Department for Farm Animals and Veterinary Public Health

University of Veterinary Medicine Vienna

Fish Health Division

(Head: Astrid Holzer)

Influence of temperature and cohabitation on the growth of the bryozoan *Fredericella sultana* in culture for transmission studies of myxozoan parasites

Bachelor thesis

University of Veterinary Medicine Vienna

Submitted by

Elea Mießen

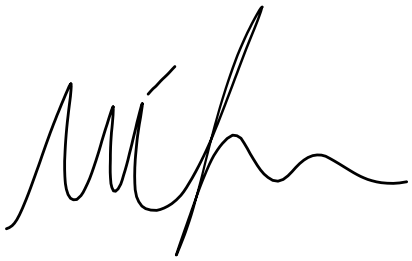
Vienna, July 2023

Supervisor: Gema Alama Bermejo, PhD

Reviewer: Hans-Peter Fuehrer, Prof

Declaration

Herby, I declare, that this thesis “Influence of temperature and cohabitation on the growth of the bryozoan *Fredericella sultana* in culture for transmission studies of myxozoan parasites” was solely written independently and has not been published elsewhere, in part or as whole. References or acknowledgments regarding citations are provided and summarized in an attached list.



Vienna, 21st of July 2023 Elea Mießen

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Abstract

Myxozoans are extremely morphologically reduced parasites that can represent serious threats to fishes. Their complex life cycles involve invertebrate hosts (annelids, bryozoans) and vertebrate hosts (usually fishes), therefore only few myxozoan life cycles are maintained in laboratories. Culturing the invertebrate hosts is challenging because little is known about their biology. Optimizing myxozoan maintenance in laboratories increases our ability to understand these parasites invasion biology.

At the Fish-Health Division, the life cycle of the myxozoan *Tetracapsuloides bryosalmonae*, the agent of proliferative kidney disease in salmonids, is maintained. The objectives of this study are 1) to determine the influence of temperature in the growth and development of the bryozoan *Fredericella sultana*, and 2) identify the effect of fish cohabitation and associated temperature change.

Bryozoan colonies were grown at three different temperatures for several weeks. Firstly, the colonies growth and survival were monitored at the different temperatures over time. Afterwards, the group was exposed for one week to PKD infected fish as well as to a temperature change for two weeks. The water was filtrated and analyzed for *T. bryosalmonae* stages with qPCR to assess the impact of the parasite on the colony's growth.

Colonies held at colder temperatures showed higher viability and smaller size but held at higher temperatures showed less viability and larger size. Cohabitation and temperature change seemed to have a negative effect on the colonies growth.

This thesis provides first insights on the optimization of *F. sultana* culture in laboratory conditions for transmission studies of a pathogenic salmonid parasite.

Zusammenfassung

Myxozoen sind extrem morphologisch reduzierte Parasiten, die ernsthafte Bedrohungen für Fische darstellen können. Ihre komplexen Lebenszyklen umfassen wirbellose Wirte (Ringelwürmer, Moostierchen) und Wirbeltierwirte (in der Regel Fische), daher werden nur wenige Myxozoan Lebenszyklen in Laboren aufrechterhalten. Die Kultivierung der wirbellosen Wirte ist herausfordernd, da wenig über ihre Biologie bekannt ist. Die Optimierung der Myxozoan Erhaltung in Laboren erhöht unsere Fähigkeit, die Invasionsbiologie dieser Parasiten zu verstehen.

In der Fischgesundheitsabteilung wird der Lebenszyklus des Myxozoans *Tetracapsuloides bryosalmonae*, dem Erreger der proliferativen Nierenerkrankung bei Salmoniden, aufrechterhalten. Die Ziele dieser Studie sind 1) die Bestimmung des Einflusses der Temperatur auf das Wachstum und die Entwicklung des Moostierchens *Fredericella sultana* und 2) die Identifizierung der Auswirkungen von Kohabitation und der damit verbundenen Temperaturänderung.

Die Moostierchenkolonien wurden mehrere Wochen lang bei drei verschiedenen Temperaturen gezüchtet. Zunächst wurde das Wachstum und Überleben der Kolonien bei den verschiedenen Temperaturen im Laufe der Zeit überwacht. Anschließend wurde die Gruppe eine Woche lang infizierten Fischen mit proliferativer Nierenerkrankung (PKD) ausgesetzt und zwei Wochen lang einer Temperaturänderung unterzogen. Das Wasser wurde filtriert und mittels qPCR auf *T. bryosalmonae* Stadien analysiert, um die Auswirkungen des Parasiten auf das Wachstum der Kolonie zu bewerten.

Kolonien, die bei kälteren Temperaturen gehalten wurden, zeigten eine höhere Lebensfähigkeit und geringere Größe, während solche bei höheren Temperaturen eine geringere Lebensfähigkeit und größere Größe aufwiesen. Die Kohabitation und Temperaturänderung scheinen eine negative Auswirkung auf das Wachstum der Kolonien zu haben.

Diese Arbeit bietet erste Erkenntnisse zur Optimierung der Kultivierung von *F. sultana* unter Laborbedingungen für Transmissionsstudien eines pathogenen Salmonidenparasiten.

Abbreviations

PKD	Proliferative kidney disease
T. bryosalmonae	*Tetracapsuloides bryosalmonae*
SPF	Specific pathogen free
F. sultana	*Fredericella sultana*
p.t.	post trigger
RT	Room temperature
∆temp	Difference of temperature
C. ovata	*Cryptomonas ovata*
S. rubescens	*Synechococcus rubescens*
SD	Standard Deviation

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1 Introduction and aim of the study

1.1 Myxozoans

Myxozoa are a group of obligate cnidarian endoparasites. They are amongst the oldest metazoan parasites on Earth (Holzer et al. 2018). These spore-forming parasites underwent morphological simplification after diverging from their free-living cnidarian ancestors. Myxozoans are known to cause important economic losses in aquaculture and wild fishes and have become an increasing concern as emerging pathogen due to global climate change (Videira et al. 2016; Strepparava et al. 2018). These parasites have complex life cycles that require two hosts: a vertebrate intermediate host, primarily fish, and an invertebrate definitive host, annelids or bryozoans.

These parasites can be divided into two classes: Malacosporea and Myxosporea. The larger class, Myxosporea, includes several genera and has as its characteristic hard shell valves. Few examples for this class are *Myxobolus cerebralis*, causative agent of the whirling disease in salmonids or *Sphaerospora molnari,* who causes gill sphaerosporosis in carps. The other class, Malacosporea, has only two genera, *Buddenbrockia* and *Tetracapsuloides,* that produce their characteristic soft-shell spores. These class uses bryozoans as their definitive host and only fish as their intermediate host (reviews by Eszterbauer et al. 2015 and Canning and Okamura 2004).

1.2 *Tetracapsuloides bryosalmonae*

The malacosporean *Tetracapsuloides bryosalmonae* causes proliferative kidney disease (PKD) in wild and farmed Salmonidae, which includes among other salmon, chars, graylings, and trout. It is manifested by severe inflammatory response, anemia, an enlarged kidney and spleen and can become systemic in most fish hosts, leading to high mortality rates (Canning et al. 1998; Feist et al. 2001). PKD is consider an emerging disease due to climate change (Ros et al. 2021).

The complex life cycle of *T. bryosalmonae* involves salmonids and bryozoans (Fig 1.1). Transmission between hosts is achieved by morphologically different waterborne spores: the malacospore and the fishmalacospore. The fishmalacospore is excreted with the urine. When this spore encounters the bryozoan, it opens and an infective amoeboid sporoplasm enters the ectoproct host via the gut epithelium and epidermis. Once inside, it undergoes

proliferation in the host coelom, leading to the development of a sac filled with malacospores. The sporoplasm of the malacospores will infect the fish host entering through the epidermis and migrating using the bloodstream to reach the target organ, the kidney. Proliferation in the kidney will lead to sporogonic stages in the tubules with the release of Fishmalacospores with the urine, completing the cycle (reviewed by Sudhagar, Kumar, and El-Matbouli 2019). Several salmonid hosts can serve as intermediate host, although only two are considered natural hosts: brown trout *Salmo trutta* and brook trout *Salvelinus fontinalis* (i.e. spore development is completed in these host) (Grabner and El-Matbouli 2008). At least 5 species of bryozoans can serve as invertebrate host for *T. bryosalmonae*, but the most preferable is *Fredericella sultana,* probably due to its widespread distribution which increases the chances and as well as the possibility for the parasite to proliferate inside (Hartikainen, Gruhl, and Okamura 2014).

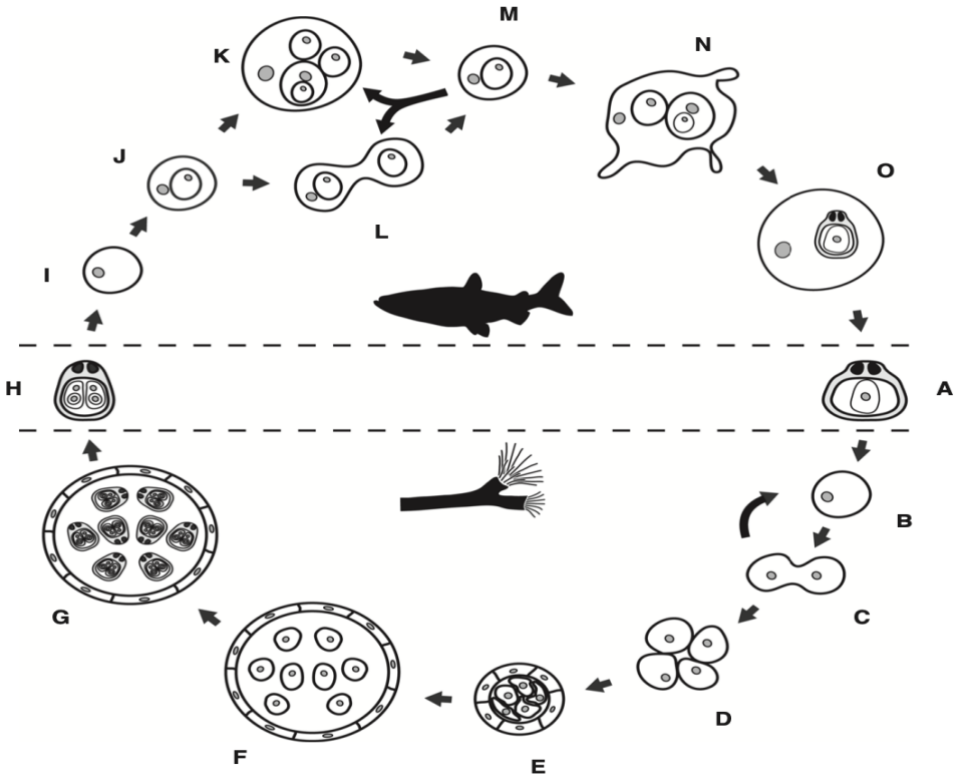

Fig 1: Life cycle of *Tetracapsuloides bryosalmonae* (Gruhl 2015) (A) Fishmalacospore. (B) Sporoplasm enters the ectoproct host via gut epithelium and epidermis. (C) Proliferation in the host coelom via mitoses. (D) Early cell cluster. (E) Early compact bilayered stage. (F) Immature sac. (G) Mature sac, filled with spores. (H) Malacospore. (I) Sporoplasm germ enters the fish host via epidermis. (J) Endogenic stage with secondary cell. (K) Proliferation by mitotic division and release of secondary cells. (L) Proliferation by mitotic division of primary and secondary cells. (M) Endogenic stage with secondary cell. (N) Sporogonic pseudoplasmodium. (O) Mature sporogonic pseudoplasmodium.

