3. Lifecycle and settlement of an Australian isolate of *Ichthyophthirius multifiliis* from rainbow trout.

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3.1 Abstract

Ichthyophthirius multifiliis Fouquet, 1876, a ciliate protozoan, is cosmopolitan and problematic parasite in cultured freshwater fish. Each geographical isolate of *I. multifiliis* has variations in lifecycle timing under different abiotic water conditions, such as temperature and salinity. We assessed the effects of salinity (1, 3, 5 and 7.5 g/L) and temperature (5, 9, 12, 17, 21, 25 and 30°C) on the development and the preferred settlement site of a temperate Australian isolate of *I. multifiliis*, infecting rainbow trout (*Oncorhynchus mykiss*). The time until theront release was significantly different between each temperature, while development time was longest at 5°C with a mean time of 188.9 h and decreased to a mean time of 11.7 h at 30°C. At 5°C our isolate produced a mean of 266.8 theronts per tomont, which increased to a mean of 493.2 theronts at 25°C and reduced to a mean of 288 theronts at 30°C. Theront length showed an inverse relationship to temperature; mean length was 62.2 µm at 5°C and 41 µm at 30°C. Our isolate reproduced faster at all temperatures and a greater sensitivity to salinity than all reported profiles for temperate isolates. Parasite abundance was highest on the dorsal region of the body of the fish. An accurate understanding of temperature-lifecycle information and optimal region to sample for surveillance will aid in the development of specific management plans for the Australian isolate of *I. multifiliis*, facilitating the strategic timing of treatments.

3.2 Introduction

Ichthyophthirius multifiliis, a ciliate parasite, has worldwide distribution (Nigrelli et al., 1976) and is a problematic parasite of cultured freshwater fish worldwide (Schäperclaus, 1992; Dickerson and Dawe, 1995). The wide geographical distribution of *I. multifiliis* is primarily due to the translocation of infected host species, the low host specificity of the pathogen and its capacity to reproduce rapidly (Matthews, 2005). The entry of *I. multifiliis* to Australia probably occurred multiple times but was first introduced on imported ornamental exotic fish (Ashburner, 1976). The first *I. multifiliis* outbreak on an Australian trout farm was associated with the release of infected goldfish *Carassius auratus auratus* in Tasmania and the parasite was introduced to Victoria with imported carp *Cyprinus carpio* (see Butcher, 1947).

The lifecycle of *I. multifiliis* is direct, with four stages: the parasitic trophont resides in the host's epidermis and develops into the tomont, which leaves the host and encysts in the aquatic environment. The tomont undergoes rapid division, usually in the cyst, into daughter cells, the tomites, which develop into theronts, the free swimming infective stage that locates the host, penetrates the epidermis and develops into a trophont (MacLennan, 1942; Matthews, 2005). The development period is influenced by water temperature (Bauer, 1958; Wagner, 1960; Noe and Dickerson, 1995; Aihua and Buchmann, 2001) and salinity (Wagner, 1960; Aihua and Buchmann, 2001). Busckiel (1910) and Nigrelli et al. (1976) suggested that subtle variations in cell morphology and temperature preferences of different isolates in different climatic zones could be due to the presence of different physiological strains of the parasite. Different strains of *I. multifiliis* are characterised by Glycosylphosphatidylinositol (GPI)-anchored membrane proteins (referred to as immobilisation antigens, or i-antigens): there are five i-antigen serotypes (Dickerson and Clark, 1996). Ecologically distinct isolates are influenced differently by temperature and salinity (Aihua and Buchmann, 2001).

Management programs for *I. multifiliis* based on European experimental data were trialled on Australian rainbow trout *Oncorhynchus mykiss* farms but resulted in lower than expected efficacy (E. Meggit, pers. comm.). Which strain(s) occur in Australia and the effect of temperature and salinity on development of Australian isolates was unknown. Detailed knowledge of the lifecycle is required to facilitate effective management of the parasite and to determine why management programs were ineffective. We therefore investigated lifecycle parameters and parasite settlement of an isolate of *I. multifiliis* from Australian trout farms, focusing on temperature and salinity, and comparing the data to other isolates.

