Towards a challenge model for the tapeworm *Eubothrium crassum*

- Incubation of cestode eggs and maintenance of a marine intermediate host, *Acartia tonsa*

By Skjalg Standal

Master thesis in Aquamedicine,

Department of Biological Sciences (BIO)

University of Bergen

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Supervisors:

Linda Andersen – The Industrial and Aquatic Laboratory (ILAB)

Erna Irene Heggland – The Industrial and Aquatic Laboratory (ILAB)

Egil Karlsbakk – University of Bergen

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Abstract

The tapeworm *Eubothrium crassum* Bloch (Cestoda: Bothriocephalidea) is a parasite that occurs mainly in fish of the genera *Salmo* in both freshwater and seawater. It is an increasing problem in farmed marine Atlantic salmon (*Salmo salar* Linnaeus) in Norway, causing larger feed intake and reduced growth of the infected fish. There are several details in the life cycle of marine *E. crassum* that are not yet fully described. However, the life cycle of *E. crassum* in marine fish farming involves a copepod first intermediate host that is directly eaten by the main host. The present study aimed to collect data towards a challenge model for marine *E. crassum* in salmon using the copepod intermediate host *Acartia tonsa* Dana to facilitate research on the parasite-host interaction. In this study *E. crassum* eggs were incubated up to four days at different salinities in autoclaved or regular seawater in addition to that some groups of eggs were treated with antibiotics to find the method with highest survival. A neutral red cell viability assay was created and combined with spectrophotometry was used to quantify the portion of viable eggs in the samples by using absorbance levels as indicator. Of the parameters tested for *E. crassum* egg incubation, only freshwater had a negative effect on the absorbance. This indicates that eggs from marine *E. crassum* should be incubated in brackish or seawater (25-34 ppt). These results also show that *E. crassum* eggs could be incubated without autoclaving the water or treating the eggs with antibiotics for at least four days without any significant decrease in survival. *A. tonsa* survival was addressed using different rearing set-ups, and copepods were challenged with *E. crassum* eggs that had been incubated at different salinities (0, 25 and 34 ppt). Copepods were successfully challenged with *E. crassum* eggs kept in 0 ppt, 25 ppt and 34 ppt water. No significant difference in prevalence between the groups challenged with eggs from 25 ppt water and 34 ppt water were registered, but both groups had a considerably higher prevalence than the group challenged with eggs kept in 0 ppt water. Decreasing copepod numbers over time were observed in all groups, regardless of set-up. Lower copepod concentrations, less handling and continuous feeding should be assessed in future studies. Fifteen salmon were orally intubated with copepods infected with *E. crassum* procercoid larvae that had developed for 224, 240 or 256 day-degrees at 16 °C. The challenged salmon were not successfully infected with *E. crassum*. There could be several causes for why an infection was not established but it is hypothesized that fish size and amount of gut contents in the fish could have had an impact, as well as number of copepods used to challenge each fish.

Sammendrag

Bendelormen *Eubothrium crassum* Bloch (Cestoda: Bothriocephalidea) er en parasitt som forekommer hovedsakelig hos laksefisker i både ferskvann og sjøvann. Den er et økende problem i norsk oppdrettslaks (*Salmo salar* Linnaeus) i sjø i Norge, og forårsaker økt fôropptak og redusert vekst hos smittet fisk. Flere detaljer i livssyklusen til marin *E. crassum* er fortsatt ikke godt nok kartlagte. Imidlertid er det kjent at livssyklusen til *E. crassum* hos marin oppdrettslaks involverer en copepode som første mellomvert, og at denne blir spist direkte av sluttverten. Denne studien har som mål å samle data for en smittemodell for marin *E. crassum* hos laks ved å bruke copepoden *Acartia tonsa* Dana som mellomvert, og på den måten tilrettelegge for forskning på interaksjonene mellom parasitt og vert. I forsøket ble *E. crassum* egg inkubert i opptil fire dager ved forskjellige vannsaliniteter, i autoklavert eller ikke-autoklavert vann og noen grupper egg ble behandlet med antibiotika som hensikt å finne metoden som ga høyest overlevelse blant eggene. Et assay ble laget der en benyttet nøytral rødt i kombinasjon med spektrofotometri for å kvantifisere andelen levende egg ved å bruke absorbans som indikator. Blant parametrene som ble testet for inkubering av *E. crassum* egg var det kun rent ferskvann som påvirket absorbans negativt. Det indikerer at egg fra marin *E. crassum* bør inkuberes i brakkvann eller sjøvann (25-34 ppt). Disse resultatene viser også at *E. crassum* egg kan inkuberes i ikke-autoklavert vann og uten antibiotikabehandling i minimum fire dager uten signifikant redusert reduksjon i overlevelsen. Overlevelse hos *A. tonsa* ble undersøkt i forskjellige inkubatorsystemer. I tillegg ble *A. tonsa* forsøkt smittet med *E. crassum* egg som var inkubert ved forskjellige saliniteter (0 ppt, 25 ppt og 34 ppt). Ingen signifikante forskjeller i prevalens mellom gruppene smittet med egg inkubert ved 25 ppt og 34 ppt ble observert, men begge gruppene hadde betydelig høyere prevalens enn den smittet med egg inkubert ved 0 ppt. Reduksjon i antall overlevende copepoder ble observert over tid i alle gruppene uavhengig av inkubatorsystem. Lavere konsentrasjon av copepodene, mindre håndtering og kontinuerlig fôring bør vurderes for framtidige oppsett for hold av copepodene. Femten laks fikk copepoder infisert med *E. crassum* procercoide larver som var utviklet i 224, 240 og 256 døgngrader ved 16 ℃ intubert oralt direkte til magesekken. Ingen av laksene ble infisert av *E. crassum*. Det er flere mulige grunner til at smitte ikke ble etablert, men fiskestørrelse og tarminnhold hos fisken har blitt foreslått som mulige faktorer, i tillegg til antall kopepoder brukt for å smitte hver fisk.

Abbreviations

BAS – Blood-agar with salt

CFU - Colony-forming units

Dpc – Days past challenge

EtOH – Ethanol alcohol

Hct – Haematocrit

PBS - Phosphate-buffered saline

Ppt – Parts per thousand (Salinity)

PSS - Physiological saline solution

UV – Ultraviolet light

1. Introduction

The production of Atlantic salmon (*Salmo salar* Linnaeus) in Norway started in the 1960s, increasing in volume over the years until stagnating around 2012 because of environmental and welfare challenges combined with stricter regulations (Misund, 2021). Atlantic salmon is an anadromous species, meaning that they spawn in freshwater and then later migrate to feeding areas in the ocean before they later return to rivers to spawn (Vøllestad, 2022). In the production of this species a wide range of pathogens in both the freshwater phase and the saltwater phase may cause a reduction in fish welfare, lower growth and mortality and thus economic losses for the farmer (Geitung, Wright, Stien, Oppedal, and Karlsbakk, 2021). The ectoparasite *Lepeophtheirus salmonis* Krøyer (Salmon louse) is a large problem for Norwegian aquaculture (Sommerset, Walde, Bang Jensen, Wiik-Nielsen, Born, Oliveira, Haukaas, and Brun, 2022). Endoparasites such as tapeworms (Cestoda, phylum Platyhelminthes) living in the intestinal tract of vertebrates may also lead to reduced growth and welfare. Some tapeworms of the genus *Eubothrium* Nybelin (order Bothriocephalidea, family Trianophoridae) are found in salmonids in the northern hemisphere (Kennedy, 1978b; Kuchta, Scholz, Brabec, and Bray, 2008) and has been a problem for decades in Norwegian farmed salmon, mainly in middle, west and south-west Norway (Berland and Bristow, 1990; Bristow and Berland, 1991; Sommerset, Walde, Bang Jensen, Wiik-Nielsen, Born, Oliveira, Haukaas, and Brun, 2022). *Eubothrium crassum* Bloch is a tapeworm that mainly matures in fish in the genus *Salmo* and has been found in freshwater and seawater in Atlantic salmon (Vik, 1963; Kennedy, 1978b). *E. crassum* has also been found in brown and sea trout (*Salmo trutta* Linnaeus) in Norway as well as Danubian salmon (*Hucho hucho* Linnaeus) from Austria, vendace (*Coregonus albula* Linnaeus) from Finland, European grayling (*Thymallus thymallus* Linnaeus) from Switzerland, lumpsucker (*Cyclopterus lumpus* Linnaeus) from Poland and rainbow trout (*Oncorhynchus mykiss* Walbaum) from Europe (Vik, 1963; Kennedy, 1978a; Chubb, 1982; Berland and Bristow, 1990; Bristow, 1993; Kráľová-Hromadová, Scholz, Shinn, Cunningham, Wootten, Hanzelová, and Sommerville, 2003; Rolbiecki and Rokicki, 2008). It was suggested by Kennedy that the species is comprised of three races; a freshwater race confined to Europe and Eurasia, a marine Atlantic race and a marine Pacific race (Kennedy, 1978b). A study on *E. crassum* from marine Atlantic salmon and from freshwater trout in Norway by Bristow and Berland (Bristow and Berland, 1989) indicated allozymes differences between worms from freshwater trout (*E. crassum*) and seawater reared salmon (freshwater and marine "races"). However, DNA sequence studies has not supported that these represent different species (Kráľová-Hromadová,

Hanzelová, Scholz, Gerdeaux, and Špakulová, 2001; Kráľová-Hromadová, Scholz, Shinn, Cunningham, Wootten, Hanzelová, and Sommerville, 2003). These findings were supported by Scholz and colleagues (Scholz, Kuchta, Shinn, Šnábel, and Hanzelová, 2003), who concluded that both the European forms are *Eubothrium crassum*. Hence, the marine and freshwater variants will in the present study be referred to as marine and freshwater forms of *E. crassum*.

E. crassum are long, segmented tapeworms that may reach over one meter in length (Berland, 1997; Ruud, 2019). As other cestodes, *E. crassum* has no mouth or gut and nutrition is absorbed directly from the environment (Støp-Bowitz and Sømme, 2022). The morphology of *E. crassum* has been described by Hanzelová and colleagues (Hanzelová, Scholz, Gerdeaux, and Kuchta, 2002) in a comparative study of *E. salvelini* and *E. crassum*. Their body consists of an elongated scolex and a main body, the strobila, consisting of several wide "segments" called proglottids, as shown in Figure 1. The scolex has a slightly convex apical disc and two elongated shallow sucking grooves situated dorsoventrally.

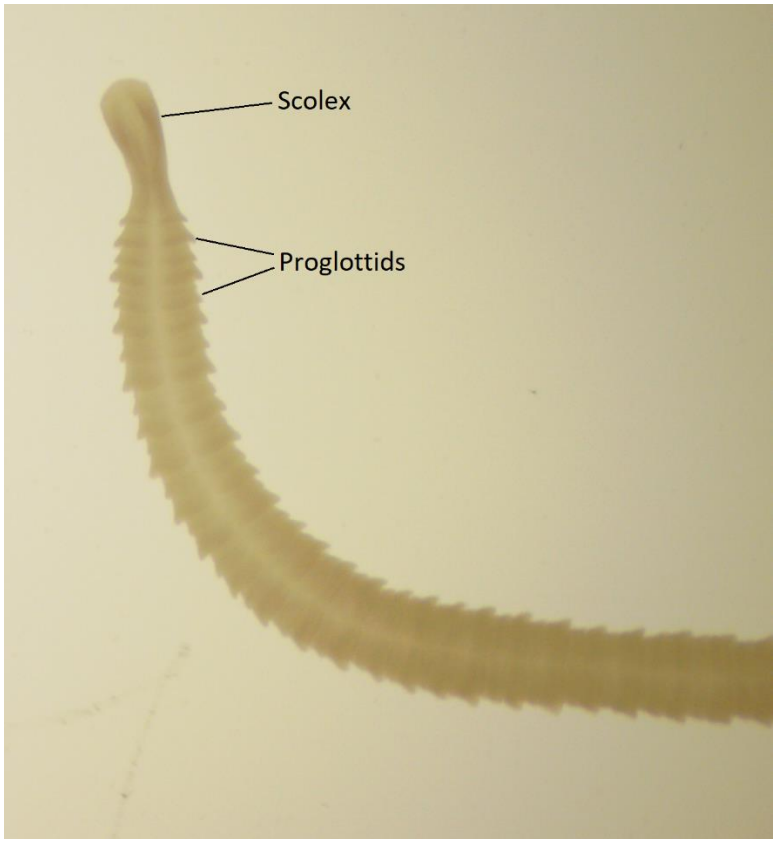

Figure 1. *E. crassum* collected from guts of farmed Atlantic salmon gut. The picture shows an elongated scolex followed by strobila consisting of proglottids.

Proglottids are formed in a neck region behind the scolex in a process called strobilization. They gradually grow and mature as they are being pushed backwards by the production of new proglottids. Therefore, mature proglottids tend to be found in the posterior strobila (Berland and Bristow, 1990). Each proglottid contains both male and female reproductive organs, shown as schematic in Figure 2, and the proglottids furthest from the scolex become filled with eggs.

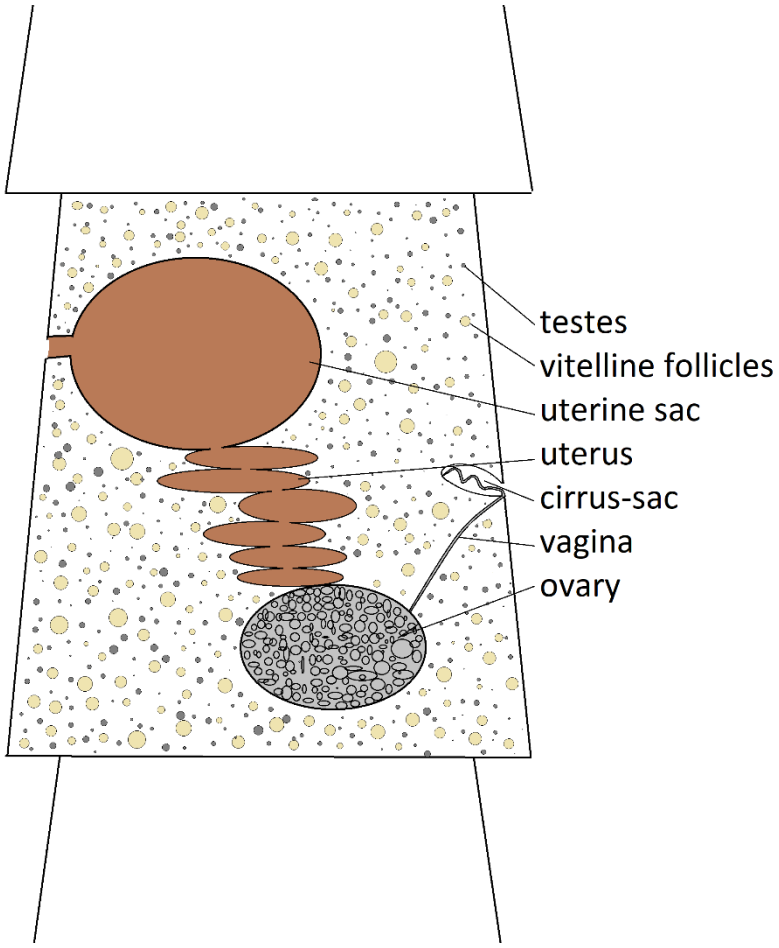

Figure 2. Schematic drawing with a ventral view of the morphology of *E. crassum* proglottids. Based on a figure by Kuchta and colleagues (Kuchta, Scholz, Brabec, and Bray, 2008).

In a study by Kuchta et al (2006), *E. crassum* eggs from Atlantic salmon and brown trout were examined (Kuchta, Shinn, Hanzelova, and Scholz, 2006). Their length was 84-109 µm and width 67-74 µm. *E. crassum* eggs are oval and have an external, scleratine capsule that encloses an embryophore and a motile oncosphere, which has three pairs of embryonic hooks, as shown

in Figure 3 (Hanzelová, Scholz, Gerdeaux, and Kuchta, 2002; Kuchta, Shinn, Hanzelova, and Scholz, 2006).

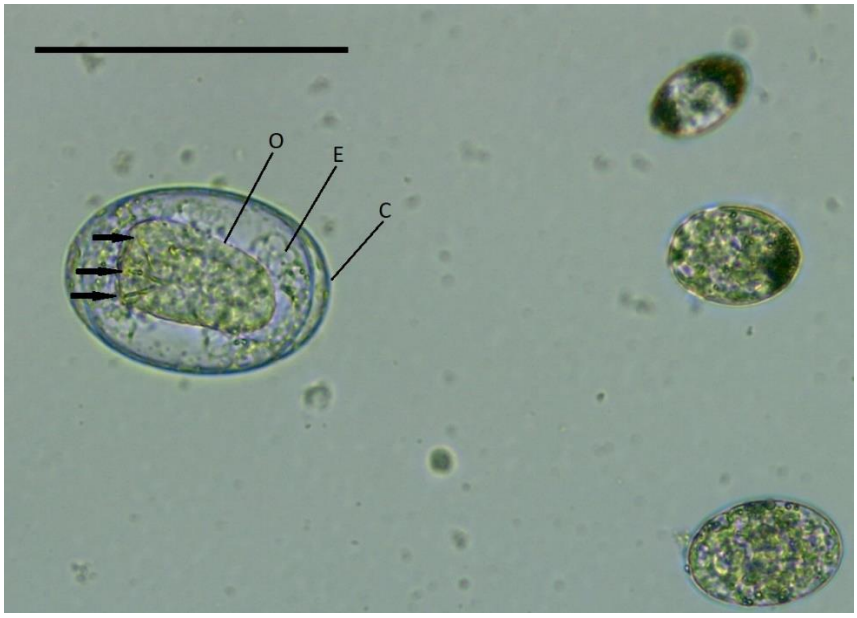

Figure 3. Mature *E. crassum* egg (left) and immature eggs (right). Visible embryonic hooks (arrows), oncosphere (O), embryophore (E) and outer capsule (C). The scale bar is 100 µm long.

There are several details in the life cycle of marine *E. crassum* that are not yet fully known. However, it was suggested by Rosen (Rosen, 1919) that the life cycle involves two intermediate hosts: a copepod and a fish (perch, *Perca fluviatilis* Linnaeus) (Kennedy, 1969). The plerocercoid *E. crassum* larvae develops in the intestinal lumen of the perch fry, or sometimes in the abdominal cavity (Kennedy, 1969). The infected perch would then be eaten by a trout which would itself become infected and in which adult *E. crassum* would develop. It has been found that the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus) could act as secondary intermediate host, or as a transport host (Vik, 1963). It has also been suggested that the life cycle of *E. crassum* in fresh water may be completed without the involvement of such a fish host, hence involving copepods only (Vik, 1963; Mulcahy and Kennedy, 1970; Wootten, 1972). Marine *E. crassum* eggs ingested by *Acartia tonsa* Dana in a study by Saksvik and colleagues were described to hatch in the copepod gut to oncosphera larvae, which penetrates the intestinal wall of the copepod (Saksvik, Nylund, Nilsen, and Hodneland, 2001). After entering the haemocoel they developed into procercoids. If a suitable host, e.g. Atlantic salmon, were to ingest an infected copepod, there is a chance that the parasite emerges from the digested copepod in the stomach or intestine and the fish becomes infected (Kennedy, 1996). In the case of only a copepod intermediate host, the larvae will develop from procercoid to adult in the final fish host, likely via a plerocercoid stage. If there is a fish secondary intermediate host, the larvae will in this develop from procercoid to plerocercoid, which then can attach and grow to

the adult in the final host. Growth of the parasite starts when the parasite has migrated from the intestine to the pyloric caeca and attached their scolex there, and their strobila may reach into the gut (Berland and Bristow, 1990; Kennedy, 1996; Berland, 1997). Eggs released from gravid worms enters the water with the fish faeces and may restart the cycle, as shown in Figure 4 (Sevatdal, 2014).

