Egg hatching in 3 species of monocotylid monogenean parasites from the shovelnose ray *Rhinobatos typus* at Heron Island, Australia

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SUMMARY

Eggs of *Neoheterocotyle rhinobatidis*, *Troglocephalus rhinobatidis* and *Merizocotyle icopae* (Monogenea: Monocotylidae) from the shovelnose ray *Rhinobatos typus* (Rhinobatidae) have a distinct hatching pattern linked to light periodicity. Larvae of these 3 monogenean species emerge only during daylight when exposed to natural illumination or when incubated in alternating 12 h periods of light and dark (light on 06.00 h, light off 18.00 h). *N. rhinobatidis* larvae emerge with a distinct peak during the first 2 h of light; this peak is not as pronounced in *T. rhinobatidis* or *M. icopae*. Eggs of *N. rhinobatidis* incubated in a reverse light/dark cycle (light on 18.00 h, light off 06.00 h) hatched only during periods of illumination, again with a peak during the first 2 h of light. Evidence suggests that the hatching patterns observed in all 3 species represent true circadian rhythms because eggs incubated in 24 h light or 24 h dark conditions continued to hatch with a rhythm. Shadows, disturbance and host tissue did not promote hatching in *N. rhinobatidis* or *T. rhinobatidis* but there were indications that host tissue may promote hatching in *M. icopae*. The hatching patterns observed are discussed with respect to their adaptive responses to host behaviour and predation pressure.

Key words: Monogenea, Monocotylidae, egg hatching, circadian rhythm, *Rhinobatos typus*, adaptive value.

INTRODUCTION

It is the task of the larva of monogenean parasites to locate its specific host and complete the life-cycle. The monumental role of the larva is exacerbated further by the fact that its average life-span ranges between 24 and 48 h and those larvae that are ciliated have a slow swimming speed relative to their hosts (Whittington, Chisholm & Rohde, 2000). To enhance the likelihood of the larva locating and infecting its specific host, the eggs of monogeneans are known to hatch in response to a variety of either environmental or host-induced cues. These cues may include light periodicity, variations in light intensity, mechanical disturbance and chemical stimulants (e.g. Kearn, 1986; Whittington *et al.* 2000).

The Monocotylidae is a family of monogeneans comprising approximately 100 species that are parasitic exclusively on sharks, rays and chimaeras. They can be found on or in a variety of sites including the skin, gills, nasal tissue, cloaca, rectum and rectal gland and 1 species, *Dictyocotyle coeliaca*, lives on the inner wall of the body cavity of *Raja* spp. (see Chisholm & Whittington, 1998*a*). The larvae of 20 species of monocotylids have now been described, but the cues which may promote egg hatching have only been studied rigorously for *D. coeliaca* (see Kearn, 1975). Eggs of *D. coeliaca* hatch continuously over a 24 h period and hatching is not induced by exposure to skin mucus or body fluids (Kearn, 1975). However, *D. coeliaca* has been considered atypical due to the unusual internal site it inhabits. We investigated hatching in 3 monocotylid species representing 3 of the 6 subfamilies from more typical sites from the same host species *Rhinobatos typus*. We studied *Neoheterocotyle rhinobatidis* (Heterocotylinae) and *Troglocephalus rhinobatidis* (Dasybatotreminae) from the gills and *Merizocotyle icopae* (Merizocotylinae) from the nasal tissue to determine what cues may trigger emergence of larvae from the eggs and whether these cues differ among monocotylids living on different sites of the same host species.

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MATERIALS AND METHODS

Source of parasites

Juvenile *R. typus* (common shovelnose ray), ranging in total length from 40 to 50 cm, were caught by seine net in Shark Bay at Heron Island on the Great Barrier Reef (23° 27′ S, 151° 55′ E). Fish were identified according to Last & Stevens (1994). *R. typus* around Heron Island are infected with *N. rhinobatidis* and *T. rhinobatidis* (both species found on the gills) and *M. icopae* (located on the nasal tissue). The

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Table 1. Egg hatching for 5 different light regime experiments for *Neoheterocotyle rhinobatidis*

(Natural (sunrise 06.00 h, sunset 18.00 h), LD 12:12 (light on 06.00 h, light off 18.00 h), DL 12:12 (light on 18.00 h, light off 06.00 h), LL (light on for 24 h) and DD (light off for 24 h). The numbers in parentheses represent the percentage of the total eggs collected.)

	Natural	LD 12:12	DL 12:12	LL	DD
Temperature (°C)	22–24	24–26	24–26	22–24	22–24
Incubation time (days)	8	9	9	9	10
Eggs hatched	289 (87)	227 (74)	366 (68)	171 (57)	146 (52)
Potentially viable eggs remaining	17 (5)	28 (9)	32 (6)	75 (25)	88 (31)
Non-viable eggs remaining	27 (8)	50 (16)	143 (26)	54 (18)	46 (16)
Total eggs	333	305	541	300	280*

* Eggs never exposed to light.

rays were kept in tanks (1·8 × 0·8 × 0·25 m) in natural light conditions for 4–6 weeks prior to dissections. Two pieces of netting were placed in the tanks to trap monogenean eggs and promote heavy parasite infections (Ernst & Whittington, 1996). Rays were fed daily on chopped pilchards.

Collection and incubation of eggs and recovery of free-swimming larvae

Infected rays were killed by pithing and adult *N. rhinobatidis*, *T. rhinobatidis* and *M. icopae* were removed from the host using fine needles and placed in separate glass Petri dishes (50 mm diameter, 14 mm depth) of filtered seawater (FSW). All dissections were carried out between 07.00 h and 08.00 h because parasites appeared to lay more eggs in the early hours of daylight. However, limited parasite numbers were available for most experiments and this apparent egg laying rhythm was not investigated further. The fecundity of these monocotylid species appeared to be lower than that of capsalid monogeneans from a teleost species in the same environment (Ernst & Whittington, 1996) and therefore as many adults as possible were collected to lay eggs. Healthy parasites reattached immediately to Petri dishes and began to lay eggs. The FSW was replaced in the Petri dishes every 4 h. Parasites were left to lay eggs for 24 h, usually in the same light regime to which the eggs were subsequently exposed (see below for exceptions). The minute eggs (mean side length 85 μm) could not be transferred easily by Pasteur pipette and were therefore collected by dragging a small loop of fine wire over the bottom of the Petri dish. The eggs adhered to the wire loop and were then transferred to a Perspex egg well (Ernst & Whittington, 1996) by flicking the end of the wire loop with a fine needle.

