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Experimental infections, using a fluorescent marker, of two elasmobranch species by unciliated larvae of *Branchotenthes octohamatus* (Monogenea: Hexabothriidae): invasion route, host specificity and post-larval development

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SUMMARY

The infection biology of *Branchotenthes octohamatus* (Monogenea: Hexabothriidae) from the gills of the southern fiddler ray, *Trygonorrhina fasciata* (Rhinobatidae), was studied using the fluorescent dye, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE). This is the first use of this technique on a monogenean species with unciliated larvae and the first for any monogenean larva infecting an elasmobranch host. CFSE-labelled post-larvae were recovered from gills of *T. fasciata* within 30 min of exposure to the host, providing strong evidence that larvae invade host gills directly and do not migrate after initial attachment elsewhere. The rapidity with which larvae settled suggests that the mode of infection may deliver larvae directly to the gills via the host's inhalant respiratory current. The specificity of *B. octohamatus* was investigated by exposing a sympatric rhinobatid host species, the western shovelnose ray, *Aptychotrema vincentiana*, to *B. octohamatus* larvae newly emerged from eggs laid by adult parasites from gills of *T. fasciata*. Experimental exposure of *A. vincentiana* to freshly hatched *B. octohamatus* larvae resulted in a persistent infection, indicating that *B. octohamatus* may not be strictly host specific. Post-larval development charted on these experimentally infected *A. vincentiana* specimens was slow. Parasites appeared to be sexually mature at 91 days at 21–25 °C. *Branchotenthes octohamatus* larvae bear only 4 pairs of hooklets on the haptor whereas all other hexabothriid larvae described so far have 5 hooklet pairs. Ontogenetic changes to the haptor revealed that it is probably hooklet pair III that is lost from *B. octohamatus* prior to larval development.

Key words: infection, larvae, development, CFSE, *Branchotenthes octohamatus*, Hexabothriidae, Monogenea, Rhinobatidae, specificity.

INTRODUCTION

Of the parasitic platyhelminths, monogeneans are widely regarded to be highly host specific (Whittington *et al.* 2000). However, obtaining a measure of the true host range of a parasite can be difficult, potentially leading to inaccurate or biased specificity estimations (Brooks and McLennan, 1993). While the evolutionary mechanisms that underlie specialization leading to specificity are not understood (Desdevises *et al.* 2002), host specificity is reinforced over time by repeated successful transmission of infective larvae to the definitive host(s). For site specificity, transmission mode may influence its maintenance for some monogenean species, depending on whether infection is active or passive. For example, ciliated larvae that can actively search for a host may settle preferentially in/at the definitive site (e.g. *Diplozoon paradoxum*: see Bovet, 1967; *Discocotyle sagittata*: see Paling, 1969; *Neoheterocotyle rhinobatidis* and *Merizocotyle icopae*: see Chisholm and Whittington, 2003). Alternatively, larvae may settle opportunistically, then migrate from the initial point of attachment to the definitive site (e.g. *Urocleidus adspectus*: see Cone and Burt, 1981; *Entobdella soleae*: see Kearn, 1984; *Benedenia lutjani*: see Whittington and Ernst, 2002), or a combined strategy may be adopted (e.g. *Heterobothrium okamotoi*: see Chigasaki *et al.* 2000). In contrast, for unciliated larvae that cannot swim, transmission is passive. These larvae can only settle opportunistically when a host presents itself. In this case, larvae may migrate to the definitive site after attachment elsewhere on the host (as for ciliated larvae above), or, importantly, the mode of transmission may deliver larvae to the definitive site directly. No study, however, has investigated

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settlement and post-infection dynamics of mono-genean species with unciliated, non-swimming larvae.

The southern fiddler ray, *Trygonorrhina fasciata* (Rhinobatidae), is type-host to the gill-dwelling monogenean *Branchotenthes octohamatus* (Hexabothriidae). Eggs of this parasite species only hatch when mechanically agitated, releasing unciliated larvae (Glennon *et al.* 2005, 2006). This provides an excellent, tractable model system to explore (1) how non-swimming larvae invade the gills of their host, (2) how quickly this is achieved and (3) the development of the parasite after settlement on the host. For the first time, the fluorescent dye, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE), is used on unciliated monogenean larvae from an elasmobranch host to visualize these otherwise near-invisible larvae on host tissue. It is also the first use of CFSE on unciliated mono-genean larvae infecting any fish. Additionally, we explore host specificity for this monogenean species by exposing a sympatric rhinobatid species, the western shovelnose ray, *Aptychotrema vincentiana*, to *B. octohamatus* larvae freshly hatched from eggs laid by adult parasites collected from *T. fasciata*.

