

Bauer, R.T., 1989. Decapod crustacean grooming: functional morphology, adaptive value and phylogenetic significance. In: Felgenhauer, B.E., Watling, L., Thistle, A.B. (Eds.), Functional Morphology of Feeding and Grooming in Crustacea. Crustacean Issues, Vol. 6. A.A. Balkema, Rotterdam, pp. 47–73.

Bresciani, J., Lützen, J., 1972. The sexuality of *Aphanodomus* (parasitic copepod) and the phenomenon of cryptogonochorism. Vidensk. Medd. dan naturhist. Foren. 135, 7–20.

Chia, F.-S., Buckland-Nicks, J., Young, C.M., 1984. Locomotion of marine invertebrate larvae: a review. Can. J. Zool. 62, 1205–1222.

Delage, Y., 1884. Évolution de la sacculiné (*Sacculina carcini* Thomps.) crustacé endoparasité de l'ordre nouveau des kentrogonides. Arch. Zool. Exp. Gén. 2 (Ser. 2), 417–736.

Glenner, H., Høeg, J.T., 1994. Metamorphosis in the Cirripedia Rhizocephala and the homology of the kentrogon and trichogon. Zool. Scripta 23, 161–173.

Glenner, H., Høeg, J.T., 1995. A new motile, multicellular stage involved in host invasion by parasitic barnacles (Rhizocephala). Nature 377, 147–150.

Glenner, H., Høeg, J.T., O'Brien, J.J., Sherman, T.D., 2000. Invasive vermigon stage in the parasitic barnacles *Loxothylacus texanus* and *L. panopaei* (Sacculinidae): closing of the rhizocephalan life-cycle. Mar. Biol. 136, 249–257.

Høeg, J.T., 1985. Cypris settlement, kentrogon formation and host invasion in the parasitic barnacle *Lernaeodiscus porcellanae* Müller (Crustacea: Cirripedia: Rhizocephala). Acta Zool. (Stockholm) 66, 1–45.

Høeg, J.T., 1987. Male cypris metamorphosis and a new male larval form, the trichogon, in the parasitic barnacle *Sacculina carcini* (Crustacea: Cirripedia: Rhizocephala). Philos. Trans. R. Soc. Lond. Ser. B 317, 47–63.

Høeg, J.T., 1992. Rhizocephala. In: Harrison, F.W., Humes, A.G. (Eds.). Microscopic Anatomy of Invertebrates, Vol. IX. Wiley-Liss, New York, pp. 313–345.

Høeg, J.T., Lützen, J., 1995. Life cycle and reproduction in the Cirripedia Rhizocephala. Oceanogr. Mar. Biol. Annu. Rev. 33, 427–485.

Høeg, J.T., Ritchie, L.E., 1987. Correlation between cypris age, settlement rate and anatomical development in *Lernaeodiscus porcellanae* (Cirripedia: Rhizocephala). J. Mar. Biol. Assoc. UK 67, 65–75.

Phillips, W.J., 1978. Some parasitic barnacles (Rhizocephala: Sacculinidae) from portunid crabs in Moreton Bay, Queensland. Mem. Queensl. Mus. 18, 255–263.

Shields, J.D., Wood, E.I., 1993. Impact of parasites on the reproduction and fecundity of the blue sand crab *Portunus pelagicus* from Moreton Bay, Australia. Mar. Ecol. Prog. Ser. 92, 159–170.

Walker, G., 1985. The cypris larvae of *Sacculina carcini* Thompson (Crustacea: Cirripedia: Rhizocephala). J. Exp. Mar. Biol. Ecol. 93, 131–145.

Walker, G., 1987. Further studies concerning the sex ratio of the larvae of the parasitic barnacle, *Sacculina carcini* Thompson. J. Exp. Mar. Biol. Ecol. 106, 151–163.

Walker, G., 1999. The cypris larvae of the rhizocephalan barnacle *Heterosaccus lunatus* with particular reference to antennular morphology. Acta Zool. (Stockh.) 80, 209–217.

Walker, G., Lester, R.J.G., 1998. The effect of salinity on the development of *Heterosaccus lunatus* larvae (Crustacea: Cirripedia: Rhizocephala). J. Crust. Biol. 18, 650–655.

Yanagimachi, R., 1961. Studies on the sexual organisation of the Rhizocephala. III. The mode of sex determination of *Peltogasterella*. Biol. Bull. 120, 272–283.

Yanagimachi, R., 1990. Fertilization and sex determination in the Rhizocephala (Cirripedia, Crustacea). In: Dale, B. (Ed.), Mechanism of Fertilization. NATO ASI Series, Vol. H45. Springer, Berlin, pp. 699–708.

Yule, A.B., 1982. The application of new techniques to the study of planktonic organisms. PhD thesis, University of Wales, Bangor.