3.3 Materials and methods

3.3.1 Culture of parasites

Rainbow trout infected with *I. multifiliis* were obtained from Snobs Creek Hatchery (Department of Environment and Primary Industries, Victoria, Australia) during the Austral summer of 2012. Infected fish were transferred to Flinders University and held in 200 L aerated aquaria containing recirculating dechlorinated municipal water (hardness 145 mg/L, alkalinity 20 mg/L as $CaCO_3$, pH 6.1), continuously filtered with biofilters. The tanks were maintained at 17 ± 1°C, with light/dark periods set artificially at 12: 12 h. The parasites were allowed to multiply in the aquaria. Any fish that showed signs of substantially affected health were removed and euthanased and replaced with naïve uninfected rainbow trout.

3.3.2 Isolation of trophonts

Temperature and salinity experiments were based on the method of Aihua and Buchmann (2001). Rainbow trout with visible trophonts were euthanased with an overdose (40 mL / 1000 L bath) of Aqui-S®, rinsed, and placed into a 600 mL beaker containing 80 mL aquarium water. Trophonts were allowed to dislodge and were collected 30 min after adding the fish using a 200 µL pipette. Eight trophonts were transferred to each well of a 24-well multidish (Corning®, New York, USA) containing 2 mL of 0.2 µm filtered (Sartorius Stedim Pty Ltd, Victoria, Australia) water from the infection tank. At the start of each experiment 8 trophonts were fixed in 10% neutral-buffered formalin (NBF) and measured.

3.3.3 Temperature trials

Multidish plates containing trophonts in wells were placed into incubators at 5, 9, 12, 17, 21, 25 and 30°C, and inspected hourly using a dissecting microscope (20 – 40 x magnification) to assess if theronts had been released, after the release of the fist theront multidish plates were inspected every 15 min. The time to the first theront release and number of tomonts that did not develop into tomocysts were recorded. Following the release of all theronts, a drop of 10% NBF and Lugol's iodine were added to each well and the number of theronts counted using a dissecting microscope. From each well, a sample was pipetted onto a slide and five randomly selected theronts were measured using a compound microscope with a calibrated ocular eyepiece (400x magnification). The experiments were repeated three times at each temperature.

3.3.4 Salinity trials

Multidish plates containing trophonts in wells with sodium chloride (Merck®, NSW, Australia, batch ref. MJ6M562652) added at 1, 3, 5, and 7.5 g/L were incubated at 12 and 17°C in separate trials and inspected hourly under a dissection microscope (20 –

40x magnification) to assess if theronts had been released, after the release of the fist theront multidish plates were inspected every 15 min.. Control groups contained wells with filtered water without sodium chloride. The protocol then followed that for temperature. The experiments were repeated three times at each salinity and temperature.

3.3.5 Detection of *I. multifiliis*

Twenty-five rainbow trout with a mean length of 6.8 ± SD 0.77 cm (Range 5.5 – 9.1 cm) were randomly selected from five different Australian trout farms. Skin scrapes are used by the Australian trout industry as the preferred surveillance technique and were therefore assessed. For each fish parasite abundance was determined for four different regions of the entire length of the body: the dorsal, ventral, lateral left and right by skin scrape using a sterile scalpel. Skin scrapes were placed onto microscope slides and viewed under compound microscope (Mag × 100) and the number of *I. multifiliis* in each region was recorded.

3.3.6 Statistical analysis

Prior to analysis, normality of the data was tested using the Kolmogorov-Smirnov test and variances were tested using Levene's test. To achieve homoscedasticity, the data for the mean time to theront production and the mean theront length were $\log_{10}$ transformed. Differences in the mean time taken to produce theronts between temperatures and salinities, the number of theronts produced at each temperature and salinity, the length of the theronts produced were analysed using a one-way ANOVA. Where significant differences were detected in the ANOVAs, post hoc comparisons were made using Tukey's tests. The statistical analysis was performed using IBM SPSS Statistics 20.0 and significance for all tests was judged at $P < 0.05$.

3.4 Results

3.4.1 Temperature trials

The trophonts produced during the temperature trials had a mean diameter of 322.7 ± standard error of the mean (SEM) 15.6 µm (range 202.5 to 502.5 µm). An increase in temperature significantly reduced the time tomonts took to release theronts (one-way ANOVA: $F_{6,\,135} = 3117.6$, $P < 0.001$). The development time was proportional to temperature and significantly different between each temperature (Table 3.1). There was a significant difference in the mean number of theronts produced by each tomocyst between each temperature (one-way ANOVA: $F_{6,\,135} = 16.223$, $P = 0.001$). Theront production was lower in cold water, significantly increasing with a higher water temperature peaking at 25°C, after which theront production decreased significantly (Table 3.1). Theront length showed an inverse relationship to the incubation temperature and was significantly different between each temperature (one-way ANOVA: $F_{6,\,133} = 26.204$, $P < 0.001$) (Table 3.1).

