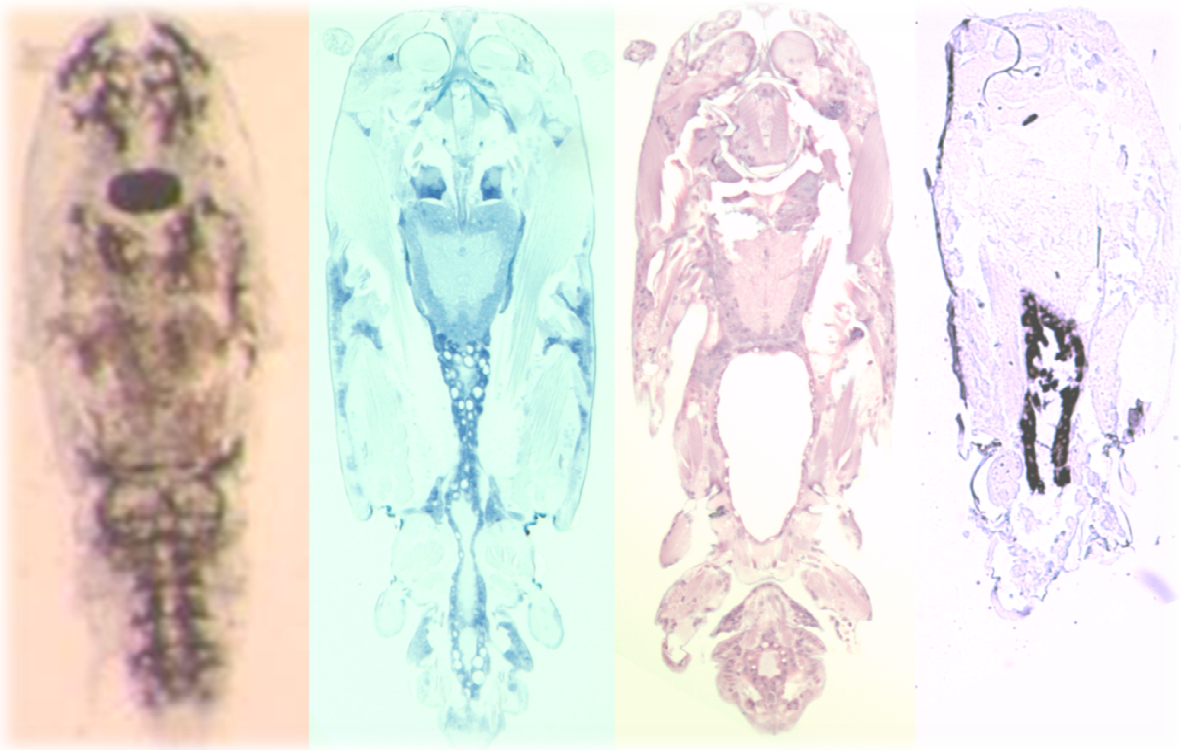
Instar Growth and Development of *Lepeophtheirus salmonis* Copepodids.



Thesis for the degree of Master of Science in Aqua medicine

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Forord

Denne hovedfagsoppgåva er ein del av mitt studie til M.S.c grada i Fiskehelse ved Institutt for Biologi, Universitetet i Bergen. Forsøk og laboratoriearbeid er utført ved Havforskningsinstituttet si avdeling i Bergen. Frank Nilsen har vore vegleiiar og eg vil rette ei hjarteleg takk for gode innspel og støtte undervegs. Eg har hatt stort utbytte av hans faglige engasjement og kommunikasjonsevner. Eg vil og takka Sussie Dalvin og Rasmus Skern for uunværleg hjelp til dei molekylærbiologiske utfordringane eg har hatt, samt til Ingrid Ulgenes Fiksdal og Anne Torsvik for innføring i histologiske arbeidsmetodar og analyse. Vidare vil eg takka instituttet, vener og medstudentar for ei flott studietid her ved Universitetet, og berre gode ønsker for flyttinga til nye lokaler og vidare utvikling av fiskehelsestudiet.
Eg er for evig takknemmeleg for at kona mi Marianne har støtta meg gjennom studietida, sjølv under svangerskap og fødselspermisjon. Du er ei utømmeleg kjelde til motivasjon og glede!

Preface

This thesis is written as a part of Integrated Master Programme in Aquamedicine at the faculty of mathematics and natural science, Department of Biology at the University of Bergen. Experiments and analyses were carried out at the Institute of Marine Research in Bergen. I would like to thank my supervisor Frank Nilsen for excellent guidance and his remarkable ability to communicate. I would also like to thank Sussie Dalvin and Rasmus Skern at IMR for helping me with the challenges in molecular biology and Ingrid Ulgenes Fiksdal and Anne Torsvik for guiding me through the histological examinations. I wish my fellow students and the Institute of Biology all best and good luck in new research facilities. This thesis could not been written without the support from my beloved wife and our beautiful newborn daughter.

1 Contents

2 Abstract

Salmon louse *Lepeophtheirus salmonis*, (Krøyer, 1837) is a caligid parasite, responsible for major economical loss and welfare issues in Norwegian marine culture of salmonid species. Infections are mainly controlled with chemical measures, but there is emerging evidence that the monotonous use of chemicals has resulted in reduced sensitivity towards the pesticides used by the industry. Although *L. salmonis* has been an area of research for several decades there is still a lack of knowledge concerning basic biological aspects of the early developmental stages. Copepodids are the infective stage and understanding the biology of this phase may allow the design of a more effective next generation combat strategy. In the present study, copepodid biology has been studied by infection trials, observation of body size, histological examinations and *in situ* hybridization. The results demonstrate that infective copepodids undergo instar growth only if they are allowed to infect a salmonid host, as no such growth has been observed in the absence of host tissue. There was no difference in growth or developmental parameters such as cuticle production or timing of molting for copepodids when they infected the host at a young (2DPM) or old (8DPM) age. This strongly indicates that host tissue and fluids constitute an essential source of nutrition for copepodids, and that the role of yolk is minimal or obsolete for the purpose of further development. Furthermore, the present study indicate that transcription rate of the salmon lice digestive enzyme *LsTryp1* increased after infection, while the opposite appears to occur in the absence of a host. No alterations wereobserved in the transcription of *LsTryp2*.

3 Introduction

Copepods (Crustacea) are common parasites on teleost fish. They occur on a wide variety of fish species but the severity of their impact depends greatly on the host-parasite relationships. Ectoparasites on teleosts are generally small and the effects are considered low unless the infection is intensive and concentrated on the gills (Kabata 1981). Intensive infection may arise in areas with a high host density (Brandal et al. 1976). High density of fish is a typical aspect of profitable production of most aquaculture species and sea farms in the northern hemisphere have especially experienced the detrimental effects of caligid Copepods (Kabata 1973; Bron et al. 1993). The genera *Caligus* and *Lepeophteirus* in the Caligidae family, jointly number more than 300 parasitic species (Kabata 1988).