1.3 Phylum Bryozoa

Bryozoans or moss animals are aquatic invertebrates widely distributed in freshwater and marine aquatic habitats. These animals form colonies of zooids that usually grow on firm substrates (e.g. rocks, shells, wood, plants). Each zooid is a single animal consisting of a cystid (body wall and exoskeleton) and its internal living parts i.e. digestive tract, musculature, nerve cell and tentacles, also known as polypide. The tentacles are arranged as a circular or horse-shoe shaped crown known as lophophore. Having a muscular and contractile body allows these animals to generate with the tentacles water currents to filter food particles i.e. plankton and bacteria. Their life cycles include phases of asexual and sexual reproduction, and usually zooids are hermaphroditic. There are >5860 species known that can be divided into three classes: Gymnolaemata (5240 spp., mostly marine), Stenolaemata (543 spp., exclusively marine) and Phylactolaemata (86 spp., freshwater dwelling) (Zhang 2013).

Most of freshwater bryozoans belong to the class Phylactolaemata. These animals are frequently found in clear, quiet, or slowly flowing water in rivers, ponds, and lakes, where they form colonies on the surfaces of submerged rocks, plants, and tree roots and branches. They can also become biofouling in water systems causing clogs and operating problems (Wood and Marsh 1999). Phylactoleamata have some characteristic traits like unmineralized cystid and colonies, interzooidal pores that allows coelomic fluid to pass freely through adjacent zooids and the generation of statoblasts, highly resistant dormant stages that persist during unfavorable conditions like drought or winter months (Ryland 2005; Francis 2001). Freshwater bryozoans are organisms of interest for myxozoan parasites research as several members of this group act as definitive hosts i.e. *Plumatella* and *Fredericella* spp. (Okamura et al. 2001).

The invertebrate host *Fredericella sultana* (Bryozoa:Phylactolaemata)

Fredericella sultana is one of the few freshwater bryozoans with a cosmopolitan distribution (Europe, Asia, North America, and Australasia) (Massard and Geimer 2008). This bryozoan forms branching tubular colonies formed by cylindrical zooids (around 200 µm long) surrounded by a chitinous outer wall and a circular lophophorae with 17-27 tentacles (Raddum and Johnsen 1983) (Fig 2 & 3). The life cycle of this bryozoan (Fig 4) involves rapid grow by asexual budding of zooids during spring and summer, with further fragmentation and re-attachment of branches; dormant stages or statoblasts and/or

regressed colonies during winter. Additionally, brief sexual reproduction occurs only in early summer, with a short-live larvae that settles to form a new colony (reviewed by Hartikainen and Okamura 2015).

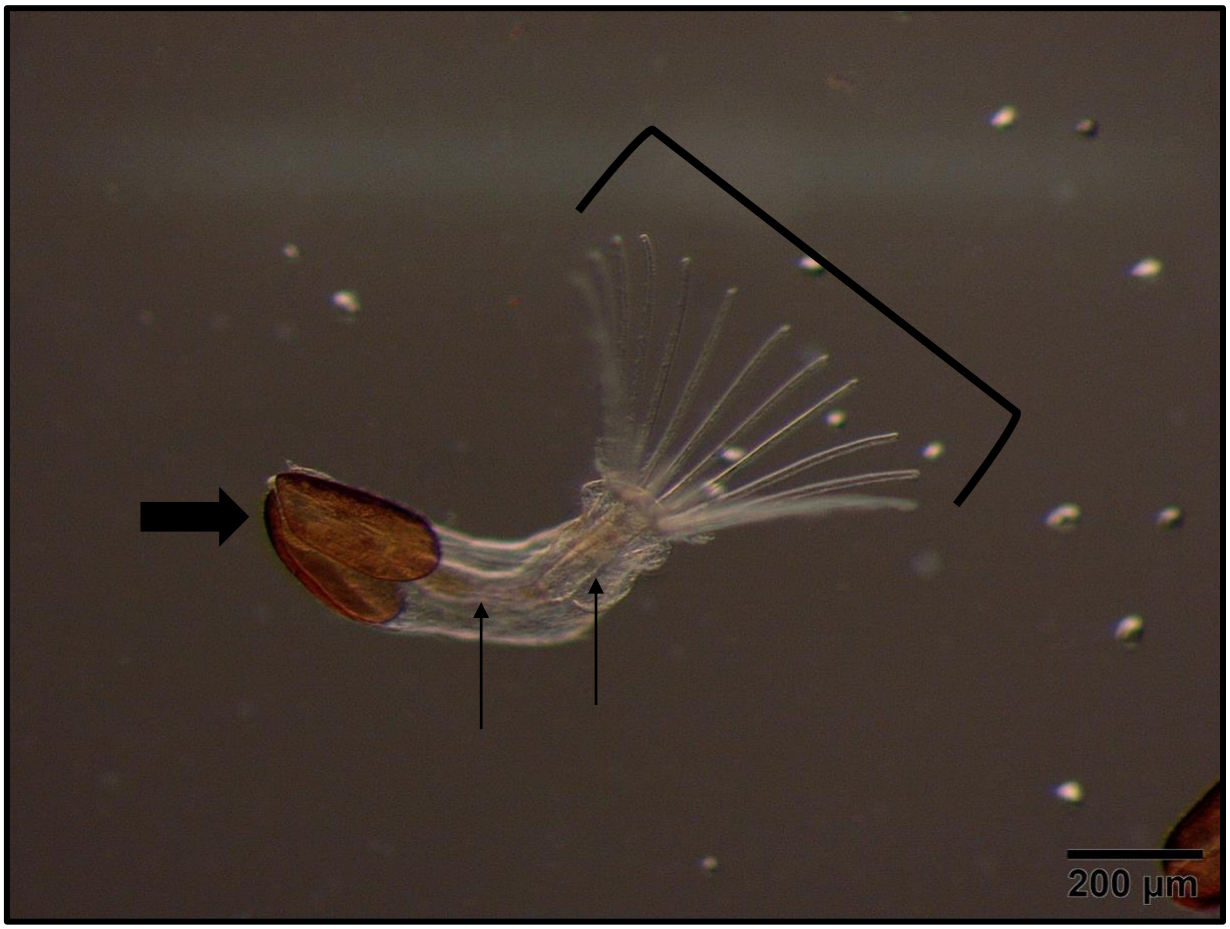

Fig 2. Hatched statoblast (block arrow) with stomach, intestines (arrows) and tentacles (brackets). Photo credit: Astrid Holzer.

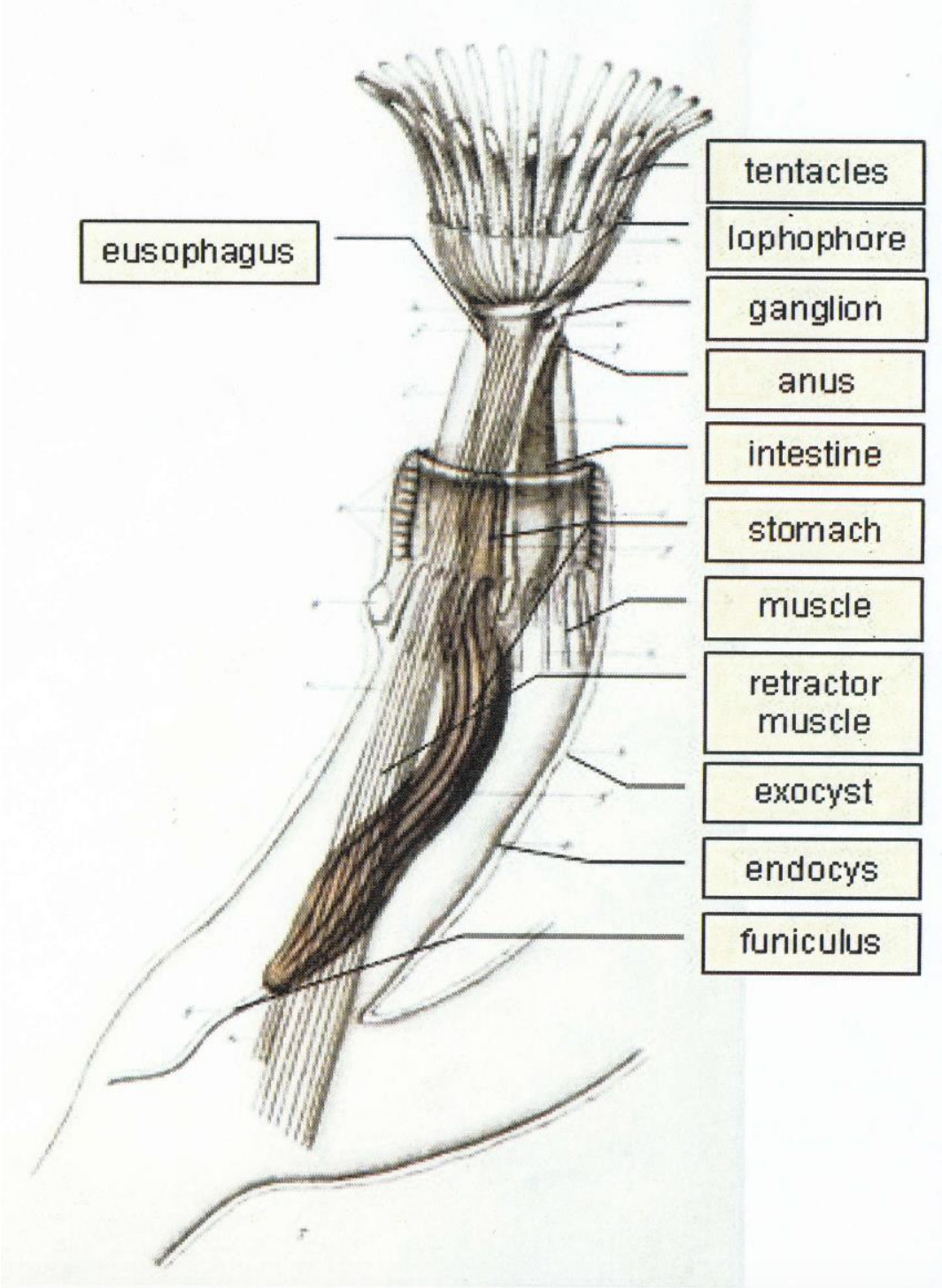

Fig 3. Anatomy of *F. sultana* main body parts of one zooid (Blumenbach 1856)