Branchotenthes octohamatus larvae are unique bearing only 4 pairs of hooklets on the haptor, whereas all other hexabothriid larvae described so far have 5 hooklet pairs (Glennon *et al.* 2005). The number and arrangement of haptoral hooklets is an important character in monogenean systematics and ontogenetic changes to the haptor have potential to offer added insight into hexabothriid relationships. Therefore, post-larval development of this unusual monogenean species is charted on experimentally infected *A. vincentiana*.

MATERIALS AND METHODS

Host collection and maintenance

Three *T. fasciata* (2 at 30 cm total length (TL), 1 at 50 cm TL) and 2 *A. vincentiana* (30 and 85 cm TL) were caught by hand in shallow water at Kingston Point, Seacliff (35°1′59″S, 138°31′29″E), near Adelaide, South Australia in January 2006. Rays were transported alive to The University of Adelaide (UA) and transferred to a 2000 l aquarium containing recirculating, aerated seawater. Lighting in the aquarium facility was regulated by a time switch to simulate a natural day/night cycle (light on at 06·00 h, light off at 18·00 h). Aquarium room ambient temperature ranged from 25 °C (January) to 21 °C (April) during the experiments. Rays were fed daily on chopped pilchard and/or prawn.

Three weeks after capture 1 female *A. vincentiana* (85 cm TL) gave birth to 16 young (∼10 cm TL) which were transferred within 24 h of birth to a separate 1000 l tank containing recirculating, aerated

seawater. Because these rays were born in a tank containing infected adult rays, the anthelminthic Praziquantel was used to rid the pups of any mono-genean parasites they may have acquired, prior to the commencement of experimental work. Two 40 h, 5 mgl^{-1} Praziquantel treatments were administered 48 h apart, according to the protocol of Chisholm and Whittington (2002).

Parasite egg collection and incubation

Branchotenthes octohamatus eggs were collected by isolating an infected *T. fasciata* specimen from the 2000 l aquarium for up to 12 h in a 60 l tank containing ∼40 l of fresh seawater aerated by an air stone. Following isolation, the ray was returned to the 2000 l aquarium. The water from the 60 l tank was filtered through a 63 μm Nitex mesh sieve and the residue examined for eggs laid by *B. octohamatus in vivo*.

Using fine needles, eggs (laid by the parasite with the appendages of adjacent eggs fused to form a chain) were transferred to small Perspex wells (internal diameter 9 mm; volume approximately 1 ml) containing fresh filtered seawater (FSW), filtered through Whatman qualitative paper. Each Perspex dish was gently immersed, using forceps, into a larger glass crystallizing dish (40 mm diameter × 30 mm deep; volume approximately 30 ml) filled with FSW and covered with a glass plate.

Eggs were incubated in a controlled temperature cabinet at 24 °C under LD12:12 (i.e. light on at 06·00 h, light off at 18·00 h) achieved by a pro-grammed timer connected to an 18W Grow-lux tube fitted to the cabinet ceiling. Eggs of *B. octohamatus* are fully embryonated after 8–10 days at 22 °C and hatching is easily promoted when eggs are mech-anically agitated (Glennon *et al.* 2006). The FSW in each dish was replaced daily for the first 5 days of incubation, after which, eggs were left undisturbed so that hatching would not be induced until larvae were required for experiments.

Labelling larvae with CFSE

A 10 mM stock solution of 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Sigma-Aldrich, Castle Hill, NSW, Australia), was prepared in 100% dimethyl sulphoxide (DMSO) and stored at 4 °C (Bronner-Fraser, 1985). The stock solution was diluted with FSW to produce a 10 μM working solution of CFSE for labelling (Yokoyama and Urawa, 1997; Chigasaki *et al.* 2000). Just prior to labelling, a fine pipette was used to extract the FSW from the Perspex wells containing freshly hatched *B. octoha-matus* larvae, so that only a film of water covered the bottom of the well. Larvae were then submerged immediately in the CFSE working solution and left

for 15 min at room temperature (∼20 °C). After labelling, the CFSE working solution was removed and the wells refilled with FSW.

Exposure of T. fasciata *to labelled larvae*

Two *T. fasciata* specimens (each ∼30 cm TL) were experimentally infected with freshly hatched CFSE-labelled larvae on separate days: 'Ray 1' was exposed to 200 larvae; 'Ray 2' was exposed to 60 larvae. On each occasion, rays were exposed to labelled larvae for 30 min in a tank containing ∼15 l of seawater with no aeration. After 30 min exposure, 'Ray 1' was transferred to a 'larvae free' tank containing aerated seawater for 1 h prior to dissection, whereas 'Ray 2' was dissected immediately.