Caligus elongatus is probably the most common parasitic copepod in the North Atlantic ocean and parasitizes a large number of hosts species including salmonids (Heuch 2007). On the other hand, *Lepeophtheirus salmonis* has a host range restricted only to salmonid species of the genera *Oncorhynchus*, *Salmo* and *Salvelinus* (Kabata 1973; Johnson and Albright 1991a). However *L.salmonis* has a higher abundance than *C.elongatus* on *Salmo salar* from the coastal waters of Norway and the Norwegian Sea (Holst et al. 1993; Heuch et al. 2005; Heuch 2007).

L.salmonis is specially adapted to reproduce when the population density is low, hence up to 11 broods can be successfully hatched following one single mating (Heuch et al. 2000). The lifecycle starts approximately 10 days after fertilization (10°C) when the eggs hatch into the nauplius I stage. The young naupliie larvae carries yolk that feeds the organism through a second nauplius stage and into the copepodid phase (Johannessen 1978; Johnson and Albright 1991a; Bron et al. 1993). The copepodid is the infective stage and must infect and settle on a suitable host before the yolk reserves are used (Johnson and Albright 1991a; Bron et al. 1993). It positions itself in the water column by swimming in the direction of light and water disturbances, thus increasing the chance of getting in close contact with a suitable host (Kabata 1981). Initial contact with a host is followed by searching phase, in which the copepodid uses its maxillipeds (fig. 1, C) to grip to the host surface (Bron et al. 1991). When a suitable location is found, the second antennae (fig. 1, C) are driven into the epidermis to create a primary attachment (Bron et al. 1991). Feeding at this stage is restricted to the small

area underneath the oral cone (fig. 1, C) and the diet involves mainly surface-epithelium and mucus (Jones et al. 1990; Bron et al. 1991; Bron et al. 1993). A preformed filament appears inside the cephalothorax of mature copepodids, but no external filament has been observed at this stage (Gonzalez-Alanis et al. 2001).

Following copepodid infection and settlement, the louse develops through four parasitic chalimi stages by feeding on mucus and skin cells from the host (Dawson et al. 1999). All chalimi stages are firmly anchored to the fish skin by a strong fibrous protein filament (Bron et al. 1991). Further development involves two free-moving preadult stages before molting into adult males and females (Johnson and Albright 1991b). These free moving stages are more virulent and feed on mucus, epidermis and blood (Brandal et al. 1976; Grimnes and Jakobsen 1996; Wagner et al. 2008). The generation time for *L.salmonis* at 10°C is 7.5-8 weeks according to Johnson et al. (1991b).

Lepeophtheirus salmonis is among the largest known parasitic copepods recorded in scientific literature (Kabata 1973), it has a dorso-ventral compressed body, well adapted to parasitize an active host such as salmon (Parker et al. 1968). The low profile allows water currents to pass the parasite without creating a drag which could cause the lice to fall off the host , a feature shared by all Caligidae (Kabata 1979). The body of mature Caligidae is composed of four tagmata (Fig. I, A) , the cephalothorax, a fourth leg-bearing segment, genital segment and the abdomen (Kabata 1979). The tagmata of early developmental stages can be hard to differentiate. *L.salmonis* copepodids have a morphology quite different from adults (Fig. I, B), with a cephalothorax and a posterior region with four segments (Johnson and Albright 1991b)

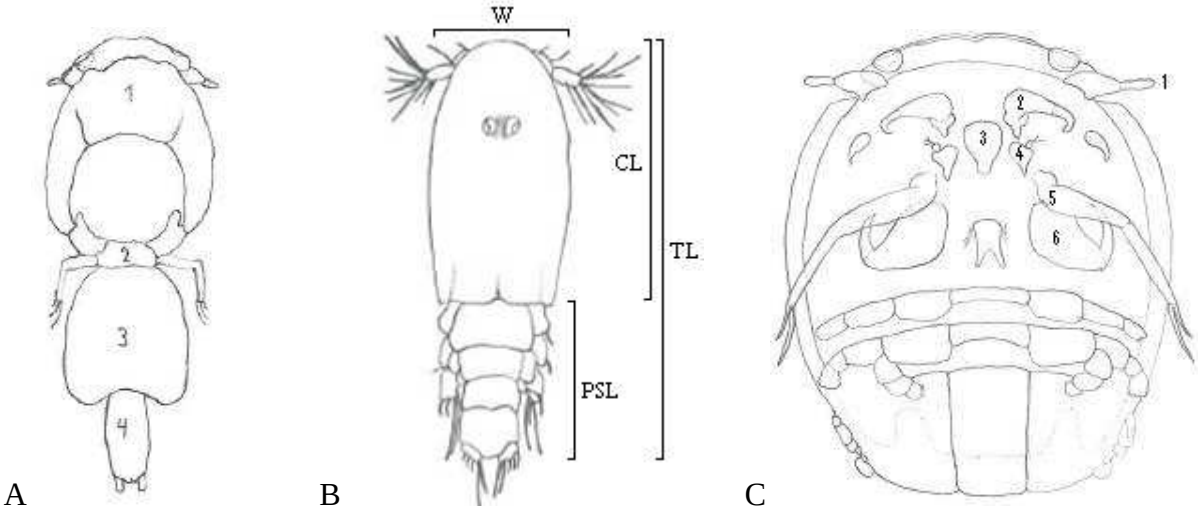

Figure I. Morphology of Caligidae. **A.** Adult Caligus (Kabata 1979).[1]Cephalotorax, [2]Fourth leg-bearing segment, [3]Genital segment, [4]Abdomen. **B.** *L.salmonis* copepodid (Johnson and Albright 1991b) Measured parameters in this study: width (W), total length (TL), cephalothorax length (CL) and posterior segment length (PSL). **C.** Ventral surface of Caligid cephalothorax (Kabata 1979). [1]First antenna, [2]Second antenna, [3]Oral cone, [4]First maxilla, [5]Second maxilla, [6]Maxilliped.

The severity of lice infections depends on infection density, developmental stage of the lice (Grimnes and Jakobsen 1996), fish species and size (Heuch et al. 2005; Wagner et al. 2008). The effects on the host can be divided into two categories: local and systemic (Kabata 1970; Skugor et al. 2008). Local effects are lesions, mechanical damage and inflammatory response caused by attachment and feeding in the vicinity of the attachment site (Kabata 1981). Such effects vary from negligible to fatal and might lead to blood loss and secondary infections which could weaken the host (Grimnes and Jakobsen 1996; Bjørn and Finstad 1997; Wagner et al. 2008). Severe local effects are mainly caused by preadult and adult lice stages, which are traditionally considered to be more virulent than the younger developmental stages (Bjørn and Finstad 1997; Skugor et al. 2008).

