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Vertical transmission of *Tetracapsuloides bryosalmonae* (Myxozoa), the causative agent of salmonid proliferative kidney disease

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1 **Vertical transmission of *Tetracapsuloides bryosalmonae* (Myxozoa), the causative agent**
2 **of salmonid proliferative kidney disease**

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20 **Short running title:** Vertical transmission of *Tetracapsuloides bryosalmonae*

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26 SUMMARY

27 The freshwater bryozoan, *Fredericella sultana*, is the main primary host of the myxozoan
28 endoparasite, *Tetracapsuloides bryosalmonae* which causes proliferative kidney disease
29 (PKD) of salmonid fish. Because spores that develop in bryozoan colonies are infectious to
30 fish, bryozoans represent the ultimate source of PKD. Bryozoans produce numerous seed-like
31 dormant stages called statoblasts that enable persistence during unfavourable conditions and
32 achieve long distance dispersal. The possibility that *T. bryosalmonae* may undergo vertical
33 transmission via infection of statoblasts has been the subject of much speculation since this is
34 observed in close relatives. This study provides the first evidence that such vertical
35 transmission of *T. bryosalmonae* is extensive by examining the proportions of infected
36 statoblasts in populations of *F. sultana* on two different rivers systems and confirms its
37 effectiveness by demonstrating transmission from material derived from infected statoblasts
38 to fish hosts. Vertical transmission in statoblasts is likely to play an important role in the
39 infection dynamics of both bryozoan and fish hosts and may substantially contribute to the
40 widespread distribution of PKD.

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42 Key words: Bryozoa, *Fredericella sultana*, statoblasts, maternal colonies, *Tetracapsuloides*
43 *bryosalmonae*, persistent infection, dispersal

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45 **KEY FINDINGS**

- 46 - Vertical transmission of *Tetracapsuloides bryosalmonae* is achieved via statoblasts
- 47 - Infected statoblasts show greater hatching success than uninfected statoblasts
- 48 - Infected statoblasts can be present in maternal colonies that have lost infection
- 49 - Dispersal of infected statoblasts likely contributes to the wide distribution of PKD
- 50 - Vertical transmission likely promotes persistent infection of bryozoan populations

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53 INTRODUCTION

54 The myxozoan parasite *Tetracapsuloides bryosalmonae*, is the causative agent of Proliferative
55 Kidney Disease (PKD) in salmonid fish (Anderson *et al.* 1999; Canning *et al.* 1999; Feist *et*
56 *al.* 2001). Mortality due to PKD in farmed fish ranges from 20% to 100%, leading to
57 increased costs of production (Ferguson and Ball, 1979; Clifton-Hadley *et al.* 1986; Feist and
58 Longshaw, 2006). The impacts of PKD on wild fish populations are generally poorly known
59 although a recent outbreak in Norway (Sterud *et al.* 2007) demonstrates the potential for mass
60 mortality and PKD is attributed to long term declines in Swiss brown trout populations
61 (Borsuk *et al.* 2006).

62 The life cycle of *T. bryosalmonae* involves exploitation of freshwater bryozoans (Phylum
63 Bryozoa, Class Phylactolaemata) as primary hosts (see Okamura *et al.* 2011, for review). The
64 parasite first develops as covert infections when single cells are associated with the bryozoan
65 body wall (Morris and Adams, 2006a). When conditions are favourable for host growth (e.g.
66 high temperatures and food levels for hosts; Tops *et al.* 2006; Hartikainen and Okamura,
67 2012) these cells detach and develop within the bryozoan body cavity to form sacs filled with
68 spores infectious to fish. Because these sacs can be observed with a stereomicroscope we refer
69 to such infections as overt (see Okamura *et al.* 2011, for review).