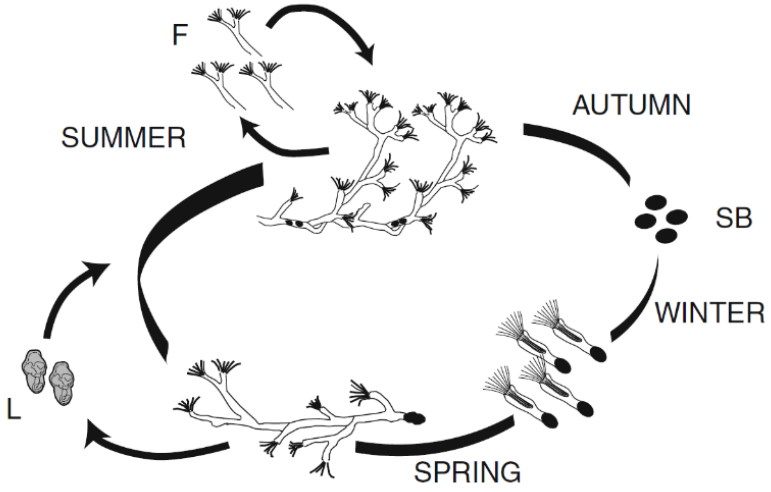

Fig 4. Seasonal life cycle of *F. sultana*. Abbreviations: L- Larvae, F- Fragmentation, SB- Statoblast (Hartikainen and Okamura 2015).

F. sultana culture for *T. bryosalmonae* laboratory infections

Few (four to five) myxozoan parasites life cycles are maintained in laboratories worldwide. *In vivo* life cycle maintenance of these parasites is essential for functional studies of disease mechanisms and design of anti-parasitic strategies. At the University of Veterinary Medicine in Vienna, the life cycle of *Tetracapsuloides bryosalmonae* is kept in the laboratory, alternating from the bryozoan *F. sultana* and brown trout *S. trutta*. Previously, other laboratories have used this invertebrate for infection studies. Known factors that influence the growth of *F. sultana* are temperature, nutrient availability, the use of different media at different pH, and water (McGurk et al. 2006; Grabner and El-Matbouli 2008; Hanna Hartikainen et al. 2009; Tops, Hartikainen, and Okamura 2009; Kumar et al. 2013). At the University of Veterinary Medicine Vienna, *F. sultana* is hatched from statoblasts, grown on ground water on petri dishes and fed using algae culture (SOPs Gorgoglione and Kotob, 2021 and further details in the MM section in this thesis) as a Specific Pathogen Free (SPF) culture. Due to the labor-intensive maintenance of these life cycles in laboratories, evaluation of culture parameters is highly desirable for the optimization of hosts maintenance and parasite transmission in the lab.

1.4 Aim and objectives of the study

The aim of this study is to provide insights into *Fredericella sultana* growth and survival in culture by investigating the influence of water temperature and of fish cohabitation on the bryozoan colonies. This study seeks to fill gaps in our knowledge on the invertebrate host culture for optimization of the parasite *T. bryosalmonae* transmission for infection studies at the Fish Health Unit (University of Veterinary Medicine Vienna).

Specific objectives

To evaluate *F. sultana* colony growth, survival and statoblast production

1) At three different water temperatures overtime.
2) Induced by temperature change and cohabitation with *T. bryosalmonae* infected fish.

Hypotheses

1.1) Warmer temperatures will increase the growth of the colonies but also cause more deaths.

1.2) Maintenance at colder temperatures will lead to reduced food consumption.

2.1) The bryozoan colonies kept in cooler conditions will growth more when exposed to fish cohabitation and higher temperature.

2.2) The viability of colonies maintained at higher temperatures will be significantly compromised compared to colder maintained colonies after cohabitation and temperature change.

2 Materials and methods

2.1 Materials

2.1.1 Equipment

Centrifuge, Eppendorf, Germany

Eppendorf tubes 1,5 mL, Eppendorf, Germany

14 mL Falcon, Eppendorf, Germany

Nanodrop, VWR, USA

Pipette tips, Sarstedt, Germany

Pipettes, Eppendorf, Germany

Thermo Cycler, BioRad, USA

Thermomixer, Eppendorf, Germany

SZX10 stereo microscope, Olympus, Japan

Vortexer, Bio Rad, USA

Heater, JBL GmbH & Co KG, Germany

Fridge, Fisher Scientific, USA

Plate reader, Thermo Fisher, USA

15 L bucket, OBI, Germany

Vacuum air pump, KNF LAB, Germany

Millipore filter holder, Millipore filter corporation, USA

Funnel with restrainer, Millipore filter corporation, USA

Filter flask, Fisher Scientific, USA

Clamps, Millipore filter corporation, USA

Hoses, OBI, Germany

5 µm Filter, Cytiva, Germany

Forceps and Scissors, Henry Schein, USA

Petri dish, Corning, USA

Carbon steel scalpel blades #15, B. Braun, Germany

Bryozoan Cage, self-made; University of Veterinary Medicine Vienna, Austria

2.1.2 Biological material

Brown trout, Glück Fischzucht GmbH, Austria

Fredericella sultana, University of Veterinary Medicine Vienna, Austria

Cryptomonas ovata, EPSAG, Germany

Synechococcus rubescens, EPSAG, Germany

2.1.3. Chemicals and Solutions

WC Medium, University of Veterinary Medicine Vienna, Austria

Phenol, AppliChem, Germany

Chloroform, Sigma-Aldrich, USA

70 % EtOH, University of Veterinary Medicine Vienna, Austria

92 % EtOH, University of Veterinary Medicine Vienna, Austria

TNES urea buffer, University of Veterinary Medicine Vienna, Austria

Proteinase K, Qiagen, Germany

Nucleinase-free water, Qiagen, Germany

2.2 Methods

2.2.1 Statoblast collection

For statoblast collection, dead *F. sultana* colonies were cut into several small pieces with a blade, then moved into a 14 mL falcon and vortexed for 6 min in order to free the statoblasts. Statoblasts are dense and heavy and settle faster than the colony leftovers so the remainder of the tissue can easily be removed with a pipette. Statoblasts were then washed once with ground water. Afterwards they were stored, fully covered in ground water, at 4 °C and until usage.

2.2.2 Bryozoan growth at different temperatures experiment

To study *F. sultana* growth at different temperatures, 576 statoblasts were taken from storage at 4 °C, spread out on petri dishes, with 32 statoblasts per dish, covered with ground water and left to hatch at RT (=trigger for growth). After three days, statoblasts had attached to the dish-surface and hatched (Fig. 2). As soon as the tentacles were visible, they were fed with an algae/ cyanobacteria mix of *Cryptomonas ovata* and *Synechococcus rubescens* (Gorgoglione & Kotob, 2021). For the first days, they were moved the minimum to reduce the risk of detachment.

After ten days they were transferred into a 15 L bucket, where they were placed at a approx. 45° angle into a wire rack, facing the bottom of the bucket. The bucket was equipped with abundant aeration. The buckets were set up at three different temperatures (six petri dishes per temperature): 16 °C in a temperature-controlled incubator, 24 °C with a heater and 20/21 °C (room temperature; RT). The choice of temperature conditions was based on specific objectives. The coldest temperature was selected to simulate natural habitat conditions, while the warmest temperature was chosen to represent conditions closer to the upper limit of their temperature tolerance range (Tops, Hartikainen, and Okamura 2009). Cultivating bryozoans at room temperature was a practical decision for laboratory settings, offering convenience.

All colonies on the petri dishes were checked weekly and fed the same amount (50 mL mix at 0.15 OD (405 nm)) of the algae *C. ovata* (90 %) and *S. rubescens (*10 %). A complete water change was performed every two weeks. The algae/cyanobacteria were fed with WC Medium (prepared according to the protocol attached in the appendix). After

eight weeks, feeding regime was adjusted, by doubling the amount to 100 mL mix at 0.15 OD (405 nm).

2.2.3 Cohabitation and temperature change experiment

For cohabitation, three brown trout (Total length = 25 cm) with a long-term infection with *T. bryosalmonae* were transferred from an outside (800 L) to an inside (180 L) tank, one week before cohabitation and protected from light or stress resulting from humans passing the aquarium, with a black foil around the tank. Temperature was set up at 18 °C; this temperature was chosen as a compromise between fish survival and parasite proliferation (Waldner et al. 2021). For protecting the *F. sultana* colonies from the fish, three special Plexiglas containers with 0.5 cm holes on five sides were made with a 3D printer and assembled (Fig 5). Bryozoans were cultivated at various temperatures as described in section 2.2.2. The experiment involved dividing the bryozoans into two groups, with three petri dishes from each temperature condition. Each temperature group was placed into a container cohabitated with fish (treated) resulting in a temperature change as follows: bryozoans cultivated at 24 °C experienced a decrease in temperature of 6 °C (Δtemp = -6); bryozoans cultivated at room temperature (RT) experienced a decrease in temperature of 2/3 °C (Δtemp = -2/3); and bryozoans cultivated at 16 °C experienced an increase in temperature of +2 °C (Δtemp = +2). The other half stayed in their buckets (controls) and was maintained at their respective temperatures. Cohabitation took place for 1 week at 18 °C, only overnight and with the water flow stopped. During the day, the bryozoan containers were moved to a 15L bucket and fed as described in 2.2.2. while fish were kept in the tank at flowthrough water and feed a regular salmonid diet of pelleted food. After cohabitation, the treated and control groups condition was assessed as described in the following section at 10 weeks p.t. Cohabitated group was checked at 12 weeks p.t. and 14 weeks p.t. for *T. bryosalmonae* stages.