Table 3.1: Temperature-dependent development of *Ichthyophthirius multifiliis* tomonts (n = 8) at different water temperatures. Different superscripts indicate significant differences using Tukey's analysis (P < 0.05).

Temp °C	Time from trophont to theront in hours. Mean (Range)	Trophonts developing into cysts (%)	Number of theronts from one cyst. Mean (Range)	Length of theronts (µm)
5	188.9 (159.45 to 206) ^{a}	71	189.2 (63 to 374) ^{a}	62.2 (39.5 to 97.5) ^{a}
9	162.3 (154 to 172) ^{b}	79	193. 9 (106 to 440) ^{a}	52.6 (39.5 to 70) ^{b}
12	46.6 (41.6 to 54.6) ^{c}	100	253 (140 to 623) ^{a,b}	48.4 (39.5 to 65) ^{b,c}
17	27.1 (23.1 to 29.4) ^{d}	88	446.4 (180 to 682) ^{c,d}	42.7 (34 to 59) ^{d,e}
21	18.3 (16.4 to 20.1) ^{e}	100	426.3 (182 to 576) ^{b,c,d}	45.4 (25 to 58.5) ^{c,d}
25	13.7 (12.6 to 16.5) ^{f}	88	493.2 (177 to 1553) ^{d}	43.8 (27.5 to 59) ^{d,e}
30	11.7 (10.2 to 14.1) ^{g}	67	288 (146 to 410) ^{a,b,c}	41 (25 to 55) ^{e}

3.4.2 Salinity trials

The trophonts produced during the salinity trials had a mean diameter of 283.8 ± SEM 9.3 µm (range 175 to 380 µm). There was a significant difference in the viability of tomonts between all salinities at 12°C (one-way ANOVA: $F_{4, 10} = 62.222$, $P < 0.001$) and 17°C (one-way ANOVA $F_{4, 10} = 61.100$, $P < 0.001$). When exposed to 7.5 g/L sodium chloride at 12 and 17°C tomonts ceased movement within 1 h, with no division or formation of tomocysts. Tomonts exposed to 5 g/L sodium chloride were unable to produce theronts, although 29 and 33% were able to form a cyst wall and initiate division at 12 and 17°C, respectively. There was no significant difference in viability of tomonts exposed to 1 g/L and controls at 12 (Table 3.2) and 17°C (Table 3.3).

Table 3.2: Salinity-dependent development of *Ichthyophthirius multifiliis* tomonts (n = 8) incubated at 12°C at different salinity levels. Different superscripts indicate significant differences using Tukey's analysis ($P < 0.05$).

Salinity (g/L)	Time from trophont to theront in hours. Mean (Range)	Trophonts encysted (%)	Trophonts with divisions (%)	Viable trophonts (%)	Number of theronts from one cyst. Mean (Range)	Length of theronts (µm)
0	53 (43.3 to 59)^{a}	83	29	83	282.1 (122 to 789) ^{a}	55.1 (40.5 to 69.5) ^{a}
1	49 (39.3 59)^{a}	92	29	92	166.4 (37 to 315) ^{a,b}	52.9 (36 to 70.5) ^{a,b}
3	97.1 (74 to 122)^{b}	50	50	33	78.5 (48 to 96) ^{b}	49.5 (39 to 70) ^{b}
5	-	29	54	0	-	-
7.5	-	-	-	-	-	-

For viable tomonts, the mean time to theront release was significantly different between all salinities at 12°C (one-way ANOVA: $F_{2, 6} = 12.071$, $P = 0.008$) and at 17°C (one-way ANOVA: $F_{2, 6} = 119.001$, $P < 0.001$). There was no significant difference between tomonts exposed to 1 g/L and controls, but release took significantly longer in tomonts exposed to 3 g/L (Table 3.2 and 3.3).