The systemic effects can be both lethal and sublethal and involves the introduction of host stress response and modulation of the immune system and physiology (Skugor et al. 2008). All infective stages are able to induce a systemic response in the host, even at low numbers (Tully and Nolan 2002; Skugor et al. 2008). A density of 11 adult salmon lice per fish or 0.75 mobile lice per gram fish weight is regarded as lethal to wild salmon smolts (Finstad et al. 2000; Wagner et al. 2008). These estimates are supported by the finding of no fish carrying more than 10 adult lice in the Norwegian sea (Holst et al. 2003). Lice infections cause cortisol release and a reduced lymphocyte-leukocyte ratio in infected sea trout post smolts (Bjørn and

Finstad 1997; Wagner et al. 2008). Elevated cortisol levels are generally found in teleost fishes in response to stressful stimuli and are therefore often used as a fish welfare indicator (Bonga 1997). The fact that salmonids experience such an increase of plasma cortisol during lice infection, indicate that the infection is experienced as a discomfortable situation (Grimnes and Jakobsen 1996; Bjørn and Finstad 1997; Moberg 1999). Infections with *L.salmonis* copepodids are known to cause modulation of the host immune system (Skugor et al. 2008). Adult salmon lice also cause a drop in hematocrit levels, elevated plasma chloride levels and cause severe osmoregulatory problems (Grimnes and Jakobsen 1996; Bjørn and Finstad 1997; Dawson et al. 1999). In summary, these physiological changes can kill the host, reduce growth or make it more susceptible to secondary infections (Grimnes and Jakobsen 1996; Bjørn and Finstad 1997).

The wild stock of Norwegian salmon and sea trout are estimated at ca. 2-2.5 million fish (Heuch et al. 2005). In contrast, standing stock of farmed salmon and trout was 231 million fish on December 2002. By other means, the aquaculture industry has increased the total number of salmonid hosts in the coastal waters of Norway by a 100 fold (Heuch et al. 2005). As a result of this, the *L.salmonis* population have increased dramatically and heavy parasite infections occasionally occur on both farmed and wild anadromous salmonids (Heuch et al. 2005). This high lice population maintained by the aquaculture industry are suggested to have a negative effect upon wild salmonid populations (Bjørn et al. 2001).

Legislation states the upper limit for sea lice abundance in Norwegian net pens (Heuch et al. 2005). If infection numbers exceed this limit, actions to reduce lice numbers are stated by law. Despite the fact that cleaner fish are used to manage lice infection, the combat strategy against salmon lice mostly relies upon the use of a few chemical delousing agents, deltametrin, cypermetrin and emamectin benzoate (Frost et al. 2006). The agents are distributed either orally or by bath treatment and contribute to major expenses by mean of man power and chemicals. Total costs due to sea lice were estimated at 500 million NOK in Norway for 1997 (Pike and Wadsworth 1999), with increased number of fish and heavier lice burden cost are probably higher today. Furthermore, treatment failure using Emmamectin Benzoate was observed in the spring 2008 in several salmon farms in Trøndelag, Norway (Nilsen and Horsberg 2008). Due to risk of resistance development, new combat strategies like

vaccination and new pharmaceutical chemicals should be developed to ensure the health and well being of farmed and wild salmonid populations (Frost et al. 2006).

A research group at the Institute of Marine Research in Bergen has for several years been using gene technology to better understand the basic biology of *L.salmonis* (Kvamme et al. 2004; Frost et al. 2006; Eichner et al. 2008). Such knowledge is crucial when designing the next generation combat strategy against this parasite. Clinical trials have shown that it is possible to reduce lice abundance by vaccination (Frost et al. 2006). The idea of vaccination is to stimulate antibody production in the host, which should mark the pathogen of interest for elimination by the host immune cells (Zinkernagel 2003). This is difficult for ectoparasite vaccines, since only a small part of the organism is in contact with the host immune system (Frost et al. 2006). However, active substances in host blood can react with components inside the parasite when the parasite withdraws blood for consumption (Brandal et al. 1976; Frost et al. 2006). Such active substances can be antibodies against certain proteins with vital function for the parasite (Frost et al. 2006). To obtain possible key-proteins that could be candidates for blocking by vaccination, detailed knowledge concerning biological processes in the lice is required (Frost et al. 2006).

Since copepodids are the link between the free-living organism and a parasite it offers the opportunity to see how *L.salmonis* initiates the parasitic life style. Firstly, it has to sense the presence of a host, act rapidly to attach to it, search the surface of it and decide whether it is suitable or not (Johannessen 1978; Bron et al. 1991). Following attachment, the copepodid have to modulate the immune system in a direction that favors the parasite (Skugor et al. 2008), a phenomenon which can be observed with the decrease in the initial melanization of the host stratum granulosum that occurs the days following infection(Jones et al. 1990). The copepodids also have to grow a new cuticle and produce an apparatus for attachment prior to the upcoming molt (Skugor et al. 2008), a process which do not occur unless the copepodid is allowed to infect a suitable host (Johannessen 1978). This strongly indicates that these processes are dependent on an external energy source in whole or as an addition to the copepodids yolk reserves (Bron et al. 1993). Such energy is obtained by feeding on the host, a process which depends on digestive enzymes to be present in the alimentary canal. F-cells or type I cells in the midgut of *L.salmonis* copepodids are believed to produce such enzymes (Nylund et al. 1992; Bron et al. 1993). Kvamme et al. (2004) have characterized five trypsin-like peptidase transcripts from the salmon louse intestine and found that there were an

increase in transcript levels between the planctonic copepodid and chalimus III stage. Low transcription levels occur in the free-living copepodid phase, and transcription levels of infective copepodids have never been studied (Kvamme et al. 2004).

Animals with exoskeleton generally grow in size by ecdysis, still increase in body dimensions without such change of cuticle (instar growth) have been reported for adult *L.salmonis females* (Eichner et al. 2008) and the related copepod *Lernaeocera branchialis* (Smith and Whitfield 1988). The authors suggest a connection between the observed instar growth and sexual maturation of the lice (Eichner et al. 2008). The underlying hypothesis in the present study is that *L.salmonis* copepodids undergoes instar growth only when parasitizing a suitable host. Production of a new cuticle and increased trypsine transcription are believed to co-occur with this event, suggesting a link between copepodid instar growth and various preparations for molting. Furthermore, yolk reserves are believed to have minimal influence on such post infection development.

In the following experiment the effects of infection and infection timing and their interaction on copepodid growth, development, molting and trypsine transcription were studied. The main objective was to compare developmental differences between copepodids that gains access to host tissue to copepodids who exclusively utilize the yolk as energy supply. This was done by measuring various growth parameters, histological examination of free-living and parasitic copepodids and use *in situ* hybridization to reveal whether the most abundant trypsine genes were active.

4 Materials and Methods

4.1 Experimental layout

The experiment consists of two major parts, an instar growth study and a trypsine transcription study. The two parts were performed separately due to workload and laboratory capacity.

4.1.1 Copepod instar growth and development study

Differences in the alterations in body dimensions between non-infective (naive) copepodids (Trial A) and copepodids which were allowed to infect salmonid hosts (Trial B) were investigated (fig. II).

Trial A was done by rearing copepodids in incubation wells without exposure to host tissue (fig. II). Five ovisacs of each strain were reared in 5 individual wells. The trial lasted until the copepodids died by lack of nutrition, approximately at 12 days post molting (DPM). Sampling was done every day during this period, but analyses were done on even number days. Ten copepodids were put on Karnovsky`s fixative (table XIX, appendix II)(Karnovsky 1965) on 4, 8 and 12 DPM .