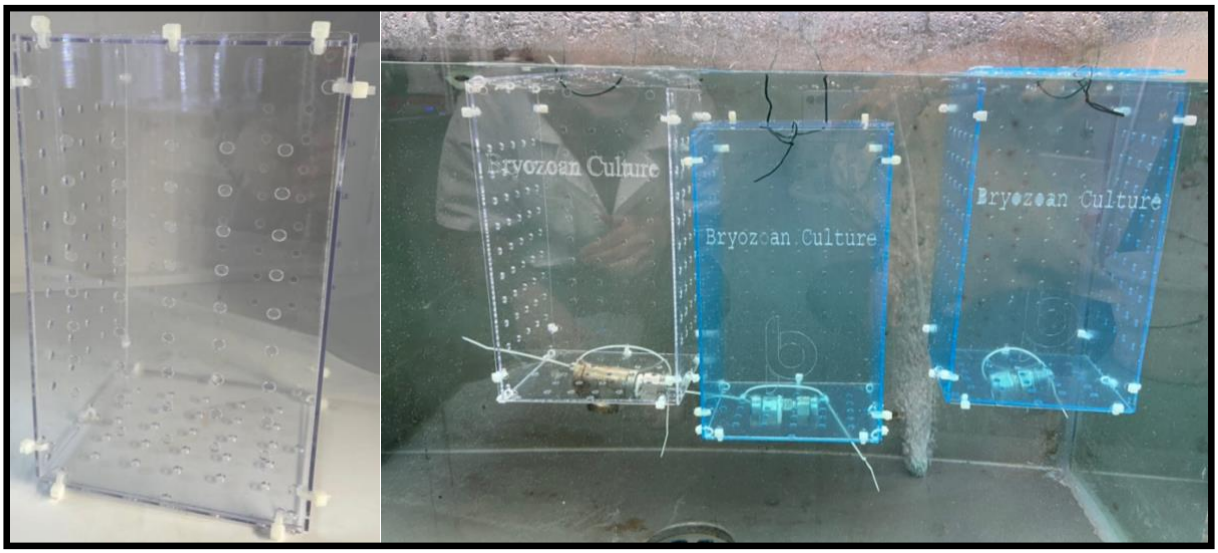

Fig 5: Container for bryozoan cohabitation with fish. The container was made of Plexiglas with 0.5 cm holes on five sides by a 3D printer and was weighted with heavy objects to be fully immersed in water.

2.2.4 Estimation of bryozoan growth and survival

After eight, ten and 12 weeks p.t., *F. sultana* colonies were observed under a stereo microscope. Firstly, the total number of hatched statoblasts were counted on each plate to determine hatching success. Thereafter, total number of newly produced statoblasts by the colonies was counted per plate.

For obtaining information of the viability of zooids per colony, number of zooids and proportion of live and dead zooids were counted. Due to the entanglement of the colonies and zooids, it was not possible to differentiate all colonies individually. Therefore, number of zooids per colony and proportion of dead vs alive zooids per colony were counted from eight representative colonies.

Total number of zooids per plate was calculated from the eight counted colonies as follows:

$$Total\ number\ of\ zooids = \ total\ number\ of\ colonies\ \times \frac{\sum zooids\ of\ 8\ counted\ colonies}{8}$$

Similarly, proportion of dead vs alive zooids was calculated for each plate.

To calculate the fraction of statoblast formation per zooid, total number of statoblasts were divided by total number of zooids per plate.

For the bryozoan growth experiment, an outlier was excluded from the RT group's data due to a distinct circumstance where the petri dish fell off the rack. This event resulted in reduced access to vital resources such as food and oxygen. Consequently, the outlier's

inclusion in the analysis could potentially introduce bias or distort the overall results. Therefore, in order to maintain the integrity of the analysis and ensure accurate interpretation, the outlier was removed from the dataset resulting in n = 2 plates.

The full experimental setup is summarized in Fig 6 for better understanding.

2.2.5 Water filtration for detection of *T. bryosalmonae* transmission stages

To obtain information about the presence of spores released by infected fish into the water, the waterflow was stopped overnight at eight and a half weeks p.t., six liters were taken out both times and filtered with a 5 µm Millipore polycarbonate filter. The water temperature of the cohabitation tank was 18 °C at the first sampling point and heated up, after cohabitation had already ended, to 20 °C for the second sampling point. Afterwards, the filter was cut in half, one part stored in 1 mL absolute EtOH and the other part in 400 µL TNES (prepared according to the protocol attached in the appendix). The part stored in EtOH was initially kept at 4 °C and then stored long-term in -20 °C to avoid evaporation. The tube containing TNES was used for DNA extraction.

2.2.6. Molecular Detection of *T. bryosalmonae*

The samples stored in 400 µL TNES buffer solution were used for DNA extraction according to Holzer et al. 2004. In brief, 661.5 µmol/L of Proteinase K was added and left to digest at 55 °C, overnight in a thermomixer. Afterwards 400 µL of phenol was added and the samples were turned over, this step was repeated after adding 400 µL of chloroform. Centrifugation was used for 5 min to separate the phases. The aqueous top layer was removed into a fresh tube containing the DNA, without disturbing the interface. In total, 330 µL were removed to a fresh tube, and 3 times the volume of 92 % ice-cold EtOH was added (990 µL) to precipitate the DNA, followed by another centrifugation step for 20 min at 4 °C. The alcohol was decanted carefully, and the last drop on the edge of the tube left on a piece of paper. DNA was washed with 1 mL of 70 % EtOH and centrifuged for 5 min. EtOH was decanted carefully again with the last drop left on a piece of paper. The tube was then left open overnight and resuspended with 60 µL of nuclease free water and concentration quantified with the Nanodrop. All centrifugation steps were performed at 15 000 x g.

For the detection of the DNA of *T. bryosalmonae* in the DNA-extracted water samples, quantitative real time PCR was performed using a TaqMan assay and following a protocol by Bettge et al. (2009). The following primers and probe were used: PKDtaqf1 (5'-GCGAGATTT-GTTGCATTTAAAAAG-3'), PKDtaqr1 (5'-GCACATGCAGTGTCCAATCG-3'), and probe-PKD (5'-CAAAATTGTGGAACCGTCCGACTACGA-3'); labelled at the 5' end with FAM and at the 3' end with TAMRA. As a positive control, a sample obtained from positive PKD-kidney tissue was included and confirmed by sequencing. The reaction volume of 25 µL contained 12.5 µL 2xQuantiTechProbePCRMix (Qiagen, Germany), 100 nM forward primer, 100 nM reverse primer, 100 nM fluorescent labelled probe, 4.5 µL PCR grade water and 5 µL of the extracted DNA. qPCR amplification was performed as follows: the amplification conditions consisted of initial denaturation at 95 °C for 5 min, followed by 45 cycles of 20 s at 95 °C (denaturation), followed by 30 s at 60 °C (annealing) and 20 s at 72 °C (elongation). The amplification cycles were preceded by another step where samples were held at 95 °C for 1 min with an increment of 0.5 °C every 10 s. The thermal cycler program finished with samples being held at 10 °C.

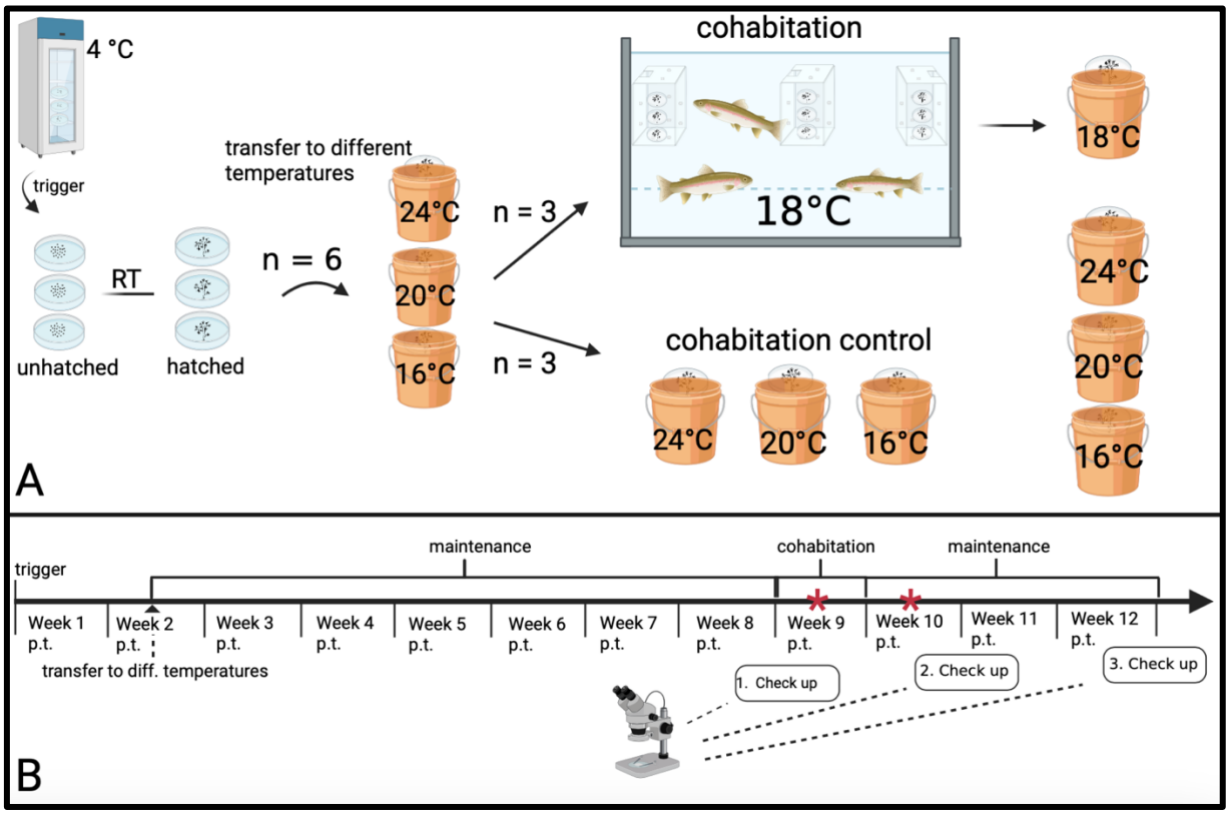

Fig 6. Experimental design. (A) Illustration of the procedure. (B) Timeline of the experiments starting the day statoblasts were removed from the fridge (=trigger for growth), maintained at different temperatures and/or

cohabitated with fish and induced temperature change. Dates of samples taken for water filtration of the fish tank, indicated with red asterisk. Statoblasts, zooids and colonies counts were performed after 8-, 10- and 12-weeks p.t with a stereo microscope. Created with BioRender.com.