Rays were killed by pithing. The dorsal and ventral skin surfaces were examined promptly for newly settled larvae using a Leica MZ16FA fluorescence dissecting microscope. Gills 1–5 from the left and right sides of the host were excised and placed separately into numbered Petri dishes containing FSW and then examined under fluorescence. The position of fluorescing post-larvae on each numbered gill was noted. Photomicrographs were taken using a Nikon DXM1200 digital camera interfaced with Nikon ACT-1 imaging software.

Host specificity and post-larval development

Sixteen, 16-day-old parasite-free *A. vincentiana* (∼12 cm TL) were exposed collectively to approximately 400 *B. octohamatus* larvae freshly hatched from eggs laid *in vivo* by adults on *T. fasciata*. During exposure to larvae, the rays were held in ∼30 l of seawater for 1 h with no aeration, then for a further 6 h with aeration. After exposure, the rays were kept in two 60 l tanks (i.e. 8 rays in each tank) containing aerated seawater. To maintain water quality and to prevent possible reinfection as time passed, rays were moved daily to clean 60 l tanks containing fresh seawater. At weekly intervals for the first 7 weeks, then at 3-week intervals thereafter, a single ray was selected at random and dissected to document the morphological development of *B. octohamatus* from newly invaded larva to adult. The interval between host dissections was increased from 1 week to 3 weeks after 'week 7' to ensure that sufficient rays would be available to complete the study because 5 rays died unexpectedly (without yielding parasite specimens) and 2 rays selected for dissection were uninfected.

Rays were killed by pithing. Gills were excised, placed in glass Petri dishes containing FSW, then examined for *B. octohamatus* using a dissecting microscope with incident light. Parasites were removed from the gills using fine forceps and transferred to a dish of FSW. Specimens were flattened and fixed in 10% formalin under cover-slip pressure,

stained in acetocarmine, dehydrated in a graded ethanol series, cleared in cedarwood oil and mounted on microscope slides in Canada balsam beneath a cover-slip. Mounted specimens were examined using a compound microscope with phase-contrast or Nomarski optics and drawings made with the aid of a drawing tube. Measurements were taken using a computerized digitizing system similar to that described by Roff and Hopcroft (1986). Measurements of the sucker sclerites follow the curve of the structure.

Specimens representing the developmental series were deposited as voucher specimens (AHC 29183) at the South Australian Museum (SAMA), Australian Helminthological Collection (AHC), North Terrace, Adelaide, South Australia 5000, Australia.

RESULTS

Invasion of T. fasciata *by CFSE-labelled larvae*

Outstanding contrast between CFSE-labelled *B. octohamatus* larvae and host tissue was achieved under fluorescence microscopy (Fig. 1). Both ray hosts were infected by labelled larvae within the 30 min exposure period. Eighty-three post-larvae (41·5%) were found on the gills of 'Ray 1' when examined 1·5 h after initial exposure to 200 larvae. Examination of 'Ray 2', 30 min after initial exposure to 60 labelled larvae revealed 38 post-larvae (63%) already settled on the gills. No post-larvae were detected on the skin surface of either host.

No pattern was evident in the distribution of post-larvae on host gills. Post-larvae were distributed evenly (close to 50%) between left and right gill chambers ('Ray 1': 51·8% left; 48·2% right; 'Ray 2': 50·2% left, 49·8% right). Post-larvae were found on the posterior and anterior faces of each gill belonging to 'Ray 1' after 1·5 h (Table 1), whereas post-larvae were absent from Gills 4 and 5 (anterior, right) and Gill 4 (anterior, left) of 'Ray 2' after 30 min (Table 2). For both host specimens, Gill 1 supported the fewest post-larvae, which can be attributed in part to this gill having only a posterior face (Tables 1 and 2). For 'Ray 1', anterior-facing gills held a slightly higher proportion of post-larvae (Table 1), whereas for 'Ray 2', post-larvae were more abundant on posterior-facing gills (Table 2). However, this difference was not significant when the unequal number of posterior versus anterior facing gills was taken into account ($\chi^2 = 3.666$; D.F. $= 1$; $\alpha = 0.05$; $P = 3.84$).