70 Freshwater bryozoans are sessile colonial invertebrates that occur in nearly all types of
71 freshwater environments (Massard and Geimer, 2008). They are suspension-feeders that
72 capture phytoplankton and other microorganisms by creating feeding currents with ciliated
73 tentacles (Massard and Geimer, 2008). Sexual reproduction is facultative, occurring briefly in
74 early summer and resulting in the release of short-lived swimming larvae (Wood and
75 Okamura, 2005). Much greater emphasis is on the asexual production of numerous dormant

76 statoblasts, which are small (<1mm), multicellular seed-like stages comprised of germinal
77 tissue surrounded by resistant chitinous valves (Wood and Okamura, 2005). Statoblasts can
78 tolerate poor conditions such as desiccation, freezing, and lack of food (Hengherr and Schill,
79 2011). When favourable conditions return statoblasts hatch and form new colony (Wood and
80 Okamura, 2005). Because of their resistant nature, small size (0.5–1 mm), and high
81 production, statoblasts likely act as a means of dispersal within and between sites. Evidence in
82 support of this includes the presence of statoblasts in waterfowl guts (Figuerola *et al.* 2004;
83 Mouranval *et al.* 2007) and faecal material (Green *et al.* 2008), gene flow patterns associated
84 with migratory waterfowl routes (Freeland *et al.* 2000; Figuerola *et al.* 2005) and the ability of
85 statoblasts to hatch after passing through duck digestive tracts (Charalambidou *et al.* 2003).
86 Statoblasts have also been found in the gut of many fish species, including largemouth bass,
87 *Micropterus salmoides* (Lacepède), crappie, *Pomoxis annularis* (Rafinesque), and common
88 carp, *Cyprinus carpio* (Linnaeus) (Marković *et al.* 2009). Statoblasts carried in ship ballast
89 tanks are likely to contribute to distributions of bryozoans worldwide (Kipp *et al.* 2010; Briski
90 *et al.* 2011).

91 Given the strong dispersal potential of statoblasts, it is of interest to know whether if *T.*
92 *bryosalmonae* can be vertically transmitted via statoblasts. In addition, vertical transmission
93 may explain the presence of infected bryozoans in sites free of salmonids (Tops *et al.* 2004) as
94 it may contribute to persistent infection in local bryozoan populations. Such vertical
95 transmission has been demonstrated for another bryozoan-malacosporean system
96 (*Buddenbrockia allmani* infecting the bryozoan, *Lophopus crystallinus*) whereby new
97 colonies developed from statoblasts were shown by PCR and sequencing to carry infection
98 (Hill and Okamura, 2007). The bizarre observation of the release of worm-like organisms
99 probably representative of *Buddenbrockia plumatellae* from statoblasts of *Plumatella fungosa*

also provides evidence for vertical transmission (Taticchi *et al.* 2004). However, until very recently investigations have failed to find evidence for infections of *T. bryosalmonae* in bryozoan statoblasts (e.g. Grabner and El-Matbouli, 2008).

It is now clear that during overt infection *T. bryosalmonae* severely reduces statoblast production, causes malformation of statoblasts and thus almost entirely castrates the infected zooids of its main bryozoan host, *Fredericella sultana* (Hartikainen *et al.* 2013). *T. bryosalmonae* infections have been detected by PCR in a fraction of the few statoblasts produced in such overtly-infected bryozoans. However, these infections were not transmitted successfully to the mature offspring hatched from the same statoblast sources, suggesting either selective mortality of infected offspring or that the infections were subsequently lost (Hartikainen *et al.* 2013). Whether vertical transmission of infection is effective thus remains an open question. The aims of the current study were to: 1) examine the occurrence and extent of vertical transmission of *T. bryosalmonae* in *F. sultana* statoblasts, and; 2) determine the effectiveness of vertical transmission by undertaking transmission studies across the *T. bryosalmonae* life cycle.

MATERIALS AND METHODS

The occurrence and extent of infection of statoblasts

F. sultana colonies associated with submerged tree roots were collected from the River Lohr, Germany (50°01'08'' N; 09°32'43'' E) on the 1st of August 2011 and from the Rivers Avon (11°27'32'' N; 41°46'29'' E) and Dun (16°86'07'' N; 43°42'63'' E) in southern England on 29th August 2012 and 5th September 2012. Material from the Lohr was used in transmission experiments and material from the Rivers Avon and Dun were used to measure infection prevalence and vertical transmission to statoblast offspring.