3 Results

3.1 Bryozoan growth at different temperatures experiment

In general, *F. sultana* colonies kept at lower temperatures seemed to form more statoblasts (Fig 7A). Colonies kept at higher temperatures looked skinny and exhibited empty intestines (Fig 7B). For the *F. sultana* colonies kept at RT, they had content in their stomachs and few statoblast formation was observed (Fig 7C).

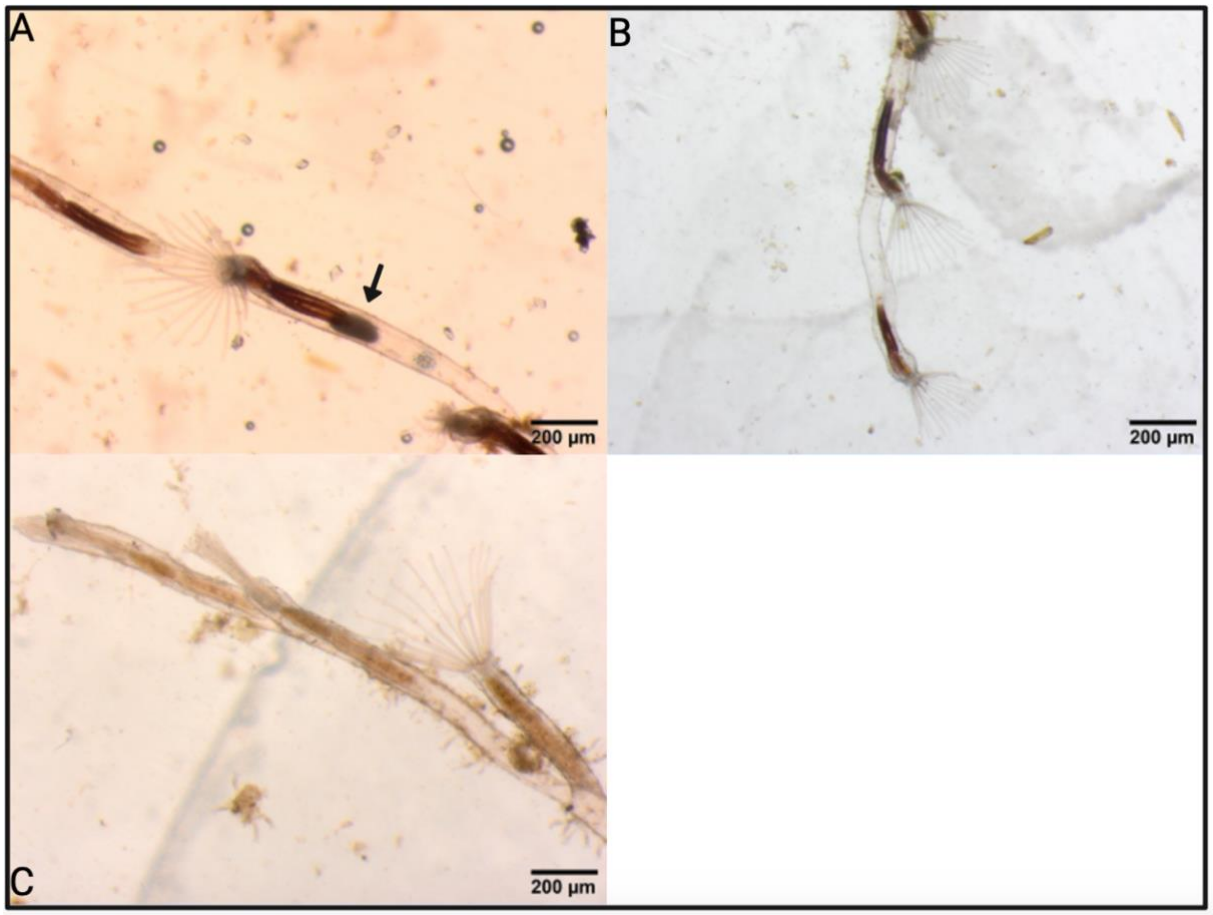

Fig 7. Stereomicroscope pictures of *F. sultana* maintained at three different temperatures. (A) Bryozoan kept at 16 °C and a statoblast indicated with arrow. (B) Bryozoan kept at 24 °C. (C) Bryozoan kept at RT. All pictures taken at 8 weeks p.t.

The mean viability and number of zooids per colony at three distinct time points was assessed. *F. sultana* colonies cultured at lower temperatures exhibited a lower number of zooids per colony compared to those cultured at higher temperatures (Tab 1, Fig 8). These

colonies also displayed a lower mortality rate compared to the colonies cultured at higher temperatures (Tab 1 & Fig 9). Colonies maintained at RT showed the highest number of zooids per colony, with an average of 13 zooids per colony at the two earliest time points and uncountable due to entanglement at the latest time point (Tab 1 & Fig 8).

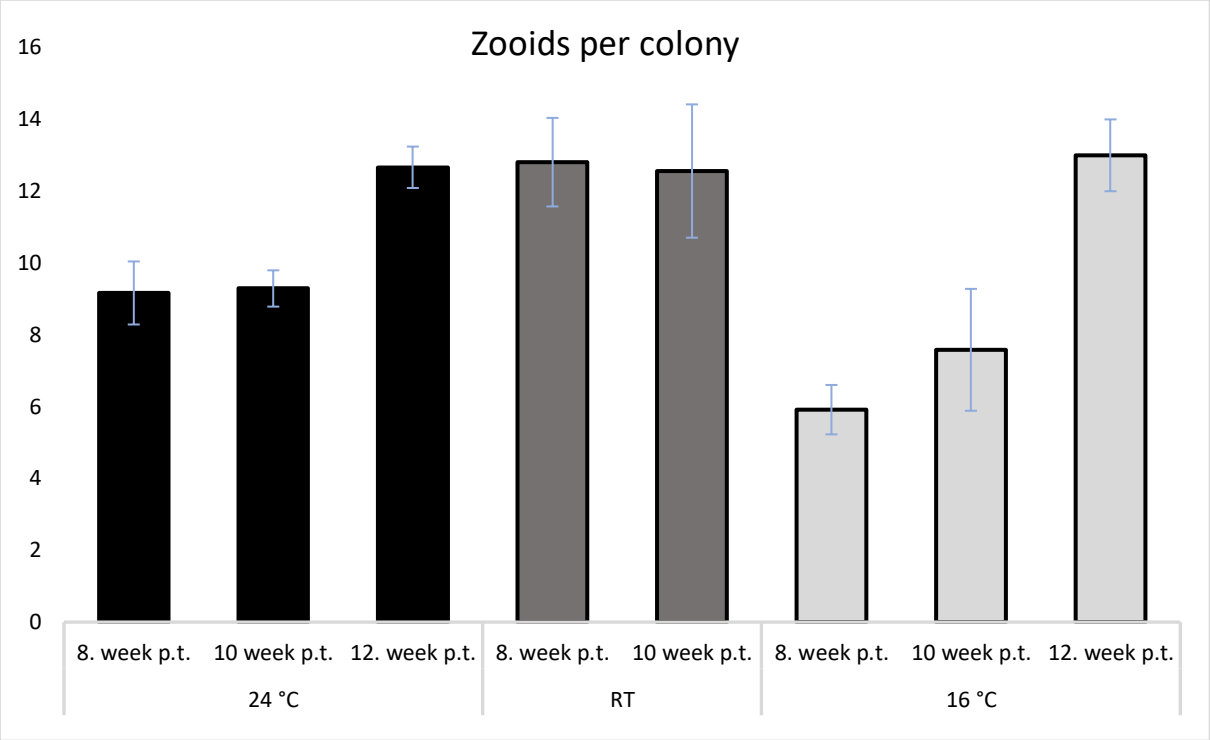

Fig 7. Average number of zooids per colony at different temperatures and different time points (n = 3 plates per temperature treatment). Error bars in blue. One outlier was removed in the RT group at both time points (n=2 plates) and RT counting at 12. week p.t. was omitted due to the excessive entanglement of the colonies.

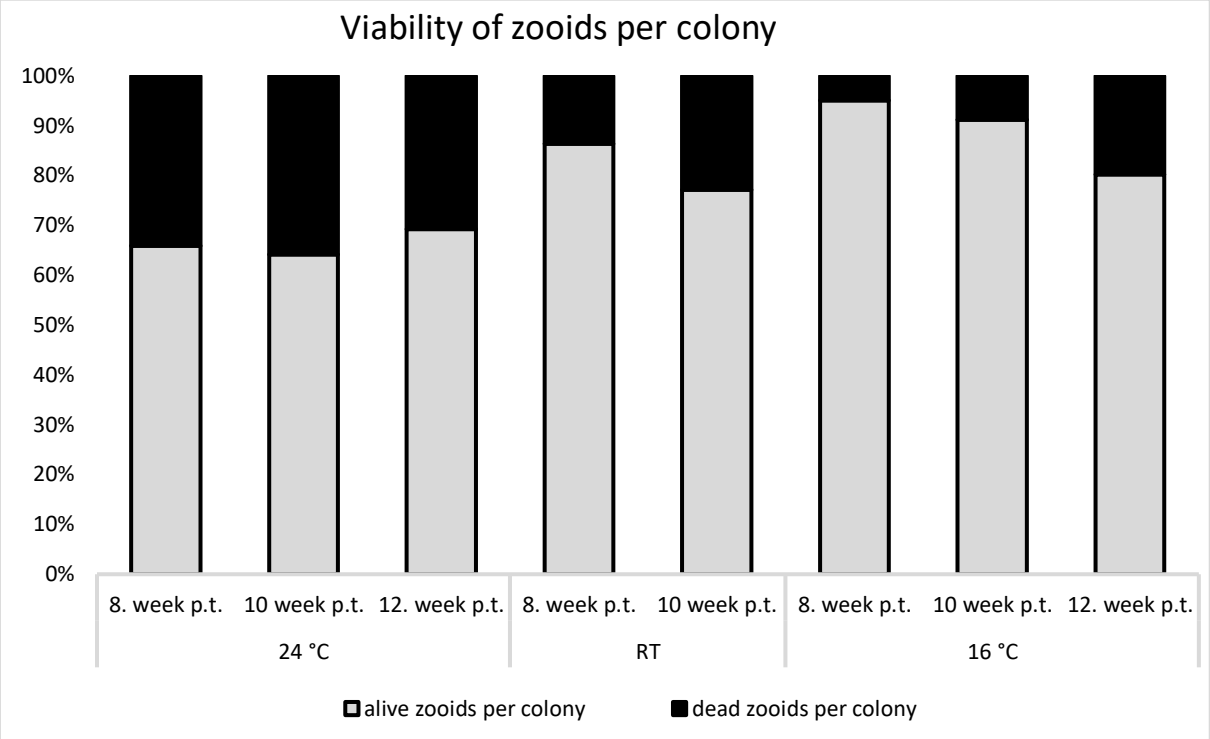

Fig 8. Proportion of live/dead zooids per colony at each time point at three different temperatures (n = 3 plates per temperature). One outliner was removed in the RT group at both time points (n=2) and RT counting at 12. week p.t. was omitted due to the excessive entanglement of the colonies.