Table 3.3: Salinity-dependent development of *Ichthyophthirius multifiliis* tomonts (n = 8) incubated at 17°C at different salinity levels. Different superscripts indicate significant differences using Tukey's analysis (P < 0.05).

Salinity (g/L)	Time from trophont to theront in hours. Mean (Range)	Trophonts encysted (%)	Trophonts with divisions (%)	Viable trophonts (%)	Number of theronts from one cyst. Mean (Range)	Length of theronts (μm)
0	25.5 (22.5 to 28.2)^{a}	100	0	100	397.7 (202 to 569) ^{a}	44.9 (35.5 to 55.5) ^{a}
1	25 (20.4 to 28.4)^{a}	96	4	96	452.1 (295 to 657) ^{a}	46 (36 to 55.5) ^{a}
3	43.6 (74 to 122)^{b}	75	25	50	204 (134 to 291) ^{b}	38.4 (30.5 to 46.5) ^{b}
5	-	33	58	0	-	-
7.5	-	-	-	-	-	-

Theront production from viable tomonts was significantly different between salinities at 12°C (one-way ANOVA: $F_{2, 46} = 8.895$, $P = 0.001$) and at 17°C (one-way ANOVA: $F_{2, 56} = 29.172$, $P < 0.001$). Theront production was not significantly different between tomonts exposed to 1 g/L and controls but was significantly lower in tomonts exposed to 3 g/L at 12°C (Table 3.2) and 17°C (Table 3.3). Theronts were largest in the control groups and significantly decreased in size with an increasing salinity at 12°C (Table 3.2) and 17°C (Table 3.3).

3.4.3 Detection of *I. multifiliis*

Mean parasite abundance was highest on the dorsal part of the fish, which was higher than the ventral part but not between the lateral sides (Table 3.4).

Table 3. 4: Mean (range) abundance of *Ichthyophthirius multifiliis* on different body regions of rainbow trout sampled from five farms.

Farm	Dorsal	Ventral	Lateral right	Lateral left	Total
1	7.8 (2 – 11)	3.6 (2 – 5)	5 (2 – 7)	4.2 (1 – 6)	5.2 (1 – 11)
2	3.4 (0 – 9)	0.4 (0 – 1)	3.4 (0 – 7)	2 (0 – 3)	2.3 (0 – 9)
3	0.4 (0 – 1)	0.2 (0 – 1)	0.2 (0 – 1)	0.8 (0 – 2)	0.4 (0 – 2)
4	1.6 (0 – 4)	1.4 (0 – 3)	2.4 (0 – 5)	0.8 (0 – 2)	1.6 (0 – 5)
5	16.6 (6 – 29)	2.8 (2 – 4)	9.4 (5 – 12)	11.2 (2 – 18)	10 (2 – 29)
Mean	6 (0 – 29)	1.7 (0 – 5)	4.1 (0 – 12)	3.8 (0 – 18)	3.9 (0 – 29)

3.5 Discussion

The time range from tomont settlement to theront release for our isolate of *I. multifiliis* is shorter than that of other isolates (Bauer, 1958; Wagner, 1960; Aihua and Buchmann, 2001), especially at ≥ 25°C (Table 3.5). Development time-temperature relationships determine timing of repeat treatments to maximize efficacy and interrupt the parasite's lifecycle. Our data confirms that different isolates of *I. multifiliis* have lifecycles that differ sufficiently to adjust treatment timing.

Table 3 5: Range of time (h) for the development of *Ichthyophthirius multifiliis* from trophont to theront release. Comparison of the present results with literature data. Adapted from Aihua and Buchmann (2001).

Temp (°C)	Present work	Aihua and Buchmann (2001)	Bauer (1958)	Wagner (1960)
5	159 – 206	192 – 228	144	168 – 192
7-9	154 – 172	84 – 108	72 – 84	48 – 120
10-12	42 – 55	46 – 58	36 – 40	48 – 60
17	23 – 29	23.5 – 35.5	23 – 26	24
20-21	16 – 21	18.5 – 23.5	18 – 20	18
25	13 – 17	16 – 27.5	14 – 15	14
30	10 – 14	16 – 25	-	-

Timing of repeat applications at different temperatures significantly alters the efficacy of a treatment regime (Lahnsteiner and Weismann, 2007). If all free-living stages are killed by a treatment, the time from trophont exit until theront release is used as the treatment interval. At 17˚C and 21˚C the treatment intervals for our isolate are 23 and