Host specificity and post-larval development

Freshly hatched *B. octohamatus* larvae from eggs laid *in vivo* by adults on *T. fasciata* established persistent infections on *A. vincentiana*. At 7, 14, 21, 28, 35, 42,
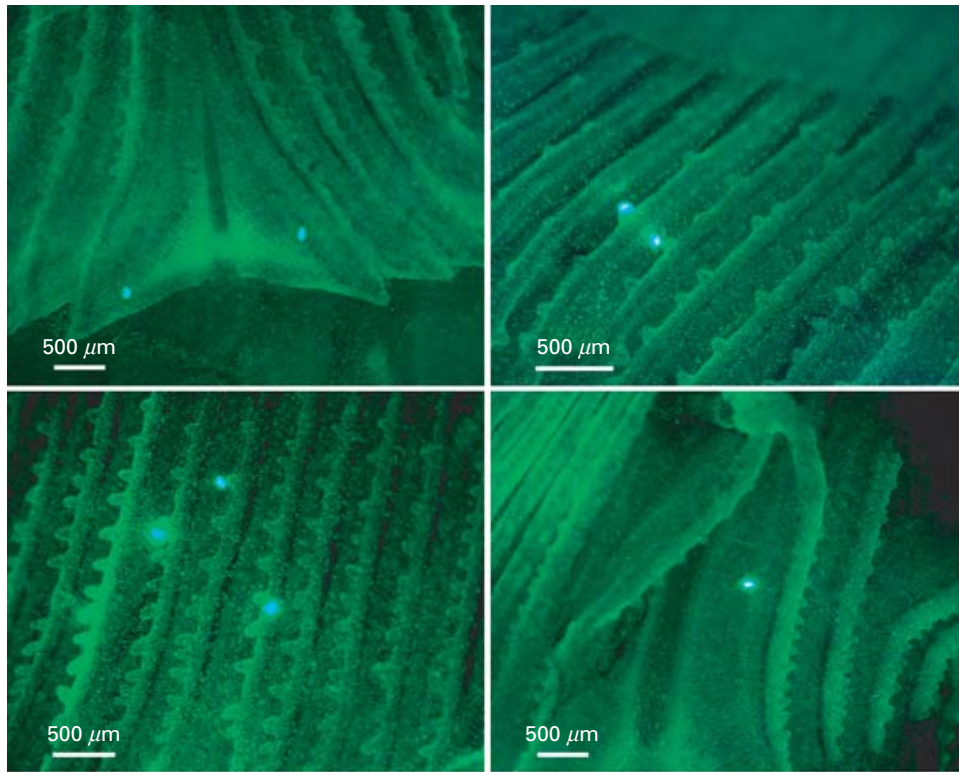
Fig. 1. Photo-micrographs of CFSE-stained *Branchotenthes octohamatus* post-larvae fluorescing on gills of *Trygonorrhina fasciata*.

49, 70 and 91 days post-infection (p.i.), a single *A. vincentiana* was selected for dissection and examined for *B. octohamatus*. A total of 26 *B. octohamatus* specimens, representing 6·5% of the initial approximately 400 larvae exposed, was recovered from the gills of 9 juvenile *A. vincentiana* over the study period. Infection intensity varied from 1 to 10 parasites per host. There was no apparent relationship between host age and infection intensity.

Post-larval development of *B. octohamatus* comprised 3 broad phases, (I) haptoral transformation, (II) allometric growth of haptor and (III) allometric growth of the anterior body and development of reproductive system. The timing and sequence of structural development within these phases is summarized in Table 3.

Few features distinguished post-larvae from larvae during the first 7 days of settlement on the host at 25 °C (Fig. 2A). The length of the post-larva remained within the length range of larvae and hamuli were yet to appear (Table 3; Fig. 2A). However, the refringent mass (presumed rudimentary gut) located posterior to the pharynx in the larva (Glennon *et al.* 2005), was divided bilaterally (Fig. 2A) and dark pigment granules visible within this region indicated that this post-larva had commenced feeding on host blood. Some differentiation of haptoral tissue on either side of the posterior-most hooklet pair, where the hamuli and unarmed suckers of the terminal appendix appear later, was also apparent (Fig. 2A). Anterior glands were clearly visible.

The formation of hamuli between 7 and 14 days at 25 °C marked the start of major changes to the haptor (Table 3; Fig. 2B). These changes included elongation of the posterior region of the haptor to form the haptoral appendix, distancing of the posterior-most hooklet pair from the other haptoral hooklets (Fig. 2B), early development of the unarmed terminal suckers of the haptoral appendix (Fig. 2B) and primordia of sucker sclerite 'Pair 1' (Fig. 2B). The paths of the intestinal caeca were also more clearly defined, forming a ring with posteriorly directed lobes that extended into the haptor (Fig. 2B).

Further transformation of the post-larval haptor to the adult form occurred with the pair-wise development of armed suckers between 21 and 28 days at 24–25 °C, and proceeded from posterior to anterior, occupying the same positions as the larval hooklets (Fig. 2C–E). Each pair of sucker sclerites attained a length of ∼40 μm before the subsequent sclerite pair started to form (Table 3). Sclerites began development prior to the suckers of each corresponding pair (Fig. 2C, D) so that early in development, hooklets could be distinguished easily from sclerites. However, as the musculature developed, the hooklets were ultimately obscured from view (Fig. 2D, E). By day 28, all suckers and sclerites were present and the unarmed terminal appendix was clearly differentiated from the rest of the haptor. Bilateral extensions to the intestinal caeca were also apparent in the haptor (Fig. 2E). Growth of the

Table 1. Distribution of 83 CFSE-labelled *Branchotenthes octohamatus* post-larvae on the gills of 'Ray 1' (*Trygonorrhina fasciata*), 1·5 h after first exposure to 200 larvae

(Values for each region are the percentage of the total number of post-larvae found on the host.)