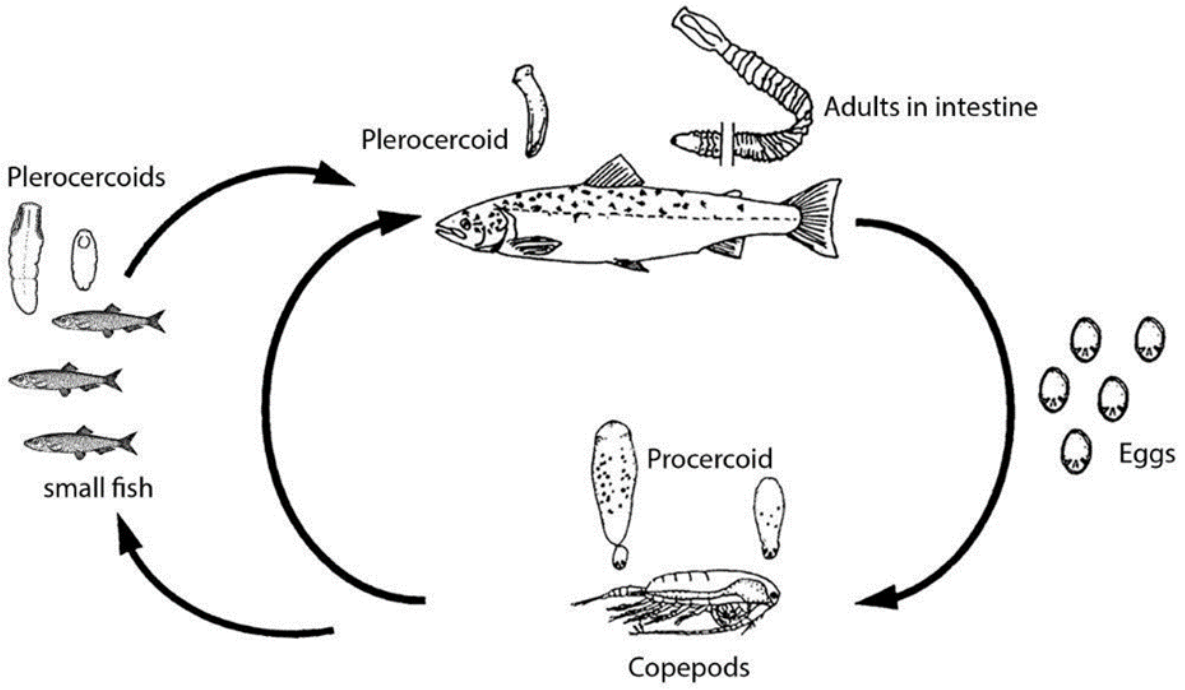

Figure 4. The life cycle of *E. crassum.* The life cycle may be completed with only a copepod as intermediate host, or both with a copepod intermediate host and a fish intermediate/transport host. Eggs are released from adult *E. crassum* in the main host and released into the water with the fish faeces to restart the cycle. Figure created by Kjartan Hodneland (1995) and modified by Egil Karlsbakk (2019).

Though not fully studied, there is a general consensus of a seasonal variation in *E. crassum* infections with the summer months showing the highest intensity (Wootten, 1972; Saksvik, Nylund, Nilsen, and Hodneland, 2001; Ruud, 2019; Sakariassen, 2019), which coincides with high copepod numbers (Gundersen, 1953; Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956; Geitung, Wright, Stien, Oppedal, and Karlsbakk, 2021). The copepods *A. clausi* Giesbrecht, *Temora longicornis Müller*, and *Pseudocalanus elongatus* Brady have been experimentally infected by eggs from marine *E. crassum* (Hodneland and Solberg, 1995). In the same study, the copepod *Calanus finmarchicus* Gunnerus was not successfully infected. Infection of the freshwater copepods *Cyclops* spp. and *Eudiaptomus* sp. with marine *E. crassum* eggs as well as the marine *A. clausi* with freshwater *E. crassum* eggs were also successful (Hodneland and Solberg, 1995). In a laboratory experiment by Saksvik and colleagues it was demonstrated that the life cycle of the marine variant could be completed

in a marine environment with only a copepod intermediate host (*A. tonsa*) and Atlantic salmon as the final host (Saksvik, Nylund, Nilsen, and Hodneland, 2001). Laboratory *A. tonsa* cultures have been maintained due to their suitability and accessibility, but also for their beneficial nutritional profile for farmed fish larvae (Støttrup, Richardson, Kirkegaard, and Pihl, 1986; Marchus and Wilcox, 2007). *A. tonsa* is a eurythermal and euryhaline calanoid copepod species (Lance, 1964; Marchus and Wilcox, 2007; Luigi, Chiara, Elisabetta, Alessandra, and Luigi, 2012) with a cosmopolitan distribution (Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956; Støttrup, Richardson, Kirkegaard, and Pihl, 1986; Sabatini, 1990; Leandro, Tiselius, and Queiroga, 2006; Marchus and Wilcox, 2007; Jørgensen, Jepsen, Petersen, Friis, and Hansen, 2019). The development of *A. tonsa* consists of six naupliar stages (NI-NVI) and six copepodite stages (CI-CVI) (Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956; Sabatini, 1990; Leandro, Tiselius, and Queiroga, 2006) with laboratory studies recording generation times between 7 and 25 days at different temperatures (Mauchline, 1998). In nature, *A. tonsa* develops from egg to adult in three to four weeks during summer and their adult life is reported to last for a month or less for most of the year, although they may live for longer as adults during winter (Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956). The general characters for practical recognition of *A. tonsa* were described by Conover and colleagues (Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956) in a comparative study of *A. tonsa* and *A. clausi*. Later, the copepodite stages of *A. tonsa* were described in more detail by Sabatini (Sabatini, 1990). Adult *A. tonsa* are approximately 1.5 mm in length, while their nauplii I are shorter than 0.1 mm (Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956; Marchus and Wilcox, 2007). The external morphology of *A. tonsa* consists of an anterior part, the cephalosome, which bears two antennae, followed by a five-segmented main body, called metasome, and at last a segmented urosome, as shown in Figure 5. The urosome differs between the male and female; it is short and three-segmented on the females while being narrower and five-segmented on the males. A

pair of swimming legs are attached to each of the five metasomal segments (Sabatini, 1990; Mauchline, 1998; Marchus and Wilcox, 2007).

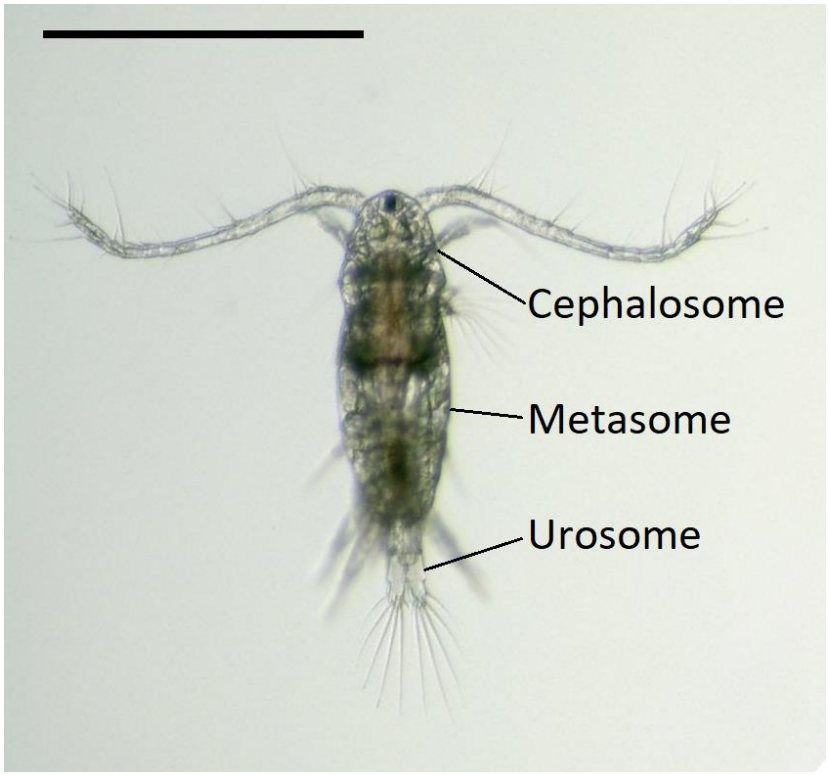

Figure 5. *A. tonsa* with two antennae connected to the cephalosome, which is followed by the metasome and the urosome. The scale bar is 0.5 mm long.

In a study by Bristow and Berland (1991) it was found that *E. crassum* infection of Atlantic salmon were directly correlated with reduced growth (Bristow and Berland, 1991). This effect was further confirmed in a laboratory experiment by Saksvik and colleagues (2001) when Atlantic salmon infected with marine *E. crassum* had a reduced growth rate of both weight and length compared to uninfected fish (Saksvik, Nilsen, Nylund, and Berland, 2001). In addition, they observed no significant weight differences between uninfected and infected fish until several months after infection. This indicates that the length of time a fish is infected may be important for the effect the parasite has on the host. Their study also showed no correlation between fish weight, length, and number of parasites, suggesting that the intensity (Busht, Laffertyt, Lotz, and Shostakll, 1997) of an *E. crassum* infection may not be significant to the growth rate of the fish. Mortality among salmon smolt associated with *E. crassum* infections was also suggested (Bristow and Berland, 1991). In addition, marine *E. crassum* have been associated with *Ichthyobodo* sp.-infections in farmed salmon in sea water in Norway (Bristow and Berland, 1991). Cestode-infected fish have also been reported to have larger feed intake compared to uninfected control fish, which may in turn increase production costs (Walkey and

Meakins, 1970; Giles, 1987). The suggested possible mortality of smolts, stunted growth, increased feed intake and possible co-infections caused by *E. crassum* infections may cause considerable economic losses for farmers (Bristow and Berland, 1991). Thus, more information on the effects of *E. crassum* infections and possible impact on coinfections is needed. By developing a challenge model for *E. crassum*, researchers will be able to study the pathogen in controlled conditions. Challenge models are important tools when studying disease development and effects of pathogens in the host, and for testing and further developing functional diets and treatments against disease.

To combat tapeworm infections, salmon farmers in Norway have used anthelminthic drugs administered in feed (mainly fenbendazole and praziquantel). However, fenbendazole is rarely used. Though effective, it has shown to cause lowered appetite, reduced growth, and has been associated with development of wounds and mortality at low temperatures (Sevatdal, 2008, 2014). Hence, praziquantel has been the most used anthelminthic. The use of praziquantel in Norway was strongly reduced in the years 2016 to 2019. Though an increase in the use was seen to increase in 2020, the amount used was considerably lower than previous years (Sommerset, Bang Jensen, Bornø, Haukaas, and Brun, 2021). Reduced effect of the anthelminthic and because the cestodes have developed resistance to it is the likely causes for the reduced use (Sevatdal and Hellberg, 2006; Sevatdal, 2008; Sommerset, Walde, Bang Jensen, Wiik-Nielsen, Born, Oliveira, Haukaas, and Brun, 2022). Maximum residue limits for drugs used for other production animals have been used to find alternatives to praziquantel, without success (Sevatdal, 2008). Oxibendazole showed some effect, but also the same side effect as fenbendazole, such as lowered appetite during and after treatment. Hence, research on new methods to prevent or treat *E. crassum* infections are required (Sevatdal, 2008).

AIM FOR THE STUDY

The aim of this study was to provide data which would further optimize a challenge model in development at The Industrial and Aquatic Laboratory (ILAB), for *E. crassum* infection of Atlantic salmon in seawater. The basic challenge model this is based on is the one used by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001). A challenge model would make research on the disease and treatments possible in a controlled environment. This study focused on testing different incubation conditions and treatments for *E. crassum* eggs. Additionally, different rearing set-ups for *A. tonsa* cultures that would allow *A. tonsa* to survive for at least 15 days were examined, as this is the period necessary for *E. crassum* to develop in this intermediate host.

Specifically, the aims were to:

1. Use neutral red staining and spectrophotometry to test the effect of different salinities and treatments (washing of eggs and antibiotics) on viability of *E. crassum* eggs
2. Improve survival of *A. tonsa* cultures
3. Perform challenge of the copepod *A. tonsa* with marine *E. crassum*
4. Perform challenge of Atlantic salmon with the marine variant of the tapeworm *E. crassum* using the copepod *A. tonsa* as intermediate host

2. Material and methods

2.1 Copepods and algae

One of the objectives for this study was to maintain copepods at high survival rates for < 21 days, as the period needed for the *E. crassum* larvae to develop in the intermediate host *A. tonsa* was reported as 15 days at 16 °C by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001). An earlier attempt at infecting salmon with marine *E. crassum* at ILAB in 2021 was unsuccessful and the main reason for this was hypothesized to be high copepod mortality and low number of infected copepods for challenge (pers. Comm. Linda Andersen, ILAB).

Copepods were provided by a commercial producer of *A. tonsa* in Norway. The copepods were delivered in 1 L bottles. The bottles were emptied into one container and the water containing copepods was gently stirred before collecting copepods for counting and before being distributed to their respective setups. Copepod stocks were maintained in either 1L Erlenmeyer flasks in incubators with or without aeration, or in small incubators with mesh bottoms (20 µm) in a flow-through system as shown in Figure 6. The flow-through systems are based on systems developed by Hamre and colleagues (Hamre, Glover, and Nilsen, 2009).

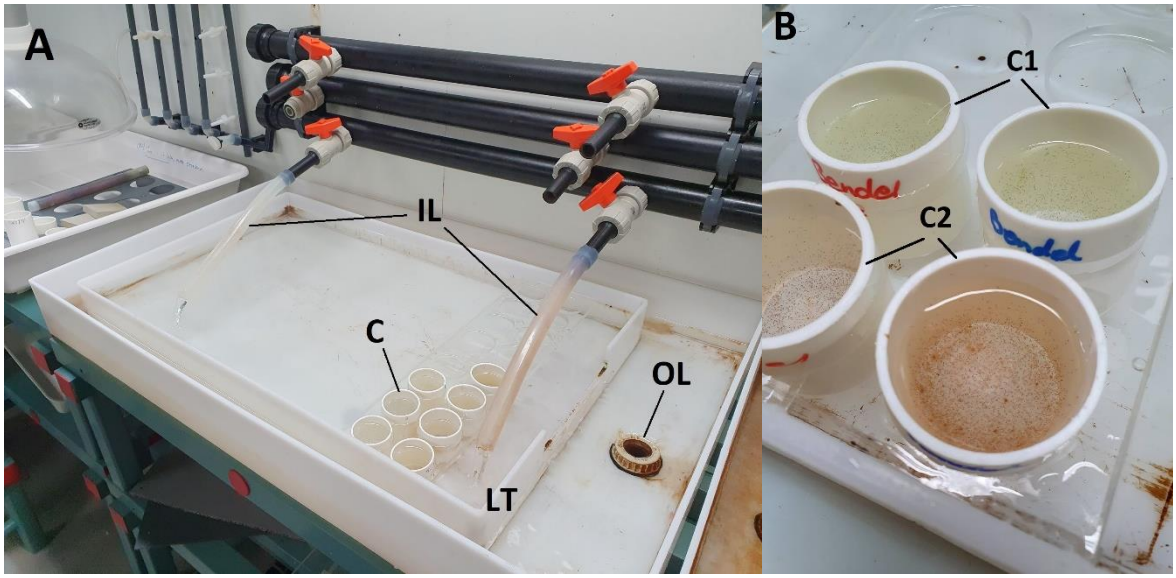

Figure 6 A) Overview of the flow-through incubator system used. Water enters the system through the water inlets (IL) with a flow of 720 L hour^{-1}. Copepod containers (C), ~ 150 mL volume, with a 20 µm mesh bottom allowing water exchange in the cup are kept in a level-tank (LT) to ensure correct water level. Water exits through the outlet (OL). B) Close-up of copepods after feeding with either algae paste (*Tetraselmis suecica* Kylin) (C1) or live *Rhodomonas baltica* Karsten (C2).

Aeration was added to one copepod culture (Table 1) using an aquarium air pump (50 L/h). The freshwater used at ILAB and in this project came from Svartediket, the lake supplying Bergen with drinking water. The water is first filtered (200 µm) before entering the water

treatment facilities on the premises. The water is treated with KitoFlokk™ (TETA VANNRENSING AS; Lørenskog, Norway) before going through a sand filter. The water is then treated with ultraviolet light (UV) and added Krystazil 40 (BIM Norway AS; Drammen, Norway) before entering ILAB's aqua facilities. Freshwater treated as described will hence in the paper be referred to as regular freshwater or just freshwater. The seawater used is pumped in from 110 m below sea surface outside of Bergen (Nordnes). The water goes through a drum filter (20 µm) and is treated with UV before entering ILAB. Seawater filtered and treated with UV as described will hence in this paper be referred to as regular seawater or just seawater. Autoclaving water to use in the present study was done with a high-pressure steam sterilizer (SX-700E; TOMY SEIKO CO., LTD.; Japan) at 120 °C for 40 minutes. The copepods were fed with either the live microalgae *R. baltica* or a commercially available algae paste (*T. suecica*), both provided by the commercial producer of copepods. Detailed amount fed to each batch is explained in their respective sections (Sections 2.1.1-2.1.6). Feeding was done twice daily (Monday-Friday), ca. 9 am and 3 pm. Copepods in incubators were fed by directly adding feed (algae) to the flasks while those in the flow-through system were fed by placing the copepod container in a flask containing feed (algae or algae paste) for 20 minutes. The water in the copepod flasks in the incubator was changed daily; 800 mL (Monday-Friday). For batch 2 this was done by filtering (20 µm) out the copepods before adding new water. To reduce risk of copepod injury through handling, the method for changing water was changed after batch 2. The new method was to lower a copepod container into the flask, allowing water to enter the container through the filter in the bottom (20 µm mesh) while detaining the copepods. Water was then sucked out from inside the container with a pipette controller (VWR® Powerpette® Pro Pipet Filler) before adding new water. Water was not replaced during weekends. Throughout the project, to estimate the number of copepods, the incubator cultures were gently stirred to achieve a homogenous distribution before pipetting 1 mL through a filter (20 µm) to remove the water. The copepods on the filter were then added to 1.5 mL 70 % ethanol (70 % pure ethanol, 30 % 0.2 µm filtered sea water) and stored at 4 °C before counting the number of copepods. The sample was then used to estimate the total number of copepods in the culture. To estimate the number of copepods in the flow-through system, the copepods in the copepod containers were moved into a beaker with 100 mL of their incubator water before homogenising the distribution and sampling in 70 % Ethanol for counting. The remaining copepods were then moved back to their copepod containers in the flow-through system. Water temperature, oxygen, salinity, and pH were measured using a handheld water quality monitor (©WTW multi 3420 Digital meter).