Trial B examines instar growth in two age groups of copepodids when feeding on a natural host. The age groups consists of a young group which are infected two days post molting and an old group which are infected 8 days post molting (fig. II). The term Days Post Infection (DPI) is used to compare these two groups. Ten fish were used to host each group in four separate fish tanks. Sampling was done every day until all copepodids had molted into the chalimus1 stage. The cumulative percentage of chalimus stages present each day was noted. Ten copepodids were put on Karnovsky`s fixative on day 1, 6 and 8 DPI.

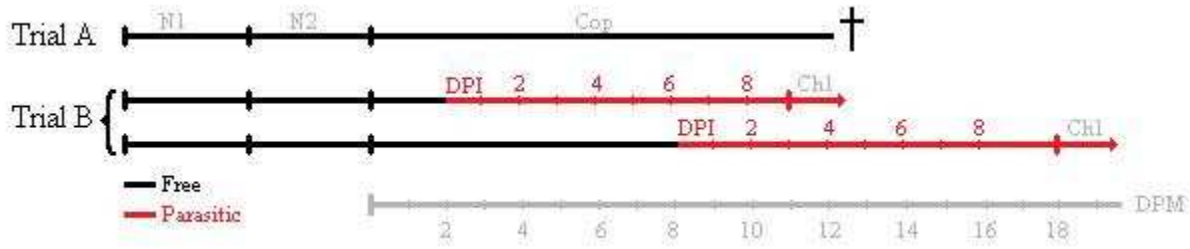

Figure II. Experimental layout for the instar growth and development study. A naive group is compared to two infective groups, infected at an early and a late point in the copepodid stage.

4.1.2 Trypsine transcription study

An additional trial was carried out to produce material for an *in situ* hybridization protocol (ISH) to see when *LsTryp1* and *LsTryp2* (Kvamme et al. 2004) are detected in copepodid sections. Half a batch of redlice copepodids of various age were allowed to infect 5 salmon in a tank. The remaining batch was reared in incubators as described for Trial A. Since neither DPI or DPM respond to both groups when we operate with mixed age groups, the day of infection were defined as day 0. Naive copepodids were sampled on day 0, 2 and 6. Infective copepodids were sampled on day 1, 3 and 6. A nauplius stage were also sampled to function as a negative control as the literature states that none of the known trypsines have been detected at this stage (Kvamme et al. 2004). All samples were fixated in 4% paraformaledhyd within 30minutes post mortem of the lice.

4.2 Lice strains

Two lice strains were used in this experiment. A 13 generation inbred line, called the Redlice strain (strain 1) was used representing low genetic variation. It lacks black pigments and appears clear red coloured to the naked eye. This colour variance made it easier to separate the different strains during handling. The second lice strain originated from Oslofjord in eastern Norway (strain 2). It had been held in the laboratory for only three generations and is expected to have a genetic composition close to a wild louse population. Strain 2 possessed normal dark pigments. Two strains were used to function as experimental duplicates and to exclude major strain dependent variances in the measured parameters.

4.3 The Fish

Atlantic salmon with an average weight of 500 gram were used as hosts in the experiment. By using large fish we were able to reduce the use of fish to 45 individuals, since a large surface area allows more copepodids to attach to each fish. Feeding and observation was carried out daily and the fish density was 10 kg/m^3 in each tank. The fish were fed a commercial Skretting diet.

4.4 Rearing facilities

All experiments were conducted in the disease laboratory at Institute of Marine Research, located in Bergen, Norway.

4.4.1 Water quality

The seawater used to incubate lice and rear Atlantic salmon were pumped from 120m depth, filtered through a sand filter (20µm) and column aerated. The temperature was approximately 10^{o}C and the salinity stable at approximately 34.00ppm. Temperature and salinity were measured daily.

4.4.2 Lice incubators

All lice material was obtained by separating egg strings from adult *L.salmonis* females, reared on Atlantic salmon at temperature and salinities close to the conditions in the incubators. Separating the egg strings from its mother has been reported not to influence the survival or hatching success of the eggs (Johannessen 1978; Johnson and Albright 1991a).

Salmon lice were hatched and reared in incubators consisting of individual wells, with water continuously administrated trough a plastic tube at the top and sealed by a 175µm filter mesh in the bottom, allowing wastewater to exit whilst preventing escapees (fig. III) (Hamre and Nilsen Submitted). Individual egg string pairs were put in separate incubation wells to allow individual monitoring of hatching success and development. Observations were done once a day during the incubation period and nauplius stages. Incubation time varies between 1 and 9 days and pigmented strings are more developed than the transparent ones. A joint hatching can therefore be archived by selecting dark egg strings. Johnson et al (1991) reports that the average duration from hatching to the copepodid stage is 87.4 hours at 10^{o}C or 36 degree days.

Figure III. Lice incubators with newly hatched *L. salmonis* nauplii larvae. Water is supplied through the nipple in centre.

4.4.3 Infection trials

A successful infection trial allows 50% of the copepodids to attach to the host. Twice the desired amount of copepodids was therefore used to obtain an abundance of 50 lice pr fish. The fish were netted and sedated, while the water level in the fish tank was lowered to approximately 10cm. When the fish were anaesthetized, it was rinsed in seawater and returned to its tank. Copepodids were then poured over the recovering fish and allowed to attach for 10 minutes before the water inlet was reopened and set to 300 L/h. Normal flow rate of 450 L/h were set one hour after infection.

4.4.4 Sampling

Copepodids were sampled with pipette from the incubation wells or by forceps from fish skin, fins, gills and mouth cavity. When sampling infective copepodids, the fish were netted and killed by a sharp blow to the head. Sedation was not used since this could lead to loss of copepodids. Furthermore, blood was withdrawn from the caudal vein to reduce bleedings and blood coagulation on the gills and fins as these were cut out. Gills and fins were examined under a microscope to better spot attached copepodids.

Sampled copepodids were kept alive in small wells filled with seawater and killed prior to measuring by adding a drop of RNA-*later* (Ambion, Applied Biosystems) to each well. This aqueous solution is commonly used to store tissue without jeopardizing the RNA quality, but is also an effective solution to kill copepodids without causing cramps that might curl the lice. A drop of water containing five dead copepodids was transferred to a microscope slide, and each individual were rinsed for mucus and orientated with the “eye” facing upward. By extracting water from the drop using cellulose fibres, the copepodids were sucked down to the microscope slide by the water surface tension, allowing calibrated pictures to be taken of the sample. Measurements were done on pictures, and total body length, cephalothorax length, width and posterior segments length (fig. I, B) were measured. A Wild Heerbrugg stereolupe with a Sony 3CCD colour video camera attached was used to examine and photograph samples at 50x magnification. Pictures were handled and measured in Image-Pro plus software.

