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VERSITA

RESEARCH NOTE

# Using the giant Australian cuttlefish (*Sepia apama*) mass breeding aggregation to explore the life cycle of dicyemid parasites

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## Abstract

Dicyemid mesozoan parasites, microscopic organisms found with high intensities in the renal appendages of benthic cephalopods, have a complex, partially unknown life cycle. It is uncertain at which host life cycle stage (i.e. eggs, juvenile, adult) new infection by the dispersive infusoriform embryo occurs. As adult cephalopods have a short lifespan and die shortly after reproducing only once, and juveniles are fast-moving, we hypothesize that the eggs are the life cycle stage where new infection occurs. Eggs are abundant and sessile, allowing a huge number of new individuals to be infected with low energy costs, and they also provide dicyemids with the maximum amount of time for survival compared with infection of juvenile and adult stages. In our study we collected giant Australian cuttlefish (*Sepia apama*) eggs at different stages of development and filtered seawater samples from the *S. apama* mass breeding aggregation area in South Australia, Australia, and tested these samples for the presence of dicyemid DNA. We did not recover dicyemid parasite cytochrome *c* oxidase subunit I (*COI*) nucleotide sequences from any of the samples, suggesting eggs are not the stage where new infection occurs. To resolve this unknown in the dicyemid life cycle, we believe experimental infection is needed.

## Keywords

Dicyemida, infusoriform embryo, cephalopod host, life cycle, dicyemid *COI* gene, *Sepia apama*

Benthic cephalopods, including the giant Australian cuttlefish, *Sepia apama*, are hosts to a group of small, worm-like parasites, known as dicyemid mesozoans (Dicyemida Van Beneden, 1876), which infect the renal appendages at high intensities (Furuya *et al.* 2004). Although 122 species of dicyemids have been described to date (Catalano 2012, 2013a, 2013b, Catalano and Furuya 2013), their life cycle remains partially unknown. It is uncertain at which host life cycle stage (i.e. eggs, juvenile, adult) new infection by the dispersive stage (infusoriform embryo) occurs. Furthermore, the route of infection this embryo takes from the environment to the renal appendages of a new host individual is unknown. Note that past experimental studies have shown there is no intermediate host and that the infusoriform embryo is released from adult host individuals into the environment (see Lapan and Morowitz 1975). For a detailed review of the life cycle of dicyemid mesozoans, including information on the two stages of development and two modes of reproduction, see McConnaughey (1951) and Furuya *et al*. (2003).

As the infusoriform embryo is microscopic with an average length of 32–36 µm, average width of 26–28 µm and average depth of 24–25 µm (McConnaughey 1951), we suggest the best chance of such a tiny embryo finding and infecting a new host individual is when there is a high host density, for example, when cephalopods aggregate to breed. However, infection of an adult individual during breeding may be unfavourable because cephalopods only live for one to two years and die shortly after reproducing (Semmens *et al.* 2007).

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Therefore another host life cycle stage that is also abundant during breeding and may provide a better chance of survival beyond the eventual death of an adult is the host's newly deposited eggs. Eggs are sessile, meaning less energy expenditure by the infusoriform embryo to find, attach to and infect this stage. Eggs also provide the longest time period for survival compared to infection of host juvenile and adult stages.

*Sepia apama*, the largest species of cuttlefish in the world, is widely known for its unique mass breeding phenomenon (Kassahn *et al.* 2003). Each year between April and July, *S. apama* congregate in their thousands on small shallow rocky reefs in Upper Spencer Gulf (USG), South Australia (SA), Australia, to spawn (Norman *et al.* 1999, Kassahn *et al.* 2003). The USG breeding aggregation is the densest cuttlefish spawning aggregation reported in the world, with up to 105 cuttlefish per 100 $m^2$ (Hall and Hanlon 2002), and subsequently represents an ideal location and opportunity to explore the dicyemid life cycle. Note that in the years 2005, 2010 and 2011, we collected *S. apama* individuals from USG and examined them for dicyemid parasites. All 32 *S. apama* individuals, with total lengths ranging from 16.5 cm to 31 cm, were found to be infected by dicyemid parasites (Catalano 2013b, Catalano *et al.* unpublished result).

Therefore to examine the hypothesis that eggs are the host life cycle stage where new infection occurs, we collected *S. apama* eggs at different stages of development from the mass breeding aggregation in USG, SA, Australia, as well as filtered seawater (SW) samples before, during and after cuttlefish arrived in the area and tested for the presence of dicyemid DNA.