Eggs were counted and the Perspex well was immersed gently into a larger glass dish (volume approximately 30 ml) of FSW and sealed with a glass plate to exclude all air. These protocols follow the methods described in detail by Ernst & Whittington (1996).

Eggs were incubated in a black cabinet 80 cm high, 80 cm wide and 50 cm deep. The front of the cabinet was equipped with a large blackout curtain which excluded all external light even while we worked in the cabinet. A housing with a lamp fitted with a 7 W bulb and covered with a blue Cinemoid sheet filter was located in the middle of the top of the cabinet (i.e. 80 cm above the floor of the box). The lamp was connected to a timer programmed to provide the desired light regime. If eggs were being incubated in darkness during daylight hours, a smaller black box was placed over the egg dishes within the cabinet to avoid possible exposure to external light when first lifting the blackout curtain to access the cabinet. Temperatures of seawater kept in the cabinet ranged between 22 and 26 °C during all experiments (Tables 1 and 2) and were within 1 °C of mean ambient water temperatures at Heron Island. A Wild M5 dissecting microscope fitted with a red filter was kept in the box so the development of eggs could be monitored with minimal disturbance. Egg wells were transferred daily into clean glass dishes filled with fresh FSW and sealed as described above. These transfers were made at random times during the day or night to avoid imposing an artificial hatching cue. Egg well transfers made in darkness were monitored using a torch fitted with a red filter because dim red light has no effect on larval hatching or behaviour (Kearn, 1973; Ernst & Whittington, 1996). Observations during this study also show that the red light had no effect on hatching.

Oncomiracidia ($\sim$ 150 μm long) began to emerge from eggs usually 8–10 days after laying. At the first sign of hatching the number of larvae was counted every 2 h until hatching was complete. Free-swimming larvae were separated from unhatched eggs by transferring the Perspex well into a new dish of FSW which was re-sealed as described above. The original dish contained the free-swimming larvae and these were killed with a few drops of formaldehyde (38 % solution) and counted. Special care was taken to check the surface of the water and sides of the dish to avoid overlooking larvae. Tests showed that no larvae were carried over in the Perspex egg well

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Table 2. Egg hatching for 3 different light regime experiments for *Troglocephalus rhinobatidis* and *Merizocotyle icopae*

(The numbers in parentheses represent the percentage of the total eggs collected. Light regimes as listed in Table 1.)

	Troglocephalus			*Merizocotyle*		
	LD 12:12	LL	DD	LD 12:12	LL	DD
Temperature (°C)	24–26	22–24	22–24	24–26	22–24	22–24
Incubation time (days)	7	8	9	8	8	9
Eggs hatched	144 (75)	18 (35)	44 (34)	71 (54)	35 (35)	5 (7)
Potentially viable eggs remaining	21 (11)	17 (34)	70 (55)	13 (10)	50 (50)	45 (64)
Non-viable eggs remaining	27 (14)	15 (30)	15 (30)	48 (36)	15 (15)	20 (29)
Total eggs	192	50	128*	132	100	70*

* Eggs transferred to DD after being laid for 24 h in LD 12:12.

during transfers. At the completion of each experiment, the eggs in the Perspex egg well were examined and classified as hatched (empty egg case), potentially viable (unhatched but with mature larvae with eyespots) or non-viable (dark brown undeveloped egg with no larva present).

Light regimes investigated

The number of eggs collected and exposed to the different experimental light regimes and the incubation temperatures are summarized for *N. rhinobatidis* (Table 1) and for *T. rhinobatidis* and *M. icopae* (Table 2).

Natural illumination

Eggs incubated in natural illumination were left on a bench in the laboratory in July 1998 but were not exposed to direct sunlight. At this time of year, sunrise was approximately at 06.00 h and sunset at 18.00 h. Natural hatching in *T. rhinobatidis* was not determined due to an absence of adult specimens on rays at this time.

LD 12:12, DL 12:12, LL and DD

Eggs of the 3 monocotylid species were incubated in the cabinet described above in 4 different experimental conditions (Tables 1 and 2) including a light/dark (LD) 12:12 regime (i.e. light on 06.00 h, light off 18.00 h), a reverse light/dark (DL) cycle (light on 18.00 h, light off 06.00 h), constant light (LL) and constant dark (DD). The same light regime was used to illuminate adult *N. rhinobatidis* while laying eggs and eggs in the process of incubation. For example if *N. rhinobatidis* eggs were designated for incubation in LD 12:12, DL 12:12 or LL, the adults were exposed to the same regime immediately after removal from the host. Those *N. rhinobatidis* eggs which were incubated in constant darkness were never exposed to light (Table 1) because after adults were removed from the gills, any eggs that had been laid were removed from the dish before it was placed in constant darkness. The eggs were later transferred to the Perspex egg wells with the aid of a microscope and stage light fitted with a red filter.

Adult *T. rhinobatidis* and *M. icopae* were usually less prevalent than *N. rhinobatidis* and laid fewer eggs. Therefore smaller numbers of eggs were available for experiments on the first 2 species (Table 2). Furthermore, when Petri dishes containing adults were put into darkness, egg laying activities almost ceased. Therefore, adults of *T. rhinobatidis* and *M. icopae* whose eggs were designated for DD experiments were incubated in LD 12:12 during the 24 h egg laying period to increase the numbers of eggs collected. Immediately after the eggs were harvested and placed into the Perspex egg wells, they were subjected to DD (Table 2).