Regarding the fraction of statoblast formation, it was observed that during elevated temperatures, formation of statoblast was relatively stagnant (2.1 % -1.7 %) over time (Fig 9). In contrast, *F. sultana* colonies cultivated at 16 °C showed an overall high production of statoblasts, with a clear decrease over time (21.8 % -10 %). Colonies maintained at RT showed an increase over time and were not countable due to entanglement of the colonies after the second time point (10-17.8 %) (Fig 9).

In the group which was heated (24 °C), the viability remained relatively stable over time, showing minimal changes (Fig. 9 & Tab 1). Similarly, the formation of statoblasts, also exhibited a consistent pattern without significant variations (Fig. 10 & Tab 1). On the other hand, the other two groups displayed a decrease in viability over time. In the group cultured at RT, there was a decrease in viability along with a concurrent increase in the formation of statoblasts over time. In contrast, the cooling group showed a decrease in statoblast formation as time progressed, as well as an overall decrease in viability (Fig. 8, 9 & Tab 1).

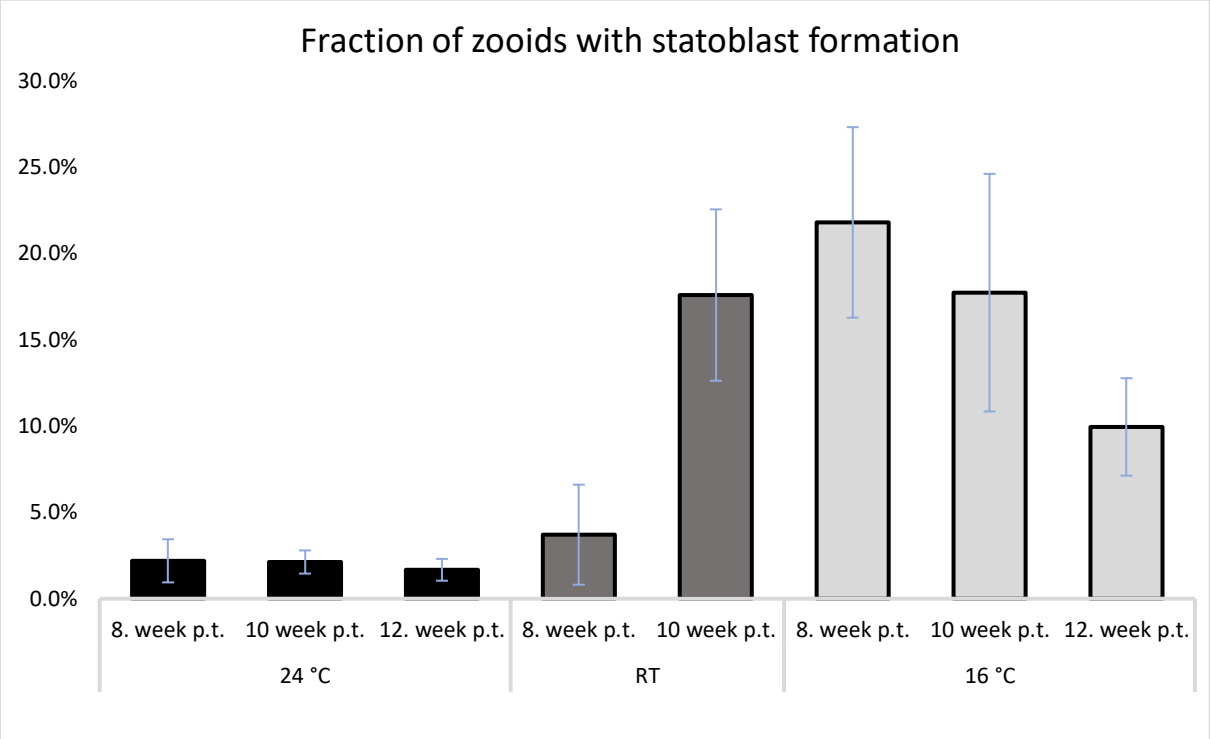

Fig 10. Fraction of total zooids with statoblast formation at each time point at three different temperatures (n = 3 plates per temperature). Error bars in blue. One outlier was removed in the RT group at both time points (n=2 plates) and RT counting at 12 weeks p.t. was omitted due to the excessive entanglement of the colonies.

Tab 1: Data set of *F. sultana* growth at 24 °C, RT and 16 °C at three timepoints. Average number of zooid per colony with SD, live and dead zooids per colony with SD and fraction of statoblasts per zooid with SD.

TEMPERATURE CONDITION	**24 °C**			**RT**		**16 °C**		
ESTIMATION OF *F. SULTANA* GROWTH / WEEK P.T.	**8 week p.t.**	**10 week p.t.**	**12 week p.t.**	**8 week p.t.**	**10 week p.t.**	**8 week p.t.**	**10 week p.t.**	**12 week p.t.**
TOTAL NUMBER OF ZOOIDS PER PLATE	240+/-24.48	242+/-40.36	310+/-39.40	246+/-77.78	243+/-88.57	166+/-29.28	212+/-46.24	366+/-58.53
NUMBER OF COLONIES	26+/-4	26+/-3	25+/-2	19+/-4	19+/-4	28+/-2	28+/-2	28+/-2
AVERAGE NUMBER OF ZOOIDS PER COLONY	9+/-0.88	9+/-0.51	13+/-0.58	13+/-1.23	13+/-1.86	6+/-0.69	8+/-1.70	13+/-1.00
LIVE ZOOIDS PER COLONY	6+/-0.69	6+/-0.26	9+/-0.20	11+/-0.10	10+/-2.21	6+/-0.75	7+/-2.19	10+/-2.16
DEAD ZOOIDS PER COLONY	3+/-0.45	3+/-0.26	4+/-0.60	2+/-1.14	3+/-0.35	0+/-0.07	1+/-0.52	3+/-1.17
FRACTION OF STATOBLAST	2.2 +/-1.2 %	2.1+/-0.7 %	1.7+/-0.6 %	3.7+/-2.9 %	17.6+/-5.0 %	21.8+/-5.5 %	17.7+/-6.9 %	10.0+/-2.8 %

3.2 Cohabitation and temperature change experiment

The number of zooids per colony decreased across all three treated (cohabitation and temperature change) temperature groups compared to the control groups (Fig 11). A general increase in mortality and statoblast formation was observed across all three treated temperature groups when compared to the control groups (Fig 12, 13 & Tab 2).

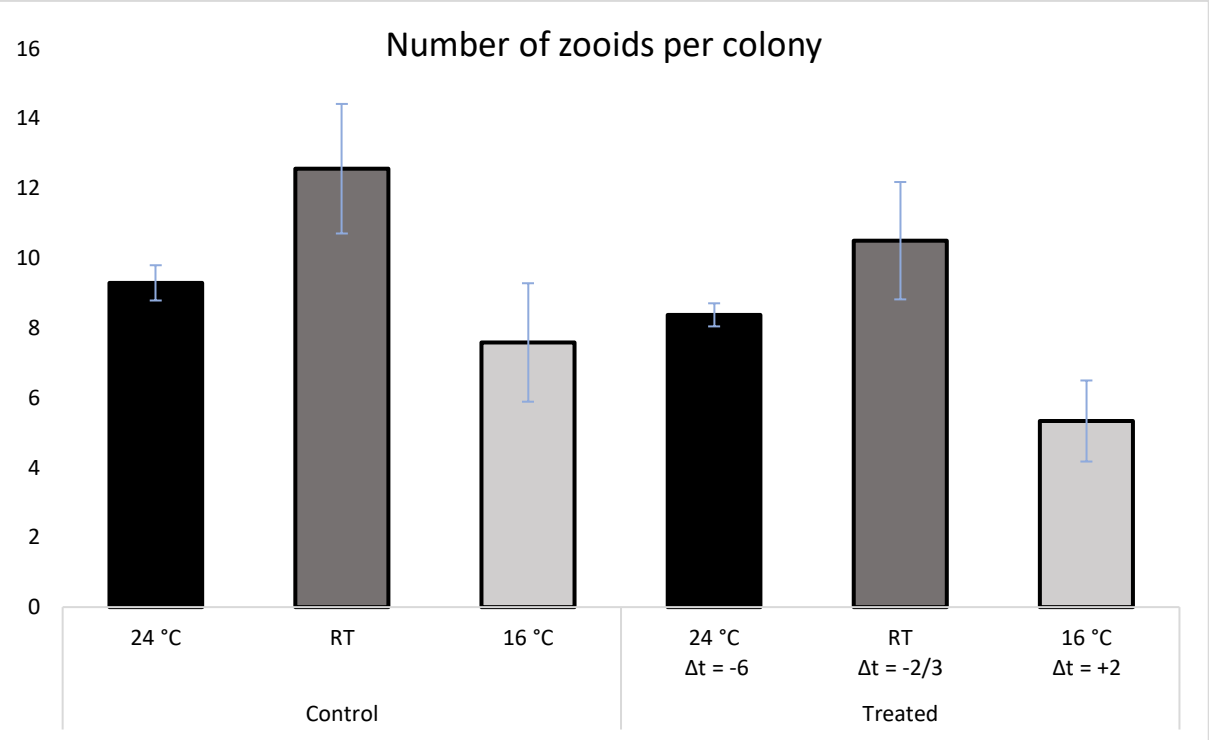

Fig 11 Number of zooids per colony between treated (cohabitation+ Δtemp change) and control groups at 10 weeks p.t. Error bars in blue.

The *F. sultana* growth in elevated temperatures (24 °C) exhibited the highest mortality rate both in control and treated groups. However, the control group maintained at cooler temperatures (16 °C) displayed lower mortality compared to the group cultured at room temperature (RT). After treatment, the RT group also showed a lower mortality rate compared to the other groups (Fig 12 & Tab 2).