R. baltica was used to feed the copepods by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001) and by the commercial copepod provider, and was hence used in this study as well. Algae cultures were received from the commercial copepod producer and maintained in parallel with each copepod batch. The algae cultures were kept in 1-2 L round flasks (21.8 ± 0.4 ℃) with continuous light and aeration. Daily (Monday-Thursday), 400 mL of the water in the algae flasks was removed and replaced with autoclaved water (~ 35.5 ppt). The same days, 240 µL of algae medium (a mixture of inorganic salts and vitamins dissolved in water, pH 3.2) was added. The algae medium was provided by the copepod producer. On Fridays 800 mL water was changed, and 480 µL algae medium added. There was no husbandry during weekends.

To estimate the daily health of the algae cultures used in this study, a "mood scheme" provided by the commercial copepod producer was used. In this scheme, the colours red, orange and green were used to describe the health of the culture depending on the culture colour. The colour red indicated that the culture was fine, as the red colour comes from the pigment phycoerythrin which is a main photosynthesising pigment for *Rhodomonas*. The colour orange indicated that the culture is less healthy as the algae have begun to break down the phycoerythrin in their cells. Lastly, a green culture indicated that algae cells are severely stressed or dead as when the water-soluble phycoerythrin dissolves into the water, the fat-soluble chlorophyll will be left as the main pigment in the algae cell. The algae were observed daily when fed with algae medium. When the algae cultures were seen to become more yellow in colour, as shown in Figure 7B, extra algae medium in addition to the regular feedings was added.

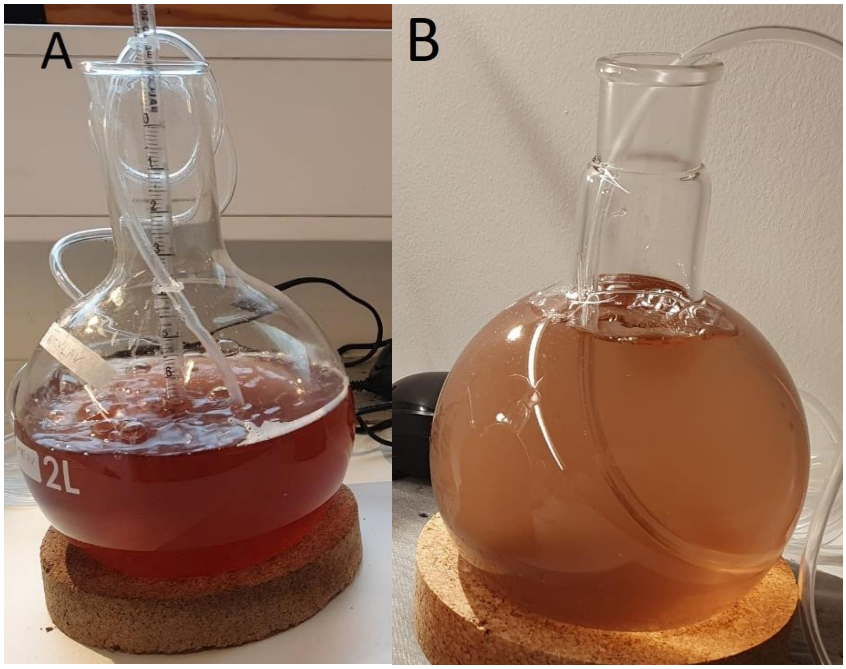

Figure 7. Algae cultures of *R. baltica*. A: Healthy algae culture with a deep red colour. B: Algae culture that had not received algae medium for 3-5 days. The algae cultures kept in this study were normally the colour shown in A.

The algae paste used consisted of *T. suecica* (Feal Fresh®; cell density: >150 M cells/mL, dry weight = 5-6 % g/L; Inalve; Nice, France) and was provided by the commercial copepod producer. The algae paste and algae medium were stored in a fridge at 4 °C in 50mL falcon tubes and weekly replaced by new ones stored in a freezer (-25 °C) to prevent spoiling.

An overview of the different copepod batches delivered to ILAB, and their respective husbandry profiles is provided in Table 1.

Table 1. Overview of the different copepod batches used in this study. The algae paste used was made of *T. suecica.* Group 1 was maintained for only two days before 100 % mortality was registered due to low oxygen. Group 2c was terminated after eight days due to an accident. Groups 3e and 3f were started later than the other groups in Batch 3.

Copepod batch #	Batch start	Batch duration (days)	Incubator system	Feed type	Aeration (yes/no)	Temperature (°C)	Salinity (ppt)	Autoclaved water (yes/no)
1	31.08.2021	2	Incubator	Algae paste	No	15	31±1	Yes
2a	07.09.2021	30	Incubator	Algae paste	No	14.6±1.0	26.3±1.8	Yes
2b	07.09.2021	30	Incubator	Algae paste	No	14.4±1.0	34.9±1.1	Yes
2c	07.09.2021	8	Incubator	Algae paste	No	15.4±1.6	33.8±1.0	No
3a	14.10.2021	13	Incubator	Algae paste	No	13±1.7	28±1.8	No

3b	14.10.2021	13	Incubator	*R. baltica*	No	12.8±1.4	27.5±2.3	No
3c	14.10.2021	13	Incubator	Algae paste	Yes	13.0±1.5	27.9±1.8	Yes
3d	14.10.2021	13	Incubator	*R. batltica*	Yes	13.3±1.9	27.4±2.5	Yes
3e	14.10.2021	5	Flow-through	Algae paste	No	16.6±1.0	33.3±0.1	No
3f	14.10.2021	5	Flow-through	*R. baltica*	No	16.6±1.0	33.3±0.1	No
4	4.11.2021	12	Flow-through	*R. baltica*	No	11.5±2.9	33.3±0.2	No
5	11.01.2022	7	Flow-through	*R. baltica*	No	8.8±0.2	33.2±0.1	No
6a	15.11.2021	15	Flow-through	*R. baltica*	No	16±0.4	33.2±0.1	No
6b	15.11.2021	16	Flow-through	*R. baltica*	No	16±0.4	33.2±0.1	No
6c	15.11.2021	17	Flow-through	*R. baltica*	No	16±0.4	33.2±0.1	No

2.1.1 Batch 1

Six 1000 mL flasks containing copepods were received from the commercial producer August 31st, 2021, and poured into a bucket where water quality was measured (Oxygen: 63 %, Temperature: 13 ℃, Salinity: 30.8 ppt), and copepods sampled for counting. The copepods were then divided into six 1000mL Erlenmeyer flasks and maintained in an incubator at 15.0 ℃ with no light except during feeding. Each flask was fed 0.25 mL of algae paste (~ 3.75 x 10^7 *T. suecica* cells) once a day. Water was not replaced in this group and 100% mortality was observed on day two. Low oxygen levels were registered; 2-10 %. Hence, the culture was discontinued.

2.1.2 Batch 2 – Copepod survival: Salinity and autoclaved water

Because salmon farms in Norway are often placed in fjords where seawater and freshwater from rivers mix and since *A. tonsa* often can be found in coastal areas, it was decided to compare copepod survival in brackish water (25 ppt) with seawater (34-35 ppt). In addition, it was examined if autoclaving the water of the copepod culture would increase survival due to less bacteria present that could affect the copepods. Seven bottles (1 L) containing copepods were received September 7th, 2021. The copepods were gently mixed, and the water parameters registered (Oxygen = 92.2 %; Salinity = 31 ppt; Temperature = 4.6 ℃; pH = 7.1) before the copepods were distributed to seven 1000 mL Erlenmeyer flasks. The copepod cultures were

maintained for 30 days. The flask water was replaced daily (Monday-Friday); 300-700 mL the first nine days. From day ten and onwards, 800 mL was replaced daily. Two flasks were given autoclaved 25 ppt water (2a), two were given autoclaved ~ 35.5 ppt seawater (2b), and three flasks were given ~ 34.5 ppt seawater (2c). The copepods were fed 1 mL algae paste (~ 1.5 x 10^8 *T. suecica* cells) twice daily Mondays-Fridays, ca. 9 am and 3 pm. The algae paste fed to the copepods quickly fell to the bottom of the flasks and accumulated over time. As this could potentially lead to build-up of bacteria and waste products, the debris on the bottom was removed daily using a pipette controller from day 14 on Mondays-Fridays. Batch 2c was terminated on day eight due to an accident with the copepod culture. Water parameters were registered with a handhold water quality monitor throughout the study and are shown in Table 2. The raw data for water qualities is provided in Appendix table 1, and copepod counts are provided in Appendix table 2.

Culture #	Culture water (ppt)	Oxygen x̄ (%)	Oxygen range (%)	Salinity x̄ (ppt)	Salinity range (ppt)	Temperature x̄ (°C)	Temperature range (°C)	pH x̄	pH range
1	25	53	26-98	26	25-28	15	14-16	7.5	7-7.8
2	25	50	26-99	26	25-28	15	14-15	7.5	7-7.9
3	35	53	36-100	35	34-36	15	14-15	7.7	7.2-8
4	35	47	22-101	35	34-36	15	14-17	7.7	7.4-8

Table 2. Water parameters registered from the copepod cultures in batch 2 throughout the study. The data have been rounded to nearest whole number, except for pH. Groups 1 and 2 were kept in autoclaved 25 ppt water, while groups 3 and 4 were kept in autoclaved full-strength seawater (35 ppt).

2.1.3 Batch 3 – Copepod survival: Incubator system

As the last group of copepods showed higher survival for those who received autoclaved 25 ppt water, this batch was used to compare survival of copepods in autoclaved 25 ppt water with copepods in 25 ppt water. In addition, it was decided partway through the test to place some of the copepods in a flow-through system which would remove the need for water exchange and thus lessen required handling. These groups were also used to compare copepod survival in groups fed with live algae (*R. baltica*), with groups fed algae paste (*T. suecica*). Six 1L containers with copepods were received October 14th, 2021, and distributed to six 1 L aerated Erlenmeyer flasks in an incubator (15 °C). The method for water exchange was altered from

Batch 2 to this batch. The new method was to lower a copepod container with a filter in the bottom (20 µm) into the flask, allowing water to enter the container from below through the mesh while detaining the copepods. Water was then sucked out from inside the container with a pipette controller before new water was added. Due to an accident with the culture eight days after culture start, the two copepod cultures that were to be moved to the flow-through system had to be discontinued. The remaining four flasks were stirred gently to get an even distribution of the copepods before distributing half of their contents to eight containers in the flow-through incubator system. Two of the copepod cultures in the incubator received 25 ppt water (3a + 3b), while the other two received autoclaved 25 ppt water (3c + 3d). The incubator copepods were fed either 1mL of algae paste (~ 1.5 x 10^8 *T. suecica* cells) (batches 3a + 3c) or 10 mL from a *R. baltica* culture (batches 3b + 3d) twice daily. In the flow-through copepod incubator system, copepods in one group (3e) were fed by filling 150 mL beakers with 100 mL of the incubator water and 0.25 mL of algae paste (~ 3.75 x 10^7 *T. suecica* cells) before moving the copepod containers into the beakers for 20 minutes twice daily. At the same time, the other group (3f) were fed in beakers filled with 50 mL incubator water and 50 mL from a *R. baltica* culture. Water parameters were registered throughout the study and are shown in Table 3. The raw data for water qualities are provided in Appendix table 3 and Appendix table 4. Copepod counts are provided in Appendix table 5.

Table 3. Water parameters registered from the copepod cultures in batch 3 throughout the study. The data have been rounded to nearest whole number, except for pH.

Culture #	Oxygen $\bar{x}$ (%)	Oxygen range (%)	Salinity $\bar{x}$ (ppt)	Salinity range (ppt)	Temperature $\bar{x}$ (°C)	Temperature range (°C)	pH $\bar{x}$	pH range
3a	75	58-98	28	26-30	14	11-15	7.7	7.4-8
3b	76	58-94	28	25-30	14	11-15	7.7	7.4-8
3c	76	58-98	29	26-30	14	11-15	7.7	7.4-8
3d	71	58-98	28	25-30	13	11-14	7.6	7.4-8
3e	N/A	N/A	33	33-34	16	14-18	N/A	N/A

3f	N/A	N/A	33	33-34	16	14-18	N/A	N/A

2.1.4 Batch 4 – Copepods for pilot challenge with *E. crassum*

Eight 1 L bottles with copepods were received from the commercial producer November 4th, 2021. The copepods were mixed and sampled in order to count the number of copepods and water parameters were read. The mixed copepods were distributed into 16 containers in a flow-through incubator system. They were fed twice daily Monday-Friday, ca. 9 am and 3 pm and once daily Saturdays + Sundays at around noon. They were fed with 50 mL *R. baltica* culture mixed with 50 mL of incubator water. After 12 days, the copepods were challenged with *E. crassum* eggs kept in either fresh water or in sea water. The method for collecting the eggs used is described in section 2.2.3 and the method for challenging the copepods is described in section 2.4.1. Water quality data are provided in Appendix table 6 .

2.1.5 Batch 5 – Copepods for challenge with *E. crassum* kept in brackish water and seawater

Salmon farms are often placed in fjords where fresh water from rivers mix with salt water from the ocean, creating brackish water. To compare the infectivity of *E. crassum* eggs released to brackish water (25 ppt) with those from sea water, two 1 L containers with *A. tonsa* was received from the commercial producer January 11th, 2022. One 1 L container with *R. baltica* was also received. After mixing and sampling the copepods for counting, they were distributed to three copepod containers in the flow-through incubator system where they were maintained similarly to copepod batch 4. The algae fed to copepods were maintained as described in section 2.1. Water quality data are provided in Appendix table 7.

2.1.6 Batch 6 – Copepods to be used as intermediate host for *E. crassum*

To use as intermediate hosts for *E. crassum* for Atlantic salmon challenge (see section 2.4.3), six 1 L containers with *A. tonsa* were received from the commercial producer November 15th, 2021. These were mixed and sampled before they were distributed to four 1 L Erlenmeyer flasks and one 2 L flask. The flasks were aerated and kept in an incubator (15 ℃) overnight before *E. crassum* challenge. After the challenge, the copepods were kept in the flow-through incubator system for 14-16 days to allow *E. crassum* procercoids to develop and become infective before the copepods were intubated Atlantic salmon. Feeding was done similarly to copepod batch 4. Additionally, to prolong feed availability, 20 mL of *R. baltica* culture was

poured directly into the copepod containers after feeding. Water quality data are provided in Appendix table 8.

2.2 Collecting *E. crassum* eggs

2.2.1 *E. crassum* eggs for viability tests using neutral red staining and chlorine treatment

Due to difficulties in retrieving *E. crassum* of marine origin at the time of the experiment, *E. crassum* from brown trout caught in freshwater was used instead. Brown trout was captured October 7th, 2021, at 2 m depth in Kalandsvatnet near Bergen, Norway. The gastrointestinal tract was removed and transferred to Petri dishes with 9 ppt physiologic saline (NaCl in dH_2O) solution (PSS) added. The pyloric caeca and intestine were cut open and any tapeworms present were moved to petri dishes together with 9 ppt PSS. Only *E. crassum* occurred. Large *E. crassum* were transferred to three separate petri dishes containing either fresh water, sea water or 9 ppt PSS and were for practical reasons kept at 4 °C overnight. Due to a low number of eggs observed the following day, terminal proglottids of the worms were cut longitudinally to release more eggs. The eggs released from the cestodes in seawater were used in a test where chlorine treatment was conducted to see if this would permeabilise the external capsule and the embryophore, allowing neutral red stain to enter the embryo, as described in section 2.3.1. After the chlorine experiment, the rest of the eggs in the three groups were incubated at 4 °C. Due to a low number of eggs in the three groups, they were collected in one Petri dish with PSS after the eggs were kept for 4 days at 4 °C, before being used for a neutral red staining test as described in section 2.3.2.

2.2.2 *E. crassum* eggs for staining and copepod challenge tests

Farmed salmon guts were received from an Aquamedicine biologist service (the fish were transported on ice directly the same day as they were collected from net pens outside Bergen) January 10th and examined for tapeworms as described in section 2.2.1. Large *E. crassum* were collected and moved to Petri dishes containing PSS. Eggs released by the cestodes were moved to separate Petri dishes with 25 ppt water or full-strength seawater. These were then moved to 50 mL centrifuge tubes and centrifuged at 200 g for 10 minutes (4 °C) before washing twice with PSS and twice with either 25 ppt water or seawater. A proportion of eggs from the dish with seawater were stained with neutral red (see section 2.3.2). The cestodes were moved to separate Petri dishes with 25 ppt water or full-strength seawater and placed in an incubator at 10 °C overnight to allow the cestodes to release more eggs. The next day the cestodes were

removed and the eggs they had released were transferred to separate 50 mL centrifuge tubes using sterile Pasteur pipettes and centrifuged at 200 g for 3 minutes at 4 ℃. The supernatant was removed, and more water of the respective water quality added, before counting the number of immature and mature eggs (maturity based on size: the mature eggs are larger and approximately 80 µm in length) using an inverted microscope (Leica DM IL LED; Leica Microsystems; Wetzlar, Germany). When the eggs placed in 25 ppt water were examined in the inverted microscope, 20.1 % were mature while 21.1 % were mature of those placed in seawater. These eggs were used in staining tests (section 2.3.2a) and copepod challenge in brackish water and seawater (section 2.4.2).

2.2.3 *E. crassum* eggs for incubation tests and challenge of copepods and Atlantic salmon

Guts from farmed Atlantic salmon (*Salmo salar*) were received November 9th, 2021, in plastic bags on ice. The fish farm was located near Bergen. The group of fish had a mean weight of 110g when they were put to sea April 24th, 2021, in Hordaland. The mean weight of the group was 1850g and the water temperature was 11.5 ℃ when the fish were sampled.

The salmon were heavily infected with *E. crassum*. The tapeworms were distributed to 5 Petri dishes. The Petri dishes received the following water qualities: i) fresh water, ii) 25 ppt seawater, iii) 30 ppt seawater, iv) 34 ppt sea water or v) PSS. The worms were left overnight in the Petri dishes at 4 ℃ because of time constraint for the downstream *in vitro* experiment (section 2.3. The following day, the eggs in the five Petri dishes were prepared and used for incubation parameter tests, an antibiotics test and challenge of copepods and Atlantic salmon, described in sections 2.3.3, 2.3.4, 2.4.1 and 2.4.3.

2.3 *In vitro* study: incubation parameters for *E. crassum* eggs

One aim of this study was to test different incubation parameters for *E. crassum* eggs to find which gave the best survival. Quantifying the number of live *E. crassum* eggs based solely on visible movement of the larvae may be a time-consuming process. Neutral red assay is a staining method that has been used for light-microscopic measurement of cell vitality for decades and the stain may be accumulated in the lysosomes of viable cells (Antal et al. 1995; Repetto, del Peso, and Zurita 2008). The uptake of neutral red is dependent on pH gradients which live cells maintain through the production of ATP (Repetto, del Peso, and Zurita 2008). If a cell stained by neutral red dies or is severely damaged, the stain will be lost (Filman, Brawn, and Dandliker 1975). Since the uptake and retention of the dye is dependent on the production of ATP, neutral red can be used to get an estimation of the number of live cells in a cell culture or cell suspension. The rationale in this study was that since dead or damaged cells lose the

neutral red stain, neutral red staining could be used to quantify the number of live eggs in a sample. In addition, a spectrophotometer could be used in combination with neutral red to quantify the number of viable eggs in several groups in a short time as well as allowing a more objective assessment.