DD after incubation in LD 12:12 and exposure to brief periods of light

A control dish of 155 *N. rhinobatidis* eggs (Batch A) was exposed to LD 12:12 conditions throughout development. Dishes containing 166 *N. rhinobatidis* eggs (Batch B) and 100 *N. rhinobatidis* eggs (Batch C) were exposed to LD 12:12 until eyespots had formed and then they were transferred to DD. When egg hatching ceased in Batch B, the remaining unhatched eggs were suddenly exposed to light in the incubation box for a period of 15 min at 12.00 h (halfway through the illumination period of the previous LD 12:12 cycle). The dish was then examined for hatched larvae. When hatching began in Batch C, the eggs were suddenly exposed to light in the incubation box for a period of 15 min commencing at 0.00 h (halfway through the dark period of the previous LD 12:12 cycle). The dish was examined for hatched larvae. The eggs in each of these dishes were then exposed for another 15 min to brighter light on the stage of the microscope and examined for larvae. This procedure was repeated for Batch C on the following 3 nights.

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Wild versus captive hosts

To test whether the hatching rhythm of monogenean eggs was altered by keeping their hosts in captivity, we examined the hatching rhythm of 200 *Neoheterocotyle* eggs which were collected from adult parasites removed from a shovelnose ray on the day it was captured. This was compared with the hatching rhythm of 305 eggs which were harvested from adult parasites removed from the gills of a ray that had been kept in captivity for 6 weeks. Both batches of eggs were incubated in LD 12:12.

Effect of egg laying duration on hatching rhythms and total experiment duration

In all experiments, adult parasites were left to lay eggs for approximately 24 h and we tested whether this influenced the hatching rhythm or the total duration of the hatching experiment. The pattern and duration of larval emergence of 70 *Neoheterocotyle* eggs collected within 6 h of removal of adults from the host and incubated in LD 12:12 was compared with results obtained from 118 eggs that were collected after the 24 h laying period and incubated in LD 12:12.

Effects of other potential hatching stimulants

Control and test batches of eggs of *N. rhinobatidis*, *T. rhinobatidis* and *M. icopae* were incubated in LD 12:12 conditions. Some test batches of eggs were mechanically disturbed by both tapping the glass container on the laboratory bench, and by exposing eggs to water currents from a glass pipette. Other test batches of eggs were exposed to either fresh or defrosted skin tissue from the ventral abdominal region of a shovelnose ray. The effect of shadows on egg hatching was tested while eggs were exposed to light in the cabinet. In each experiment comparisons were made with control batches which were not manipulated.

RESULTS

Hatching in N. rhinobatidis

Natural and LD 12:12. In natural conditions, eyespots formed after 5 days and hatching commenced after 8 days (Table 1). Eggs hatched during daylight hours only, between 06.00 h and 18.00 h (Fig. 1A). Few larvae emerged on the first day of hatching but on Day 2 there was a distinct peak in larval emergence during the first 2 h period of light, between 06.00 h and 08.00 h (Fig. 1A). Hatching continued for a total of 5 successive days and 87% of the eggs hatched successfully (Table 1). A similar hatching pattern was observed for eggs incubated in

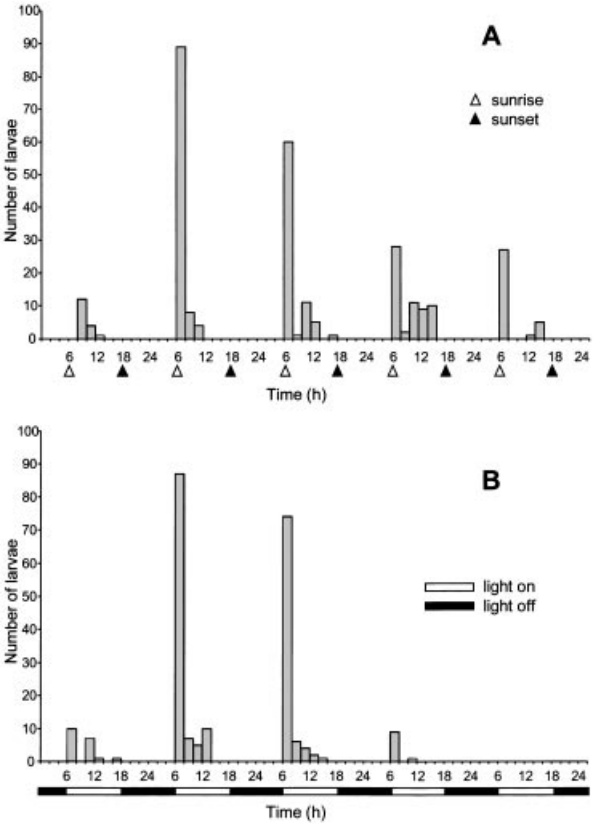

Fig. 1. Number of larvae of *Neoheterocotyle rhinobatidis* hatching at 2 h intervals from (A) 333 eggs exposed to natural daily illumination and (B) 305 eggs exposed to LD 12:12. △ and ▲ indicate the times of sunrise and sunset, respectively. Bottom panel in (B) indicates alternate 12 h periods of light (white) and darkness (black).

experimentally controlled conditions of LD 12:12. Eyespots formed after 5 days and hatching, which started after 9 days (Table 1), was restricted to periods of illumination. Again, few larvae hatched on the first day but a strong hatching peak during the first 2 h period of light was observed on the 2 subsequent days (Fig. 1B). Hatching continued for a total of 4 successive days and 74% of the eggs hatched successfully (Table 1).

Origin of host and duration of egg laying

The pattern of egg hatching for 305 *N. rhinobatidis* eggs collected from parasites from a shovelnose ray that had been held for 6 weeks was similar to that observed for 200 eggs from parasites obtained from a ray dissected immediately after capture (Fig. 2A). The absence of the large emergence peak on Day 3 for eggs from parasites of the wild host compared with eggs from parasites from the captive host (Fig. 2A) may be because fewer eggs were available for the experiment. The hatching pattern and duration of hatching were similar for 80 eggs of *N. rhinobatidis* which had been collected within 6 h after removal of the parasites from the host and for 118 eggs collected