123 Tree roots with attached colonies from the River Lohr were glued to Petri dishes, placed in an
124 aerated 10 L bucket filled with Bryozoan Medium C (BMC) (Kumar *et al.* 2013) and
125 maintained at 18 °C (± 2 °C) by feeding with cultured algae as described in Grabner and El-
126 Matbouli (2008) . Briefly, 6 algal species (*Cryptomonas ovata*, *Cryptomonas*
127 spp., *Chlamydomonas* spp., *Chlamydomonas reinhardtii*, *Synechococcus* spp., *Synechococcus*
128 *leopoliensis*) were cultured in sterile Guillard's WC-Medium. *C. ovata* comprised 80% of
129 food provided to *F. sultana* colonies and the remaining 20% was comprised of the other algae.
130 Food was provided daily by adding 500 ml of the algal culture (concentration of 6.5 to 7.5 x
131 10⁶ cells/ml) to the bucket and the water was changed every week.

132 After 49 days, microscopical examination of the bryozoans in BMC medium revealed cells
133 swirling in the host body cavity and the subsequent development of overt *T. bryosalmonae*
134 infections in 28% of colonies observed (n=100). Thirty statoblasts dissected from overtly-
135 infected laboratory-cultured colonies originally collected from the field were retained for PCR
136 assays. To minimise the possibility of contamination, statoblasts were incubated in distilled
137 water at 4 °C for 24 h (spores degrade in < 24 h at 18 °C; De Kinkelin *et al.* 2002) and were
138 then washed several times with distilled water prior to preserving in RNA Later until DNA
139 extraction.

140 Colonies from the Rivers Avon and Dun were collected by detaching several branches from
141 each colony with forceps and placing these branches together in a tube specific for that colony
142 and maintained at room temperature (20 °C) for 24 h prior to collecting all the mature
143 statoblasts that were present in the material. Mature statoblasts were identified by the dark
144 brown colour of valves. Statoblasts were dissected from bryozoans using a stereomicroscope
145 (100 x magnification) which also enabled determining whether *T. bryosalmonae* sacs were
146 present within the bryozoan body cavity. Colonies lacking overt infections were subject to

147 **PCR to test for the presence of covert infections.** Statoblasts from the same brood (i.e. from
148 the same maternal colony) from the Rivers Avon and Dun were placed in 1.5 ml tubes with
149 deionised water and stored at 4 °C (Rivers Avon and Dun) for five months. After five months,
150 the statoblasts of the same brood (i.e. originating from a single maternal colony) were placed
151 in Petri dishes filled with artificial pond water and were allowed to hatch at 20 °C over a
152 period of seven days. The hatched statoblasts were preserved in 100% ethanol for PCR assays
153 for detection of infection of individual statoblasts. The tissue of maternal colonies was also
154 preserved in ethanol to determine infection status. Associations between river, maternal
155 colony infection, statoblast hatching status and statoblast infection were tested using Chi-
156 square (for two-way analyses) and G tests (three-way analyses).

157 DNA extractions used either a QIAamp DNA mini Kit (QIAGEN) (Lohr samples) or a
158 modified hexadecyltrimethylammonium bromide (CTAB) protocol (Tops and Okamura,
159 2003) (Avon and Dun samples). Two *T. bryosalmonae*-specific PCR assays were used. 1)
160 Statoblasts from the River Lohr were diagnosed using a nested PCR with 5F (5'-
161 CCTATTCAATTGAGTAGGAGA-3') and 6R (5'-GGACCTTACTCGTTTCCGACC-3')
162 (Kent *et al.* 1998) in the first round, followed by a second round PCR using PKD-real F (5'-
163 TGTCGATTGGACACTGCATG-3') and PKD-real R (5'-ACGTCCGCAAACCTTACAGCT-
164 3') (Grabner and El-Matbouli, 2009). The final 166 bp amplicon was verified to originate
165 from *T. bryosalmonae* via purification from 1.5% agarose gels, cloning and sequencing.
166 Briefly, the amplicon was purified using a MinElute gel extraction kit (QIAGEN), cloned into
167 the pCR4-TOPO vector (TOPO TA, Invitrogen) and the recombinant plasmids purified from
168 *Escherichia coli* using a QIAprep Miniprep (QIAGEN). Cloned PCR products were
169 sequenced in a commercial sequencing laboratory (LGC Genomics GmbH, Berlin, Germany).
170 2) Statoblasts from the Rivers Avon and Dun were subjected to PCR with the primers 514F

new (5'-ATTCAGGTCCATTCGTGAGTAACAAGC-3') and 776R (5'-CTGCCCTTAATTGGGTGTATCAGC-3') (diagnostic amplicon size 244 bp, PCR conditions as in Hartikainen *et al.* 2013). The PCR products were visualised on a 1.5% agarose electrophoretic gel and five positive samples were verified by direct sequencing on ABI Prism 3700xl using BigDye v1.1 chemistry.