Specifically, between 15 and 30 eggs were collected each month from the underside of flattened rocks from July to October 2011 at three localities in False Bay, USG, SA, following Cronin and Seymour (2000) (see Table I). One hundred litres of filtered SW were also collected from Stony Point (see Table I) on five occasions in 2011 and six occasions in 2012 corresponding to periods before, during and after cuttlefish breed. Ten circular test sieves of 20 cm diameter were stacked upon each other vertically in descending order from coarse to fine mesh sizes (4 mm, 2 mm, 500 µm, 250 µm, 125 µm, 68 µm, 53 µm, 38 µm, 30 µm and 20 µm). The SW was slowly poured through the sieve stack and samples were collected from the 53 µm, 38 µm, 30 µm and 20 µm mesh size sieves. Filtered SW was stored in a 50 mL Falcon tube to which 45 mL of 100% undenatured ethanol was added.

DNA was extracted from eggs and filtered SW. For the eggs, a total of 20 egg membranes, 20 inside yolk sacs (from newly deposited eggs with undeveloped larvae; July and August 2011 collections) and 30 miniature cuttlefish at different stages of development (from eggs collected in September and October 2011) were extracted following the Gentra Kit (Gentra Systems) protocol, with a final elution volume of 50 µl in TLE. Total lengths were recorded for each of the fully formed cuttlefish from which DNA was extracted for September and October 2011 (see Table II). Dissection equipment was cleaned and sterilized in ethanol for each new egg to avoid cross contamination. For the filtered SW samples, each tube was spun at 1000 g for 10 min at room temperature, braking speed of 2, in a low speed bucket centrifuge (Centrifuge 5810R, Rotor A-4-81, Eppendorf). Excess ethanol was removed with a Pasteur pipette, 5 mL of 10 mM Tris was added and tubes were inverted 20 times to re-suspend the pellet. The spin and Tris wash steps were repeated twice, and then the pellet was re-suspended in 1 mL 10 mM Tris before transfer to a labeled 1.5 mL eppendorf tube. All remaining extraction steps followed the Gentra Kit protocol, with a final elution volume of 50 µl in TLE.

Each extract diluted 1:100 was tested with two cytochrome *c* oxidase subunit I (*COI*) primer pairs that are known to amplify partial dicyemid parasite DNA (from *S. apama* kidney samples): M1425 5'-GTTTTTTGGACATCCTGAGGT-3' and reverse M1426 5'-AGGACATAGTGGAAGTGTGCTACAAC-3' from Watanabe *et al.* (1999) (400 bp fragment), and newly designed primer pair M1435 5'- GCCTTATTTTAGTACAGTGTGC-3' and reverse M1436 5'-CGAGTATCAATATCTATACCAGATG-3' (1,000 bp fragment). Amplification reactions were conducted in a final volume of 25 µl containing 2.5 µl of GeneAmp 10x PCR Buffer II (Applied Biosystems, Inc. [ABI]), 4 µl of 25 mM $MgCl_2$ (ABI), 2 µl of 10 mM dNTP, 1 µl of each primer at 5 mM, 0.10 µl of AmpliTaq® Gold (ABI), 0.10 µl of BSA at 200 ng/µl and 2.5 µl gDNA extract. Cycling conditions were 95°C for 10 min with 35–40 cycles of amplification (94°C for 45 s, 50–52°C for 45 s and 72°C for 1–2 min). Negative controls were included in

**Table I.** Collection and locality data for monthly *Sepia apama* egg sampling. Abbreviations: SA, South Australia; USG, Upper Spencer Gulf

| Collection | Date collected | Locality | Latitude<br>Longitude |
|---|---|---|---|
| 1 | 12th July 2011 | 1.1 km east of Black Point, False Bay, USG, SA | 32°59′ 36.7″S<br>137°43′ 56.4″E |
| 2 | 11th August 2011 | Stony Point, False Bay, USG, SA* | 32°59′ 45.1″S<br>137°45′ 6.08″E |
| 3 | 14th September 2011 | Black Point, False Bay, USG, SA | 32°59′ 28.73″S<br>137°43′ 14.38″E |
| 4 | 12th October 2011 | Black Point, False Bay, USG, SA | 32°59′ 28.73″S<br>137°43′ 14.38″E |

*Locality of filtered seawater collections

**Table II.** Total lengths (mm) for extractions of miniature fully formed *Sepia apama* from September and October 2011 egg samples. Abbreviation: BC, Baby Cuttlefish