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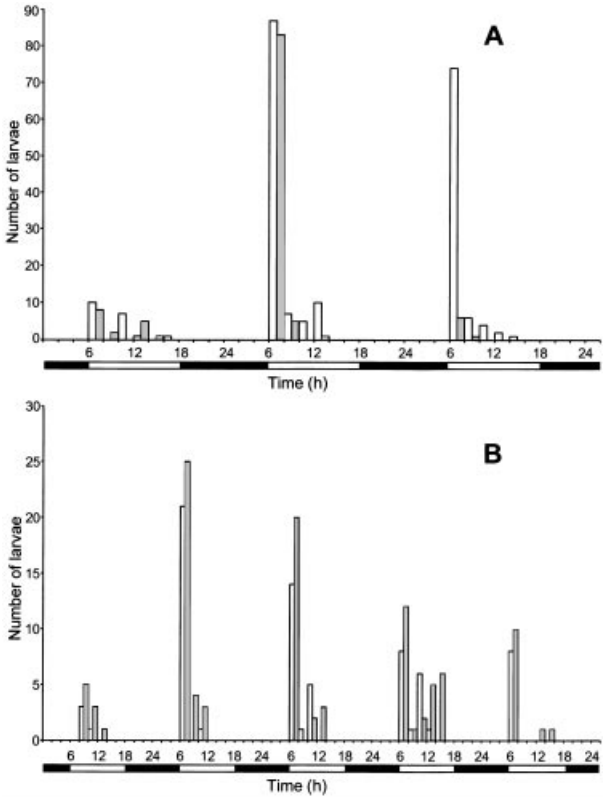

Fig. 2. Number of larvae of *Neoheterocotyle rhinobatidis* hatching at 2 h intervals from (A) 305 eggs laid by parasites removed from a host kept in captivity for 6 weeks (white bars) versus 200 eggs laid by parasites removed from a wild host on the day of capture (grey bars) and (B) 80 eggs collected 6 h after being laid (white bars) versus 118 eggs collected 24 h after being laid (grey bars). Bottom panels as in Fig. 1B.

within 24 h after the adults were removed from the host (Fig. 2B). Hatching success for both egg batches was 88%. Therefore, using eggs that had been laid over a 24 h period had no effect on the final hatching results.

DL 12:12, LL, DD

When the light regime was reversed to DL 12:12, 92% of the larvae that hatched emerged during periods of illumination (Fig. 3). A pattern similar to that described above for the LD 12:12 experiment was found. Eggs began hatching after 9 days (Table 1). Only a few larvae emerged during the first day of hatching, but a strong emergence peak occurred during the first 2 h period of light on each of the 4 subsequent days (Fig. 3). Few eggs (8%) hatched during periods of darkness, usually during the 2 h period immediately before the light came on (16.00 h–18.00 h) (Fig. 3). Hatching success was 68% and only 32 (6%) potentially viable eggs remained (Table 1).

Eggs incubated in LL began to hatch after 9 days and continued to hatch with a distinct pattern (Fig. 4A). With the exception of 2 larvae, all emerged

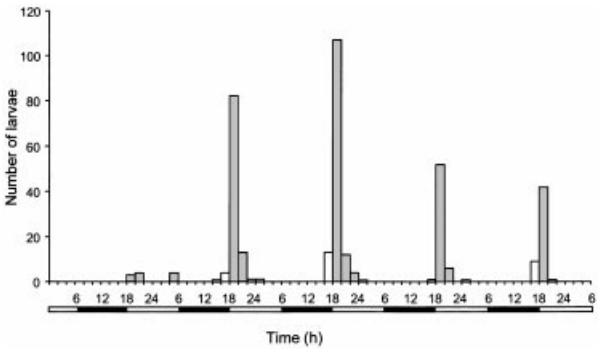

Fig. 3. Number of larvae of *Neoheterocotyle rhinobatidis* hatching at 2 h intervals from 541 eggs incubated in DL 12:12. White bars in histogram indicate larvae that hatched during hours of darkness. Bottom panel as in Fig. 1B.

during the hours corresponding to the natural light period (i.e. between 06.00 h and 18.00 h) (Fig. 4A). Few larvae hatched on Day 1 and no distinct peak in hatching was observed during the period corresponding to the first 2 h of illumination (Fig. 4A). Furthermore, there was a shift in larval emergence with more eggs hatching later in the morning and in the afternoon than seen in the LD 12:12 experiments (compare Figs 1B and 4A). The hatching success was 57% but at the end of the experiment 75 potentially viable eggs (25%) remained unhatched (Table 1).

Eggs of *N. rhinobatidis* incubated in DD (i.e. these eggs had never been exposed to light) also demonstrated a distinct rhythmical pattern of larval emergence. Hatching commenced 10 days after egg collection and larvae only emerged during the time corresponding to the period between dawn and dusk (Fig. 4B). As seen in the LL experiment, the large peak in larval emergence between 06.00 h and 08.00 h (the first 2 h of the natural illumination) highlighted earlier was absent and there was a shift in hatching to later in the morning and in the afternoon. At the completion of the experiment 88 potentially viable eggs (31%) remained and hatching success was 52% (Table 1). We also observed that when the dishes containing eggs were removed from the dark cabinet at the completion of the DD experiment, many larvae emerged immediately from these unhatched eggs. Egg hatching appeared to be stimulated by the sudden exposure to light and therefore we tested this further in the experiment described below.

DD after LD 12:12 and exposure to brief periods of light

The control dish, Batch A (155 eggs), hatched 9 days after collection for 4 successive days with the hatching pattern observed in the LD 12:12 experiment described above (e.g. Fig. 1B). Batch B (166 eggs), which was incubated in LD 12:12 but