Is vertical transmission in statoblasts effective?

We conducted a laboratory-based transmission study using bryozoan colonies originated from the River Lohr to determine whether infection of statoblasts results in effective vertical transmission to colonies that subsequently develop and that these in turn can promote further transmission of infection to fish. Material for this study was gained by cohabiting two-month old specific pathogen free (SPF) colonies for 8 h per day for 14 days with 5 brown trout that had been infected by exposure to naturally infected *F. sultana* colonies from the River Lohr eight weeks earlier. The cohabitation experiment was conducted several times in order to obtain as many statoblasts as possible, using different SPF *F. sultana* colonies. A total of 19 colonies (originating from the River Lohr) were present on the petri dishes and at the beginning of exposure each had at least three zooids. In between bouts of cohabitation fish were transferred back to their aquarium and fed with commercial fish feed. *F. sultana* colonies were maintained in culture systems (buckets; see above) after the 8 h of cohabitation. These colonies were then allowed to grow on following exposure to fish until they produced statoblasts. Statoblasts were collected from these colonies, washed and placed in distilled water for 24 h at 4 °C.

Statoblasts produced by colonies exposed to infected fish were used in a study to address whether infection was successfully vertically transmitted to daughter colonies that hatched from these statoblasts and whether this infection could in turn be transmitted to fish. For this

study we cohabited two colonies derived from these statoblasts and that were observed to have spores and sacs of *T. bryosalmonae* with five SPF brown trout (4 cm in length) for eight hours daily for two weeks. For the control, five SPF brown trout fish were cohabited with SPF *F. sultana* colonies (two colonies comprising 18 zooids) from long-term laboratory stock. The aquaria were checked daily for fish mortality and extraordinary behaviour. After cohabitation, fish were placed in 20 L aquaria with flow-through water. Eight weeks post-infection, all five fish from each group were dissected and kidney, liver and spleen were sampled for DNA extraction and *T. bryosalmonae*-specific PCR assays using 5F and 6R primer sets.

Immunohistochemistry was performed on 30 statoblasts collected from the colonies exposed to fish (described above) and from uninfected colonies deriving from our laboratory stock. Statoblasts were washed with distilled water, incubated for 24 h at 4 °C and fixed in 10% neutral buffered formalin and paraffin embedded 4 µm sections were labelled with *T. bryosalmonae* specific monoclonal antibody P01 (Aquatic Diagnostics LTD). Endogenous peroxidase activity in deparaffinised sections was blocked with 3% H₂O₂ in phosphate buffered saline (PBS) for 10 min. Following rinses with Tris-buffered saline (TBS), non-specific protein binding sites were blocked with normal sheep serum (1:10 dilution in TBS) for 30 min. The sections were then incubated for 60 min in a humid chamber at room temperature with the reconstituted P01 antibody (20 µg/mL in PBS). After three washes with TBS for 2 min each, the sections were incubated for 30 min with 100µL of goat anti-mouse polyclonal antibody (IgG) conjugated with horseradish peroxidase (Dako EnVision+ System-HRP AEC kit). After four washes with TBS for 2 min each, slides were incubated with the AEC substrate for 4–5 min until a pink color appeared. The reaction was stopped by

immersion of the slides in distilled water for 3–4 min. Sections were then mounted and examined microscopically.

RESULTS

The occurrence and extent of infection of statoblasts

The *T. bryosalmonae*-specific PCR assays demonstrated parasite presence in statoblasts produced by colonies collected from all three rivers as well as in statoblasts collected from SPF *F. sultana* colonies exposed to infected brown trout (e.g. see Fig. 1). Sequencing of positive samples showed 99% similarity to the 18S rRNA gene of *T. bryosalmonae* (GenBank accession numbers, FJ981823 and AF190669).

