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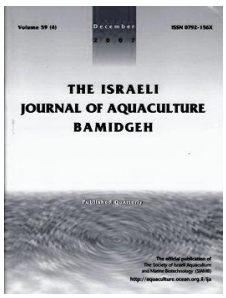

The Development of a Small-scale Laboratory System to Study *Cryptocaryon irritans* Infection in Seawater-adapted Guppies, *Poecilia reticulata*

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Keywords: Cryptocaryon irritans, guppy, sea-water adaptation, histopathology

Abstract

The protozoan parasite *Cryptocaryon irritans* is responsible for significant economic losses of commercially farmed marine fishes. In this study we report, for the first time, *C. irritans* infection in the seawater-adapted guppy *Poecilia reticulata*. We describe a simple method for experimental propagation and maintenance of the infection in a laboratory, without apparent loss of infectivity, for15 months. Fish were gradually adapted to increasing concentrations of artificial seawater by successive transfer to incremental steps of 10 ppt every 2 days and then transferred to disease propagation aquaria (salinity 30 ± 2 ppt) where the infection was maintained. Between 2-3 fish were held at any one time in each aquaria, each fish being replaced once it succumbed to the infection and died. Gross observations of heavily infected fish revealed a uniform distribution of trophonts visible on the skin and fins as white surface lesions or 'spots' after 4-5 days. Death of the host usually occurred after 7-8 days. Heavily infected fish were fixed and processed using routine histological techniques and histological examination revealed infective trophonts, round or pyriform in shape, invading the fish epithelium as well as larger, feeding trophonts located beneath the epithelium. A thickening of the epithelial layers and a proliferation of mucous secreting cells was evident around embedded parasites.

Introduction

Cryptocaryon irritans (Brown) is a marine ciliated protozoan parasite causing 'white spot disease' (Colorni and Burgess, 1997). It has a very wide host range and can infect almost all marine teleosts (Colorni, 1985). Its life cycle is temperature dependent and comprises of four morphologically distinct developmental stages (Colorni, 1987; Burgess and Matthews, 1994). The trophont or parasitic stage resides within the epidermis of the fish and feeds on the epithelial layer of the skin, fins and gills. It leaves the fish host upon maturity as a free-swimming prototomont, and, settling on a suitable substrate, it then transforms to the tomont or cyst stage by secreting a double layered cyst wall. Inside the tomont numerous small theronts are produced and released as the free-swimming infective stage which contact fish skin and penetrate into the epidermis, where

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they settle as trophonts and complete the life cycle. Elevated numbers of trophonts will cause mass mortalities due to disruption in osmotic balance, asphyxiation and secondary bacterial infection (Diamant *et al.*, 1991; Dickerson, 2006). With the growth of the mariculture industry it has become one of the most common and persistent diseases causing significant economic losses. However few effective treatments and control measures have been developed against *C. irritans* infection (Yoshinaga *et al*., 2011). Therefore a simple, laboratory-scale system that allows the mass propagation of this parasite will benefit studies that are aimed at developing relevant treatments and control measures.

Guppies (*Poecilia reticulata* (Peters)), native to parts of the Caribbean and the South America continent, are small, live-bearing, tropical freshwater fish. Due to their diversified colouration and ease of culture they have become one of the most commercially important aquarium fish in the ornamental aquaculture industry. Guppies are euryhaline and their ability to withstand elevated salinities has previously been reported (Gibson and Hirst, 1955; Depeche and Schoffeniels, 1975; Chervinski, 1984; Shikano *et al.,* 2001). Laboratory propagation of *C. irritans* has hitherto been described in juvenile sea bream (*Sparus aurata*) (Colorni, 1985), the salt-water adapted black molly (*Poecilia latipinna*) (Yoshinaga and Dickerson, 1994), the grey mullet (*Chelon labrosus*) (Burgess and Matthews, 1994) and the Pompano (*Trachinotus ovatus*) (Dan *et al*., 2006). Whilst the development of an *in vitro* technique using a medium for propagating *C. irritans* through the four stages of its lifecycle has made advances into removing the need for a fish model (Yoshinaga *et al.*, 2007), limitations in the methodology were reported, such as a loss of viability of the parasites during the process and the requirement for live fish for initial parasite inoculation of the medium.

The current study reports a simple and efficient system that allows the passage and propagation of the marine parasite *C. irritans* using the sea-water adapted guppy as a model. In addition we describe host-parasite interactions at the tissue level in guppies as a result of heavy infection with *C. irritans*, of which there is currently limited histological data (Dickerson, 2006).