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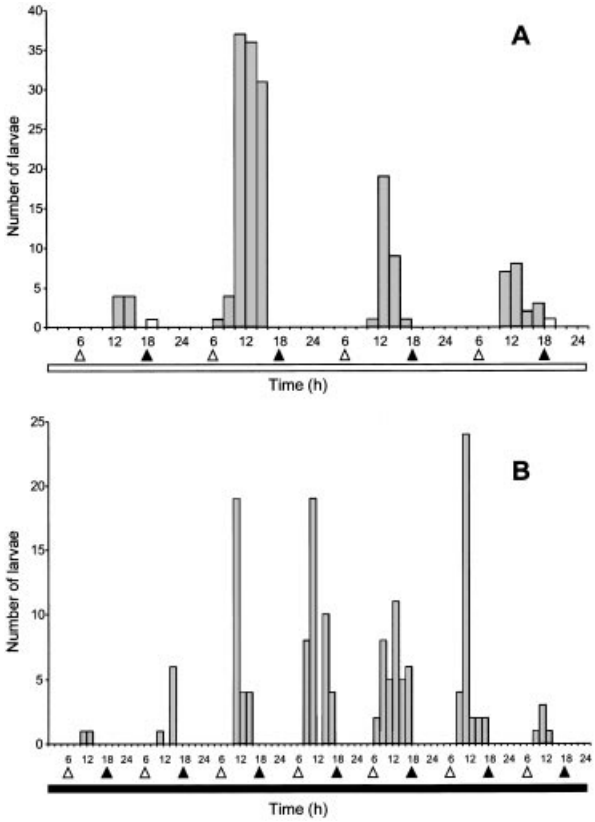

Fig. 4. Number of larvae of *Neoheterocotyle rhinobatidis* hatching at 2 h intervals from (A) 300 eggs exposed to LL and (B) 280 eggs incubated in DD. Note that eggs incubated in LL had never been exposed to darkness and those incubated in DD had never been exposed to light. White bars in histogram indicate larvae that hatched during hours of darkness. Symbols and bottom panels as in Fig. 1.

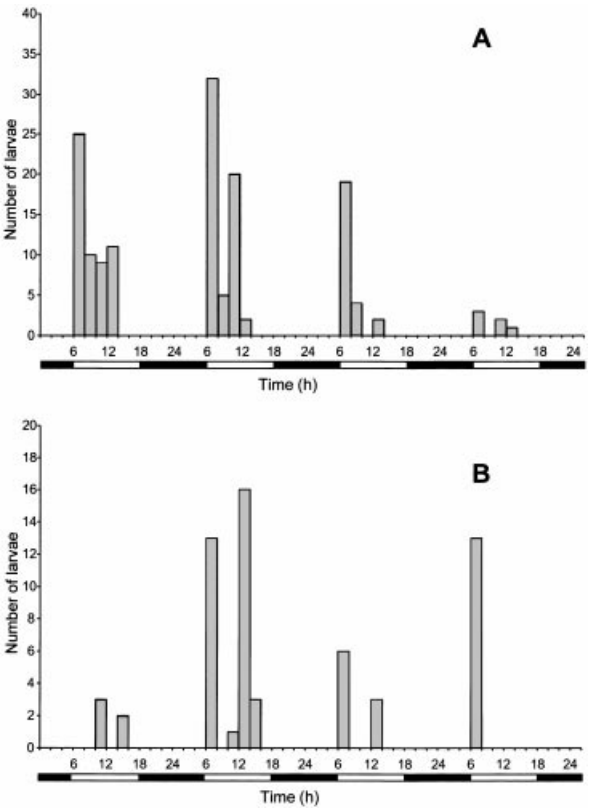

Fig. 5. Number of larvae hatching at 2 h intervals from (A) 192 *Troglocephalus rhinobatidis* eggs incubated in LD 12:12 and (B) 132 *Merizocotyle icopae* eggs incubated in LD 12:12. Bottom panels as in Fig. 1B.

transferred to DD when eyespots were observed, also started hatching 9 days after collection. The hatching pattern was similar to that seen in the DD experiments outlined above (e.g. Fig. 4B). Hatching occurred only during the period corresponding to daylight (between 06.00 h and 18.00 h) for 4 consecutive days. When no further hatching occurred, 55 potentially viable eggs remained unhatched. These eggs were exposed to light in the cabinet at 12.00 h and 31 larvae emerged during a 15 min period. Eggs in Batch C (100 eggs) were incubated similarly to Batch B and began to hatch 9 days after collection. Hatching occurred only during the period corresponding to light and at the end of the experiment, 40 potentially viable eggs remained. The remaining eggs were exposed to light in the cabinet at midnight (0.00 h) for 15 min and stronger light from the stage of a microscope for 15 min, but no hatching occurred. This was repeated on the following 3 nights but still no hatching occurred.

Hatching in T. rhinobatidis *and* M. icopae

Fewer adults of *T. rhinobatidis* and *M. icopae* were available. Furthermore, these parasites laid smaller numbers of eggs than *N. rhinobatidis* and therefore not all experimental light regimes were tested with eggs of these species.

Natural and LD 12:12

Eggs of *M. icopae* exposed to natural light regimes hatched only during daylight hours. The hatching rhythm of *T. rhinobatidis* was not determined when exposed to natural illumination. Eggs of *T. rhinobatidis* and *M. icopae* incubated in LD 12:12 hatched only during daylight hours (Fig. 5A and B, respectively). Although most larvae emerged during the first 2 h of light, the initial hatching peak was not as prominent as that observed for *N. rhinobatidis*. *T. rhinobatidis* eggs began to hatch after 7 days and 75% of the eggs hatched by the completion of the experiment (Table 2). *M. icopae* eggs commenced hatching after 8 days and 54% of the eggs hatched (Table 2).

LL and DD

The effect of the reverse light/dark regime (DL 12:12) was not examined for these 2 species. Eggs of *T. rhinobatidis* and *M. icopae* laid and incubated in LL hatched only during times that would have been

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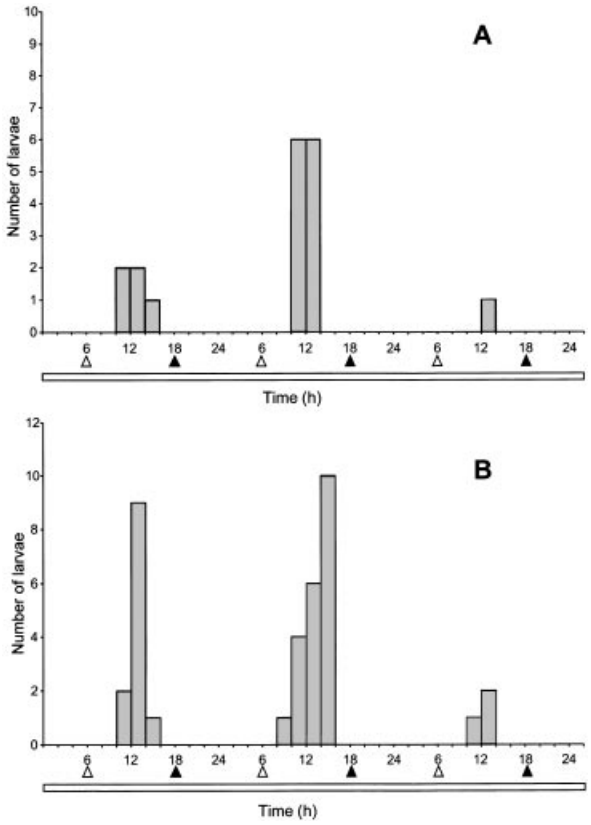