4.5 Histology

Histological methods were used for the ISH protocol and examine the production of chalimus I cuticle in copepodids. Copepodids are too small to fit a normal embedding cassette (WLAS-

1060), and had to be placed in a lens paper formed as an envelope prior to further treatment. Infiltration, embedding, slicing and dying were performed according to normal histological procedures (appendix III). Histowax sections of 5µm thickness were used for the ISH-trial and resin sections of 3µm were used in the morphology study. The resin sections were stained with toloudine blue or hematoxyline-erythrosine dye (appendix III).

4.6 *In situ* hybridization

In situ hybridization protocol of Kvamme et al. (2004) was used to locate *LsTryp1* and *LsTryp2* transcript in copepodids. In brief, the principle behind ISH is that a labeled complementary RNA strand (probe) hybridizes to target sequences (mRNA) at elevated temperatures. Post hydrolysis by RNAase, washing and manipulation with parameters such as temperature, salt and detergent concentrations removes unhybridized probe and thus reducing the risk of background staining. The labeled probe is detected by anti label antibodies, and visualized by chromogenesis. A correct ISH is performed with a sense probe parallel to the complementary antisense probe. The sense probe will function as a control as no specific binding should occur between sense probe and the target sequence (i.e the mRNA).

4.6.1 RNA probe synthesis

Digoxigenin (DIG) labeled single stranded sense and antisense RNA probes were produced according to protocol and under strict hygienic conditions as such probes are vulnerable for degrading by RNAse (Kvamme et al. 2004). Vector NTI advance® software was used to design inserts of approximately 600bp and make specific primers (table XX and XXI, appendix II) for probe amplification. Forward and reverse primers with and without SP6promotors were ordered and mixed as shown in table I. The inserts were amplified by polymerase chain reaction (PCR) (table XXII, appendix II) using specific primers flanking the SP6 region and product was verified in 1% agarose gel. A Macherey-Nagel Nucleospin® Extract II kit was used for rinsing and elution of the PCR products prior to probe synthesis. Synthesis and labelling of the probe (table XXIII, appendix II) was performed with a Roche DIG RNA labeling kit (SP6/T7). The pre-probes were evaluated by a RNA 6000 Nano Assay together with two standard solutions of 100 ng/µl and 25 ng/µl. The probes were stored at ÷70 ^{o}C until use.

Table I. Mixing of primers in order to produce sense and antisense PCR product. Identification letter in ().

	LsTryp1	**LsTryp 2**
Sense	Tryp1F-SP6 + Tryp1R (D)	Tryp2F-SP6 + Tryp2R (B)
Antisense	Tryp1F + Tryp1R-SP6 (C)	Tryp2F + Tryp2R-SP6 (A)

4.7 Statistical analysis

Instar growth data were tested for normal distribution with a Kolmogorov-Smirnov test (Zar 1998) and homogeneity of variances were tested using Levene`s F test. All statistical analysis was performed with Statistica software and difference among the groups were tested with Student-Newman-Keuls test (Zar 1998).

5 Results

5.1 Rearing condidtions

Recorded data on water quality is presented in table II and fig. XII (appendix I) and show stable values for all the trials.The average temperature was 9.1 (± 0.4)°C and the average salinity were 33.1 (± 0.9)‰ (± standard deviation of mean). A slightly reduced temperature occurred during trial B (table II).

Table II. Average temperature and salinity for each trial (±standard deviation of mean).. The conditions were stable, but slightly lower temperatures occurred in trial B.

	T (°C)	Sal (‰)
Trial A	9,3 (±0,2)	33,3 (±0,6)
Trial B	8,8 (±0,3)	33,0 (±1,0)
ISH trial	9,4 (±0,1)	33,4 (±0,8)

Infection success in trial B varied between 40 and 50 % attachment of the total number of copepodids added to the salmon. The results from the ISH trial were on the other hand very low, and as few as 10 % of the copepodids were recollected from the fish.

5.2 Instar growth

A total number of 2022 copepodids were measured from trial A and B. All length data were normal distributed and had acceptable F-values (table XVII and XVIII, appendix I). No significant increase in copepodid body dimensions was observed in trial A and all copepodid groups exposed to host tissue showed significant instar growth during trial B (Fig. IV- V and table III-VI, appendix I). This overall trend is reflected in all groups. The increase in total body length was between 11.8% and 14.1% , and largest width increase varied from 25.3% to 35.4%. Length of the four posterior segments increased between 14.9% and 39.8% whilst the cephalothorax length showed an increase between 3.4% and 7.0%.

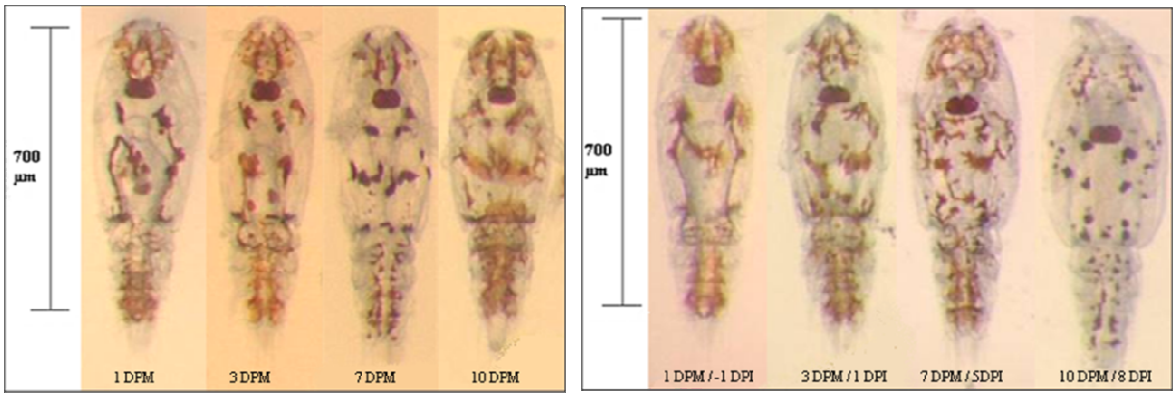

Figure IV. Redlice copepodids from trial A (left) and trial B (right). The term Days Post Molting (DPM) indicates the age of the copepodids and refers to both trials whilst the term Days Post Infection (DPI) describes time from infection point and refers exclusively to trial B copepodids. Visible changes in length and width appear as the infective copepodids of trial B develops toward the end of the copepodid stage. No such changes occur in the naïve groups of trial A.

5.2.1 Total body length

The two copepodid groups of trial A did not experience any significant increase in total length when reared in incubators until death by starvation, but the naïve Redlice group had a significant decrease in total body length (fig. V and Table VII, appendix I). On the other hand, the four copepodid groups of trial B had an increase in total length shortly after initial contact with host tissue (fig. V and Table VII and VIII, appendix I). Significant increase occurs as early as two DPI for Oslofjord groups and three to four DPI for the Redlice groups (Table VII and VIII, appendix I). The infective copepodids in trial B grew significantly larger ($P < 0.001$) than the naïve copepodids of trial A (Table XV, appendix I). The amount of sampled copepodids (n) decreased at the end of the trials due to molting and mortalities, resulting in reduced number of copepodids measured on the last sampling dates (Yellow markers in fig. V).