Ray 1 (*n* = 83 larvae)

	Posterior facing			Anterior facing			
	Left	Right	Total post	Left	Right	Total ant	Total/gill
Gill 1	1·3	3·6	4·9	n/a*	n/a*	—	4·9
Gill 2	8·4	3·6	12·0	8·4	2·4	10·8	22·8
Gill 3	8·4	2·4	10·8	7·2	6·0	13·2	24
Gill 4	8·4	6·0	14·4	4·8	14·6	19·4	33·8
Gill 5	3·6	3·6	7·2	1·3	6·0	7·3	14·5
Totals	30·1	19·2	**49·3**	21·7	29·0	**50·7**	**100**

Gills numbered from anterior to posterior.
* Gill 1 has no anterior face.

Table 2. Distribution of 38 CFSE-labelled *Branchotenthes octohamatus* post-larvae on the gills of 'Ray 2' (*Trygonorrhina fasciata*), 30 min after first exposure to 60 larvae

(Values for each region are the percentage of the total number of post-larvae found on the host.)

Ray 2 (*n* = 38 larvae)

	Posterior facing			Anterior facing			
	Left	Right	Total post	Left	Right	Total ant	Total/gill
Gill 1	5·3	7·9	13·2	n/a*	n/a*	—	13·2
Gill 2	5·3	10·4	15·7	5·3	2·6	7·9	23·6
Gill 3	5·3	5·3	10·6	5·3	7·9	13·2	23·8
Gill 4	7·9	10·4	18·3	—	—	—	18·3
Gill 5	7·9	5·3	13·2	7·9	—	7·9	21·1
Totals	31·7	39·3	**71**	18·5	10·5	**29**	**100**

Gills numbered from anterior to posterior.
* Gill 1 has no anterior face.

haptor was allometric between 28 and 49 days p.i. (Phase II) by which time each pair of armed suckers had attained approximately equal size (Table 3, Fig. 2H). Some differentiation of tissue within the intercaecal space of the anterior body indicated early development of the reproductive system (Fig. 2H). Ensuing growth of the juvenile worm was allometric with respect to the anterior body while reproductive development advanced (Phase III) (Fig. 2I). By day 70 most male and female reproductive structures had formed, except for the vitellarium (Fig. 2I). By day 91, vitellarium was present and the worm appeared to be sexually mature, although no eggs were observed in the uterus of the single specimen we examined (see Figure 1 in Glennon *et al.* 2005 for illustration of adult).

DISCUSSION

We have developed the first parasite-host model to study the biology of an unciliated monogenean larva infecting an elasmobranch host. This unique model has also allowed us to investigate questions of host specificity and explore ontogenetic development. Labelling of *B. octohamatus* larvae with CFSE before experimental infection of *T. fasciata* enabled rapid and accurate inspection of dissected gill tissue for the presence of tiny, newly settled post-larvae. Discovery of labelled post-larvae on *T. fasciata* gills within 30 min of exposure and the absence of post-larvae from other host body surfaces provides strong evidence that unciliated *B. octohamatus* larvae infect the gills of their host directly and do not migrate to

Table 3. Temporal sequence of structural changes in *Branchotenthes octohamatus* during development on gills of *Aptychotrema vincentiana*

(All measurements are in micrometres: range with sample size in parentheses.)

Days p. i.	Phase*	Fig.	Total length (TL)	Haptor length (HL)	Ratio TL:HL	Hamulus length	Sucker sclerite length			Haptoral sucker diameter			Temp. (°C)
							Pair 1	Pair 2	Pair 3	Pair 1	Pair 2	Pair 3	
7	I	2A	140 (n=1)		2:1								25
14	I	2B	214–238 (n=2)	107–120 (n=1)	1·4:1	49–55 (n=4)	20–26 (n=3)						25
21	I	2C, D	212–276 (n=10)	111–174 (n=10)	1:1	50–64 (n=12)	34–61 (n=12)	30–42 (n=7)		34–51 (n=12)			25
28	I	2E	326–384 (n=4)	202–267 (n=5)	1:1	50–70 (n=8)	67–90 (n=8)	51–70 (n=7)	31–59 (n=8)	44–60 (n=7)	34–50 (n=6)	37 (n=1)	24
35	II	2F	460 (n=1)	271 (n=1)	1:1·7	65–66 (n=2)	84–85 (n=2)	70–72 (n=2)	52–53 (n=2)	66–70 (n=2)	60–63 (n=2)	40–41 (n=2)	24
42	II	2G	784 (n=1)	493 (n=1)	1:1·5	70–76 (n=2)	176–178 (n=2)	133–141 (n=2)	134–143 (n=2)	117–125 (n=2)	104–118 (n=2)	77–82 (n=2)	24
49	II	2H	970–1830 (n=5)	600–1400 (n=5)	1:1·5	55–62 (n=5)	271–304 (n=10)	258–305 (n=10)	235–292 (n=10)	120–147 (n=6)	135–158 (n=6)	125–147 (n=5)	23
70	III	2I	4562 (n=1)	1052 (n=1)	1·3:1	63–64 (n=2)	501–505 (n=2)	490–501 (n=2)	505 (n=2)	277 (n=2)	276–285 (n=2)	248–271 (n=2)	22–23
91	III		5376 (n=1)	1760 (n=1)	1·5:1	57–58 (n=2)	650 (n=1)	660–663 (n=2)	660–670 (n=2)	280 (n=1)	302 (n=1)	250 (n=1)	21–22