We collected 181 statoblasts from 47 *F. sultana* colonies from the River Avon and 57 statoblasts from 27 colonies from the River Dun. Only one overtly-infected colony (with 11 *T. bryosalmonae* sacs) was observed (River Avon). This colony had two mature uninfected statoblasts. A high proportion of the remaining colonies were covertly infected (68% from River Avon and 74% from River Dun) (see Fig. 2) but infection status (covertly infected vs. uninfected) at the time of field collection was not dependent on river (Chi-Square Test: $\chi^2=0.30$, df=1, p=0.59). Infected statoblasts were obtained from colonies that were both covertly infected and uninfected (presumably having lost their infection recently) at the time of collection in both sites (see Fig. 3). Higher proportions of infected statoblasts were collected from the River Dun material (35% and 45% of statoblasts collected from covertly-infected and uninfected maternal colonies, respectively) than from the River Avon material (29% and 19% of statoblasts collected from covertly-infected and uninfected maternal colonies, respectively) (Fig. 3) (statoblast infection status was not dependent on river: G=1.8, df=1, p=0.18). Notably, the highest proportions of infected statoblasts were collected from

uninfected maternal colonies from the River Dun (45%) but from covertly-infected maternal colonies from the River Avon (29%) (Fig. 3) (statoblast infection status was not dependent on maternal colony status: $G=0.30$, $df=1$, $p=0.58$). There was also no association between river, maternal colony infection status and statoblast infection (2x2x2 G test: $G=3.6$, $df=4$, $p=0.463$).

We assessed the frequency of vertical transmission by considering the infection status of pooled statoblasts produced by all infected colonies. Here we included statoblasts produced by uninfected maternal colonies that had produced at least one infected statoblast. Although there was a slightly higher proportion of infected statoblasts (as revealed by PCR) in material collected from the River Dun than in the River Avon (39% and 30%, respectively: see Fig. 4) infection status was not dependent on river (Chi-Square Test: $\chi^2=1.51$, $df=1$, $p=0.22$). The percentages of these infected statoblasts that hatched were 94% ($n=46$) and 100% ($n=21$) for the Rivers Avon and Dun, respectively. The uninfected colonies with no infection in any of the statoblast offspring produced 15 statoblasts in the Avon and 3 statoblasts in the Dun. The percentage of statoblasts that hatched were 87% and 100%, respectively.

There were notably higher proportions of hatched statoblasts that were infected (50% on the River Dun, 30% on the River Avon) relative to unhatched infected statoblasts (0% on the River Dun, 21% on the River Avon) (Fig. 5) and the effect was greatest for material from the River Dun. These patterns are reflected by the dependency of statoblast infection on hatching status (2x2 G test: $G=6.56$, $df=1$, $p=0.0104$) (uninfected statoblasts did not hatch as well as infected ones) but not on river (2x2 G test: $G=1.6$, $df=1$, $p=0.2059$), although statoblast hatching status was dependent on river (2x2 G test: $G=6.62$, $df=1$, $p=0.0101$) (Fig. 5). Overall, therefore, river, statoblast infection and hatching status were inter-dependent (2x2x2 G test: $G=22.68$, $df=4$, $p<0.0001$).

Is vertical transmission in statoblasts effective?

Six of the 30 statoblasts collected for hatching from laboratory-infected *F. sultana* colonies (20 %) successfully hatched. We confirmed that infection was successfully vertically transmitted to two of the six hatched colonies (33%) by observing free spores and mature sacs in two colonies. These infected colonies were each comprised of 2–3 zooids and were subsequently used in exposures to fish. The SPF brown trout that were cohabitated for two weeks with these two infected colonies didn't show any abnormal behaviour or mortalities during the whole trial, and they were positive for *T. bryosalmonae* after eight weeks post-infection (Fig. 6). Band intensities suggest the *T. bryosalmonae* stages were most abundant in the kidney but were also present in liver and spleen. No DNA was amplified from control fish.

The use of *T. bryosalmonae* specific monoclonal antibodies revealed the presence of cryptic stages in the infected statoblasts collected from laboratory infected *F. sultana* colonies (Fig. 7), which appeared as small red signals (arrows, Fig. 7). No such signal was observed in the negative control (Fig. 8).