2.3.1 Treatment of *E. crassum* eggs with chlorine for permeabilization

Chlorine has been used to strip the chorionic layers (dechorionation) of *Drosophila* eggs (Lemosy and Hashimoto, 2000; Rand, Kearney, Dao, and Clason, 2010). To test if this was also necessary for *E. crassum* eggs for allowing neutral red staining, eggs with and without chlorine treatment were stained with neutral red using a combination of described methods (Lemosy and Hashimoto, 2000; Benedicenti, Secombes, and Collins, 2019). A chlorine dilution series was created by diluting a sodium hypochlorite solution (15 % Sodium hypochlorite solution; CAS-nr.: 7681-52-9; Hjelle Kjemi AS; Bergen, Norway) with PBS (Phosphate-buffered saline). This series consisted of four 1.5mL centrifuge-tubes containing 100 µL of 1.25 %, 2.5 %, 5 % and 10 % sodium hypochlorite dilutions. To each tube, 50 µL from the *E. crassum* eggs in seawater prepared in section 2.2.1 were used. A control tube with eggs in seawater without chlorine was also used. Eggs from the Petri dishes with seawater were added to each of the tubes with chlorine. The eggs were washed twice by centrifuging at 200 g for 2 minutes at 4 ℃ and replacing the supernatant with PBS (sea water for the control) up to 100 µL. To each tube, 1.4 µL of sterile filtered (0.2 µm) 6.25 mg/mL neutral red solution (Neutral red Gurr (C.I. 50040); Cat. No: 340564A; VWR International; Leuven, Belgium) was added. The eggs were washed once after 10 minutes before being observed in an inverted microscope.

2.3.2 Staining of eggs with neutral red and trypan blue

After the chlorine treatment test, the remaining eggs in the three groups were incubated for four days at 4 ℃. Due to a low number of eggs, the three groups were combined in a 15 mL centrifuge-tube with PSS and centrifuged at 300 x *g* for 1.5 minutes at 4 ℃. From this solution with eggs, 50 µL was added to each of five centrifuge-tubes, four test tubes and one control. The tubes were washed twice by centrifuging at 200 g for 2 minutes at 4 ℃ and replacing the supernatant with PBS. The final volume in the tubes was 100 µL. Four of the five tubes were stained with 1.4 µL sterile-filtered (0.2 µm) neutral red solution (6.25 mg/mL) for 10, 20, 30 and 40 minutes based on methods used in previous studies for fly eggs and amoebae (Lemosy and Hashimoto, 2000; Benedicenti, Secombes, and Collins, 2019). The eggs were observed during the incubation time. After the allotted time the tubes were washed twice with PBS and

observed in an inverted microscope. Because of a low start number of eggs and loss during the washing procedure, no eggs remained. Due to this, it was decided to try the staining method using eggs from the control group. Eggs were stained for 40 minutes and washed using the same method as previously described. The eggs were observed during staining and after staining and washing.

2.3.2a Staining live and dead eggs

Due to the low number of eggs when staining was first tested, it was decided to retry the staining method. To do this, guts from farmed Atlantic salmon were acquired January 10th and eggs from *E. crassum* in the fish guts were collected (see section 2.2.2). The day the eggs were collected, some of those incubated in seawater were stained with neutral red to count the number moving embryos among the stained eggs. The eggs were stained using the method described in section 2.3.2. The eggs were then observed in an inverted microscope for any movements immediately after the incubation period ended. The staining methods described in this section were performed on eggs up to nine days after the eggs were collected.

Trypan blue is a dye that stains dead cells and not living cells with intact cell membranes, as opposed to neutral red (Strober, 2015). It was hypothesized that trypan blue could be used to validate the neutral red assay as a method for quantifying the number of viable eggs in a sample. Sterile filtered (0.2 µm) 0.4 % trypan blue solution (VWR™; Solon, Ohio) was used. Live eggs treated with seawater (see section 2.2.2) were stained one and nine days after collection with neutral red using the method described in section 2.3.2 and with trypan blue one day after collection using the method described by Strober (Strober, 2015).

To compare the effect of dyes on live eggs with dead eggs, some of the eggs from section 2.2.2 were killed by either freezing (-25 ℃ for 40-120 minutes), immerison in 70 % EtOH (40-120 minutes) or with CASy blue before staining with neutral red and trypan blue. Freezing and ethanol immersion before staining with neutral red was done one, six and nine days after collecting the eggs. The same was done with trypan blue one and six days after egg collection, while CASy® blue was only used on eggs before staining six days after they were collected.

To kill the eggs with CASY® blue (Innovatis AG; CASY®-Technology; Reutlingen, Germany), 200 µL of the eggs in seawater were used. The eggs and 800 µL CASY® blue were gently mixed using a pipette in a 15 mL centrifuge tube and incubated for 2 minutes at room temperature (approximately 22 ℃). After incubation, 9 mL CASY℃ ton (Innovatis AG; CASY®-Technology; Reutingen, Germany) was added to the solution and gently mixed with a pipette. The eggs in the solution were then stained with neutral red or trypan blue as described.

Nine days after collecting the eggs, a small-scale neutral red staining test of eggs was conducted on an unspecified number of eggs. The aim of this test was to determine if a shorter neutral red incubation time would make it easier to distinguish between different types of eggs, e.g., big, and mature vs small and immature. A small amount (5-20 µL) of the solution with eggs was placed on an object glass. The tip of a pipette needle was gently touched into neutral red dye before stirring the eggs gently with it. After 20 minutes, the stained solution was carefully removed with a pipette without removing any eggs, before PSS was added. This was repeated 3-5 times until the solution was clear or barely pink before observing the eggs in an inverted microscope. This method was completed twice on live eggs and once on dead eggs (frozen or 70 % EtOH).

2.3.3 *E. crassum* eggs incubation under various salinities and treatments

One of the aims of the study was to test different incubation salinities and other treatments to find which would give the highest survival of *E. crassum* eggs when incubated < 4 days. The other treatments were 1) washing/not washing the eggs, 2) incubating the eggs in autoclaved/regular water, and 3) adding antibiotics/not adding antibiotics to the eggs. At the same time, the effect on bacterial growth when adding antibiotics to incubated eggs was examined. To use for these tests, *E. crassum* eggs were collected from cestodes in farmed Atlantic salmon November 9th, 2021, and kept in five different Petri dishes, described in section 2.2.3.

A cell scraper was used on Petri dishes i) – iv) to stir up the unwashed eggs before they were transferred to four 1.5 mL centrifuge tubes. The eggs in the Petri dish with PSS were diluted 1:5 with PSS and the number of viable eggs, based on size, was counted in 5x3 µL drops using an inverted microscope. The concentration of eggs was 3.97 eggs µL^{-1}.

The eggs in the Petri dish with PSS were stirred up with a cell scraper before transferring the solution to a 50 mL centrifuge tube. The eggs were washed twice with PSS (centrifuged at 200 x *g* for 10 minutes at 4 ℃) before being distributed to eight 1.5 mL centrifuge tubes. The eggs in the tubes were then washed twice (centrifuged at 200 x *g* for 10 minutes at 4 ℃) with their respective water qualities. The water qualities used were freshwater, 25 ppt seawater, 30 ppt seawater and 34 ppt seawater as well as autoclaved 0 ppt freshwater, 25 ppt seawater, 30 ppt seawater and 35 ppt seawater.

The number of viable eggs in each of the 12 groups (four unwashed, four washed with autoclaved water, four with regular water) was counted in an inverted microscope by placing

5x3 µL of the solutions on glass slides. Due to too few eggs gathered for the unwashed 25 ppt group, this group could not be included. The groups were moved to 15 mL falcon tubes and diluted to a concentration of 2 eggs μL^{-1} with their respective water quality. By pipetting, three 96 well plates were filled with 8x100 µL replicates from each group as shown in (Figure 8) for a total number of 200 viable eggs per well. The remaining eggs were pipetted back to separate Petri dishes and kept in an incubator at 10 °C to be used for copepod and Atlantic salmon challenges, described in sections 2.4.1 and 2.4.3.

The groups were also used to test the effect of antibiotics on bacterial growth in wells with *E. crassum* eggs. To do this, an antibiotic solution (Antibiotic-Antimycotic 100x; a mixture of Streptomycin sulphate 1 - < 2.5 %, CAS-nr.: 3810-74-0 and Penicillin G sodium salt < 1 %, CAS-nr.: 69-57-8; Biowest; Nuaillé, France) was diluted 1:4 with PBS. One µL of this solution was added to rows E, F, G and H for a 1:400 dilution. One µL PBS was added to rows A, B, C and D. The plates were incubated at 10 °C for 16 hours, 38 hours and 110 hours respectively.

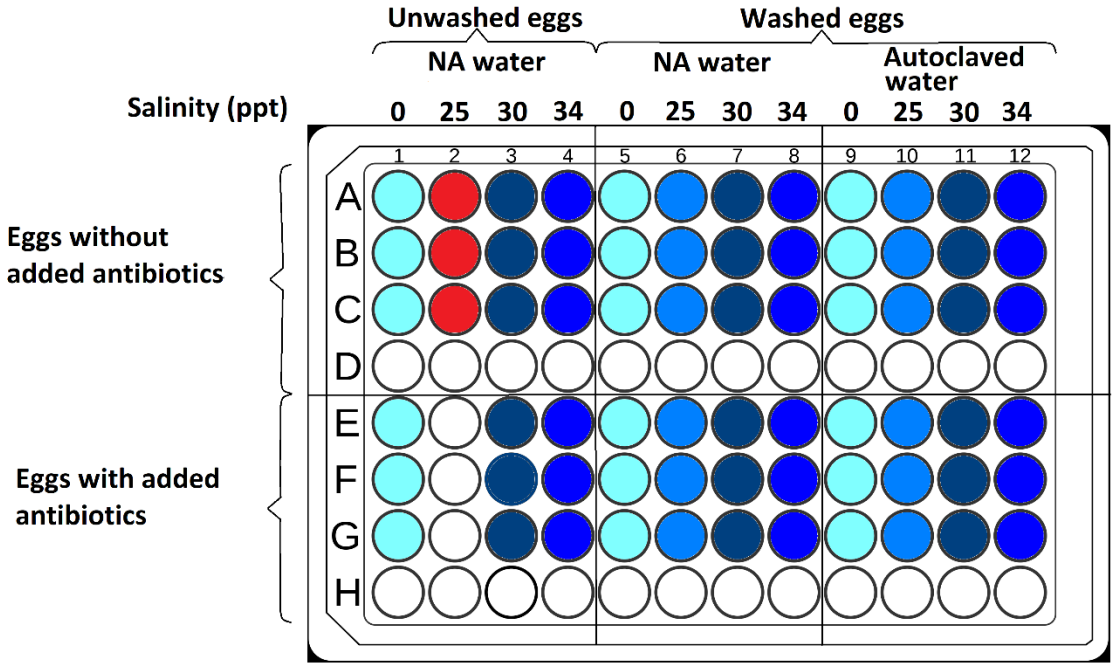

Figure 8. Setup of water qualities and antibiotic-treatment for the *in vitro* experiment of *E. crassum* egg survival during incubation for < 4 days. Columns 1-8 had eggs incubated with regular water. No eggs were placed in column 2 due to too few eggs gathered for the unwashed 25 ppt water group. Wells 2A-C were control groups with 100 µL PSS + 1.4 µL filtered neutral red. Rows D and H were not stained with neutral red.

After incubation, plates 1 and 2 were treated the same way: 1.4 µL filtered neutral red stain (6.25mg/mL) was added to all rows except the negative controls, rows D and H, in which 1.4 µL PBS was added. The final volume of the wells was 101.4 µL and those dyed had a neutral red concentration of 86.3 µg/mL. The plate was left to incubate at room temperature (approximately 22 ℃) for 40 minutes before washing three times with PSS. The wells were washed by centrifuging the plate at 200 g for 3 minutes (4℃), removing the supernatant and adding PSS before repeating the process for a total of three times. After washing, the wells each had PSS added to a volume of 100 µL. To wells 2A-C 100 µL PSS and 1.4 µL neutral red stain was added as positive controls. Absorbance was read using a SPARK® Multimode microplate reader together with the software Tecan – Spark control dashboard.

The three plates each had an absorbance peak. As a measure of the degree of neutral-red staining, the absorbance levels in the range between the lowest and highest peak of the three plates were used. Variation in absorbance was examined with Main Effects Analysis of Variance (ANOVA), for each time. Post-Hoc Newman-Keuls tests were used to identify significantly different treatments. The data was checked for heteroscedasticity using Levene's test for homogeneity of variances. All statistical tests were performed in the software Statistica (64) 13 (TIBCO Software).

2.3.4 Antibiotics and bacteriology

To test the growth of bacteria on incubated *E. crassum* eggs over time, two replica of 96-well plates were prepared; from the unwashed eggs kept in seawater, washed eggs kept in seawater and washed eggs kept in autoclaved seawater, 50 µL was added to two wells for a total of six wells filled on each plate. From the 1:4 antibiotic-antimycotic solution used earlier, 0.5 µL was added to one well of each group. To the other wells, 0.5 µL PSS was added. The plate covers were then put on the two plates and sealed with parafilm wrapping before being set to incubate at 10 ℃ for 13 hours and 36 hours respectively. After incubation, 1:100 and 1:1000 dilutions were created using PBS for each of the six filled wells. From these dilutions, 70 µL were sown on BAS (2 %) and set to incubate at 10 ℃ for four days (~ 95-98 hours). After this incubation period, the bacteria colony morphology and CFU were registered. Using a plastic wire needle, bacterial colonies were gently moved from the agar plates and mixed in PBS on an object glass to be examined in a microscope. The method was replicated using the 96 well plate incubated for 110h prepared in section 2.3.3. Using PBS, 1:100 and 1:1000 dilutions were created from wells 4A+H, 8A+H and 12A+H. From these dilutions, 70 µL were sown on BAS before incubating at 10 ℃ for 3 days.

CFU/mL in each well was calculated using the following formula:

$$\frac{CFU}{mL} = \frac{(no.\,of\ colonies\ \times\ dilution\ factor)}{volume\ of\ culture\ on\ plate}$$

The two dilution factors used were 100 and 1000. The volume of culture sown on plates was 700 µL.

2.4 Challenge of copepods and Atlantic salmon with *E. crassum*

2.4.1 Pilot challenge of copepods with *E. crassum*

After keeping the batch 4 copepods for 12 days, a number of the copepods were moved to two Petri dishes. *E. crassum* eggs that had been incubated for five days in fresh water were added to one dish, and eggs incubated with seawater were added to the other (see sections 2.2.3). The Petri dishes were then covered with a cloth mat. After one hour, some of the copepods were moved to a new Petri dish for observation with an inverted microscope. To hold the copepods still without crushing them, a cover glass supported by Vaseline drops was put on top of the water droplet with copepods. Since no infections were immediately observed, the copepods were moved to containers in the flow-through incubator system and observed once more the following day.

2.4.2 Copepod challenge with *E. crassum* eggs kept in brackish water and seawater

To compare the infectivity of the *E. crassum* eggs kept in 25 ppt water and 34 ppt water (see section 2.2.2) over time, the batch 5 copepods were challenged 1, 2 and 5 days after the eggs were collected. The day they were challenged, each copepod container was split in two 1 L Erlenmeyer flasks containing 800 mL from the incubator system water and placed in an incubator at 15 ℃. To each flask, an estimated number of ~ 20000 mature eggs were added. Eggs held in 25 ppt water were added to one copepod flask and eggs held in full-strength seawater (34 ppt) to the other. The copepods were challenged over three hours. To keep the eggs from settling on the bottom and thereby reducing their exposure to the copepods, a cycle of ten minutes aeration and 30 minutes without was used, allowing the copepods to ingest the eggs. After three hours, the copepods were moved back to the flow-through incubator system. The copepods were examined for procercoids one day after challenge with eggs using the method described in the pilot challenge test (see section 2.4.1).

2.4.3 Atlantic salmon challenge with *E. crassum* using *A. tonsa* as intermediate host

To use as intermediate hosts for *E. crassum* larvae before infecting salmon, the batch 6 copepods were used.

After six days of incubation at 10 ℃, the *E. crassum* eggs kept in seawater (see sections 2.2.3 and 2.3.3) were moved into a 50 mL falcon tube before being centrifuged at 200 x *g* for 10 min at 4 ℃. The eggs were washed once with PSS and 3 times with seawater. The number of mature eggs was counted (~ 13670 eggs/mL) before 1.5 mL of the solution with eggs (~ 20500 embryonated eggs) was added to each of the four 1 L flasks. The estimated total number of copepods challenged was ~ 26.000 and estimated total number of added *E. crassum* eggs ~ 82000 for an average of 3.2 mature eggs per copepod. The challenge lasted for three hours with cycling aeration before the copepods were distributed to eight copepod containers in the flow-through incubator system (Temperature: 16 ℃). The day after challenge, a 16 % prevalence of procercoids was observed from 232 copepods examined with an inverted microscope. After 13 days, all the copepods were collected to a Petri dish to count infection prevalence using Vaseline and cover slips. The copepods were then moved back to the flow-through system and distributed to four copepod containers. Prior to challenging the Atlantic salmon, 121 copepods were examined, of which 42 were infected for a prevalence of 35 %. A mean intensity of 1.14 (range 1-2) was registered from the 48 larvae observed.

The fish used for experiments were specific-pathogen free and reared and held at ILAB. Five uninfected fish were examined prior to the experiment. They were euthanized by an overdose of 200 mg/L Finquel vet. (FINQUEL® vet.; Tricaine mesylate 1000 mg/g; Intervet International B. V.; Boxmeer, Netherlands) in a bucket with water from the fish tank. The fish were then weighed ($\bar{x}$ = 248 g, range 174 – 345 g), the fork-length measured ($\bar{x}$ = 26.7 cm, range 24.5 - 29.5 cm), and blood sampled with sterile 1 mL syringes to register haematocrit (Hct) levels ($\bar{x}$ = 45.4 %, range 39 - 53 %). For the hct values, blood from the syringes was transferred to micro hct tubes and sealed with wax. The hct tubes were centrifuged at 16060 x *g* for 3 minutes at 4 ℃ before reading the hct values with a hct card reader. Tissues from heart, spleen and kidney were sampled for PCR and histology. The histology samples were placed in a Formaldehyde 4 % buffered aqueous solution (Formalin 10 %; CAS Number: 50-00-0; VWR International; Leuven, Belgium) while the samples for PCR were placed in RNA*later*™ Stabilization Solution and frozen at -25 ℃. The guts were emptied, and contents and guts were carefully examined for worms using a stereoscope. None were found.

In a challenge model used by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001), *A. tonsa* used as intermediate hosts were infected by *E. crassum* 15 days x C (240 day-degrees) before being intubated to Atlantic salmon. As this experiment is based on their study, and to increase chance of successful infection, the fish in this experiment were infected over three days with copepods at 224, 240 and 256 day-degrees past infection with *E. crassum*. Before the fish challenge, 1 mL sterile single use syringes were filled with copepods (infection prevalence 35 %). Sterile soft feeding needles (Bit protection feeding needle; Fuchigami; Japan) were added to the syringes to prevent injury of the fish while orally intubating.

At the infection time, the mean fish weight was ~ 250 grams. The fish were kept in a 500L tank and starved for 24 hours before challenge. Before challenge, the fish were anesthetized. This was done by tapping the tank for water down to a total volume of 200 L before adding 2 mL Aqui-S (AQUI-S vet.; isoeugenol 540 mg/mL; Intervet International B. V.; Boxmeer, Netherlands). Fish were then moved to a bucket containing 0.75 g Finquel vet. dissolved in 10 L of water from the fish tub. When the fish showed reduced reaction to physical stimuli, they were weighed, length measured and tagged using visible implant elastomer (VIE) tags before 100 µL of the solution with copepods was intubated as shown in Figure 9. On one occasion, the opening of the feeding needle was sealed by a mass of copepods when trying to intubate one of the fish. A tweezer was used to push the copepods back into the syringe before the intubation could continue as normal.