5.2.2. Specific segment growth

The growth of cephalothorax and posterior segments followed the same trend as seen for the total length. None or little alteration in body dimensions occurred during trial A (fig. XIII-XV and table IX-XIV, appendix I). During trial B, greatest width and the length of posterior segments continuously increased whilst the cephalothorax length stagnated after a few days of growth (fig. XIII-XV, appendix I). However, all growth in body sizes observed in trial B copepodids were statistically significant (table IX-XIV, appendix I). The increase in total body length is mainly caused by the increase in the length of the posterior segments.

5.2.3 Age differences

There was high resemblance in growth patterns between the copepodids infected early (2DPM) versus those that were infected late (8DPI) in trial B. A Tukey HSD test of the trial B data (Zar 1998) show no significant difference between age groups of same strain 6DPI (Oslofjord : $P = 0.311$ and Redelice :$P = 0.997$) (Table XVI, appendix I) . A Student-Newman-Keuls test (Zar 1998) performed on the same data set showed a marginal significant difference ($P = 0.040$) between young and old Oslofjord copepodids (Table XV, appendix I) The size difference between the Oslofjord age groups is 11.83 µm at this time (6DPI).

5.2.5 Strain differences

There were significant differences ($P < 0.001$) in the post infection growth patterns of the two strains used in trial B (table XV and XVI, appendix I). The Oslofjord strain grows larger than

the Redlice strain independent of infection date. Such strain dependent growth differences were not observed in the naïve copepodids of trial A (P = 0.283) (table XV, appendix I). The variance within the Oslofjord population is larger than for the Redlice strain (Fig. V).

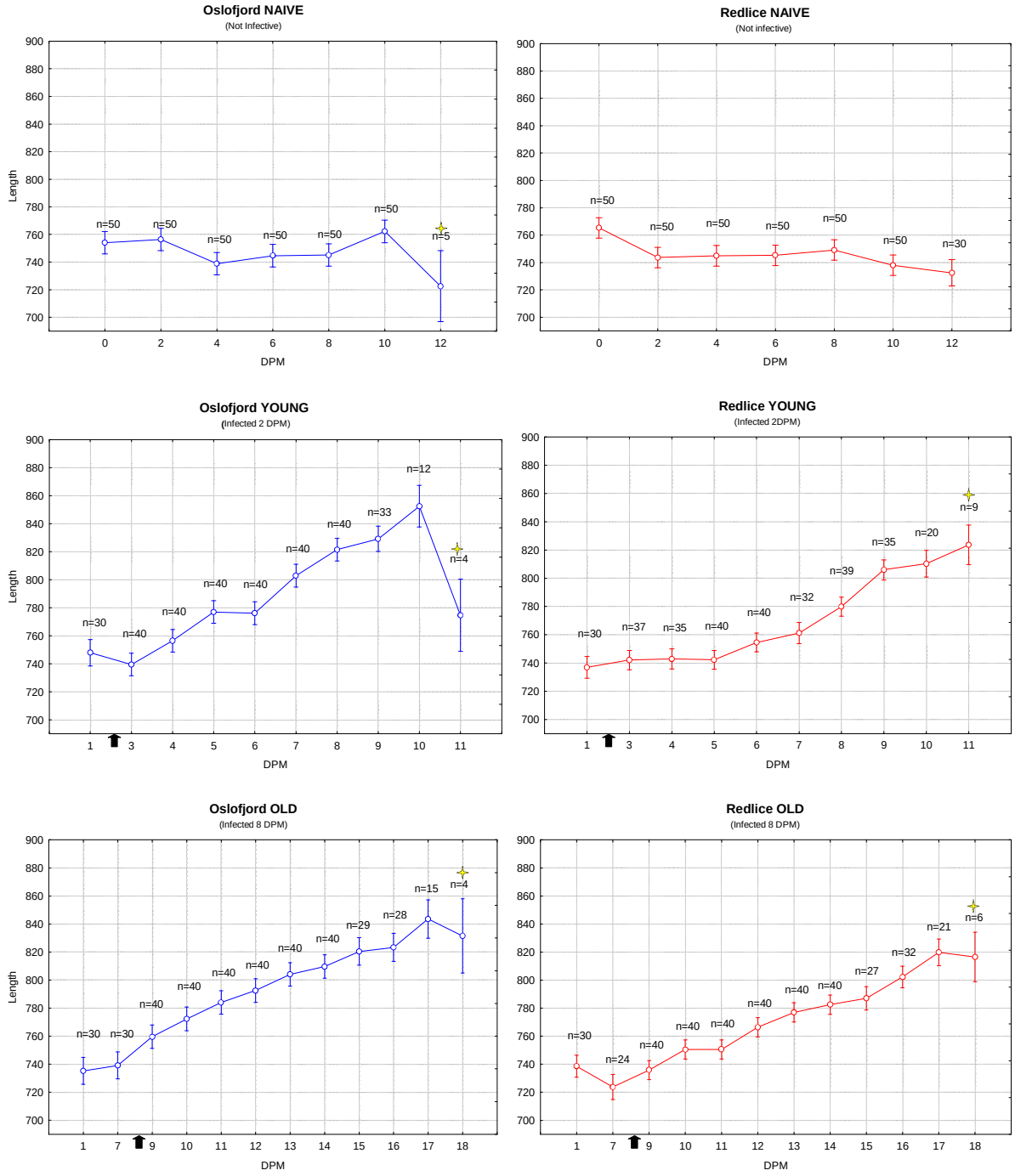

Figure V. Total length (µm) at different time points of copepodids in trial A and B. Arrows indicate infection point. Vertical bars denote 0.95 confidence intervals. Number of measured individuals (n) is displayed above each sample point. Yellow markers indicate suboptimal sample size. ANOVA results are presented in table III, appendix I.

5.3 Molting

The first copepodids to molt were observed on 7DPI in trial B, independent of age prior to infection (fig. VI). Young copepodids of the Redlice strain initiated molting 8 DPI, one day later than the three other groups. Thus, this overdue molt was very united and resulted in high numbers of chalimus-I stages in this group on 8DPI (fig. VI). The results reflect a trend, where Oslofjord strains molt at a higher frequency than Redlice strain reflecting the growth study data. No observations are available for the young Redlice group on 10DPI due to the loss of a salmonid host in this group during the trial.

A heavy expansion of cephalothorax width can be observed the day prior to molting (fig. VII, B). No external frontal filament of copepodids has been observed, but the second antennae are commonly bent out as a response to the copepodid being removed from the fish skin (fig. VII). The characteristic frontal filament of the chalimus stages can be observed immediately after molting.

Histological examintaions reveal structural alterations in the cuticle as the copepodid infects a host and develops towards molting. The cuticle of young naïve copepodids is typically swollen, stains prominently by hematoxyline-erythrosine and is partly infiltrated with nuclei (fig. VIII, A). The cuticle gets more condensed as the naïve copepodid matures (fig. VIII, B-C). Mature copepodids on the host have a two layered cuticle from 6DPI, where the inner cuticle (i.e. the chalimus I cuticula) is similar to that of naïve copepodids (fig. VIII, E-F). The outer cuticle appears to be acellular and is less stained by hematoxyline-erythrosine (fig. VIII, E-F). This two layered structure were first observed 6DPI, and becomes more evident closer to molting. A proliferation of subcuticular takes place with the observed alterations in cuticle composition. No such structural changes or cell proliferation were observed in naïve copepodids of same age (fig. VIII, C vs.F).