16 hours, respectively. Tomonts, however, are more resistant to chemical treatments than theronts (Heinecke and Buchmann, 2009). Sodium percarbonate is also less effective at lower temperatures: SPC at 128 mg/L for 1 h at 17°C is 100% effective against all free-living life stages of our isolate of *I. multifiliis* but is only 50% effective against tomonts at 12°C (Chapter 4). Strategic treatment at lower temperatures requires each cohort of tomonts to be treated at least twice, with a second pair of strategic treatments at the time from trophont exit until theront release to maximally disrupt the lifecycle. Treatments at 12˚C should be applied 12, 42 and 54 hours after the initial treatment. Some reinfection will occur, but this treatment pattern maximises efficacy. Treatment, however, is rarely required at 12˚C (M. Landos, unpublished).

Theront production in our isolate of *I. multifiliis* was highest at 25°C. In a Danish *I. multifiliis* isolate, Aihua and Buchmann (2001) found higher mean production of theronts per tomocyst than we observed in our isolate at all temperatures except 25°C. Theront production per tomocyst ranges from 64 (MacLennan, 1937) to 3000 (Wagner, 1960) and is influenced by the age and size of tomonts (Ewing et al., 1986) and temperature (Aihua and Buchmann, 2001). The age of the theronts used in this study was unable to determined but the size was relatively uniform, however, this may had an influence individual variation in theront production. The present results suggest optimum range for theront production of the Australian isolate is 17-25°C and production was significantly reduced at 4°C and 30°C. A Danish isolate produced most theronts at 11.6-21°C and was also inhibited at 5°C and 30°C (Aihua and Buchmann, 2001). This variation may reflect the higher average water temperatures encountered on Australian trout farms compared to European farms. We observed that low theront production was associated with tomonts dividing prior to undergoing encystment at low (4°C) and high (30°C) temperatures. Few of these daughter tomonts were viable and formed tomocysts, and those that did were smaller than normal tomocysts and produced fewer theronts. This probably reflects the temperature tolerance range of the parasite. *Ichthyophthirius multifiliis* outbreaks in Australia do not follow the same course as described for European outbreaks in rainbow trout and the higher reproductive capacity of the European isolate at 9-17˚C is likely to influence this infection dynamic.

Salinity had a significantly reduced the viability of the Australian isolate of *I. multifiliis* at 3 g/L and above and completely prevented theront production at 5 g/L. Wagner (1960) and Aihua and Buchmann (2001) reported that theront production continued at 5 g/L salinity but production occurred over a longer period than at lower salinity. Aihua and Buchmann (2001) observed tomocyst formation at 7.5 g/L and Wagner (1960) reported survival and theront production at 10 g/L after 63 hours. Mifsud and Rowland (2008) reported effective control of *I. multifiliis* infecting silver perch (*Bidyanus bidyanus*) in Australia using 2 - 3 g/L sodium chloride. Direct comparisons of isolates are difficult to make due to differences in experimental design, but these results indicate that Australian isolates are probably more sensitive to salinity than other isolates. The sensitivity of the Australian isolate to salinity has treatment and prevention applications where sodium chloride use is feasible, such as in small volume recirculation systems.

Management of *I. multifiliis* relies on early detection of the parasite to facilitate decision making, such as whether to treat, based on the temperature and re-infection. Microscopic examination of skin scrapes is routinely used on trout farms for *I. multifiliis* monitoring. We found that the dorsal region of rainbow trout had the highest abundance of *I. multifiliis*. Hines and Spira (1973a) reported a higher abundance of *I. multifiliis* on the dorsal part of mirror carp experimentally infected with *I. multifiliis*. The dorsal region is probably the preferred settlement site for *I. multifiliis*, and that the dorsal part of the fish is the most informative part of the fish to sample when monitoring *I. multifiliis*.

Our Australian isolate of *I. multifiliis* reproduces more rapidly but is more sensitive to salinity than other described isolates. Molecular analysis would complement the ecological profiles and further characterise this Australian isolate and provide further points of comparison to other geographical isolates. Assessing development time from settlement to exit from the host at different temperatures is also required to compliment the lifecycle-temperature relationships for the free-living stages, to maximise capacity to strategically time treatments.

3.6 Acknowledgments

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