DISCUSSION

Patterns of infection of maternal colonies and statoblasts

We obtained evidence for surprisingly high levels of covert infection in the maternal colonies that were collected from both the Rivers Avon and Dun (68% and 74%, respectively) relative to levels that were monitored during a previous study that examined temporal prevalences of infection in bryozoan populations in the River Cerne, also in southern England (the highest

prevalence of infection being ~50%; Tops, 2004). There are several factors that may contribute to explaining these high levels of covert infection including: the development of new primers in this study, if bryozoans in these systems are more susceptible to infection, if a greater number of infectious spores are present or if there is a greater degree of vertical transmission of infection to new colonies produced from infected statoblasts or infected colony fragments thus promoting the spread of infection through populations (Okamura *et al.* 2011).

We found that infected statoblasts were produced in maternal colonies that were both covertly infected and uninfected during the time the statoblasts were collected. These results suggest that some colonies lose infection but nevertheless pass infection to progeny produced during a prior period of infection. This may have happened, for instance, if the colony had effectively sealed off infected regions containing statoblasts by inward pinching of the peritoneum as was observed to occur in a colony of *Plumatella fungosa* infected with *Buddenbrockia plumatellae* (Canning *et al.* 2002). An alternate explanation is failure in DNA extraction from the maternal colony or that there was insufficient *T. bryosalmonae* DNA to form a clear band on the agarose gel. A possible explanation for apparent rescue of some statoblasts from infection is the inclusion of infected and uninfected portions of the colony in the same DNA extraction, giving a mixture of infected and uninfected statoblasts from an infected parent.

A substantial proportion of statoblasts produced by bryozoans from both the Rivers Avon and Dun were infected (30% and 39%, respectively), a finding further corroborated by detection of cryptic stages in infected statoblasts in our monoclonal antibody study. We also found that most infected statoblasts hatched and that this effect was greatest for material from the River Dun (94% and 100% in the Rivers Avon and Dun, respectively). Indeed, the hatching success was significantly associated with infection, with more infected than uninfected statoblasts

successfully hatching. This may indicate complex interactions between the colony ability to support both infections and produce viable statoblasts or parasite mediated stimulation of statoblast hatching. Our laboratory studies complemented our above findings by demonstrating that the presence of *T. bryosalmonae* in statoblasts can indeed result in subsequent overt infections in colonies that develop from statoblasts and that these infections, in turn, are transmitted to fish.

Implications of vertical transmission via statoblasts

Statoblasts function as dormant propagules that allow bryozoans to persist during unfavourable periods. In addition, there is good evidence that buoyant statoblasts are likely to be dispersed over great distances by waterfowl and ship ballast. Our demonstration that vertical transmission via statoblasts is effective provides evidence that *T. bryosalmonae* will be dispersed in space or time by hitchhiking along with its host, thereby similarly evading unfavourable conditions and achieving colonisation of new habitats. *F. sultana* statoblasts are not buoyant, yet vertical transmission via statoblasts and dispersal with bryozoan hosts may explain the widespread distribution of PKD in the northern hemisphere. Indeed, long distance dispersal achieved by infected statoblasts may explain phylogeographic patterns that suggest Europe may have been colonised by *T. bryosalmonae* from North America (Henderson and Okamura, 2004).

The capacity for vertical transmission via statoblasts demonstrated here for *T. bryosalmonae* appears to be a general characteristic of malacosporeans since it has been shown in *Buddenbrockia allmani* (Hill and Okamura, 2007) and appears to be achieved by *Buddenbrockia plumatellae* (Taticchi *et al.* 2004). This capacity should enable malacosporeans to persist indefinitely in local populations of bryozoans (Hill and Okamura,

2007) and, in terms of PKD, is likely to contribute to the regular annual outbreaks observed in many fish farms (El-Matbouli and Hoffmann, 2002; Okamura *et al.* 2011). Vertical transmission via colony fragmentation (Morris and Adams, 2006; Hill and Okamura, 2007) provides another mechanism by which malacosporean infections can be amplified within local populations. Significantly, in these colonial hosts, the mechanisms of vertical transmission documented to date are achieved by infection of clonal propagules (asexually-produced statoblasts, fragments of maternal colonies). Thus, it will achieve persistent infection of clonal genotypes over time and space which, in turn, will spread the risk of loss of infection from clonal genotypes.