Materials and methods

Seawater adaptation of guppies. Guppies (0.4–0.06 g), used as experimental hosts in this study, were obtained from a commercial aquaculture farm in the Arava valley, Israel. Experimental salinities were prepared using freshwater, de-chlorinated with the use of 50 mg/L of sodium thiosulphatepentahydrate (William Blythe, Accrington, UK) with the addition of artificial salt (Red Sea Fish Pharm Ltd., Eilat, Israel). Fish were gradually adapted to increasing concentrations of artificial seawater by successive transfer through aquaria equipped with individual biological filters and aeration in incremental steps of 10 ppt every 2 days. Fish were finally transferred to a standard marine aquarium (temp. 28 ºC; salinity 30 ± 2 ppt) where they remained for a minimum of 2 days before being transferred to disease propagation aquaria. Water quality was monitored daily.

Parasite isolation and propagation. Cryptocaryon irritans theronts were obtained from Dr. Angelo Colorni, Israel Oceanographic and Limnological Research (IOLR), Eilat, Israel and were used to infect seawater-adapted guppies. The infection was propagated in three 10 L aquaria (temp. 28 ºC; 30 ± 2 ppt) equipped with individual biological filters and aeration; between 2-3 fish were held at any one time in each aquaria each fish being replaced once it succumbed to the infection and died.

Histopathology. A total of 15 heavily infected fish were examined macroscopically under a dissecting microscope. Five fish were then euthanised in 0.025% clove oil (Kildea *et al*. 2004), fixed in 10% buffered formalin and processed using routine histological techniques. The slides were stained with H&E and photographed using a Zeiss Axiocam MRc5 fitted to a Zeiss microscope (Axioskop).

Results

Propagation of C. irritans on guppies. C. irritans was successfully maintained for 15 months in successive cycles on sea-water adapted guppies (temp. 28 ºC; salinity 30 ± 2

ppt). Gross observations revealed a uniform distribution of large round to ovoid shaped ciliates or trophonts on both body and fins of heavily infected fish 4-5 days after introduction to the infection aquaria, with each 'spot' representing a developing trophont within an epithelial capsule or vesicle (Fig. 1A and B). Wet mounts of gills did not reveal the presence of any parasites, suggesting that in guppies the infection may be limited to the skin. As the disease progressed, fish became lethargic, stopped feeding and moved to the bottom of aquaria, displaying increased opercular movements. Mucous was observed streaming off the fins and tail of the fish. Death of the host usually occurred after 7-8 days.

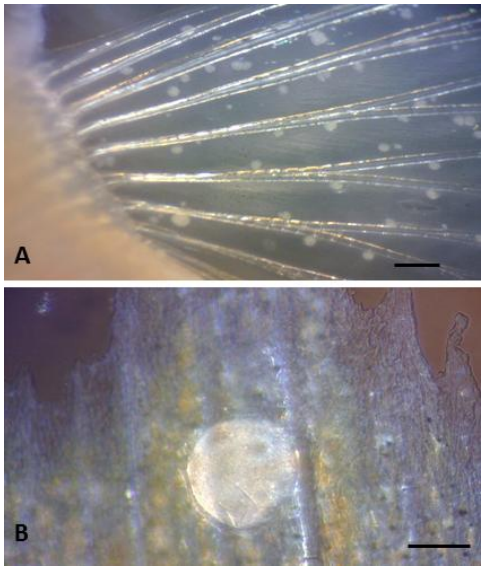

Figure 1 Fresh mount of a pectoral fin of guppy (*P. reticulata*) having been exposed to *C. irritans* for 5 days at 28 ºC. **A)** Each white spot represents a single trophont - *note* variations in trophont sizes. Bar = 400 µm; **B)** Higher magnification of a single trophont on a pectoral fin. Bar = 200 µm.

Histopathology. Histological examination revealed infective trophonts invading the fish epithelium (Fig. 2A). They appeared round or pyriform in shape, approx. 25-60 µm in length, with dense ciliation and a distinctive quadripartite macronucleus (Fig. 2B). Feeding trophonts, located beneath the epithelium reach diameters of 60- 450 µm (Fig. 3) and were observed to move with a rotating motion, creating a tissue space within the epithelial layers as they feed. The macronucleus of the trophont has four lobes arranged into a crescent (Fig 4) – each lobe is approx. 10 µm long by 8 µm wide with 2 nucleoli (Colorni & Diamant, 1993). In Figure 4 a mature trophont has lifted the epithelial cell layers forming a raised bubble or bulge that is visible macroscopically. A thickening of the epithelium and a proliferation of mucous secreting cells was visible (Fig.4).

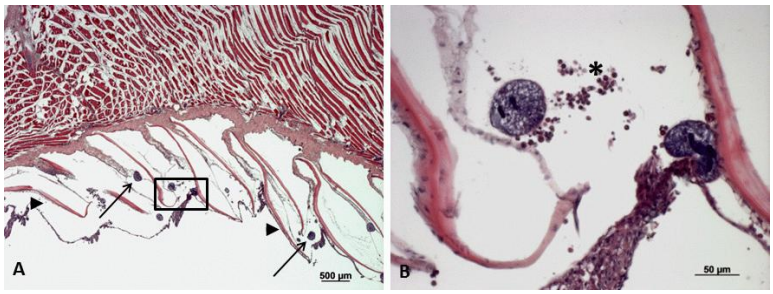

Figure 2 *Cryptocaryon irritans* in the caudal peduncle of guppy, 5 day post-infection. **A)** Trophonts (arrows) have penetrated the epithelium and can be seen between raised scales (arrowheads). **B)** Higher magnification of the boxed area from **A** showing densely ciliated trophonts, surrounded by infiltrating leucocytes (asterisk).