* I=haptoral transformation; II=allometric growth of haptor; III=allometric growth of anterior body and development of reproductive system.
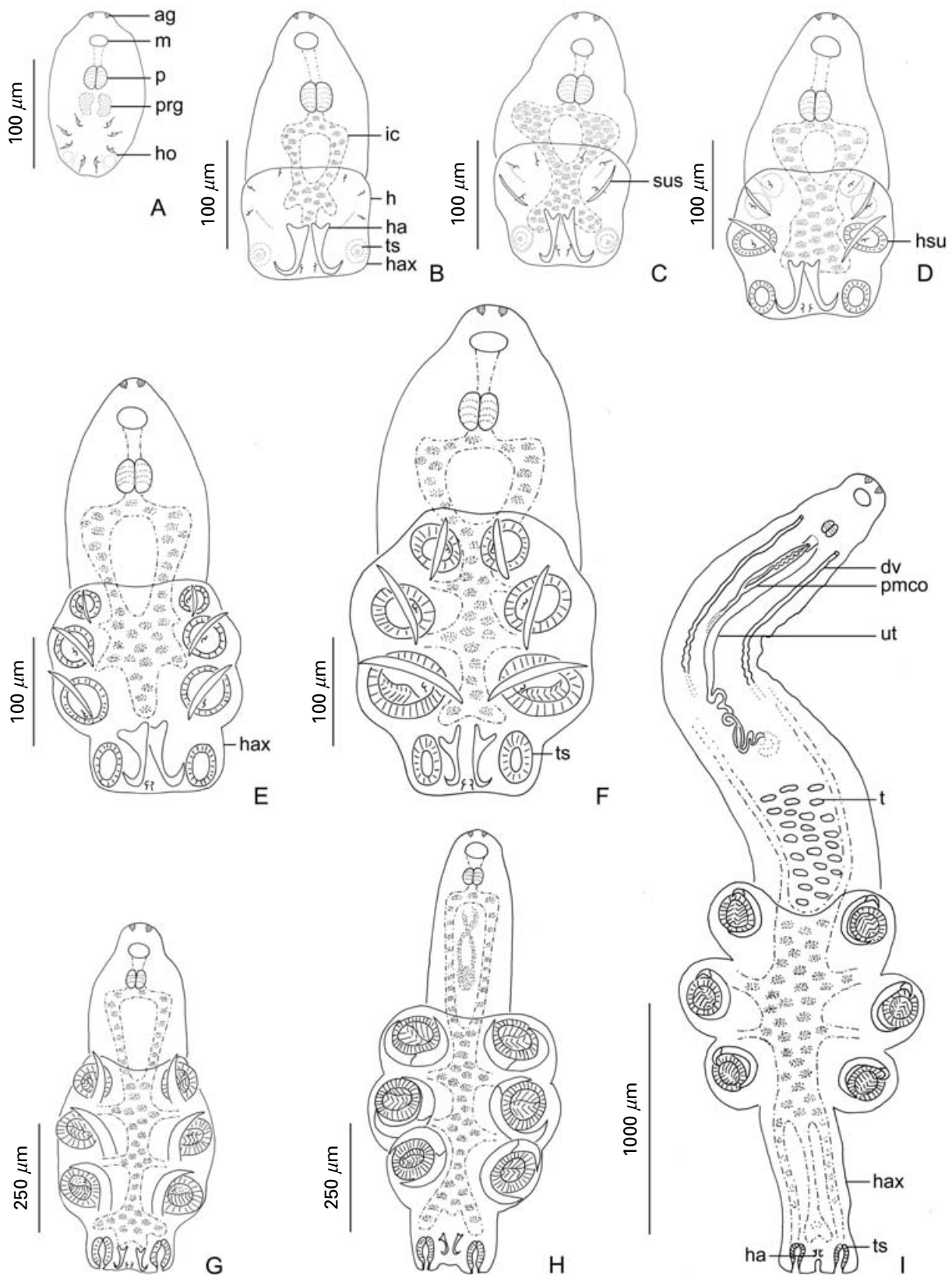
Fig. 2. Development of *Branchotenthes octohamatus* on gills of *Aptychotrema vincentiana*. (A) 7 days. (B) 14 days. (C–D) 21 days. (E) 28 days. (F) 35 days. (G) 42 days. (H) 49 days. (I) 70 days. Abbreviations: (ag) anterior gland duct opening; (dv) distal part of vagina; (h) haptor; (ha) hamulus; (hax) haptoral appendix; (ho) hooklet; (hsu) haptoral sucker; (ic) intestinal caecum; (m) mouth; (p) pharynx; (pmco) proximal part of male copulatory organ; (prg) presumed rudimentary gut; (sus) sucker sclerite; (t) testis; (ts) terminal unarmed sucker; (ut) uterus.