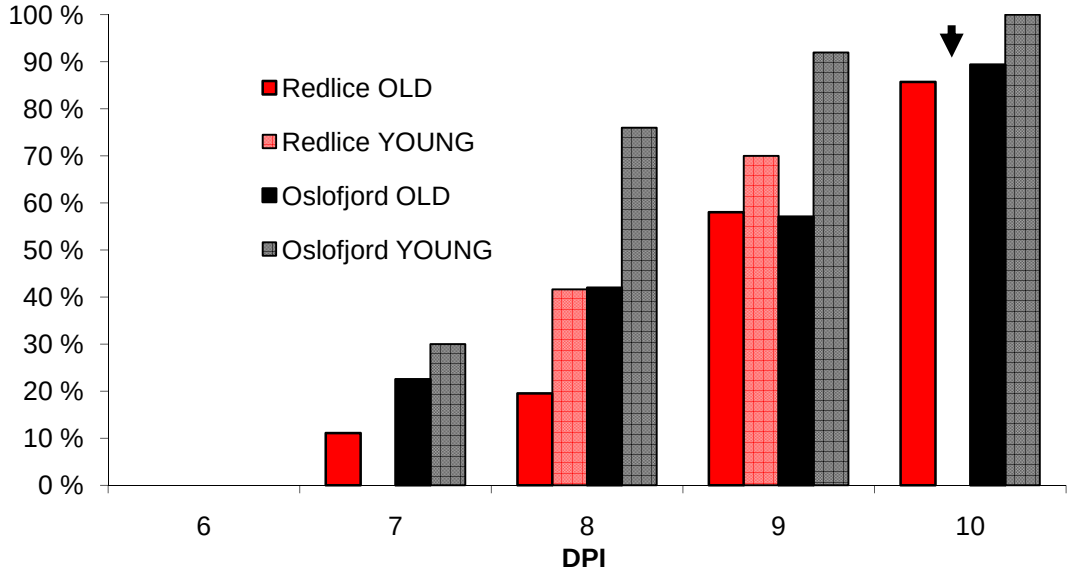

Figure VI. Cumulative percentage of chalimus I stages present at the end of trial B. Molting is initiated on 7 DPI except for the young Redlice group which start to molt on 8DPM. No data exist for the young Redlice group (arrow) due to the loss of a salmon host.

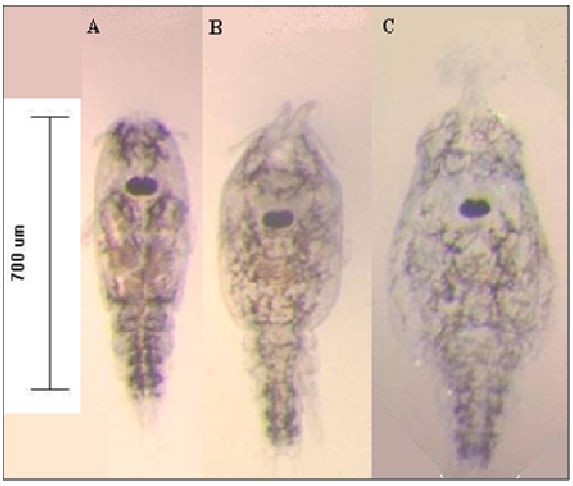

Figure VII. Morphological differences within the copepodid stage compared to a chalimus I stage. **A.** Free-living copepodid – Mobile and active behavior, slim body shape and approximately 720µm long. **B.** Mature infective copepodid – Relatively inactive, wide cephalothorax and > 800 µm long. Notice the secondary antennae bent out anterior. **C.** Chalimus I –Mostly immobile, irreversible attached via the frontal filament. The length of chalimus I exceeds 1mm (Johnson and Albright 1991b).

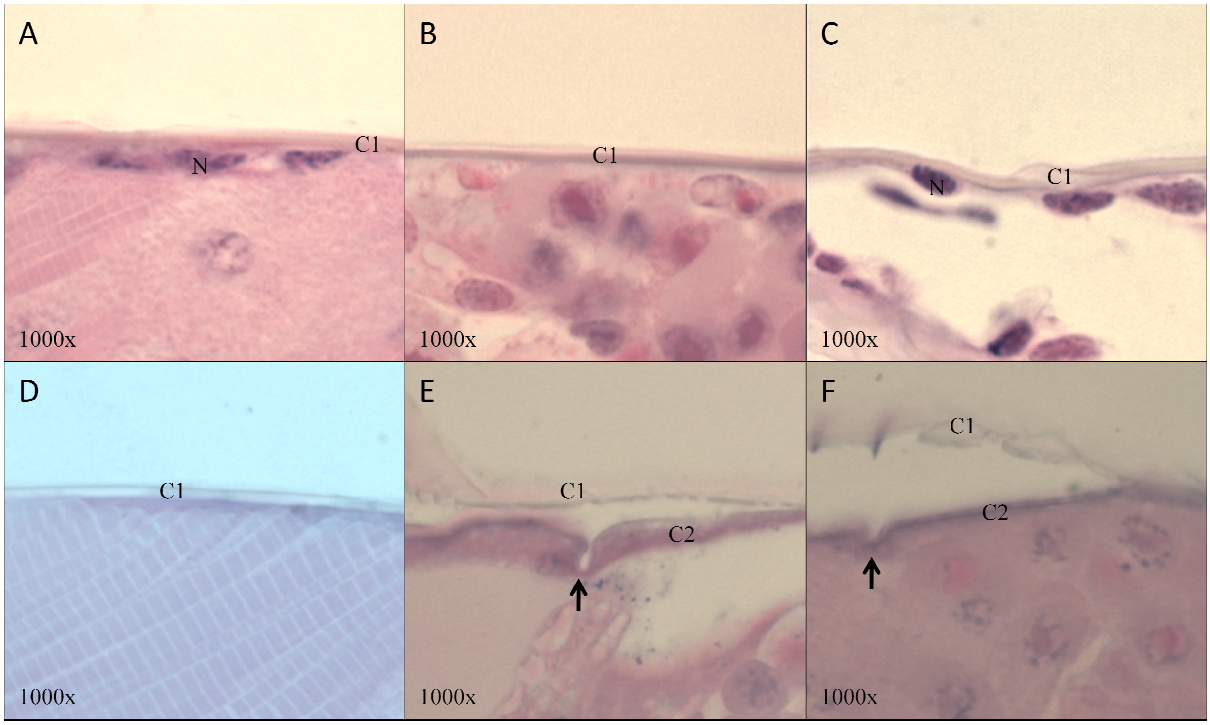

Figure VIII . Hematoxyline-erythrosine stained resin sections of copepodid cuticle. **A-C**. Naive Copepodids. Condensation of copepodid cuticle (C1), no two layered structures observed. **A.** 4 DPM - The cuticle is swollen, stains HE-positive and is infiltrated with nuclei (N). **B.** 8DPM - The cuticle is condensed and partly stained. **C**. 12DPM - Condensed and nearly transparent copepodid cuticle with infiltrated nuclei, note the low density of subcuticular cells. **D-F.** Infective copepodids. Copepodid cuticle is condensed and almost transparent. Two cuticles can be observed from 6DPI. **D.** 1DPI – Condensed and transparent cuticle. **E.** 6DPI – Chalimus cuticle (C2) present underneath the copepodid cuticle (C1). The new cuticle is folded (arrow) whilst the old is partly shed. **F.** 8DPI – Two cuticles present (C1 + C2), the new chalimus cuticle stains violet and are folded (arrow). A proliferation of subcuticular cells can be observed.