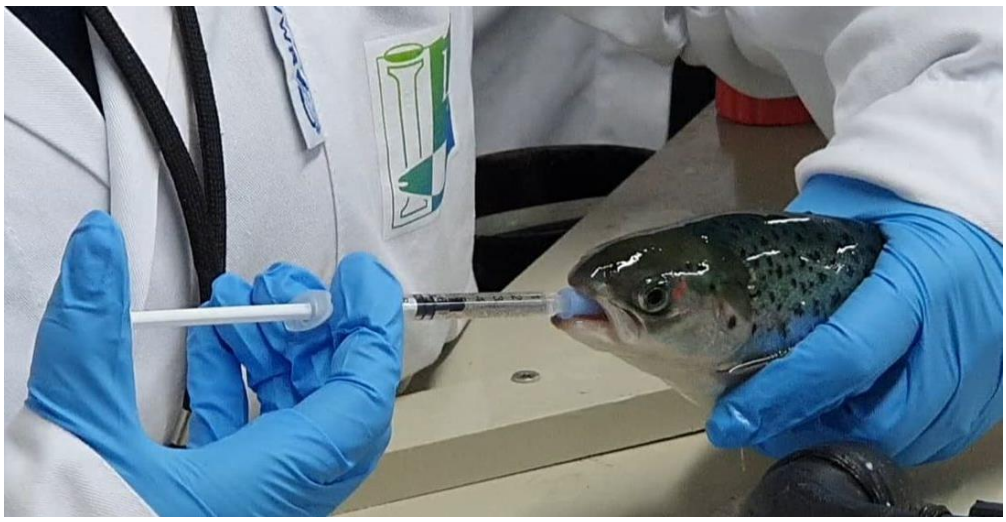

Figure 9 Intubation method. The syringes used had soft feeding needles to avoid injury of the fish while intubating infected copepods directly into the stomach. In this picture a red VIE tag is shown posterior to the eye.

Five groups of fish were used in this experiment as shown in Table 4.

Table 4. Copepod groups used in fish challenge. The number of copepods used to infect each fish differed due to difficulty in filling each syringe with the same number of copepods. The exact number of copepods used for the bath treatment was not registered. However, the prevalence of the infected copepods used was 35 %.

	Infected copepods				Uninfected copepods (controls)
Infection method	Intubated			Bath	Intubated
Day-degrees past copepod infection	224	240	256	256	N/A
Number of fish in group	5	5	5	10	10
Average number of copepods fed per fish	19	7	37	N/A	45

After intubation, the fish were moved to an oxygenized tub for awakening before they were moved into a new tank. This process was similar for the three groups intubated with infected copepods and the control with non-infected copepods. For the bath treatment, 10 fish were anesthetized, measured, and tagged the same way as the other groups before they were moved to an aerated tub containing copepods for two hours. Half the control and bath treated fish were sampled after ~ 2 months (55-57 days), the rest after ~ 3 months (83-85 days).

The fish were kept for < 3 months before sampling. The fish tank temperature in this period was 12.1 ± 0.7 °C except for a period when the temperature was 8.9 ± 0.2 °C. This change in temperature was due to miscommunication with technical staff and started 43 days after challenge and lasted for 19 days before returning to set temperature. The fish were kept at salinity: 34.2 ± 0.8 ppt; O_2: 90 ± 10 %; flow: 1000 L/h with lighting 12 hours on/12 hours off. Daily 130 g of feed (Nutra Olympic; 4 mm; Skretting) was added to the tank, increasing to 250 g through the period. No mortality was observed throughout the period.

After 2 months, five fish from the control and bath groups were examined for infection. The fish were euthanized and dissected. Weight and length were measured before blood was collected using 1 mL sterile syringes. The blood was used to measure haematocrit and collect plasma. To collect plasma, blood was transferred to 1 mL centrifuge tubes and centrifuged at 600g for 10 minutes at 4 °C before plasma was carefully removed from the tubes with a 1 mL pipette to new 1 mL centrifuge tubes and kept at 4 °C. The spleen was sampled for PCR and histology using the same method as earlier. After 3 months (83-85 days, depending on when the fish was infected) the rest of the fish in all groups were euthanized and examined the same

way as previously described. The guts and gut contents were carefully examined for worms using a stereoscope.

In this paper, the terms prevalence, abundance and intensity are used in accordance with (Busht, Laffertyt, Lotz, and Shostakll, 1997).

3. Results

3.1 Copepod survival tests

In batch 2, the copepods maintained in autoclaved 25 ppt or 35 ppt water decreased in number over time (ANOVA, $P<0.001$), but there was no difference in survival related to salinity ($P=0.1$) (Figure 10).

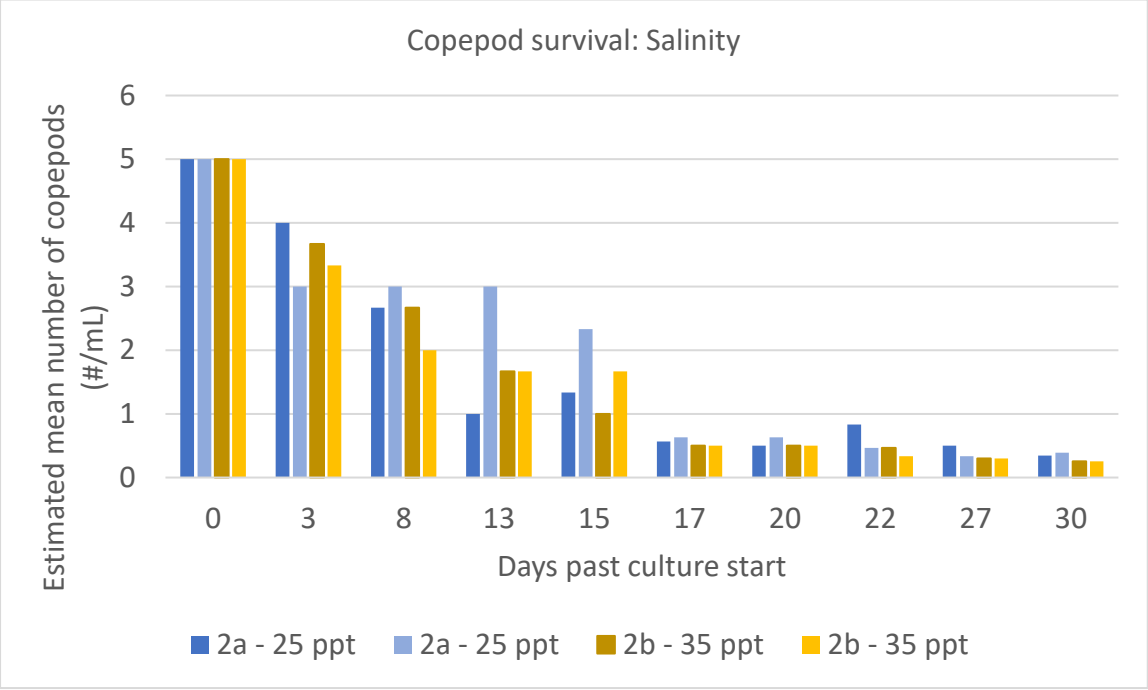

Figure 10. Estimated mean number of copepods per mL in batch 2. The sample size used for counting the number of copepods for days 0 – 15 were 3 x 1mL. This was increased to 3 x 10mL from day 17 to increase copepod counting accuracy. Day 30 past culture start, the sample size was again increased to 3 x 30mL.

In batch 3, the copepods kept in the flow-through system and fed with live algae (*R. baltica*) had higher survival than those fed algae paste (*T. suecica*) as well as those kept in an incubator regardless of feed, shown in Figure 11.

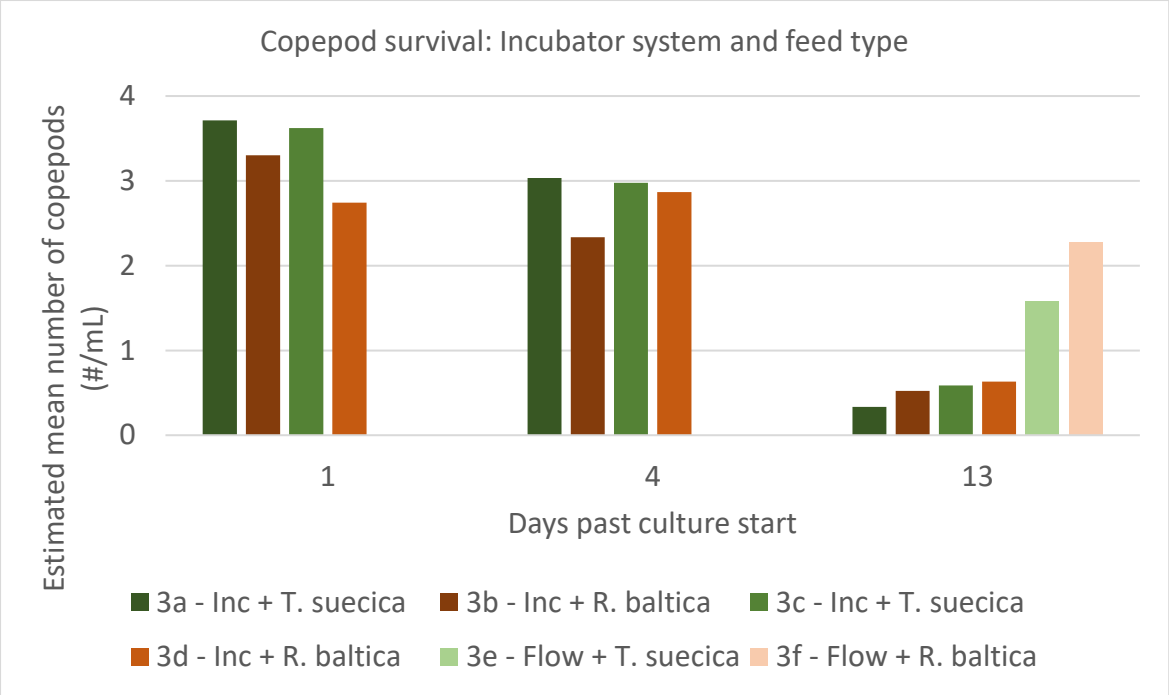

Figure 11. Estimated mean number of copepods per mL for batch 3. On day eight after culture start, half of the copepods in the incubator (groups 3a-d) were moved to start the groups in the flow-through system (groups 3e+f).

The *R. baltica* cultures kept throughout the whole study were observed daily Mondays-Fridays and the culture colour was used as indication of the culture health. The colour of the cultures was mainly deep red, which, according to the commercial producer of copepods who also supplied the algae, meant the cultures were healthy.

3.2 *In vitro* study: Incubation parameters for *E. crassum* eggs

3.2.1 Incubation of *E. crassum* eggs with various salinities and treatments

When collecting *E. crassum* from the fish guts, mucus and other organic matter followed as well. The debris was observed to absorb/trap the neutral red stains in the groups of eggs that were not washed and thus affected the absorbance levels. Therefore, the neutral red results from unwashed eggs could not be analysed based on absorbance. Washed eggs were incubated for 16h, 38h or 110h in regular water or autoclaved water, with or without added antibiotics and in different salinities (0, 25, 30 and 34 ppt). Incubation time, antibiotics or autoclaving the water did not have a significant effect on the absorbance levels (ANOVA). However, salinity

always had a significant impact (ANOVA, P<0.001), and Post-Hoc testing revealed that this was due to lower absorbance in the 0 ppt incubations than in the other salinities (16h P<0.001, 48h: P<0.001; 110h: P<0.05). The absorbance levels are provided in Appendix table 9.

3.2.2 Antibiotics and bacteriology

The agar plates from the different salinity and treatment groups from the *in vitro* study were examined three or four days after sowing on agar plates. Unexpectedly, only the groups that were incubated with autoclaved seawater showed bacterial growth with monomorphic colonies after incubation (Figure 12). The bacteria were comma shaped and motile, but not identified further.

Figure 12. The morphology of bacteria colonies grown on BAS. Only the groups that were incubated with autoclaved seawater had bacterial growth. The morphology of the bacteria colonies from all the groups were similar: they were circular, raised, light grey in colour and with a smooth surface.

The estimated number of colony-forming units mL^{-1} in the different groups based on BAS agar plate cultures are shown in Table 5. All the agar plates that received diluted water from the 110 hour-plate with colonies were overgrown. The CFU/mL in the *E. crassum* egg incubation wells increased with time. The addition of antibiotics (a mixture of Streptomycin sulphate and Penicillin G sodium salt).

Table 5. CFU/mL in *E. crassum* egg samples with and without antibiotics. The 13h and 36h groups were incubated on agar for four days before examination. The 110h group was incubated on agar for three days before examination. OG: overgrowth. The number of colonies counted were A: 11, B: 215, C: 3, D: 5.

	Dilution			
	No antibiotics		Antibiotics	
Incubation time (h)	1:100	1:1000	1:100	1:1000
13	$1.6*10^{4A}$	0	$4.3*10^{3C}$	0

36	OG	$3.1*10^{6B}$	$7.1*10^{3D}$	0
110	OG	OG	OG	OG

E. crassum eggs treated with chlorine were stained with neutral red in parallel with eggs not treated with chlorine to determine if chlorine was needed to allow for staining. Both eggs with and without chlorine treatment were stained, showing that chlorine treatment was not necessary prior to staining.

3.2a Staining live and dead eggs

Immediately after staining with neutral red, 16 *E. crassum* eggs were observed over 30 minutes. In these eggs, two larvae were observed moving. No control with unstained eggs was examined. When staining 'live' (untreated) and 'dead' (frozen, EtOH or CASy treated) *E. crassum* eggs with neutral red and trypan blue, the results were conflicting. Live eggs were stained (

Table 6) with neutral red one and nine days after collection, while trypan blue was used only one day after collection.

Table 6. Live, mature *E. crassum* eggs stained with neutral red or trypan blue. All eggs observed nine days after collection, regardless of size and maturity, were stained with neutral red. None of the eggs were stained with trypan blue one day after collection.

Days after egg collection	**Neutral red**			**Trypan blue**		
	N eggs	Stained	Stained (%)	N eggs	Stained	Stained (%)
1	236	79	34	226	0	0
9	200	200	100			

E. crassum eggs were killed by either freezing, immersing in ethanol, or treating with CASy blue before staining with neutral red/trypan blue (Table 7).

Table 7. Results from neutral red/trypan blue staining of mature *E. crassum* eggs right after they were killed using ethanol, freezing or CASy blue. All eggs killed by any method and stained with neutral red six and nine days after collection were stained, regardless of size and maturity. None of the eggs were stained with trypan blue. Neutral red was used to stain eggs 1, 6 and 9 days after collecting the eggs, while trypan blue was used to stain eggs 1 and 6 days after collecting the eggs. The numbers in the table are presented as neutral red/trypan blue.

Days after egg collection	**Ethanol immersion**			**Freezing**			**CASy blue**		
	Stained	Total	Stained (%)	Stained	Total	Stained (%)	Stained	Total	Stained (%)
1	104/0	134/41	78/0	93/0	107/75	87/0			

6	112/0	112/151	100/0	137/0	137/103	100/0	68/0	68/51	100/0
9	~ 150	~150	100	~150	~150	100			

The two small-scale tests at staining live eggs with neutral red using an unspecified number of eggs and stain gave conflicting results. In the first small-scale test, embryonic hooks were visible in most of the eggs that were stained (Figure 13). In the second test, most eggs, including small immature eggs and eggs without visible embryonic hooks, were stained. When a similar procedure was done on eggs killed with ethanol, all eggs were stained. Of the eggs killed by freezing, only a few eggs were stained while most were not.

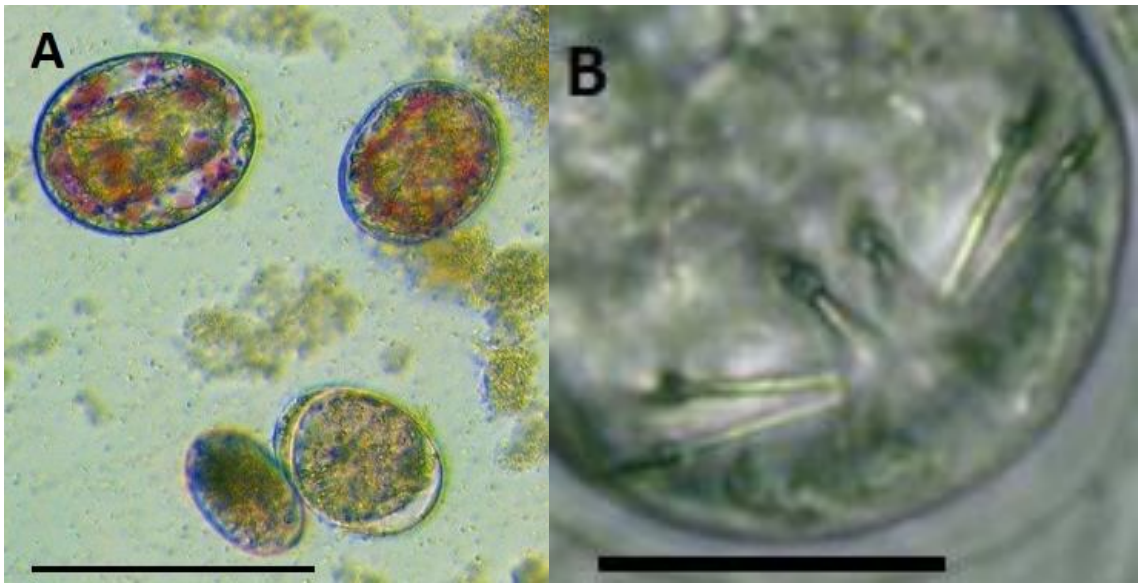

Figure 13. A) *E. crassum* eggs in seawater incubated for 15 minutes with neutral red nine days after collecting the eggs. Embryonic hooks are seen in the two stained eggs. B) Oncosphera larvae with six embryonic hooks (three pairs). Scale bars used: A) 100 µm long, B) 50 µm long.

3.3 Challenge of copepods and Atlantic salmon

3.3.1 Pilot challenge results

Out of 84 copepods observed after challenge with eggs of marine Atlantic salmon *E. crassum* origin that had been kept in freshwater, 5 *E. crassum* larvae were observed in only 3 infected copepods (prevalence = 3.6 %; mean intensity = 1.7). Of the 60 copepods observed after challenge with eggs kept in seawater, 19 *E. crassum* larvae were observed in 13 infected copepods (prevalence = 21.7 %; mean intensity = 1.5). A copepod infected with two *E. crassum* larvae is show in Figure 14.