the definitive site after initial attachment elsewhere on the host. These data further suggest that the unciliated larvae are carried passively in the inhalant respiratory current. Inspiration of larvae via the host's inhalant respiratory current has also been observed for ciliated larvae of *Diplozoon paradoxum* (see Bovet, 1967). These otherwise active larvae stop swimming in a host's inhalant current and are carried passively into the buccal cavity (Bovet, 1967). Inspiration by the host has also been proposed as the mechanism of infection for *Discocotyle sagittata* (see Paling, 1969; Gannicott and Tinsley, 1998). Glennon *et al.* (2006) found that *B. octohamatus* eggs hatch when mechanically agitated and the newly hatched unciliated larva thrashes about from side to side. Rays frequently disturb sediment as they forage or settle on the sea floor, providing the kind of physical disturbance that eggs of this monogenean species need to hatch. If *B. octohamatus* eggs are stimulated to hatch by a foraging host, then prompt inspiration of larvae by the host is highly probable. While being drawn through the gill chamber by the ventilating current, the sharp, thrashing movements of *B. octohamatus* larvae may facilitate the penetration or 'grabbing' of gill tissue by the larval hooklets and provide anchorage and prevent expulsion of the larvae from the ray via the exhalant current. Such opportunistic attachment to the gills is supported by the absence of any pattern in the settlement distribution of *B. octohamatus* post-larvae on gills of *T. fasciata*.

We have demonstrated that freshly hatched *B. octohamatus* larvae from eggs laid by adults on the type-host *T. fasciata* are capable of establishing persistent infections on the western shovelnose ray, *A. vincentiana*. Both rhinobatid species occur sympatrically in South Australia and across southern Australia to Perth in Western Australia. We have found a hexabothriid that appears morphologically similar to *B. octohamatus* on *A. vincentiana* in Western Australia (unpublished data). Our infection studies suggest that the hexabothriids from these 2 rhinobatid species are conspecific and molecular studies are currently underway to test this suggestion.

Following host invasion, *B. octohamatus* post-larvae invest early in attachment structures. The hamuli are first to develop, presumably assuming an important role in anchoring the post-larvae while the unarmed suckers of the terminal appendix and then the armed suckers of the haptor proper form. Only after this haptoral development has occurred does the anterior part of the body undergo significant growth. So far, 2 studies have investigated morphological development of hexabothriid species: *Squalonchocotyle torpedinis* from the marbled electric ray, *Torpedo marmorata* (see Euzet and Raibaut, 1960) and *Rajonchocotyle emarginata* from *Raja clavata* (see Wiskin, 1970). Like *B. octohamatus*, the

sequence of haptoral sclerite and sucker development is from posterior to anterior. While Euzet and Raibaut (1960) related worm development to time, they did not chart development of *S. torpedinis* to sexual maturity and provided no information about the temperature at which the study was conducted. Direct comparisons with *B. octohamatus* are therefore limited. However, we have established that *B. octohamatus* appear to be sexually mature by 91 days p.i. at 22–25 °C and the oldest specimens of *S. torpedinis* examined by Euzet and Raibaut (1960) were at 55 days (see their Figure 5). By this time (at an unknown temperature), *S. torpedinis* had attained a level of development equivalent to *B. octohamatus* specimens at 28 days p.i. at 24 °C (see our Fig. 2E), indicating that development of *S. torpedinis*, like that of *B. octohamatus*, is protracted.