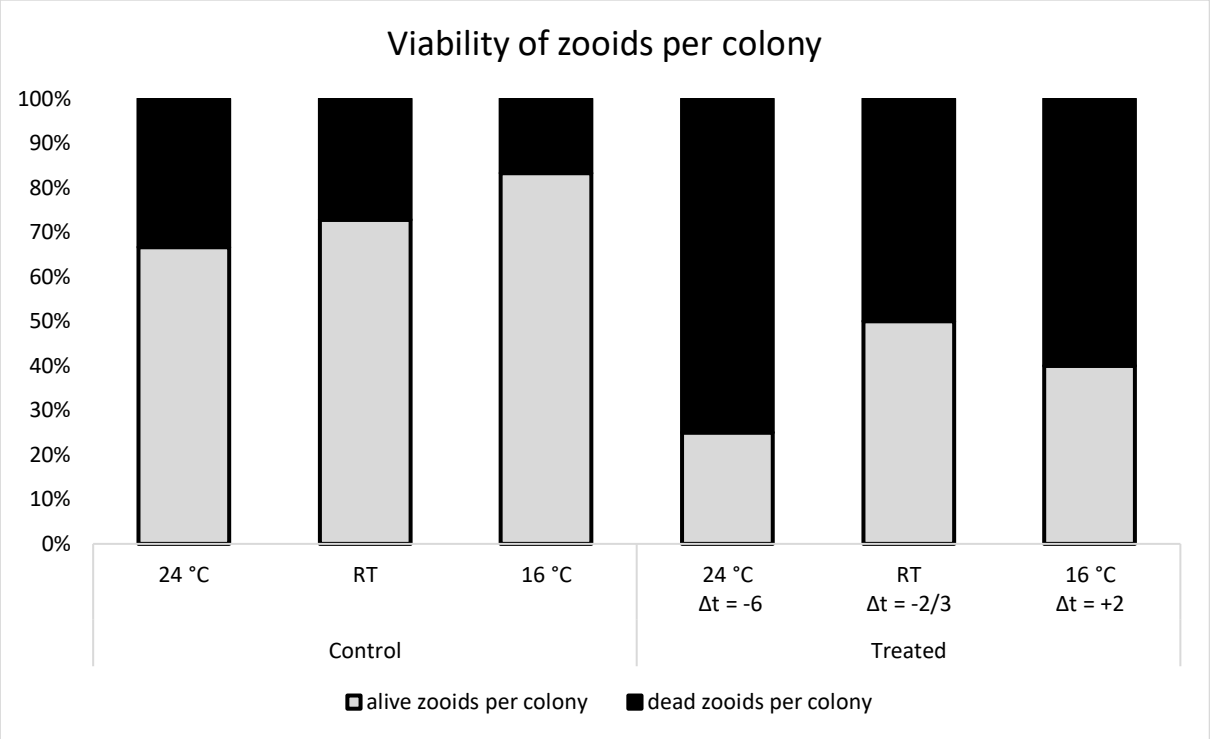

Fig 12. Proportion of live/dead zooids per colony between treated (cohabitation and ∆temp change) and control at 10 weeks p.t.

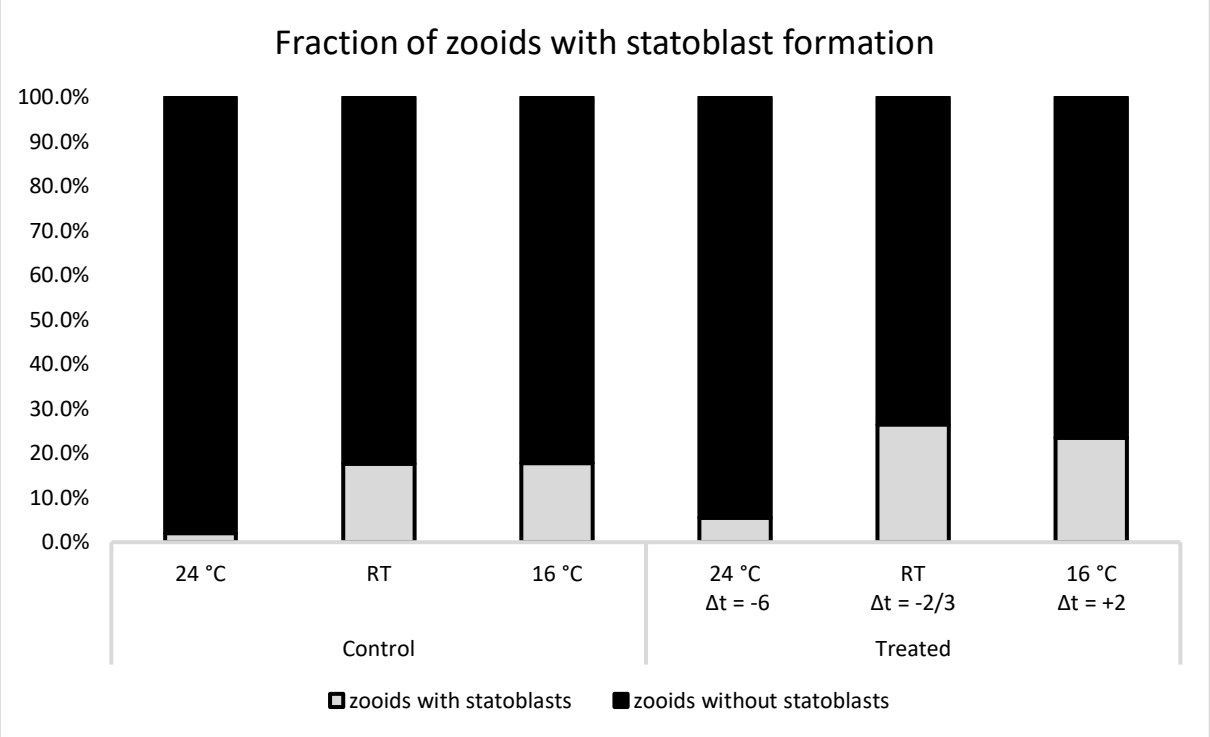

Fig 13. Fraction of zooids with statoblasts and without statoblasts between treated (cohabitation and ∆temp change) and control group at 10 weeks p.t.

Tab 2: Data set of *F. sultana* growth at 24 °C, RT and 16 °C of control and treated group (cohabitation and Δtemp change). Average number of zooid per colony with SD, live and dead zooids per colony with SD and fraction of statoblasts per zooid with SD.

CONDITION	CONTROL			TREATED		
BRYOZOAN GROWTH ESTIMATION/ GROUP	24 °C	RT	16 °C	24 °C Δtemp = -6	RT Δtemp = -2/3	16 °C Δtemp = +2
TOTAL NUMBER OF ZOOIDS PER PLATE	242+/-40	243+/-89	212+/-46	209+/-17	255+/-47	123+/-48
NUMBER OF COLONIES	26+/-3	19+/-4	28+/-2	25+/-2	24+/-3	22+/-5
AVERAGE NUMBER OF ZOOIDS PER COLONY	9+/-0.51	13+/-1.86	8+/-1.7	8+/-0.33	11+/-1.68	5+/-1.16
LIVE ZOOIDS PER COLONY	6+/-0.26	8+/-2.21	5+/-2.19	2+/-0.45	5+/-0.50	2+/-0.07
DEAD ZOOIDS PER COLONY	3+/-0.26	3+/-0.35	1+/-1.38	6+/-0.43	5+/-1.38	3+/-1.21
FRACTION OF STATOBLAST	2.1+/-0.7 %	17.6+/-5.0 %	17.7+/-6.9 %	5.5+/-2.0 %	26.5+/-5.3 %	23.4+/-10.3 %

Detection of *T. bryosalmonae* in donor infected fish in cohabitation

qPCR showed a positive signal for positive control in both runs (Fig. 14A: Ct of 29.08; B: Ct of 30.32). Sample taken at 18 °C showed a negative result (Fig 14B) and sample taken at increased temperature showed a slight signal after more than 40 cycles (Ct of 42.96). No infection was detected for the cohabitation groups at 12 weeks p.t. and 14 weeks p.t. with the stereo microscope.

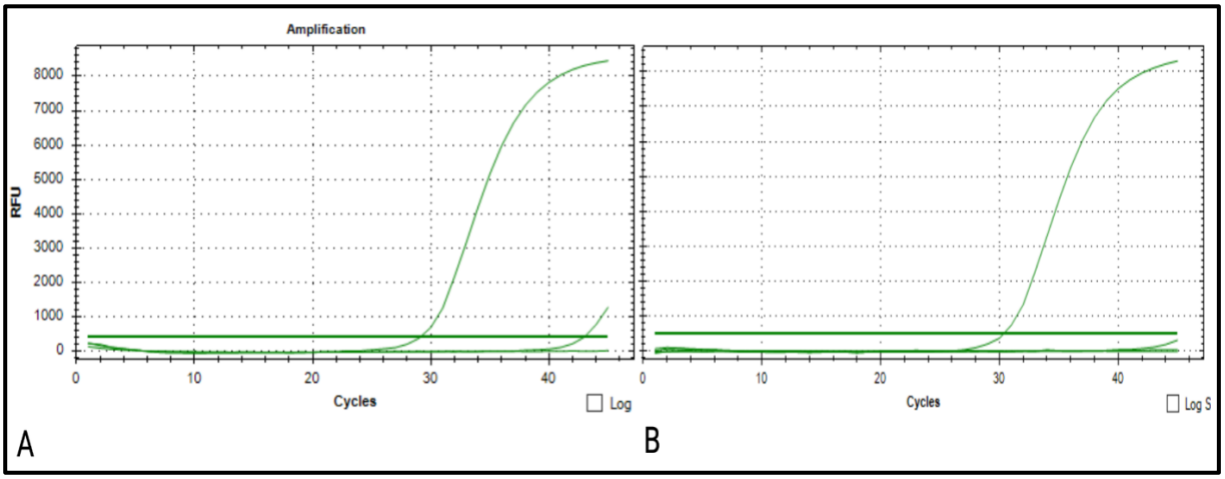

Fig 14. Number of amplification cycles of the parasite *T. bryosalmonae* detection. (A) water samples taken at 20 °C. (B) Water samples taken at 18 °C.

4 Discussion

4.1 Potential trade-off between reproductive investment and survival strategies in Bryozoan colonies at different temperatures

This study provided compelling evidence supporting a direct correlation between the water temperature of bryozoan cultures and the statoblast formation in *F. sultana*. Highest statoblast production occurred at the colonies maintained at the coldest temperature. These results align with the research conducted by Hartikainen and Okamura in 2015, which demonstrated that statoblast formation predominantly occurs during winter months when temperatures are colder.