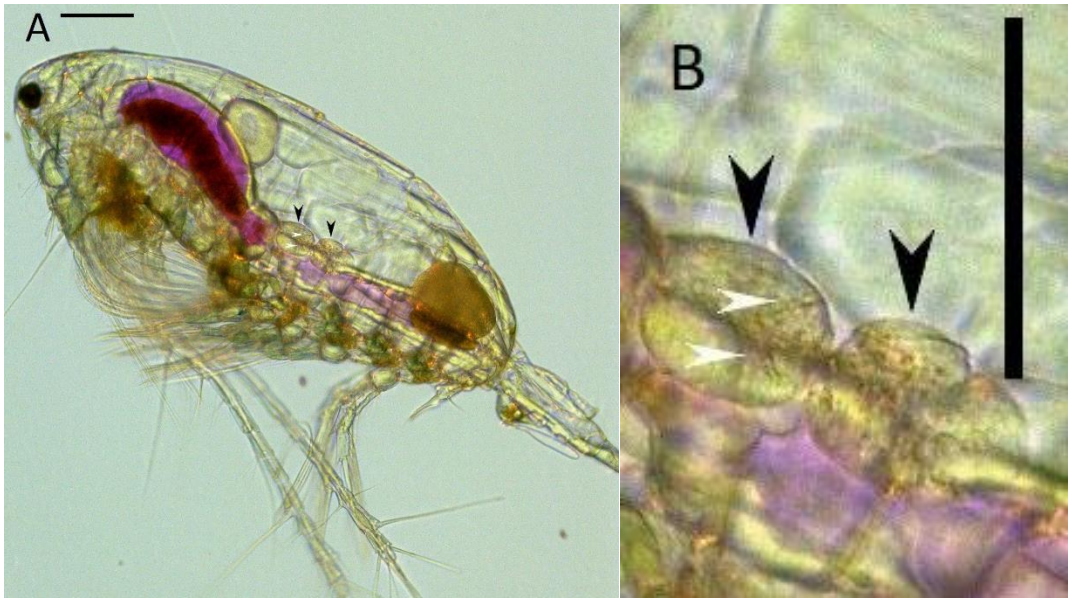

Figure 14. *A. tonsa* infected with two *E. crassum* larvae two days after exposure to eggs. Black arrows point at the two *E. crassum* procercoid larvae. White arrows point at the embryonic hooks in one of the larvae. The gut contents are pink from digesting *R. baltica*. The scale bar is 100 µm long in both A and B

3.3.2 Copepod challenge with *E. crassum* eggs kept in brackish water and seawater

As the prevalence of infected copepods (Table 8) did not change over time (days) ($0.14<G_H<2.9$; $0.23<P<0.94$), total prevalence was used to compare the two groups. No significant difference was registered in prevalence ($\chi^2_{(1df)} = 1.25$; $P = 0.26$).

Table 8. Number of infected copepods in 25 ppt water and full-strength seawater (34 ppt) when observed 1, 2 and 5 days after challenge.

25 ppt water	Days past challenge	Examined copepods #	# Infected copepods	Mean intensity	Prevalence %
	1	75	11	1	14.6
	2	92	13	1	14.1
	5	94	12	1	12.7
	Total	261	36	1	13.8
34 ppt water	1	89	9	1	10.1
	2	87	13	1	14.9
	5	97	7	1	7.2
	Total	273	29	1	10.6

3.3.3 Challenge of Atlantic salmon with *E. crassum*

For the Atlantic salmon challenge, the prevalence of *E. crassum* in the intermediate host *A. tonsa* used was 35 %, mean intensity was 1.14 and range 1-2. The fish were intubated with 7-37 copepods each. No infections were observed in any of the challenged fish examined 2 and 3 months after challenge. Histology samples, PCR samples and blood samples taken from the

fish were not examined due to that no infections were found. Weight, fork-length and haematocrit levels of the fish examined in this study are provided in Appendix table 10.

4. Discussion

Incubation of *E. crassum* eggs with various salinities and treatments.

Washed eggs were incubated in regular seawater or autoclaved water, with or without antibiotics and in different salinities (0, 25, 30 and 34 ppt). The incubation time, use of antibiotics or whether the water was autoclaved or not did not have a significant effect on the absorbance levels. Nor did the saltwater qualities tested (25-34 ppt). However, freshwater incubations clearly decreased the neutral red absorbance. These results indicate that eggs from marine *E. crassum* have a lower survival in freshwater compared to higher salinities. This is further supported by the copepod challenge tests where the copepods challenged with eggs kept in freshwater had a lower prevalence of *E. crassum* (3.6 %) than those challenged with eggs kept in 25 ppt water (12.7 - 14.6 %) and 34 ppt water (7.2 – 35 %). Kennedy reported that eggs from *E. crassum* in resident trout died of osmotic stress within 48 h in salinities greater than 33 % seawater (Kennedy, 1978a). In the present study, the eggs incubated in freshwater were incubated at 10 ℃ for five days before copepod challenge, showing that some eggs from marine *E. crassum* may survive and still be infective for at least five days after release into freshwater. This may suggest that *E. crassum* with a marine life cycle has a higher chance at infecting hosts at lower salinities than *E. crassum* with a freshwater life cycle has at infecting hosts in a higher salinity environment. According to the results in the present study, incubation of *E. crassum* eggs in 25 ppt and 34 ppt water for up to five days at 10 ℃ should not cause a significant increase in egg mortality, allowing studies on live eggs to be performed within this time frame. Additionally, treating the eggs with antibiotics or incubating them in autoclaved water are not necessary precautions against microbial growth for this time frame. However, these treatments were not correlated with any increase in mortality and may thus still be recommended to avoid microbial growth. The absorbance levels from the groups of *E. crassum* eggs that were not washed due were not used because the staining of mucus, slime and other debris together with the eggs influenced absorbance levels. This debris was hypothesized to capture/absorb the neutral red stain, which probably would have led to higher absorbance levels that would not present a correct estimate of the number of live eggs in the samples.

To study the microbial growth on egg samples over time (13h-110h), blood agar plates were used. Solutions with *E. crassum* eggs incubated in either regular water or autoclaved water, with or without added antibiotics was diluted and sown on BAS and colony-forming units mL^{-1} were counted. Surprisingly, only the groups incubated with autoclaved water showed growth.

Since this was observed across different egg groups, it is unlikely that the eggs themselves were the source of the bacteria, suggesting that the autoclaved water used was contaminated. As no controls with only water on BAS were prepared, the water could not be controlled for contamination.

The BAS culturing showed increased growth of bacteria over time, and all plates sown from the plate incubated for 110h was overgrown. The groups that were treated with antibiotics had clearly lower growth of bacteria compared to the groups that were not treated with antibiotics. Of the groups examined, some had very few colonies (3, 5 and 11). Because of the low numbers, the calculated CFU/mL is probably not as reliable. This indicates that more dilutions should have been used. Additionally, the agar plates should have been examined earlier before they were overgrown.

Neutral red as quantitative method

The neutral red assay is a staining method that has been used for light-microscopic demonstration of cell vitality for decades, and the uptake is dependent on pH gradients that live cells maintain through the production of ATP (Antal, Sipka, Surányi, Csipo, Seres, Maródi, and Szegedi, 1995; Repetto, del Peso, and Zurita, 2008). If a cell stained by neutral red dies or is severely damaged, the stain will be lost (Filman, Brawn, and Dandliker, 1975). As dead or damaged cells will not be stained, neutral red staining was chosen for quantifying the number of live eggs in a sample of *E. crassum* eggs. Neutral red staining combined with spectrophotometry could allow quantifying viable eggs in several groups in a short time as well as allowing a more objective assessment of the groups. In the present study, the neutral red staining method was based on two studies for *Drosophila* eggs and amoebae (Lemosy and Hashimoto, 2000; Benedicenti, Secombes, and Collins, 2019). As opposed to neutral red, Trypan Blue is a dye that stains dead cells, and not live cells with intact cell membranes (Strober, 2015). By staining samples of *E. crassum* eggs with either neutral red or trypan blue, the number of stained eggs should complement each other as neutral red should stain live cells and trypan blue dead cells. However, the staining results were conflicting as neutral red stained both live eggs and eggs that should be dead, while trypan blue stained neither. This is in accordance with the results in a study by Forson et al. (2019), who used neutral red to assess viability of the eggs of another platyhelminth parasite, Schistosoma dermatobium. They found that eggs considered dead also stained, albeit weaker (Forson, Tetteh-Quarcoo, Ahenkorah, Aryee, Okine, Afutu, Djameh, Agyapong, Anang, and Ayeh-Kumi, 2019). Both the neutral red and trypan blue staining results on the “dead eggs” in the present study

suggests that they were in fact alive. While this is unlikely, a problem in interpreting the neutral red staining of "dead" eggs result in a lack of a control of the ability of treatments to kill embryos inside. Microscopy for embryo movement, or challenge of copepods with such eggs could have been done instead to substantiate their status. However, some of the egg material used in these tests were suboptimal as some eggs had been kept for up to 9 days before use. Additionally, the staining material used was made approximately two months earlier and may thus have influenced the results. As the staining methods used in the present study were conflicting, the methods should be optimized further as it did not clearly differentiate between the different types of eggs (mature, immature), and tests should be performed with a fresh batch of eggs and freshly prepared stains. The trypan blue staining method did not differentiate between viable and unviable eggs and may be unsuitable for *E. crassum* egg differentiation. Although the neutral red method needs further optimization (shorter incubation times could be tested for neutral red in order to see if this can better separate between mature and immature eggs), the present study shows that chlorine is not needed to stain *E. crassum* eggs with this stain.

Factors impacting copepod survival

In a study by Saksvik and colleagues it was shown that 15 days of development of the marine *E. crassum* procercoids in the copepod *A. tonsa* at 16 °C was enough to become infective for salmon (Saksvik, Nylund, Nilsen, and Hodneland, 2001). One of the goals in the present study was to improve the survival of copepod cultures to allow for high copepod survival rates for < 21 days to allow for procercoid development in addition time for setup of cultures and fish challenge.

In batch 2, the copepods maintained in autoclaved 25 ppt or 35 ppt water decreased in number over time (ANOVA, $P<0.001$), but there was no difference in survival related to salinity ($P=0.1$). Copepods in batch 3 held in the flow-through incubator system and fed *R. baltica* was shown to have highest survival. However, the copepods were kept in the flow-through system for only a few days (five) and the number of copepods was only counted once before the batch was terminated. Hence, the results from this group could be misrepresentative due to few and small sample sizes. Additionally, the survival of the copepods in all groups declined strongly throughout the study, regardless of feed type given, salinity, or incubator system, suggesting that other parameters than feed, salinity and incubator system may play a significant role. At the time of termination, the copepod batches 2 and 3 had concentrations of approximately 500-2000 copepods L^{-1} and 1000-3000 copepods L^{-1}, respectively. These concentrations are

considerably higher than those used in the studies by Saksvik and colleagues (50-150 copepods L^{-1}) (Saksvik, Nylund, Nilsen, and Hodneland, 2001) and Støttrup and colleagues (<100 copepodite stages IV-VI L^{-1}) (Støttrup, Richardson, Kirkegaard, and Pihl, 1986). In the study by Støttrup and colleagues, they observed high mortality at higher concentrations of copepods than 100 adults L^{-1}. This may suggest that to increase the survival of copepods for laboratory use, the copepod density should be lowered. Additionally, the copepod cultures in the present study were kept in considerably smaller containers (1 L or 150 mL) compared to those in the studies by Saksvik and colleagues (75 L) and Støttrup and colleagues (200 L and 450 L). Thus, for future work on the challenge model, larger copepod containers could be used to achieve a lower density while at the same time the total number of copepods is increased. Marchus and Wilcox (2007) suggested that *A. tonsa* does not respond well to rough handling or the crushing effect of gravity (Marchus and Wilcox, 2007). The method for changing water in the copepod incubator flasks in batches 1 and 2 may have caused the copepods to be crushed by either the water flow or gravity itself, and thus this may have been a significant stressor, and perhaps induced mortality. This method was changed to a more gentle handling method later in the study. All copepods kept in the flow-through incubator system was fed the same way; by moving the copepod container out of the incubator water and over into a 150 mL beaker containing water and feed. It is possible that when moving the container out of the water, the effect of gravity may have caused stress, injury and perhaps mortality in the copepod population. In the present study, the copepod batches fed algae paste received up to 1.5×10^8 cells twice daily Mondays-Fridays. However, the algae paste was observed to quickly fall to the bottom of the copepod containers and this may have reduced the availability of the feed. In the present study, the densities of the *R. baltica* cultures maintained throughout the study were not examined. In the study by Saksvik and colleagues (2001) they provided 400 mL feed daily (200 mL *R. baltica* and 200 mL *Isochrysis galbana*) to each copepod tank (mean number of *A. tonsa* per tank estimated to 5600) (Saksvik, Nylund, Nilsen, and Hodneland, 2001). They kept the *I. galbana* cultures at a density of $2\text{-}3 \times 10^6$ cells per mL and *R. baltica* cultures at a density of $1\text{-}2 \times 10^6$ cells per mL. In the study by Støttrup and colleagues (1986), *A. tonsa* cultures were also fed daily with either 8×10^8 *R. baltica* cells or 1.6×10^9 *R. baltica* cells, depending on the size of the copepod container (Støttrup, Richardson, Kirkegaard, and Pihl, 1986). Similarly, the copepod producer that supplied *A. tonsa* to the present study, feed their copepods continuously. As copepod survival was seen to decline throughout the study, it is possible that feed was not supplied in high enough amounts, or that feed was not available for long enough periods. Another factor to consider is that the batches of copepods received from the

commercial provider may have contained copepods of varying ages, which may have led to differences in survival rates between otherwise similar groups.

Challenge of copepods with *E. crassum*

In the present study marine *E. crassum* eggs incubated in freshwater (0 ppt), brackish water (25 ppt) and full-strength seawater (34 ppt) were used to successfully infect *A. tonsa*. *E. crassum* eggs with a length of approximately 80 µm or longer were counted when calculating the concentrations of mature eggs for copepod challenges and for the *in vitro* study of the eggs. Copepods used for the brackish water test were challenged with *E. crassum* eggs incubated for 1, 2 or 5 days in 25 ppt water or 34 ppt water. No significant difference was registered between these groups. *A. tonsa* is often found in coastal water and estuaries with varying salinities (Conover, Riley, Deevey, Conoyer, Wheatland, Harris, and Sanders, 1956; Marchus and Wilcox, 2007; Luigi, Chiara, Elisabetta, Alessandra, and Luigi, 2012) and thus should be able to cope with 25 ppt brackish water. A higher prevalence was observed in the intermediate host copepods challenged with eggs incubated in seawater for six days than those from the brackish water test incubated in either 25 ppt or seawater for 1, 2 or 5 days. A reason for this may be that the copepods used as intermediate hosts in the Atlantic salmon challenge were observed for a longer time after challenge than those in the brackish water test. The copepods in the brackish water test were observed one day after challenge only, while those used as intermediate hosts for the Atlantic salmon were observed both the day after challenge, and two weeks later. The prolonged time before the second observation could have allowed the *E. crassum* procercoid larvae time to develop and thus be easier to spot. The prevalence of the copepods used as intermediate hosts increased from 16 %, observed one day after challenge, to 35 % two weeks after challenge, supporting this explanation. The number of mature eggs per copepods in the copepod challenges could have influenced the difference in prevalence. As the number of copepods challenged in the brackish water test was not counted prior to challenge, no conclusions may be made in this regard. However, 3.2 mature eggs were provided per copepod for the copepods used as intermediate hosts in the Atlantic salmon challenge. This number is considerably lower than the 55 ripe eggs given to each adult in the study by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001). Despite the difference in number of mature eggs per copepod, the prevalence of infected copepods in the present study was higher (35 %) compared to in the study by Saksvik and colleagues (26 %). This shows that it is not necessary to use more than 4 mature eggs per copepod for future studies. In conclusion,

marine *E. crassum* eggs that are to be used for *A. tonsa* challenge may be kept in either 25 ppt water or full-strength seawater for at least five days before challenge and still be infective.

Challenge of Atlantic salmon with *E. crassum*

The present study aimed to collect data to further improve a challenge model in development at The Industrial and Aquatic Laboratory (ILAB) for *Eubothrium crassum* in Atlantic salmon using the copepod *A. tonsa* as intermediate host.

In the present study Atlantic salmon were not successfully infected with *E. crassum*. This is despite that the intermediate host *A. tonsa* was infested with *E. crassum* procercoids of an age that should be infective, and also of various day-degrees of development. The challenge model used in the present study is based on that presented by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001). In that study, the fish challenged was considerably smaller (weight $\bar{x}$: 83g compared to $\bar{x}$: 248g in the present study) and a larger number of copepods per fish was used (~ 60 copepods fish^{-1} compared to 7-37 copepods fish^{-1} in the present study). Saksvik et al. (2001) achieved a 100 % prevalence of infection. Additionally, Hodneland and Solberg (1995) performed a similar study where ten smolts (weight $\bar{x}$: 55.4 g) of Atlantic salmon were orally intubated with 5-10 *A. clausi* (infected with 15-day old marine *E. crassum* procercoids) (Hodneland and Solberg, 1995). In their case infection in one individual only was observed, suggesting that a higher number of copepods per fish is necessary to ascertain an infection. However, it is unlikely that the lower number of copepods used to infect each fish in the present study is the single cause for that an infection was not established. Although each fish was challenged with fewer copepods than by Saksvik and colleagues, the mean prevalence of *E. crassum* in the copepods used in the present study was higher; 35 % in the present study compared to 26 % in the study by Saksvik and colleagues. This suggests that there should be other differences in the two studies that caused the difference in success. As to the difference in size of the fish used, an increase in prevalence of *E. crassum* with fish age has been reported in resident Rainbow trout (Wootten, 1972) and Brown trout (Vik, 1963), but in nature fish transport hosts may also be involved in transmission. In seawater aquaculture that is unlikely to happen. Ruud (2019) observed that juvenile *E. crassum* in farmed salmon mostly occurred in fish less than 35 cm of length (Ruud, 2019). Since some worms may remain as juveniles for months (Saksvik, Nilsen, Nylund, and Berland, 2001), that

indicates that it is at the post smolt stage that most infections occur. Since in both the previous studies where infections were obtained used post smolt salmon, fish size could be a possible explanation for the present negative results. In pen-reared Atlantic salmon a negative correlation is generally seen between *E. crassum* abundance and fish size (Ruud, 2019; Sakariassen, 2019). Ruud and Sakariassen suggested that this negative correlation may be a result of smaller fish being more prone to feeding on small items, such as copepods, and that this behaviour reduces as the fish grows. In addition, they suggested that the gill rakers in larger fish may be too big to filter out copepods, leading to less copepods being ingested. This could have influenced that an infection was not established in any of the bath-challenged fish in the present study. However, these suggestions are unlikely to be relevant for the orally intubated fish in the present study as they had copepods intubated directly into their stomach. The copepod *A. clausi* was challenged with eggs from marine *E. crassum* in the study by Hodneland and Solberg (Hodneland and Solberg, 1995). In their study, developing bothria of the *E. crassum* procercoids were discernible 13-14 days after copepod infection, and they presumed the procercoids to be infective for fish at that stage. The copepods were used to challenge Atlantic salmon 15 days after copepod infection. Similarly, Saksvik and colleagues (2001) found that 15 days of development in the intermediate host *A. tonsa* at 16 °C should be enough for the procercoids to become infective (Saksvik, Nylund, Nilsen, and Hodneland, 2001). In the present study, *E. crassum* procercoids were allowed to develop for 14-16 days in *A. tonsa* before fish challenge to increase the chance of infection. Thus, it should be safe to assume that at least some of the procercoids in the present study were infective, as both Hodneland and Solberg, and Saksvik and colleagues were successful in infecting fish with 15-day old procercoids. The growth, measured as a two-dimensional size, and development of *Bothriocephalus claviceps* procercoids in copepods were studied by Dupont and Gabrion (Dupont and Gabrion, 1987). They observed that the growth of the procercoids was negatively correlated with the density of the parasites in the host, while the development was independent of the density. This is further supported by the findings of Wedekind (1997), who studied the infection of the cestode *Schistocephalus solidus* in the copepod *Macrocyclops albidus* (Wedekind, 1997). Hodneland and Solberg (1995) observed that the survival of infected *A. clausi* had a negative correlation with procercoid load, with the proportion of copepods with multiple infections decreasing drastically over time post infection (Hodneland and Solberg, 1995). However, in their study they did not specify the mean, range, or intensity of procercoid infections. This observations is in contrast to those in a study by Wedekind (1997), where no significant difference in mortality was found between *M. albidus* infected by the cestode

Schistocephalus solidus and uninfected *M. albidus* (Wedekind, 1997). Additionally, Wedekind found that the intensity of infections had no significant difference for the mortality of the copepods. In the study by Wedekind, the copepods were well fed and kept singly. This was assumed to prevent intra-specific food competition and other stress factors that could increase the difference between infected and non-infected copepods. In the study by Saksvik and colleagues the mean intensity of procercoids in the copepods was 1.1 ± 0.24 and intensity range 1-3. A mean intensity of 1.14 and range 1-2 was observed in the copepods used for fish challenge in the present study, with high similarity to the study by Saksvik and colleagues (Saksvik, Nylund, Nilsen, and Hodneland, 2001). As in the study by Saksvik and colleagues, in the present study, the procercoid loads were not observed over time, and the intensity and range were only recorded just before fish challenge. However, the recorded intensity means and ranges in both studies show that most of the copepods were infected by only one procercoid 15 days post infection. This indicates that the procercoid intensity in the present study was not the cause for a lack of established infection in the fish groups.