Post-larval development patterns of several other (non-hexabothriid) polyopisthocotylean monogeneans have also been studied (e.g. *Diclidophora denticulata* (Diclidophoridae): see Frankland, 1955; *Polystoma indicum* (Polystomatidae): see Dutta and Tandon, 2000), although times to sexual maturity were not quantified. Rubio-Godoy and Tinsley (2002) observed sexually mature *Discocotyle sagittata* (Discocotylidae) on gills of experimentally infected rainbow trout maintained at 13 °C, 63 days after a single exposure event to larvae. However, their study also showed development to be affected by the number of larvae used and the mode of infection, i.e. whether single or trickle (multiple) exposure events (Rubio-Godoy and Tinsley, 2002). In contrast, development periods are known for a number of monopisthocotylean monogeneans. Several capsalid species reach sexual maturity after about 14 days p.i. at 24–25 °C (e.g. *Neobenedenia girellae*: see Bondad-Reantaso *et al.* 1995; *Benedenia lutjani*: see Whittington and Ernst, 2002). Chisholm and Whittington (2003) reported a slightly longer time to sexual maturity for 2 monocotylid species at 25 °C: *Neoheterocotyle rhinobatidis* (∼21 days) and *Merizocotyle icopae* (undetermined but >21 days). It is reasonable that developmental variation between species may reflect familial differences, as these differences ultimately reflect other life-history parameters such as the degree of transformation from larva to adult, adult size, the site occupied by the parasite on the host (e.g. skin or gills), the mode of nutrition (monopisthocotyleans – epithelial feeders; polyopisthocotyleans – blood feeders) and the nature of the host (e.g. teleost or elasmobranch). Hexabothriids are large, gill-dwelling, blood-feeding monogeneans of elasmobranchs that undergo considerable morphological change from larva to adult. The slow development time we have determined for *B. octohamatus* may be a feature of hexabothriids generally and may be governed in part by the aforementioned factors. However, more data are needed to confirm this.

Infection intensity of *B. octohamatus* was very low (1–2 individuals) for the majority of experimentally infected *A. vincentiana* dissected. However, where more than 1 specimen was recovered from a host (14, 21, 28 and 49 days p.i.), considerable variation in size and level of development was apparent between specimens (e.g. Fig. 2C and D at 21 days p.i.). Although the amount of pressure applied to specimens during fixation may explain some morphometric variation, it cannot account for the morphological variation observed. Chisholm and Whittington (2003) also noted variability between specimens of *N. rhinobatidis* from the gills of *Rhinobatos typus* within a single infection cohort and speculated that the immune response of non-naïve hosts may have had a part to play. Our study and that of Euzet and Raibaut (1960) used very young hosts so it is unlikely that acquired host immunity would have exerted significant influence on developmental progress for *B. octohamatus* and *S. torpedinis* respectively. However, it is also possible that variable development reflects a strategy to extend the effective duration of a single infection event (Chisholm and Whittington, 2003). With respect to *B. octohamatus*, many larvae can potentially hatch at the same time if the appropriate mechanical hatching stimulus is received, as eggs are laid end-to-end forming a chain. Therefore, for this monogenean species, single infection events involving multiple (possible sibling) larvae are likely. If host encounters are low, then the adaptive value of variable post-larval development may be important. On the other hand, the fact that *B. octohamatus* larvae used to infect *A. vincentiana* in our study were hatched from eggs laid by adults on *T. fasciata*, raises the question of possible host preference. *Aptychotrema vincentiana* may be less suitable as a host for *B. octohamatus* than *T. facsiata* and this may have had a negative effect on both parasite infection intensity and post-larval development.

Eight hooklet pairs are generally believed to represent the ancestral condition in monogenean larvae (Bychowsky, 1957; Llewellyn, 1970; Boeger and Kritsky, 1993). All larval Hexabothriidae described, except for *B. octohamatus*, have 5 hooklet pairs and are considered to have lost the 2 anterior-most hooklet pairs (VII and VIII) and the posterior-most hooklet pair (I), retaining only hooklet pairs II–VI (numbered posterior to anterior) (Llewellyn, 1963). The presence of only 4 hooklet pairs in *B. octohamatus* represents a unique condition among hexabothriids studied so far (Glennon *et al.* 2005). According to Llewellyn (1963), during development of the characteristic hexabothriid haptor, hooklet pairs IV–VI are replaced by the armed suckers of the haptor proper and hooklet pair III replaced by the unarmed suckers of the terminal appendix (e.g. *S. torpedinis*: see Euzet and Raibaut, 1960; *R. emarginata*: see Wiskin, 1970). Development of

the armed haptoral suckers of *B. octohamatus* corresponds to the positions of hooklet pairs IV–VI, conforming to the characteristic hexabothriid arrangement, although the hooklets do persist and are not replaced during development. However, the unarmed suckers of the terminal appendix of *B. octohamtaus* do not develop in correspondence with a pair of hooklets but arise between the 2 posterior-most hooklet pairs, indicating that it is hooklet pair III that is lost from *B. octohamatus* prior to larval development.

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