A body of work now demonstrates that *T. bryosalmonae* undergoes developmental cycling between covert and overt infections dependent on host condition. The former cause no detectable effect on bryozoan host fitness and are expressed when hosts are in poor condition while overt infections temporarily effectively castrate bryozoan hosts and are expressed when hosts are in good condition (Tops *et al.* 2006; Hartikainen and Okamura, 2012). The overall driver of such developmental cycling appears to be a strategy for *T. bryosalmonae* to enable long term persistence in bryozoan populations by minimising virulence while occasionally undergoing horizontal transmission to fish. Such low virulence is anticipated when the reproductive interests of both host and parasite are aligned (Bull *et al.* 1991) as is the case for vertical transmission. Previous to this study it was predicted that vertical transmission of *T. bryosalmonae* by statoblasts must occur (e.g. Tops *et al.* 2004; Grabner and El-Matbouli, 2008; Tops *et al.* 2009). By providing the first evidence that such vertical transmission is apparently extensive and effective, our study significantly contributes to our understanding of infection dynamics in both bryozoan and fish hosts.

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For Peer Review

Figure captions

Figure 1 PCR amplification of *Tetracapsuloides bryosalmonae* DNA from statoblasts collected from *T. bryosalmonae* infected *Fredericella sultana* colonies under laboratory conditions: Lane 2 to 4 first round PCR amplified by 5F-6R primers and lane 7 to 9 nested PCR amplified by PKD-real F-R primers. Lane 1 and 5: 100bp DNA ladder (Invitrogen); lane 2: infected statoblasts DNA; lane 3: positive control; lane 4: SPF statoblasts DNA; Lane 6 and 10: 50bp DNA ladder (Fermentas); Lane 7: infected statoblasts DNA; Lane 8: positive control; Lane 9: SPF statoblasts DNA.

Figure 2 The percentage of maternal colonies that were covertly-infected and uninfected at the time of collection from Rivers Avon and Dun. One overtly-infected colony collected from the River Avon is not included. N values above bars are the numbers of colonies in each infection category per river.

Figure 3 The percentage of statoblasts that were infected according to the infection status of their maternal colony (covertly-infected or uninfected at the time of collection from the field) from the Rivers Avon and Dun. Two uninfected statoblasts from an overtly-infected colony collected from the River Avon are not included. N values above bars are the numbers of statoblasts collected from uninfected and covertly infected maternal colonies per river.

Figure 4 The percentage of infected and uninfected statoblasts collected from infected colonies (including covertly-infected maternal colonies and maternal colonies with infected statoblasts) from the Rivers Avon and Dun. Two uninfected statoblasts from an overtly-infected colony collected from the River Avon are not included. N values above bars are the numbers of statoblasts according to their infection status per river.

Figure 5 The percentages of hatched and unhatched infected statoblasts collected from infected colonies (including covertly-infected maternal colonies and maternal colonies with infected statoblasts) from the Rivers Avon and Dun. Numbers above bars are n-values.

Figure 6 PCR amplification of *Tetracapsuloides bryosalmonae* DNA from brown trout (*Salmo trutta*) samples cohabitated with *F. sultana* raised under laboratory conditions from infected statoblasts: Lane 1: 100 bp DNA ladder (Invitrogen); Lane 2-4: infected kidney, liver and spleen DNA samples of brown trout, respectively; Lane 5: negative control; Lane 6: positive control.

Figure 7 Horizontal section of infected statoblast of *Fredericella sultana* with *Tetracapsuloides bryosalmonae* monoclonal antibody, revealed the presence of cryptic stages inside the statoblast (arrows), enlarged section (lower left) in small windows focusing on cryptic stages of *T. bryosalmonae* (arrows), and intact statoblast (upper left). The image shows the dark brown chitinous valve of the statoblast containing vitellogenic material and putative stained parasite stages.

Figure 8 Specific pathogen free *Fredericella sultana* statoblast with *Tetracapsuloides bryosalmonae* monoclonal antibody

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