Another observation emerging from the *F. sultana* growth experiment was that colonies maintained at the lowest temperatures exhibited a reduced growth and reduced mortality rates, but an increased statoblast formation. This finding further accentuates the intricate relationship between temperature and bryozoan colony dynamics. The decrease in zooid abundance but increase in statoblast formation at lower temperatures suggests a potential trade-off between reproductive investment and survival strategies within *F. sultana* populations. It is plausible that in colder environments, allocating resources towards statoblast formation takes precedence over zooid production. This strategic reallocation of resources may be a result of the physiological and metabolic adaptations required for bryozoans to endure and overcome the challenges imposed by low temperatures.

Trade-offs between reproductive investment and survival strategies has been reported as it was shown that declining food resources reduced growth, increased partial mortality and provoked statoblast production in *F.sultana* and almost all uninfected colonies at low food levels started making statoblasts (Hartikainen and Okamura 2012).

The reduced mortality rate observed in *F. sultana* colonies at lower temperatures can be attributed to multiple factors. Firstly, lower temperatures may result in reduced metabolic rates, slowing down physiological processes and potentially extending the lifespan of individual zooids within the colony. This decreased metabolic activity may lead to a reduction in energy expenditure, promoting overall colony health and longevity. The relationship between low temperatures and prolonged lifespan has been observed in many other organisms like rotifers (Yoza 2018) or nematodes (Vakkayil and Hoppe 2022). Secondly, colder temperatures may also contribute to a decrease in the prevalence of

pathogens or parasites that could potentially harm or compromise the colonies. Many pathogens and parasites exhibit optimal growth and reproduction rates at higher temperatures, and this has become particularly noticeable in aquatic environments with the increase risks for aquatic animal health due to global warming (Ros et al. 2021; Combe et al. 2023). A colder environment may create a less hospitable condition for these harmful organisms, and this could explain the consequent reduced mortality rates in *F. sultana* colonies.

In comparison to the colonies maintained at lower temperatures, the colonies subjected to higher temperatures exhibited the lowest statoblast formation but the highest mortality rates. Increased mortality rates of *F. sultana* colonies held at higher temperatures were also observed by Tops, Hartikainen, and Okamura 2009. This finding further supports the hypotheses of a potential trade-off between reproductive investment and survival strategies in *F. sultana* colonies: elevated temperatures can accelerate metabolic rates and increase energy demands within the colonies. As a result, resources that could potentially be allocated for statoblast production may be diverted towards sustaining essential physiological functions. The reduced allocation of resources to reproductive strategies, such as statoblast formation, may compromise the long-term survival and persistence of the colonies (Giaimo and Traulsen 2019). Furthermore, higher temperatures can also impose stress on colonies, making them more susceptible to various environmental challenges. Increased metabolic rates and elevated physiological activity can lead to higher energy expenditure, potentially straining the resources available for maintaining colony health and resilience. Additionally, higher temperatures may support the growth and reproduction rates of pathogens and parasites (Ros et al. 2021; Combe et al. 2023), further jeopardizing the well-being and survival of the colonies.
Fredericella sultana higher mortality rate observed in colonies maintained at higher temperatures could also be impacted by the fact that in the experimental setup, all colonies were initially fed the same amount for the first five weeks, regardless of the temperature they were exposed to. It was only after observing their physical condition, specifically their skinnier appearance and empty stomachs, that the colonies were provided with increased feeding. Bryozoans biomass is greatly influence by the nutrient availability in the environment (Hartikainen et al. 2009). Further feeding regimes are being currently tested to determine the optimal feeding conditions for *F. sultana* culture at University of Veterinary Medicine Vienna.

4.2 Cohabitation and temperature effect *F. sultana* colonies growth

This study shows the effect of cohabitation and temperature change on viability, statoblast formation and number of zooids per colony. In all respects, the findings indicate a detrimental effect on the *F. sultana* colonies.

During the night, when cohabitation took place, the *F. sultana* colonies were exposed to all the excretions released by the fish as well as leftover food residue discarded by the fish. This exposure to fish waste could have had a negative impact on the colonies and contributed to their overall decline. The presence of fish excretions and food remnants in the vicinity of the colonies could have introduced various contaminants and altered the water chemistry, leading to unfavorable conditions for the bryozoans' survival and reproductive processes (Svobodova et al., 1993)

While the primary reason for this effect could be attributed to fish cohabitation and temperature change, it is crucial to consider additional factors such as the handling process. The frequent transition of the *F. sultana* colonies between the tank and their designated buckets on a daily basis may have induced a significant level of stress, thereby resulting in a diminished quality of life for the colonies, since disturbance can affect bryozoans (Bone and Keough 2005). However, it is also important to acknowledge that the room temperature varied during course of the experiment, and the colonies maintained at RT were the most impacted by that variability. In contrast, colonies held at higher or lower temperatures were consistently regulated by the use of a heater or fridge. It could be due to that reason that the colonies cultivated at RT prior to treatment experienced less mortality rates compared to the group cultivated at fewer temperatures, because they could have developed an effective stress response allowing them to cope with the fluctuations (Somero 2020). This additional stressor, coupled with the other factors mentioned previously, likely contributed to the observed negative effects on the viability, statoblast formation, and zooid numbers within the bryozoan colonies.

Parasite *T. bryosalmonae* infection can affect *F. sultana* growth, especially at environmentally extreme conditions (like high temperatures) (Tops, Hartikainen, and Okamura 2009). In this thesis, the molecular detection of *T. bryosalmonae* in the water was extremely low (Ct 42.09) and no infection was observed in any zooid at any temperature and time points, which suggests that the parasite influence in the colonies growth could be consider negligible.

For future experiments, it is advisable to test the two conditions, temperature change and fish cohabitation, separately in order to better understand their individual effects on *F. sultana* colonies. One approach would be to maintain *F. sultana* colonies at different temperatures and afterwards subject them to a temperature change, assessing the colony's response and any associated changes in behavior, physiology, or reproductive strategies. Another approach would involve maintaining *F. sultana* colonies at the same temperature as the fish species with which they are intended to cohabitate. This would allow researchers to investigate the specific interactions and potential effects of cohabitation on the colonies.

Additionally, in future experiments, it would be beneficial to utilize a reduced number of statoblasts for hatching initially. In the present study, only data from eight representative colonies were counted, due to the heavy entanglement of the colonies. This limited sample size may have compromised the representativeness of the data and could have influenced the overall analysis. A reduced number of statoblasts would result in minimized entanglement and overcrowding of zooids, facilitating growth monitoring of *F. sultana* colonies.

4.3. Conclusions

Fredericella sultana culture is maintained at the University of Veterinary Medicine Vienna for transmission studies of the salmonid parasite *Tetracapsuloides bryosalmonae. F. sultana* colonies maintained at colder temperatures have the highest viability and least food consumption, but colonies are also smaller with less available zooids for parasite infection. At higher temperatures, the colonies are larger, but mortality increased substantially; however, the food may be a limiting factor at these temperatures. The setup of bioreactors in the lab for increased algae production may support the growth of larger bryozoans' colonies at higher temperatures.

In this study we showed that cohabitation and a sudden temperature change affects *F. sultana* colonies negatively despite their previous maintained temperature conditions. Since *T. bryosalmonae* stages are only detectable after some weeks post cohabitation, viability of the colonies is an important factor. Therefore, it could be recommended to cultivate at RT since it showed the highest viability after cohabitation.

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Appendix

Figures & Tables

TNES buffer preparation:

10 mM Tris-HCl + 125 mM NaCl + 10 mM ethylenediaminetetraacetic acid (EDTA) + 0.5 % sodium dodecyl sulphate (SDS) + 4 M urea

Algae WC-medium preparation:

Stock solutions
Dissolve each separately in 500 mL distilled water and autoclave:

Reagents	Amount	Storage number
$CaCl_2 \cdot 2H_2O$ (Calcium chloride dihydrate)	8.4 g	057B
$MgSO_4 \cdot 7H_2O$ (Magnesium sulfate heptahydrate)	18.5 g	061B
$NaHCO_3$ (Sodium bicarbonate)	6.3 g	005B
$K_2HPO_4 \cdot 3H_2O$ (Dipotassium hydrogen phosphate trihydrate)	5.7 g	065B
$NaNO_3$ (Sodium nitrate)	42.5 g	274B
$Na_2SiO_3 \cdot 5H_2O$ (Sodium metasilicate pentahydrate)	10.6 g	070B

Trace elements stock solution
Dissolve all together in 1 L distilled water:

Reagents	Amount	Storage number
Na_2EDTA (Titriplex III)	4.36 g	036B
$FeCl_3 \cdot 6H_2O$ (Iron(III) Chloride Hexahydrate)	3.15 g	056B
$CuSO_4 \cdot 5H_2O$ (Copper(II) sulfate pentahydrate)	0.01 g	277B
$ZnSO_4 \cdot 7H_2O$ (Zinc sulfate, Heptahydrate)	0.022 g	074B
$CoCl_2 \cdot 6H_2O$ (Cobalt (II) chloride hexahydrate)	0.01 g	276B
$MnCl_2 \cdot 4H_2O$ (Manganese(II) Chloride Tetrahydrate)	0.18 g	275B
$Na_2MoO_4 \cdot 2H_2O$ (Sodium Molybdate dihydrate)	0.006 g	071B
H_3BO_3 (Boric acid)	1 g	077B

Vitamin mix stock solution
Dissolve all together in 1 L sterile distilled water:

Reagents	Amount	Storage number
Thiamin HCl (B1)	0.1 g	264B
Biotin (H)	0.0005 g	072B
Cyanocobalamin (B12)	0.0005 g	263B

Final preparation of Algae WC medium:
To each bottle with 2 L of distilled water, add 2 ml of each stock solution and trace elements, 0.23 g of TES buffer ($C_6H_{15}NO_6S$) (079B), mix well and autoclave. Work under sterile conditions, to keep the stock solutions sterile.
After autoclaving the medium, let it cool at room temperature and add 2 ml of vitamin mix to the 2 l medium bottle.

When preparing several bottles of medium at once, a master mix solution can be prepared to accelerate the whole process of medium preparation. In each big autoclave, we can fit 7 bottles of 2 L, there are 2 autoclaves.
In this case, in a 500 mL glass bottle, add the volume of stock solutions, trace elements and TES buffer accordingly to the final number of bottles desired (make sure you prepare for 1 extra bottle, to compensate for errors in pipetting), see the next table for examples. Mix well the master mix solution, and then take the volume needed to each bottle with 2 L of distilled water.
Do not forget to add the vitamins mix after autoclaving the medium.

Master mix preparation Components:	Amount of medium in preparation		
	2 L (1 bottle)	16 L (8 bottles)	30 L (15 bottles)
Stock solutions	2 ml	16 ml	30 ml
Trace elements stock solution	2 ml	16 ml	30 ml
TES buffer (079B)	0.23 g	1.84 g	3.45 g