Fig. 6. Number of larvae hatching at 2 h intervals from (A) 50 *Troglocephalus rhinobatidis* eggs incubated in LL and (B) 100 *Merizocotyle icopae* eggs incubated in LL. Note that these eggs were never exposed to darkness. Symbols and bottom panels as in Fig. 1.

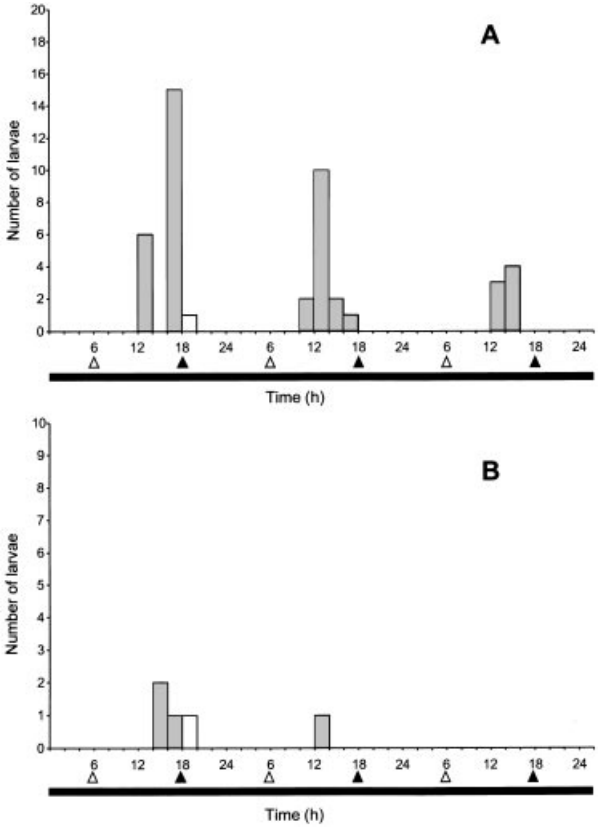

Fig. 7. Number of larvae hatching at 2 h intervals from (A) 128 *Troglocephalus rhinobatidis* eggs incubated in DD and (B) 70 *Merizocotyle icopae* eggs incubated in DD. Note that these eggs were moved into DD after being laid for 24 h in LD 12:12. Symbols and panels as in Fig. 1.

light in the natural regime (Fig. 6A and B, respectively). Hatching times shifted from early morning to later in the morning or early afternoon as seen for *N. rhinobatidis* and the hatching success rates were low (35%) for both species (Table 2). Adult *T. rhinobatidis* and *M. icopae* laid very few eggs in darkness and therefore adults used for the DD experiments were exposed to a LD 12:12 light regime during the 24 h laying period; the eggs were then transferred directly to DD. Only 1 *T. rhinobatidis* larva emerged during what would have been darkness and all others (hatching success 34%; Table 2) emerged between 06.00 h and 18.00 h (Fig. 7A). Hatching success for *M. icopae* eggs in DD was very low (7%) and only 5 of 70 eggs collected hatched (Table 2). Although 4 of the 5 larvae emerged between 06.00 h and 18.00 h, no significant patterns could be determined because of the poor hatching success (Fig. 7B). We were unable to repeat this experiment.

Effect of other potential hatching stimulants on the 3 monocotylid species

After the regular spontaneous and rhythmical daily hatching had commenced, test batches of eggs were exposed to disturbance or host tissue between 18.00 h and 06.00 h when hatching had been shown not to occur (i.e. during the hours of darkness) to determine if any of these cues could induce hatching. Other test batches were subjected to shadows between 16.00 h and 18.00 h when hatching had been found to occur rarely in LD 12:12 experiments (see above). There was no indication that shadows or disturbance induced hatching in any of the 3 species examined. There was no hatching in *N. rhinobatidis* or *T. rhinobatidis* when eggs were exposed to host tissue at night but 2 *M. icopae* larvae emerged when eggs were exposed to tissue from the abdominal region of the host during darkness.

DISCUSSION

Although larvae of 20 monocotylid species are described, this is only the second study to investigate specifically egg hatching in monocotylid monogeneans. We have determined that eggs of *N. rhinobatidis*, *T. rhinobatidis* and *M. icopae* hatch with a rhythm linked to light periodicity and that larvae emerge only during daylight hours. There is a distinct hatching peak during the first 2 h of light for *N. rhinobatidis*, but this peak is not as pronounced in the other 2 species. This is the first indication that