| 14th September 2011 egg samples | | |
|---|---|---|
| **Number** | **BC number** | **Total length** |
| 1 | BC7 | 9 |
| 2 | BC8 | 10 |
| 3 | BC9 | 11 |
| 4 | BC10 | 9 |
| 5 | BC11 | 8 |
| 6 | BC12 | 9 |
| 7 | BC13 | 14 |
| 8 | BC14 | 12 |
| 9 | BC15 | 6 |
| 10 | BC16 | 9 |
| 11 | BC17 | 10 |
| 12 | BC18 | 7 |
| 13 | BC19 | 13 |
| 14 | BC20 | 5 |
| 15 | BC21 | 6 |
| **12th October 2011 egg samples** | | |
| **Number** | **BC number** | **Total length** |
| 1 | BC1 | 28 |
| 2 | BC2 | 26 |
| 3 | BC3 | 28 |
| 4 | BC4 | 25 |
| 5 | BC5 | 22 |
| 6 | BC6 | 20 |
| 7 | BC22 | 17 |
| 8 | BC23 | 19 |
| 9 | BC24 | 16 |
| 10 | BC25 | 20 |
| 11 | BC26 | 18 |
| 12 | BC27 | 23 |
| 13 | BC28 | 10 |
| 14 | BC29 | 10 |
| 15 | BC30 | 21 |

each reaction. PCR products were visualized with UV following agarose gel (1.5%) electrophoresis and staining for 30 min with GelRed (Biotium). Products were cleaned using a Multi Screen Vacuum Manifold and 384-well Multi Screen Filter Plate (Millipore), then sequenced with the forward primer only using dye terminator chemistry (BigDye Terminator v3.1 cycle-sequencing kit, ABI). Sequence alignments, error correction and similarity analysis (neighbour-joining trees) were performed using Geneious Pro 5.3.4 (Drummond *et al.* 2010 – Biomatters Ltd).

All egg membranes, yolk sacs, miniature cuttlefish and filtered SW samples tested with dicyemid specific primers M1435/M1436 did not produce PCR products. Using dicyemid specific primers M1425/M1426, 26 samples were positive corresponding to 1 egg membrane (0/10 July 2011, 1/10 August 2011), 8 yolk sacs (0/10 July 2011, 8/10 August 2011), 12 miniature cuttlefish (0/15 September 2011, 12/15 October 2011) and 5 filtered SW samples (20 µm, 38 µm April 2012; 38 µm, 53 µm August 2012; 38 µm September 2012; the remaining 39 filtered SW samples were negative). The 353 bp of *COI* sequences for these 26 samples were identical (representative sequence deposited in GenBank™, accession no. JX983108. However, when compared to sequences on GenBank™ and those obtained by the authors from dicyemid-infected *S. apama* kidney samples (Catalano *et al.* unpublished results), these sequences were clearly highly divergent from dicyemid *COI*. Rather this short fragment was 86% homologous to *COI* of the soil bacterium, *Mycobacterium rhodesiae.*

With no amplification of dicyemid DNA from any of the collected samples, our finding tends to refute the hypothesis that new infection by the infusoriform embryo is at the egg life cycle stage. However, in the years we sampled (2011 and 2012), cuttlefish numbers were reduced in USG compared to previous years, indicating dicyemid density in the environment may also have been low and may explain our result of not detecting dicyemid DNA in filtered SW samples. If these parasites were transmitted vertically though, we would expect to detect dicyemid DNA in the egg samples irrespective of the reduction in numbers at the mass breeding site. Nonetheless, bacterium DNA was amplified and the observed result may simply be explained by saturation of bacteria in the sample compared to parasites, meaning the detection of dicyemid DNA has been masked and subsequently missed even though they are present. Clearly a more reliable method is needed to address the challenging unknowns that still surround the dicyemid life cycle. A potential infection route survey could be undertaken, with different host tissues tested using the same dicyemid specific primers in this study. However, we do not believe such a test would be informative or enlightening. Larger sized adults may not be continuously infected, but instead infected when quite small and not re-infected since. Therefore such a route survey may return negative results, when instead the wrong sized host is being sampled. To shed further light on this conundrum, the first requirement is to establish when infection occurs and at what host life cycle stage. Then this stage could be targeted to establish the unknown route.

Lapan and Morowitz (1975) exposed uninfected *Sepia*, raised from eggs in isolated aquaria, to infusoriform embryos and found small vermiforms in the renal coelom. Successful infections, however, were in the order of 10% compared to 100% in nature, and they explicitly stated that their observations 'are not intended to represent firm experimental evidence'. Nonetheless, based upon this observation, we believe future research should follow on from Lapan and Morowitz (1975) to complete the life cycle of dicyemid parasites. Experimental infection in tanks is required. Such an experiment would provide a meaningful assessment of both the host life cycle stage whereby new infection occurs as well as the entry

route, allowing the complete life cycle of this bizarre group of poorly understood parasites to be resolved.

### Ethical Note

Over one spawning season, each female cuttlefish can lay hundreds of eggs. From a past study by Naud *et al.* (2004), conservative estimates of fecundity and female population size yielded a total egg production of approximately 9,500,000 eggs per annum for an estimated 28,000 females. This equates to approximately 339 eggs per female. Hence, the total maximum number of 120 eggs collected in this study represents a very small proportion of the total egg production (over 200 eggs less than what one female may lay based upon the conservative estimates above) and should have little, if any, effect on population viability.

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