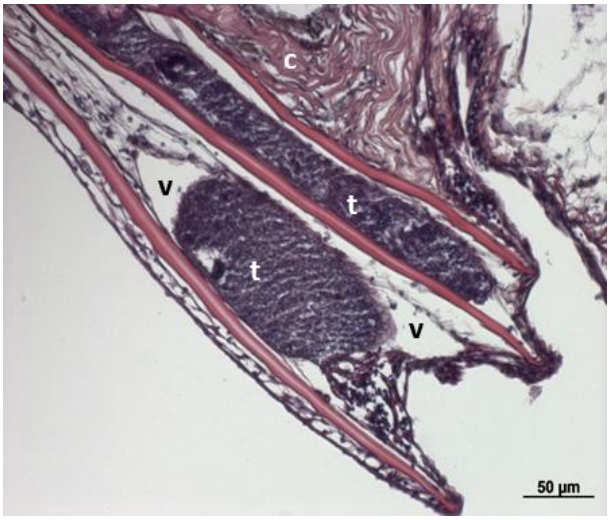

Figure 3 Feeding trophonts (t) of *C. irritans* located under the epithelium between lepidotrichia of guppy pelvic fin at 5 days post-infection. (v = vacuolar spaces; c = connective tissue).

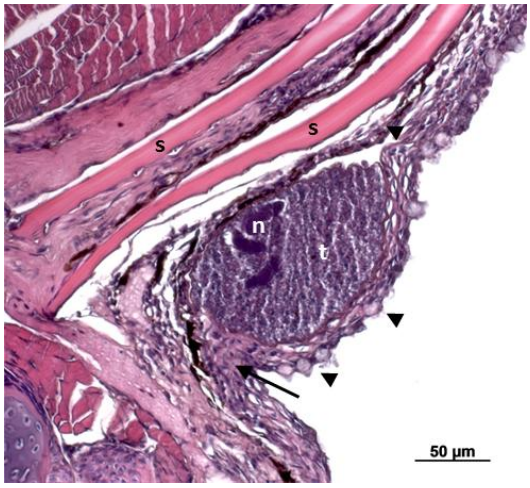

Figure 4 A feeding trophont (t) of *C. irritans* located in the epithelia of the dorsal fin-fold of a guppy, at 5 days post-exposure. *Note* proliferation of mucous cells (arrowheads) and thickening of epithelium (arrow). (n = lobed macronucleus; s = scales).

Discussion

In this study we describe a simple method for experimental propagation and maintenance of *C. irritans* infection using the sea-water adapted guppy as a model in the laboratory for 15 months. A well-established obstacle to the systematic study of this ubiquitous parasite is the difficulty in maintaining infection and collecting sufficient parasites (Yoshinaga and Dickerson, 1994). However, the established ease of culture of the live-bearing guppy and its suitability for adaptation to elevated salinities prepared using artificial salt allows the repeated passage of the disease under laboratory conditions with minimal water requirements, especially beneficial in areas where there is no access to marine systems. Furthermore the ability for these small fish to be held in self-contained aquaria units reduces the risk of spread of the disease to other experimental systems. In addition, in the present study, the use of freshwater guppies precludes the risk of fish possessing a previously acquired immunity to the marine parasite *C. irritans* and this naivety appears to result in high individual infection levels with no apparent loss of infectivity over time. A loss of parasite viability has been previously observed; Burgess and Matthews (1994) reported that none of the strains of *C. irritans* progressed beyond 34 cycles of propagation in the grey mullet (*C. labrosus*). Such observations have similarly been reported in the propagation of *Ichthyophithirius multifiliis* (Houghton and Matthews, 1986; Noa and Dickerson, 1995).

In the current study we describe observations of the host-parasite interaction during the course of the experimental infection at a histological level – *i.e.* mature trophonts established within the epithelial layer. The reported histo-pathological changes are similar to those previously reported for the freshwater *I. multifiliis* infection in various host fishes by Ventura and Paperna (1985). The proliferation of mucous secreting cells in the epithelium in this study, as reported in the histological findings, is consistent with gross observations made of elevated amounts of mucous seen streaming off heavily infected fish. This observed physiological response to *C. irritans* infection has been previously reported in marine fish (Nigrelli and Ruggieri, 1966; Wilkie and Gordin, 1969).

In conclusion, an efficient system allowing the passage and propagation of the parasite using the guppy as a model is described, as are histopathological changes as a result of heavy infection with *C. irritans*, of which there is currently limited information.

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