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light periodicity is an important hatching cue in monocotylids. Egg hatching rhythms have now been reported for 16 monogenean species including the 3 species investigated by us here (Whittington *et al.* 2000). In some instances, these rhythms have been linked to host behaviour and larval emergence occurs when the chances of meeting the specific host are enhanced. For example, Kearn (1973) showed that the larvae of *Entobdella soleae* emerge during the first 4 h of daylight when the flatfish host, *Solea solea*, is known to be inactive. *Polystoma integerrimum* eggs are deposited in shallow waters of ponds and hatch during the day when the tadpole hosts congregate in the warm shallow waters (Macdonald & Combes, 1978). *Discocotyle sagittata* larvae emerge during the first 2 h of darkness when the host, *Oncorhynchus mykiss*, is known to be inactive (Gannicott & Tinsley, 1997). In this case, however, Gannicott & Tinsley (1997) demonstrated that hatching could also occur during daylight when eggs were exposed to host tissue. Therefore, host-induced hatching could override the strict nocturnal hatching rhythm. Unfortunately, we cannot relate the hatching rhythm we have demonstrated for the 3 species of monocotylids to the behaviour of their host *R. typus* because there is no information on the daily activity pattern of the shovelnose ray. However, if hatching is an adaptive response to host behaviour, we can infer from our results that these rays may be inactive early in the day. This remains to be tested. Alternatively, predation may be a selective force behind the hatching patterns we have found. Ernst & Whittington (1996) suggested that the daily hatching rhythms, seen in the capsalid monogeneans *Benedenia lutjani* and *B. rohdei*, could be the result of predation pressure exerted by invertebrate filter feeders. They are prolific on coral reefs and have been shown to increase their feeding activities at night (Porter, 1974; Sebens, 1977). The study by Ernst & Whittington (1996) was the first on hatching rhythms of monogeneans from reef fish and they suggested that if monogeneans from other reef hosts exhibited a similar hatching rhythm, then this might be further support for the predation hypothesis. Our data, therefore, support this hypothesis, but further studies of monogenean hatching patterns from typical reef fishes are required. We have also determined that eggs from the monocotylid *Empruthotrema quindecima* appear to hatch only when exposed to tissue from the blue-spotted fantail ray (Chisholm & Whittington, unpublished data). This is a different hatching strategy, but in this instance, the ciliated larvae have little risk of being preyed upon because they will hatch in close proximity to the host and likely infect it immediately.

It is noteworthy that egg hatching in the monocotylid species examined here occurs over a number of consecutive days. This has also been recorded in other monogenean species and could be an adaptive response to the behaviour of the host. Whether a host is in close proximity to eggs when rhythmical hatching occurs is likely to be unpredictable. Hatching over a number of days, therefore, may confer an advantage to the parasite and increase the probability of larvae coming in contact with a potential host. Unfortunately there is no information on the behavioural pattern or distributional range of shovelnose rays around Heron Island. We have observed that juvenile shovelnose rays usually congregate in Shark Bay on a rising tide, but it is not known where they go at other times of the day. Alternatively, hatching over consecutive days could be the result of genetic variability in the development time of eggs laid by individual worms. However, we found that eyespots usually appeared in all eggs collected over a 24 h period on the same day.

The eggs of the monocotylid *Dictyocotyle coeliaca* were shown to hatch spontaneously, but there was no evidence of a daily hatching rhythm because larvae emerged continuously over a period of 7 weeks during both day and night periods (Kearn, 1975). Only 1 other monogenean species from an elasmobranch, the hexabothriid *Rajonchocotyle emarginata* from a number of *Raja* species, has been shown to hatch with a diurnal rhythm, but in this species hatching occurs mostly during darkness (Whittington & Kearn, 1986). Indirect evidence suggests, however, that light may play an important role in egg hatching for many other monocotylid species. In our own studies, we have found that eggs of *Clemacotyle australis* (see Beverley-Burton & Whittington, 1995), *Decacotyle floridana* (see Chisholm & Whittington, 1998*b*), *Dendromonocotyle ardea* (see Chisholm & Whittington, 1995), *Heterocotyle capricornensis* (see Chisholm & Whittington, 1996*a*), *Merizocotyle australensis*, *Monocotyle helicophallus*, *M. spiremae* (see Chisholm & Whittington, 1996*b*), *Neoheterocotyle rhinobatis* and *N. rhynchobatis* (see Chisholm & Whittington, 1997) all hatch spontaneously with greater numbers of larvae present in the dishes early in the mornings (Chisholm & Whittington, unpublished observations). These eggs do not require stimulation by disturbance, shadowing or host secretions. Furthermore, larvae of these species continue to emerge for a number of days after the initial hatch. The presence of a hatching rhythm in these species needs to be investigated further. Kearn (1968) also noted that eggs of *Merizocotyle* sp. (= *M. undulatae*) hatched spontaneously, but that there were too few eggs to establish a hatching pattern. *Monocotyle pricei* and *Calicotyle quequeni* also appear to hatch spontaneously (see Kingston, Dillon & Hargis, 1969 and Suriano, 1977, respectively).

The eggs of monocotylid species from the shovelnose ray were also exposed to shadows, disturbance and host tissue/secretions. Eggs of neither *N. rhinobatidis* nor *T. rhinobatidis* showed

any hatching response to these cues, but 2 larvae of the merizocotyline *M. icopae* did emerge when exposed to host tissue during a period of darkness. This warrants further investigation because there are also indications that eggs from other members of the Merizocotylinae hatch in the presence of host tissue. Eggs of *Empruthotrema raiae* appeared to be fully developed after 20 days, but hatching did not occur spontaneously and larvae had to be extracted from eggs by cover-slip pressure before they could be described (Kearn, 1976). These eggs did not hatch when manipulated, which suggests that disturbance is not a cue and therefore perhaps exposure to host secretions may induce hatching. As mentioned above, preliminary investigations have revealed that *E. quindecima* from nasal tissue of the blue-spotted fantail ray, *Taeniura lymma*, hatches only in the presence of fresh host tissue (Chisholm & Whittington, unpublished observation). Mechanical disturbance may induce hatching in *Dendromonocotyle kuhlii*. Young (1967) described spontaneous hatching of *D. kuhlii* with larvae emerging after 5 days at 70–80 °F (= 21–27 °C). Kearn (1986), however, reported an incidental observation that *D. kuhlii* eggs hatched rapidly when disturbed with a fine needle, but stated that this required more rigorous investigation.