Animal feeding needle tips were used to reduce the chance of fish injury during oral challenge of the fish. On one occasion the tip of the feeding needle was clogged by copepods, and they had to be pushed back with tweezers before they could be ejected again. Hence, it cannot be ruled out that for a few individuals, the copepods containing larvae could have been crushed during oral injection, reducing the chance of fish infection. Additionally, the feeding needles used are designed to be used for veterinary practices involving small animals like rats or hamsters and may thus not be as suitable for all studies involving fish. Thus, other intubation methods and equipment could be examined in future studies. Prior to the challenge, the fish were starved for 24 hours. Thus, it is possible that the larvae would have been able to establish an infection in the fish if the fish had not been starved as the stomach contents might have given some protection from the gastric juice. Coughing or regurgitation of the copepods in the recovery batch was not observed, but it cannot be ruled out that this occurred for some individuals, and this may have reduced the chance of infection. As there could be several causes for why an infection was not established in the salmon in this study, further research is required for establishment of the challenge model.

Future perspectives

In the present study, infection of Atlantic salmon with *E. crassum* was not successful. Recommendations for future work on the challenge models follows. Other equipment and procedures for oral challenge should examined. Additionally, the influence of fish size on

infection chance and whether the fish were starved before challenge or not, and the impact on infection success, should be studied further.

When *E. crassum* eggs are used in future studies, the present results show that they can be incubated in water with salinity 25 ppt or 34 ppt. Additionally, washing the eggs after they are collected from the tapeworm is recommended as mucus and other organic materials could increase microbial growth. However, further studies are needed to determine if precautions against microbial growth are needed when incubating the eggs for longer than four days. The water the eggs are incubated in should be autoclaved as a precaution, and eggs washed and treated with antibiotics.

To improve the survival of *A. tonsa*, different rearing set-ups should be studied. Specifically, larger copepod containers with lower copepod concentrations should be addressed. Additionally, handling should be kept at a minimum due to the fragility of the copepods, and feed should be supplied in ample amounts, and preferably continuously.

To quantify the proportion of viable *E. crassum* eggs in a sample, the neutral red cell viability staining assay should be optimized to separate mature and immature *E. crassum* eggs, or another method for assessing viability should be used. Copepods could be challenged with a determined number of mature eggs, and the infection prevalence in the copepods used to estimate the portion of infective eggs in the sample. Another suggestion is that the smallest eggs can be filtered out so that only the eggs that are largest and most likely to be mature and infective remains in the sample.

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6. Appendix

Appendix table 1. Water qualities from copepod batch 2.

	Salinity (ppt)					Temperature (°C)			
Date/Flask	**1**	**2**	**3**	**4**	**Date/Flask**	**1**	**2**	**3**	**4**
07.09.21	28.1	27.9	33.8	33.9	07.09.21	14.2	14.2	14.6	14.9
08.09.21	27.9	27.8	33.7	33.7	08.09.21	15.6	15.2	15	15.1
09.09.21	28	27.9	33.7	33.7	09.09.21	14	14.7	14.8	15.1
10.09.21	27	27	34.3	34.4	10.09.21	14.6	14	14.1	13.9
13.09.21	25.6	25.5	35.4	35.2	13.09.21	14.1	14	13.9	14.8
14.09.21	25.2	24.7	35.8	36	14.09.21	14	14.2	14	13.8
15.09.21	24.7	24.5	35.6	35.5	15.09.21	14.7	14.6	14.5	14.7
17.09.21	24.8	24.8	35.6	35.7	17.09.21	14.8	15.2	15.4	16.7
20.09.21	24.7	24.7	35.5	35.7	20.09.21	14.2	14.5	13.8	14.3
24.09.21	24.8	25	35.5	35.8	24.09.21	13.6	13.8	13.9	13.9
27.09.21	24.9	25.1	35.7	35.9	27.09.21	14	13.8	13.7	14
29.09.21	24.5	24.9	34.8	35.5	29.09.21	13.6	13.7	13.4	13.7
	Oxygen (%)					**pH**			
Date/Flask	**1**	**2**	**3**	**4**	**Date/Flask**	**1**	**2**	**3**	**4**
07.09.21	98.3	98.8	99.9	100.6	07.09.21	7.6	7.7	7.7	7.7
08.09.21	76.9	78.7	83.3	77.2	08.09.21	7	7	7.2	7.4
09.09.21	62.4	60.2	51.5	52.2	09.09.21	7.6	7.5	7.7	7.7
10.09.21	26	37.6	35.8	36.4	10.09.21	7.5	7.4	7.6	7.5
13.09.21	55.5	32.5	40.2	30.3	13.09.21	7.2	7.3	7.5	7.4
14.09.21	36.2	26	48.9	41.2	14.09.21	7.6	7.7	7.9	7.8
15.09.21	41.5	29.3	38.5	26.6	15.09.21	7.5	7.7	8	8
17.09.21	35.8	40.6	36.4	22	17.09.21	7.8	7.9	8	7.9
20.09.21	41.2	48.8	44.5	37.6	20.09.21	7.6	7.6	7.9	7.9
24.09.21	43	34.5	44.3	36	24.09.21	7.9	7.9	7.9	8.1
27.09.21	41.8	50.2	37.7	34.3	27.09.21	7.4	7.6	7.8	7.7
29.09.21	41.1	33.4	67.1	39.8	29.09.21	8.1	7.9	7.7	8

Appendix table 2. Copepod counts from copepod batch 2.

Days past copepod culture start	Flask # (1-4)	Salinity (25 ppt/34 ppt)	Sample size (mL)	Copepods counted	Copepods per mL
0	1	25 ppt	1	9	9
0	1	25 ppt	1	3	3
0	1	25 ppt	1	3	3
0	2	25 ppt	1	9	9
0	2	25 ppt	1	3	3
0	2	25 ppt	1	3	3

0	3	34 ppt	1	9	9
0	3	34 ppt	1	3	3
0	3	34 ppt	1	3	3
0	4	34 ppt	1	9	9
0	4	34 ppt	1	3	3
0	4	34 ppt	1	3	3
3	1	25 ppt	1	5	5
3	1	25 ppt	1	3	3
3	1	25 ppt	1	4	4
3	2	25 ppt	1	0	0
3	2	25 ppt	1	6	6
3	2	25 ppt	1	3	3
3	3	34 ppt	1	5	5
3	3	34 ppt	1	2	2
3	3	34 ppt	1	4	4
3	4	34 ppt	1	4	4
3	4	34 ppt	1	1	1
3	4	34 ppt	1	5	5
8	1	25 ppt	1	0	0
8	1	25 ppt	1	5	5
8	1	25 ppt	1	3	3
8	2	25 ppt	1	5	5
8	2	25 ppt	1	3	3
8	2	25 ppt	1	1	1
8	3	34 ppt	1	3	3
8	3	34 ppt	1	3	3
8	3	34 ppt	1	2	2
8	4	34 ppt	1	1	1
8	4	34 ppt	1	3	3
8	4	34 ppt	1	2	2
13	1	25 ppt	1	2	2
13	1	25 ppt	1	1	1
13	1	25 ppt	1	0	0
13	2	25 ppt	1	3	3
13	2	25 ppt	1	4	4
13	2	25 ppt	1	2	2
13	3	34 ppt	1	3	3
13	3	34 ppt	1	1	1
13	3	34 ppt	1	1	1
13	4	34 ppt	1	1	1
13	4	34 ppt	1	1	1
13	4	34 ppt	1	3	3
15	1	25 ppt	1	2	2
15	1	25 ppt	1	1	1

15	1	25 ppt	1	1	1
15	2	25 ppt	1	4	4
15	2	25 ppt	1	1	1
15	2	25 ppt	1	2	2
15	3	34 ppt	1	1	1
15	3	34 ppt	1	1	1
15	3	34 ppt	1	1	1
15	4	34 ppt	1	3	3
15	4	34 ppt	1	1	1
15	4	34 ppt	10	1	0.1
17	1	25 ppt	10	5	0.5
17	1	25 ppt	10	6	0.6
17	1	25 ppt	10	6	0.6
17	2	25 ppt	10	8	0.8
17	2	25 ppt	10	8	0.8
17	2	25 ppt	10	3	0.3
17	3	34 ppt	10	2	0.2
17	3	34 ppt	10	5	0.5
17	3	34 ppt	10	8	0.8
17	4	34 ppt	10	2	0.2
17	4	34 ppt	10	10	1
17	4	34 ppt	10	3	0.3
20	1	25 ppt	10	3	0.3
20	1	25 ppt	10	8	0.8
20	1	25 ppt	10	4	0.4
20	2	25 ppt	10	4	0.4
20	2	25 ppt	10	7	0.7
20	2	25 ppt	10	8	0.8
20	3	34 ppt	10	7	0.7
20	3	34 ppt	10	4	0.4
20	3	34 ppt	10	4	0.4
20	4	34 ppt	10	0	0
20	4	34 ppt	10	6	0.6
20	4	34 ppt	10	9	0.9
22	1	25 ppt	10	8	0.8
22	1	25 ppt	10	9	0.9
22	1	25 ppt	10	8	0.8
22	2	25 ppt	10	4	0.4
22	2	25 ppt	10	5	0.5
22	2	25 ppt	10	5	0.5
22	3	34 ppt	10	2	0.2
22	3	34 ppt	10	5	0.5
22	3	34 ppt	10	7	0.7
22	4	34 ppt	10	4	0.4

22	4	34 ppt	10	1	0.1
22	4	34 ppt	10	5	0.5
27	1	25 ppt	10	5	0.5
27	1	25 ppt	10	5	0.5
27	1	25 ppt	10	5	0.5
27	2	25 ppt	10	4	0.4
27	2	25 ppt	10	6	0.6
27	2	25 ppt	10	0	0
27	3	34 ppt	10	3	0.3
27	3	34 ppt	10	4	0.4
27	3	34 ppt	10	2	0.2
27	4	34 ppt	10	2	0.2
27	4	34 ppt	10	2	0.2
27	4	34 ppt	10	5	0.5
30	1	25 ppt	30	15	0.5
30	1	25 ppt	30	10	0.33333333
30	1	25 ppt	30	6	0.2
30	2	25 ppt	30	9	0.3
30	2	25 ppt	30	16	0.53333333
30	2	25 ppt	30	10	0.33333333
30	3	34 ppt	30	5	0.16666667
30	3	34 ppt	30	13	0.43333333
30	3	34 ppt	30	5	0.16666667
30	4	34 ppt	30	13	0.43333333
30	4	34 ppt	30	6	0.2
30	4	34 ppt	30	4	0.13333333

Appendix table 3. Water qualities from the incubator cultures in copepod batch 3.

	Salinity (ppt)							**Temperature (°C)**					
Date/Flask	**1**	**2**	**3**	**4**	**5**	**6**	**Date/Flask**	**1**	**2**	**3**	**4**	**5**	**6**
14.10.21	29.7	29.7	29.7	29.7	29.7	29.7	14.10.21	11.4	11.4	11.4	11.4	11.4	11.4
15.10.21	29.9	29.9	29.8	29.9	30	30	15.10.21	14.5	13.9	13.9	14.1	14.5	14
18.10.21	29.8	28.6	28.1	28	27.7	28.4	18.10.21	14	13.9	14.5	14.5	15.3	15.4
22.10.21	26.4	25.2	26.1	24.8			22.10.21	14.7	14.1	14.5	15.2		
	Oxygen (%)							**pH**					
Date/Flask	**1**	**2**	**3**	**4**	**5**	**6**	**Date/Flask**	**1**	**2**	**3**	**4**	**5**	**6**
14.10.21	57.5	57.5	57.5	57.5	57.5	57.5	14.10.21	7.4	7.4	7.4	7.4	7.4	7.4
15.10.21	60.5	64	59.1	58.2	62.7	60.9	15.10.21	7.4	7.5	7.5	7.5	7.5	7.5
18.10.21	88	63.6	84.5	92.8	<10	<10	18.10.21	7.8	7.5	7.9	8	7.2	7.3

22.10.21	98	97.8	98.1	94.4			22.10.21	8	8	8	7.8		

Appendix table 4. Water qualities from the flow-through incubator system the copepod cultures from batch 3 was kept in.

	Temperature (℃)	**Salinity (ppt)**
18.10.21	16.5	33.4
19.10.21	16.6	33.4
20.10.21	16.7	33.4
21.10.21	16.6	33.4
22.10.21	16.6	33.4
23.10.21	15.6	33.3
24.10.21	16.1	33.3
25.10.21	17.5	33.3
26.10.21	17.6	33.3

Appendix table 5. Copepod counts from copepod batch 3.

Days after start	Batch #	Autoclaved water (yes/no)	Feed type (R. baltica / T. suecica)	Copepod count	Sample size	Copepods/mL
1	3a	no	R. baltica	109	30	3.6333333
1	3a	no	R. baltica	107	30	3.5666667
1	3a	no	R. baltica	118	30	3.9333333
1	3b	no	T. suecica	125	30	4.1666667
1	3b	no	T. suecica	81	30	2.7
1	3b	no	T. suecica	91	30	3.0333333
1	3c	yes	R. baltica	109	30	3.6333333
1	3c	yes	R. baltica	94	30	3.1333333
1	3c	yes	R. baltica	123	30	4.1
1	3d	yes	T. suecica	99	30	3.3
1	3d	yes	T. suecica	74	30	2.4666667
1	3d	yes	T. suecica	74	30	2.4666667
1	3e	no	R. baltica			
1	3e	no	R. baltica			
1	3e	no	R. baltica			
1	3f	no	T. suecica			
1	3f	no	T. suecica			
1	3f	no	T. suecica			
4	3a	no	R. baltica	110	30	3.6666667
4	3a	no	R. baltica	86	30	2.8666667
4	3a	no	R. baltica	77	30	2.5666667
4	3b	no	T. suecica	60	30	2
4	3b	no	T. suecica	73	30	2.4333333
4	3b	no	T. suecica	77	30	2.5666667
4	3c	yes	R. baltica	92	30	3.0666667

4	3c	yes	R. baltica	91	30	3.0333333
4	3c	yes	R. baltica	85	30	2.8333333
4	3d	yes	T. suecica	85	30	2.8333333
4	3d	yes	T. suecica	95	30	3.1666667
4	3d	yes	T. suecica	78	30	2.6
4	3e	no	R. baltica			
4	3e	no	R. baltica			
4	3e	no	R. baltica			
4	3f	no	T. suecica			
4	3f	no	T. suecica			
4	3f	no	T. suecica			
13	3a	no	R. baltica	10	30	0.3333333
13	3a	no	R. baltica	6	30	0.2
13	3a	no	R. baltica	14	30	0.4666667
13	3b	no	T. suecica	10	30	0.3333333
13	3b	no	T. suecica	21	30	0.7
13	3b	no	T. suecica	16	30	0.5333333
13	3c	yes	R. baltica	23	30	0.7666667
13	3c	yes	R. baltica	16	30	0.5333333
13	3c	yes	R. baltica	14	30	0.4666667
13	3d	yes	T. suecica	19	30	0.6333333
13	3d	yes	T. suecica	19	30	0.6333333
13	3d	yes	T. suecica	19	30	0.6333333
13	3e	no	R. baltica	29	10	2.9
13	3e	no	R. baltica	26	10	2.6
13	3e	no	R. baltica	24	10	2.4
13	3e	no	R. baltica	12	10	1.2
13	3f	no	T. suecica	14	10	1.4
13	3f	no	T. suecica	23	10	2.3
13	3f	no	T. suecica	12	10	1.2
13	3f	no	T. suecica	14	10	1.4

Appendix table 6. Water qualities from the flow-through incubator system cultures in which copepod batch 4 was held

	Temperature (℃)	Salinity (ppt)
4.11.21	12.2	33.4
5.11.21	9	33.2
6.11.21	9.2	33
7.11.21	9.1	33.2
8.11.21	9.2	33.1
9.11.21	12	33.3
10.11.21	12.1	33.2
11.11.21	12.2	33.2
12.11.21	9	33.2

13.11.21	9.1	33.4
14.11.21	9.1	33.5
15.11.21	9.2	33.3
16.11.21	9.1	33.3

Appendix table 7. Water qualities from the flow-through incubator system cultures in which copepod batch 5 was held.

	Temperature (℃)	Salinity (ppt)
16.01.22	9	33.1
17.01.22	9	33.1
18.01.22	9	33.1
19.01.22	9	33.1
20.01.22	8.8	33.1
21.01.22	8.9	33.1
22.01.22	8.9	33.1
23.01.22	8.9	33.3
24.01.22	8.8	33.1
25.01.22	8.9	33.1
26.01.22	8.8	33.1
27.01.22	8.8	33.1
28.01.22	8.7	33.2
29.01.22	8.8	33.1

Appendix table 8. Water qualities from the flow-through incubator system cultures in which copepod batch 6 was held.

	Temperature (	Salinity (ppt)
15.11.21	15.8	33.1
16.11.21	15.8	33.2
17.11.21	15.6	33.1
18.11.21	15.8	33.1
19.11.21	16	33.2
20.11.21	16.4	33.3
21.11.21	16.2	33.2
22.11.21	16	33.1
23.11.21	16.2	33.1
24.11.21	16.1	33.1
25.11.21	16	33.1
26.11.21	15.8	33.1

27.11.21	15.9	33.2
28.11.21	16	33.1
29.11.21	16.1	33.1
30.11.21	16.1	33.1
01.12.21	16.2	33.1
02.12.21	16.2	33.1

Appendix table 9. Absorbance readings used to determine the significance of the different *E. crassum* egg incubation parameters and treatments. The absorbance peaks of the three 96-well plates examined were 514, 524 and 530 and the mean absorbance between the highest and lowest of the three were used. Background absorbance (the levels read from negative controls) was removed from the mean absorbance levels.