Circadian rhythms have an endogenous nature. They persist in constant environmental conditions and can be phase-shifted by altering the light-dark cycle (Goldbetter, 1996). Thus, the hatching rhythm we have reported for 3 monocotylid species can be considered a true endogenous circadian rhythm. The rhythm persists even in eggs that have been laid and incubated in total light or darkness, but can be altered by changing the pattern of the light/dark cycle as demonstrated by the reverse light/dark (DL 12:12) experiment. Ernst & Whittington (1996) also found that eggs of *B. lutjani* and *B. rohdei* hatched with a rhythm even when maintained in total darkness. They discuss in detail the ramifications of this finding and hypothesize that *Zeitgebers* other than light periodicity may be involved in synchronizing the circadian clocks (Ernst & Whittington, 1996). Our investigation and the study by Ernst & Whittington (1996) suggest strongly that eggs of at least some monogenean species can monitor more than 1 environmental cue. If this is the case, whatever cue entrains the circadian rhythm must have the same periodicity as the LD cycle. Other *Zeitgebers* that could assume importance for monogenean eggs may include tidal amplitude and phase (Ernst & Whittington, 1996) and ambient temperature variation. In the present study there was no correlation between tidal phase and egg hatching. Our experiments were conducted at 22–26 °C which was within the ambient water temperature range around Heron Island (21–27 °C) for 1998–1999. Perhaps natural day and night differences in ambient seawater temperature around Heron Island provide a cue, but we have no data for this and expect differences would be small. Other cues that may act as *Zeitgebers* in the environment where monogenean eggs are found are unknown, but a hierarchy of different cues that gain dominance in the absence of more important and common *Zeitgebers* such as LD periodicity, may exist. The identity and importance of these other *Zeitgebers* remain to be identified for monogeneans. Alternatively, Ernst & Whittington (1996) suggested that components of the monogenean egg may already possess the circadian rhythm from their parents. In the absence of light periodicity, this inherited rhythm may control egg hatching (Ernst & Whittington, 1996). This is a conceivable scenario since circadian rhythms exist at every level of cellular organization (Edmunds, 1988).

Gannicott & Tinsley (1997) determined that *Discocotyle sagittata*, parasitic on the gills of the rainbow trout *Oncorhynchus mykiss*, also hatched with a distinct rhythm linked to light. The majority of larvae incubated in LD 12:12 hatched during the first 2 h of darkness, but hatching became arhythmic when eggs incubated in LD 12:12 were transferred to DD when hatching commenced. The rhythm seemed to be maintained when eggs were incubated in LD 12:12 and then transferred to LL when hatching began. It is unknown why this occurred. Hatching of *Polystoma integerrimum* is rhythmical when eggs are incubated in LD 12:12 with eggs hatching during daylight hours (Macdonald & Combes, 1978). This hatching rhythm can be entrained because eggs first incubated in LD 12:12 and then transferred to LL or DD continued to hatch with a rhythm. However, the rhythm was lost when eggs were laid and incubated in 24 h light or 24 h dark (Macdonald & Combes, 1978). In these 2 monogenean species, therefore, the observed hatching pattern does not appear to be endogenous because the rhythm is not maintained in constant environmental conditions and can be modified by exogenous light regimes.

Larval emergence was delayed by 1 month when eggs of *P. integerrimum* were incubated in total darkness at low temperatures (Macdonald & Combes, 1978). Similarly, Bychowsky (1957) found that hatching was delayed when eggs of a number of *Axine* species were incubated in DD. There was no indication in the present study that incubation of eggs in DD delayed hatching in any of the 3 species examined. However, we did observe that hatching continued for 7 days and that hatching success was reduced greatly in eggs incubated in darkness. Although the daily hatching rhythm is maintained, many potentially viable eggs that remained failed to hatch. When the remaining apparently viable eggs of *N. rhinobatidis* from DD experiments were exposed to light at 12.00 h, over 50% hatched. However,

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when unhatched eggs were exposed to light at 0.00 h no hatching occurred. These results indicate that hatching of *N. rhinobatidis* may be stimulated by exogenous light cues, but only if the cue is provided during what would have been a light period in the natural regime. This hatching strategy may impart an advantage to the parasite. If the eggs were buried deep in sediments at the seabed and not exposed to light, it might be detrimental to hatch during the normal light cycle since larvae may be trapped in the interstices. If the sediments were disturbed (for example, by a shovelnose ray burying itself at the seabed) and eggs were resuspended to the surface of the seabed or into the seawater, hatching may then be advantageous. This scenario may only be beneficial during daylight since other adaptive responses, which may relate to host behaviour or predation pressure as discussed above, are obviously dominant.

The similarity in the hatching strategies of *M. icopae* from the nasal tissues and *N. rhinobatidis* and *T. rhinobatidis* from the gills of the same host species is noteworthy. Hatching patterns have been investigated for a number of monogenean species which inhabit different sites on the same host. Eggs of *B. lutjani* from the skin and *B. rohdei* from the gills of yellow stripey, *Lutjanus carponatatus*, hatch spontaneously during daylight hours (Ernst & Whittington, 1996). Similarly, eggs of the microbothriid *Leptocotyle minor* from the skin and the hexabothriid *Hexabothrium appendiculatum* from the gills of the dogfish, *Scyliorhinus canicula*, hatch only in response to the presence of host tissue (Whittington, 1987). In these 2 instances, therefore, the pattern of hatching or the hatching cue is the same for monogenean species from the same host species. However, this is not always the case. *Dictyocotyle coeliaca* from the body cavity and *Acanthocotyle lobianchi* from the skin can share the same host species, *Raja naevus*, but the unciliated larvae of *A. lobianchi* emerge only in the presence of host skin mucus (Macdonald, 1974). As discussed above, eggs of *D. coeliaca* hatch spontaneously and arhythmically over a 7-week period. Kearn (1975) hypothesized that the host, *R. naevus*, may lack a distinct daily activity pattern and suggested that both of these hatching strategies reflect an adaptation to this arhythmical pattern. Eggs of *B. seriolae* and *Heteraxine heterocerca* from the skin and gills respectively of the yellowtail, *Seriola quinqueradiata*, hatch in response to light periodicity, but their hatching patterns differ (Kearn, Ogawa & Maeno, 1992). When incubated in identical conditions, *B. seriolae* eggs hatched during daylight hours whereas eggs of *H. heterocerca* had a distinct hatching peak at dusk and continued to hatch throughout the night. Kearn *et al.* (1992) suggested that the difference in hatching strategies may reflect potential differences in the larval invasion sites. This hypothesis assumes that the observed hatching patterns are an adaptive response to host behaviour and that the best time for invading gills may not be the best time for invading skin. Following this reasoning, perhaps the similarity between the hatching strategies for the 3 species of monocotylids from the shovelnose ray indicates that they may all have a similar route of infection. This remains to be investigated.

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