Incubation time	+/- Antibiotics	Washed/unwashed and autoclaved water/normal water	Salinity	Well #	Mean absorbance (between 514-530 nm)	Mean absorbance negative control	Mean absorbance minus background absorbance (negative control)
16h	No antib.	Unwashed	0	A1	0.186211111	0.062166667	0.124044444
16h	No antib.	Unwashed	0	B1	0.193666667	0.062166667	0.1315
16h	No antib.	Unwashed	0	C1	0.241777778	0.062166667	0.179611111
16h	No antib.	Unwashed	25	A2			
16h	No antib.	Unwashed	25	B2			
16h	No antib.	Unwashed	25	C2			
16h	No antib.	Unwashed	30	A3	0.081788889	0.062166667	0.019622222
16h	No antib.	Unwashed	30	B3	0.074366667	0.062166667	0.0122
16h	No antib.	Unwashed	30	C3	0.080055556	0.062166667	0.017888889
16h	No antib.	Unwashed	34	A4	0.0704	0.062166667	0.008233333
16h	No antib.	Unwashed	34	B4	0.060844444	0.062166667	-0.001322222
16h	No antib.	Unwashed	34	C4	0.062011111	0.062166667	-0.000155556
16h	No antib.	Washed + norm.	0	A5	0.0639	0.062166667	0.001733333
16h	No antib.	Washed + norm.	0	B5	0.062477778	0.062166667	0.000311111
16h	No antib.	Washed + norm.	0	C5	0.060733333	0.062166667	-0.001433333
16h	No antib.	Washed + norm.	25	A6	0.112177778	0.062166667	0.050011111

16h	No antib.	Washed + norm.	25	B6	0.1208	0.062166667	0.058633333
16h	No antib.	Washed + norm.	25	C6	0.156877778	0.062166667	0.094711111
16h	No antib.	Washed + norm.	30	A7	0.138711111	0.062166667	0.076544444
16h	No antib.	Washed + norm.	30	B7	0.161222222	0.062166667	0.099055556
16h	No antib.	Washed + norm.	30	C7	0.137222222	0.062166667	0.075055556
16h	No antib.	Washed + norm.	34	A8	0.136488889	0.062166667	0.074322222
16h	No antib.	Washed + norm.	34	B8	0.143133333	0.062166667	0.080966667
16h	No antib.	Washed + norm.	34	C8	0.198944444	0.062166667	0.136777778
16h	No antib.	Washed + auto	0	A9	0.092488889	0.062166667	0.030322222
16h	No antib.	Washed + auto	0	B9	0.1081	0.062166667	0.045933333
16h	No antib.	Washed + auto	0	C9	0.0858	0.062166667	0.023633333
16h	No antib.	Washed + auto	25	A10	0.127033333	0.062166667	0.064866667
16h	No antib.	Washed + auto	25	B10	0.134288889	0.062166667	0.072122222
16h	No antib.	Washed + auto	25	C10	0.121888889	0.062166667	0.059722222
16h	No antib.	Washed + auto	30	A11	0.119777778	0.062166667	0.057611111
16h	No antib.	Washed + auto	30	B11	0.095144444	0.062166667	0.032977778
16h	No antib.	Washed + auto	30	C11	0.079144444	0.062166667	0.016977778
16h	No antib.	Washed + auto	34	A12	0.100366667	0.062166667	0.0382
16h	No antib.	Washed + auto	34	B12	0.105033333	0.062166667	0.042866667
16h	No antib.	Washed + auto	34	C12	0.116677778	0.062166667	0.054511111
16h	Antib.	Unwashed	0	E1	0.245822222	0.055590741	0.190231481
16h	Antib.	Unwashed	0	F1	0.155766667	0.055590741	0.100175926
16h	Antib.	Unwashed	0	G1	0.195922222	0.055590741	0.140331481
16h	Antib.	Unwashed	25	E2			
16h	Antib.	Unwashed	25	F2			
16h	Antib.	Unwashed	25	G2			
16h	Antib.	Unwashed	30	E3	0.083055556	0.055590741	0.027464815
16h	Antib.	Unwashed	30	F3	0.067411111	0.055590741	0.01182037
16h	Antib.	Unwashed	30	G3	0.094833333	0.055590741	0.039242593

16h	Antib.	Unwashed	34	E4	0.076088889	0.055590741	0.020498148
16h	Antib.	Unwashed	34	F4	0.067922222	0.055590741	0.012331481
16h	Antib.	Unwashed	34	G4	0.075366667	0.055590741	0.019775926
16h	Antib.	Washed + norm.	0	E5	0.082188889	0.055590741	0.026598148
16h	Antib.	Washed + norm.	0	F5	0.075155556	0.055590741	0.019564815
16h	Antib.	Washed + norm.	0	G5	0.0598	0.055590741	0.004209259
16h	Antib.	Washed + norm.	25	E6	0.099944444	0.055590741	0.044353704
16h	Antib.	Washed + norm.	25	F6	0.102266667	0.055590741	0.046675926
16h	Antib.	Washed + norm.	25	G6	0.081966667	0.055590741	0.026375926
16h	Antib.	Washed + norm.	30	E7	0.130466667	0.055590741	0.074875926
16h	Antib.	Washed + norm.	30	F7	0.106444444	0.055590741	0.050853704
16h	Antib.	Washed + norm.	30	G7	0.109433333	0.055590741	0.053842593
16h	Antib.	Washed + norm.	34	E8	0.120988889	0.055590741	0.065398148
16h	Antib.	Washed + norm.	34	F8	0.134833333	0.055590741	0.079242593
16h	Antib.	Washed + norm.	34	G8	0.118222222	0.055590741	0.062631481
16h	Antib.	Washed + auto	0	E9	0.081022222	0.055590741	0.025431481
16h	Antib.	Washed + auto	0	F9	0.092655556	0.055590741	0.037064815
16h	Antib.	Washed + auto	0	G9	0.089644444	0.055590741	0.034053704
16h	Antib.	Washed + auto	25	E10	0.1124	0.055590741	0.056809259
16h	Antib.	Washed + auto	25	F10	0.099644444	0.055590741	0.044053704
16h	Antib.	Washed + auto	25	G10	0.113933333	0.055590741	0.058342593
16h	Antib.	Washed + auto	30	E11	0.098111111	0.055590741	0.04252037
16h	Antib.	Washed + auto	30	F11	0.1001	0.055590741	0.044509259
16h	Antib.	Washed + auto	30	G11	0.099655556	0.055590741	0.044064815
16h	Antib.	Washed + auto	34	E12	0.171122222	0.055590741	0.115531481
16h	Antib.	Washed + auto	34	F12	0.099333333	0.055590741	0.043742593
16h	Antib.	Washed + auto	34	G12	0.095888889	0.055590741	0.040298148

48h	No antib.	Unwashed	0	A1	0.208255556	0.073124074	0.135131481
48h	No antib.	Unwashed	0	B1	0.174866667	0.073124074	0.101742593
48h	No antib.	Unwashed	0	C1	0.210666667	0.073124074	0.137542593
48h	No antib.	Unwashed	25	A2			
48h	No antib.	Unwashed	25	B2			
48h	No antib.	Unwashed	25	C2			
48h	No antib.	Unwashed	30	A3	0.0877	0.073124074	0.014575926
48h	No antib.	Unwashed	30	B3	0.079988889	0.073124074	0.006864815
48h	No antib.	Unwashed	30	C3	0.085788889	0.073124074	0.012664815
48h	No antib.	Unwashed	34	A4	0.056066667	0.073124074	-0.017057407
48h	No antib.	Unwashed	34	B4	0.061544444	0.073124074	-0.01157963
48h	No antib.	Unwashed	34	C4	0.0696	0.073124074	-0.003524074
48h	No antib.	Washed + norm.	0	A5	0.077722222	0.073124074	0.004598148
48h	No antib.	Washed + norm.	0	B5	0.081166667	0.073124074	0.008042593
48h	No antib.	Washed + norm.	0	C5	0.080122222	0.073124074	0.006998148
48h	No antib.	Washed + norm.	25	A6	0.123966667	0.073124074	0.050842593
48h	No antib.	Washed + norm.	25	B6	0.114955556	0.073124074	0.041831481
48h	No antib.	Washed + norm.	25	C6	0.130011111	0.073124074	0.056887037
48h	No antib.	Washed + norm.	30	A7	0.140422222	0.073124074	0.067298148
48h	No antib.	Washed + norm.	30	B7	0.131477778	0.073124074	0.058353704
48h	No antib.	Washed + norm.	30	C7	0.156655556	0.073124074	0.083531481
48h	No antib.	Washed + norm.	34	A8	0.150477778	0.073124074	0.077353704
48h	No antib.	Washed + norm.	34	B8	0.139311111	0.073124074	0.066187037
48h	No antib.	Washed + norm.	34	C8	0.126333333	0.073124074	0.053209259
48h	No antib.	Washed + auto	0	A9	0.094388889	0.073124074	0.021264815
48h	No antib.	Washed + auto	0	B9	0.089255556	0.073124074	0.016131481
48h	No antib.	Washed + auto	0	C9	0.086355556	0.073124074	0.013231481
48h	No antib.	Washed + auto	25	A10	0.1374	0.073124074	0.064275926

48h	No antib.	Washed + auto	25	B10	0.084188889	0.073124074	0.011064815
48h	No antib.	Washed + auto	25	C10	0.117755556	0.073124074	0.044631481
48h	No antib.	Washed + auto	30	A11	0.116933333	0.073124074	0.043809259
48h	No antib.	Washed + auto	30	B11	0.111233333	0.073124074	0.038109259
48h	No antib.	Washed + auto	30	C11	0.1111	0.073124074	0.037975926
48h	No antib.	Washed + auto	34	A12	0.114877778	0.073124074	0.041753704
48h	No antib.	Washed + auto	34	B12	0.140888889	0.073124074	0.067764815
48h	No antib.	Washed + auto	34	C12	0.152222222	0.073124074	0.079098148
48h	Antib.	Unwashed	0	E1	0.217066667	0.071487963	0.145578704
48h	Antib.	Unwashed	0	F1	0.212133333	0.071487963	0.14064537
48h	Antib.	Unwashed	0	G1	0.2149	0.071487963	0.143412037
48h	Antib.	Unwashed	25	E2			
48h	Antib.	Unwashed	25	F2			
48h	Antib.	Unwashed	25	G2			
48h	Antib.	Unwashed	30	E3	0.092566667	0.071487963	0.021078704
48h	Antib.	Unwashed	30	F3	0.080555556	0.071487963	0.009067593
48h	Antib.	Unwashed	30	G3	0.080422222	0.071487963	0.008934259
48h	Antib.	Unwashed	34	E4	0.067922222	0.071487963	-0.003565741
48h	Antib.	Unwashed	34	F4	0.091822222	0.071487963	0.020334259
48h	Antib.	Unwashed	34	G4	0.062833333	0.071487963	-0.008654630
48h	Antib.	Washed + norm.	0	E5	0.069377778	0.071487963	-0.002110185
48h	Antib.	Washed + norm.	0	F5	0.068977778	0.071487963	-0.002510185
48h	Antib.	Washed + norm.	0	G5	0.073788889	0.071487963	0.002300926
48h	Antib.	Washed + norm.	25	E6	0.116377778	0.071487963	0.044889815
48h	Antib.	Washed + norm.	25	F6	0.110255556	0.071487963	0.038767593
48h	Antib.	Washed + norm.	25	G6	0.093811111	0.071487963	0.022323148
48h	Antib.	Washed + norm.	30	E7	0.139355556	0.071487963	0.067867593

48h	Antib.	Washed + norm.	30	F7	0.131666667	0.071487963	0.060178704
48h	Antib.	Washed + norm.	30	G7	0.131766667	0.071487963	0.060278704
48h	Antib.	Washed + norm.	34	E8	0.143111111	0.071487963	0.071623148
48h	Antib.	Washed + norm.	34	F8	0.127577778	0.071487963	0.056089815
48h	Antib.	Washed + norm.	34	G8	0.093811111	0.071487963	0.022323148
48h	Antib.	Washed + auto	0	E9	0.0958	0.071487963	0.024312037
48h	Antib.	Washed + auto	0	F9	0.091877778	0.071487963	0.020389815
48h	Antib.	Washed + auto	0	G9	0.088122222	0.071487963	0.016634259
48h	Antib.	Washed + auto	25	E10	0.109422222	0.071487963	0.037934259
48h	Antib.	Washed + auto	25	F10	0.1091	0.071487963	0.037612037
48h	Antib.	Washed + auto	25	G10	0.121611111	0.071487963	0.050123148
48h	Antib.	Washed + auto	30	E11	0.119277778	0.071487963	0.047789815
48h	Antib.	Washed + auto	30	F11	0.117177778	0.071487963	0.045689815
48h	Antib.	Washed + auto	30	G11	0.112222222	0.071487963	0.040734259
48h	Antib.	Washed + auto	34	E12	0.158755556	0.071487963	0.087267593
48h	Antib.	Washed + auto	34	F12	0.146533333	0.071487963	0.07504537
48h	Antib.	Washed + auto	34	G12	0.116611111	0.071487963	0.045123148
110h	No antib.	Unwashed	0	D1	0.180866667	0.06764537	0.113221296
110h	No antib.	Unwashed	25	D2	0.054211111	0.06764537	-0.013434259
110h	No antib.	Unwashed	30	D3	0.103533333	0.06764537	0.035887963
110h	No antib.	Unwashed	34	D4	0.128788889	0.06764537	0.061143519
110h	No antib.	Washed + norm.	0	D5	0.081211111	0.06764537	0.013565741
110h	No antib.	Washed + norm.	25	D6	0.118188889	0.06764537	0.050543519
110h	No antib.	Washed + norm.	30	D7	0.152811111	0.06764537	0.085165741
110h	No antib.	Washed + norm.	34	D8	0.147344444	0.06764537	0.079699074
110h	No antib.	Washed + auto	0	D9	0.095277778	0.06764537	0.027632407
110h	No antib.	Washed + auto	25	D10	0.115777778	0.06764537	0.048132407

110h	No antib.	Washed + auto	30	D11	0.103533333	0.06764537	0.035887963
110h	No antib.	Washed + auto	34	D12	0.111388889	0.06764537	0.043743519
110h	Antib.	Unwashed	0	E1	0.165011111	0.063539352	0.101471759
110h	Antib.	Unwashed	25	E2	0.054511111	0.063539352	-0.009028241
110h	Antib.	Unwashed	30	E3	0.098688889	0.063539352	0.035149537
110h	Antib.	Unwashed	34	E4	0.0869	0.063539352	0.023360648
110h	Antib.	Washed + norm.	0	E5	0.0791	0.063539352	0.015560648
110h	Antib.	Washed + norm.	25	E6	0.215911111	0.063539352	0.152371759
110h	Antib.	Washed + norm.	30	E7	0.133888889	0.063539352	0.070349537
110h	Antib.	Washed + norm.	34	E8	0.135966667	0.063539352	0.072427315
110h	Antib.	Washed + auto	0	E9	0.080144444	0.063539352	0.016605093
110h	Antib.	Washed + auto	25	E10	0.128311111	0.063539352	0.064771759
110h	Antib.	Washed + auto	30	E11	0.147577778	0.063539352	0.084038426
110h	Antib.	Washed + auto	34	E12	0.134766667	0.063539352	0.071227315

Appendix table 10. Weight, fork-length, and haematocrit-levels of the fish used in the challenge.

Fish group/ day-degrees past copepod infection	Fish nr.	Date	Weight (g)	Fork-length (cm)	Haematocrit (%)
Base	1	02.12.21	345	29.5	42
Base	2	02.12.21	300	28	53
Base	3	02.12.21	195	24.5	48
Base	4	02.12.21	174	25	45
Base	5	02.12.21	227.6	26.5	39
Control	1	02.12.21	191.2	25	N/A
Control	2	02.12.21	259.4	27.5	N/A
Control	3	02.12.21	221.8	25.5	N/A
Control	4	02.12.21	210	25	N/A
Control	5	02.12.21	225.2	26	N/A
Control	6	02.12.21	157.6	22.5	N/A
Control	7	02.12.21	193.4	25	N/A
Control	8	02.12.21	227.6	28	N/A
Control	9	02.12.21	254	27	N/A
Control	10	02.12.21	234.4	27	N/A
Control	1	26.01.22	400	31	42

Control	2	26.01.22	520	33.5	41
Control	3	26.01.22	460	32	32
Control	4	26.01.22	640	35	35
Control	5	26.01.22	490	32	32
Control	6	23.02.22	590	35.5	49
Control	7	23.02.22	690	37.5	43
Control	8	23.02.22	690	37	53
Control	9	23.02.22	760	38	43
Control	10	23.02.22	305	29.5	45
Bath (256 day-degrees)	1	02.12.21	243.6	26	N/A
Bath (256 day-degrees)	2	02.12.21	252	27	N/A
Bath (256 day-degrees)	3	02.12.21	300	28.5	N/A
Bath (256 day-degrees)	4	02.12.21	182.8	24.5	N/A
Bath (256 day-degrees)	5	02.12.21	251.6	26	N/A
Bath (256 day-degrees)	6	02.12.21	221.2	26	N/A
Bath (256 day-degrees)	7	02.12.21	231.8	27.5	N/A
Bath (256 day-degrees)	8	02.12.21	301.6	27.5	N/A
Bath (256 day-degrees)	9	02.12.21	266.4	27.5	N/A
Bath (256 day-degrees)	10	02.12.21	199.8	24.5	N/A
Bath (256 day-degrees)	1	26.01.22	635	34.5	44
Bath (256 day-degrees)	2	26.01.22	535	35	40
Bath (256 day-degrees)	3	26.01.22	475	32	42
Bath (256 day-degrees)	4	26.01.22	410	31.5	33
Bath (256 day-degrees)	5	26.01.22	605	34.5	47
Bath (256 day-degrees)	6	23.02.22	800	38.5	48
Bath (256 day-degrees)	7	23.02.22	745	38.5	45
Bath (256 day-degrees)	8	23.02.22	825	39	50
Bath (256 day-degrees)	9	23.02.22	795	37.5	50
Bath (256 day-degrees)	10	23.02.22	745	37.5	43
224 day-degrees	1	30.11.21	328	29	N/A
224 day-degrees	2	30.11.21	268	27	N/A
224 day-degrees	3	30.11.21	259.2	26.5	N/A
224 day-degrees	4	30.11.21	251.4	26.5	N/A
224 day-degrees	5	30.11.21	297.4	27.5	N/A

224 day-degrees	1	23.02.22	820	37.5	48
224 day-degrees	2	23.02.22	930	39	50
224 day-degrees	3	23.02.22	620	35.5	47
224 day-degrees	4	23.02.22	645	37	46
224 day-degrees	5	23.02.22	615	35.5	45
240 day-degrees	1	01.12.21	269.8	27.5	N/A
240 day-degrees	2	01.12.21	276.2	28	N/A
240 day-degrees	3	01.12.21	269.2	27	N/A
240 day-degrees	4	01.12.21	269.8	27	N/A
240 day-degrees	5	01.12.21	240.2	26	N/A
240 day-degrees	1	23.02.22	780	38	47
240 day-degrees	2	23.02.22	615	36	46
240 day-degrees	3	23.02.22	765	37.5	50
240 day-degrees	4	23.02.22	705	37.5	46
240 day-degrees	5	23.02.22	735	37.5	45
256 day-degrees	1	02.12.21	181.4	25	N/A
256 day-degrees	2	02.12.21	248.6	26.5	N/A
256 day-degrees	3	02.12.21	244.6	26	N/A
256 day-degrees	4	02.12.21	238	25.5	N/A
256 day-degrees	5	02.12.21	240.4	26.5	N/A
256 day-degrees	1	23.02.22	617	35.5	46
256 day-degrees	2	23.02.22	670	37	46
256 day-degrees	3	23.02.22	430	32.5	52
256 day-degrees	4	23.02.22	740	36.5	44
256 day-degrees	5	23.02.22	755	37.5	46