5.4 Trypsine transcription

The ISH probes tested to be of high quality by agarose gel and nano assay verification (fig. XVI and XVII, appendix I). A single band of correct size was observed for all probes. The nano assay confirmed the agarose gel results and determined that the RNA concentration in the probes varied between 75ng/µl and 204ng/µl. These concentration differences were normalized by diluting the probe with highest concentration with DEPC water to obtain sense-antisense probe set of approximately the same RNA concentration.

Hybridization using *LsTryp1* antisense probes showed the presence of target gene m-RNA in the cytoplasm of copepodid intestine epithelial cells. Detectable levels were found in both infective copepodids and naïve copepodids on day 0 and day 2. However, even though this is not a quantitative method, the signals are without doubt stronger in the infective copepodids (fig. IX). No detectable levels could be observed in the naïve copepodids on day 6 (fig. IX).

No signals occurred in the slides hybridized with the *LsTryp1* sense probe, indicating that the antisense probe hybridization is specific for the mRNA of interest.

Figure IX. *In situ* hybridization using LsTryp1 probes in copepodid histowax sections. **A-C.** Naïve copepodids have weak or no signals. **A.** Naïve day O – Weak but positive signal. **B.** Naïve day 2 – Weak but positive signal. **C.** Naïve day 6 – No signal. **D-F.** Infective copepodids have strong signals. **D.** 1 DPI – Positive signal. **E.** 3 DPI – Positive signal. **F.** 6 DPI– Positive signal.

The examination of the related trypsine gene, *LsTryp2*, did not show any change in transcription rate post infection as no *LsTryp2* mRNA were detected in neither free-living or

infective copepodids. On the other hand, hybridization with the sense probe resulted in heavy signals in the cytoplasm of a variety of cells all over the copepodid body, except muscle cells (fig. XI). Since the signals were restricted to the cytoplasm in a selection of cells, it cannot simply be explained as background staining. To further test the *LsTryp2* probes, hybridization were carried out on sections of adult *L.salmonis* females. The antisense probe worked as expected and strong signals occurred in the gut associated epithelial cells of the adult sea lice (fig. X). Sections of adult lice hybridized with the sense probe resulted in signals in most cells, thus not in muscle cells, cuticle or connective tissue. The same pattern was observed when a third *in situ* hybridization was carried out on the copepodid sections. As signals arise from hybridization with the *LsTryp2* sense probe, it cannot function as a valid control in this experiment.

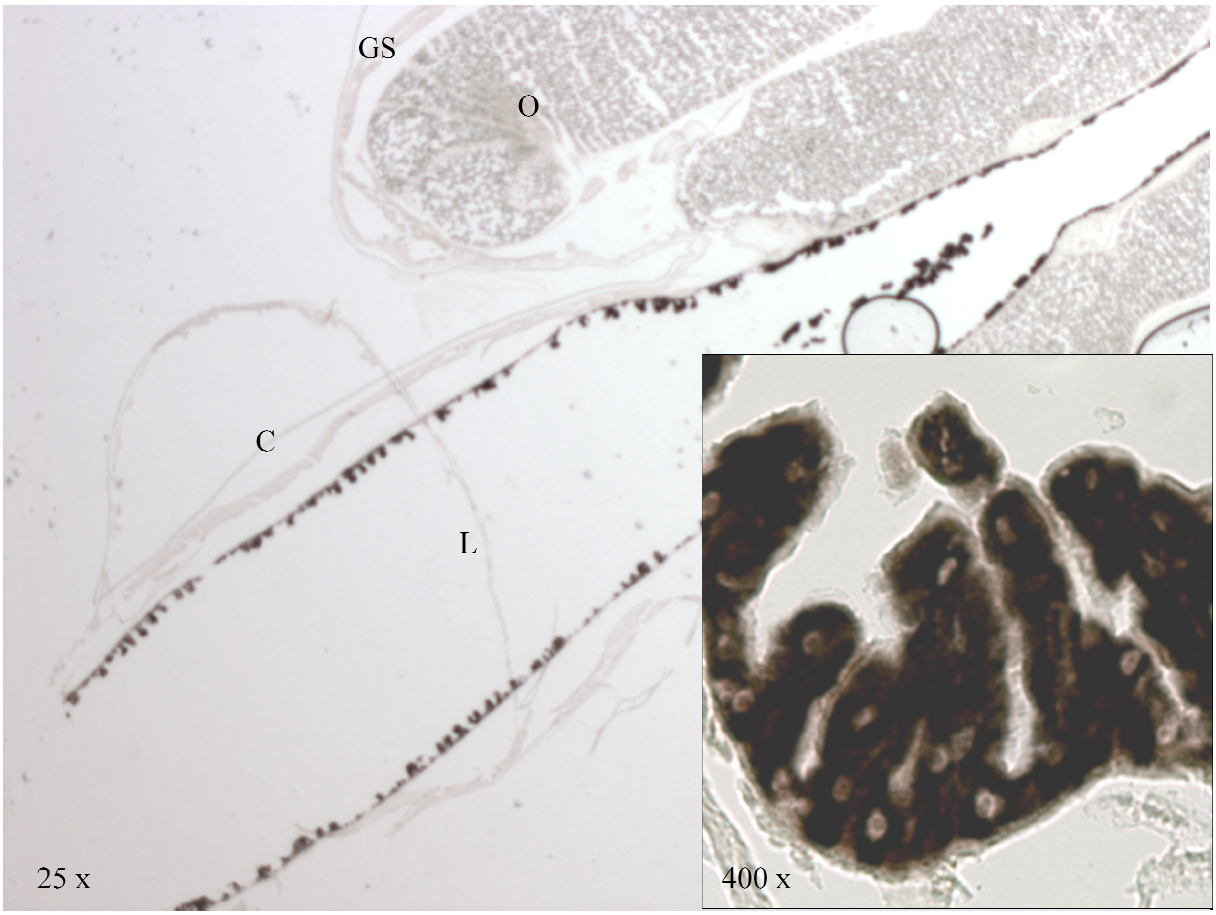

Figure X. Section of an adult *L.salmonis* female, hybridized with LsTryp2 antisense probe. Genital segment (GS), Oocytes (O), Cuticle (C), Lumen (L).. A strong signal occurs in gut associated epithelial cells.

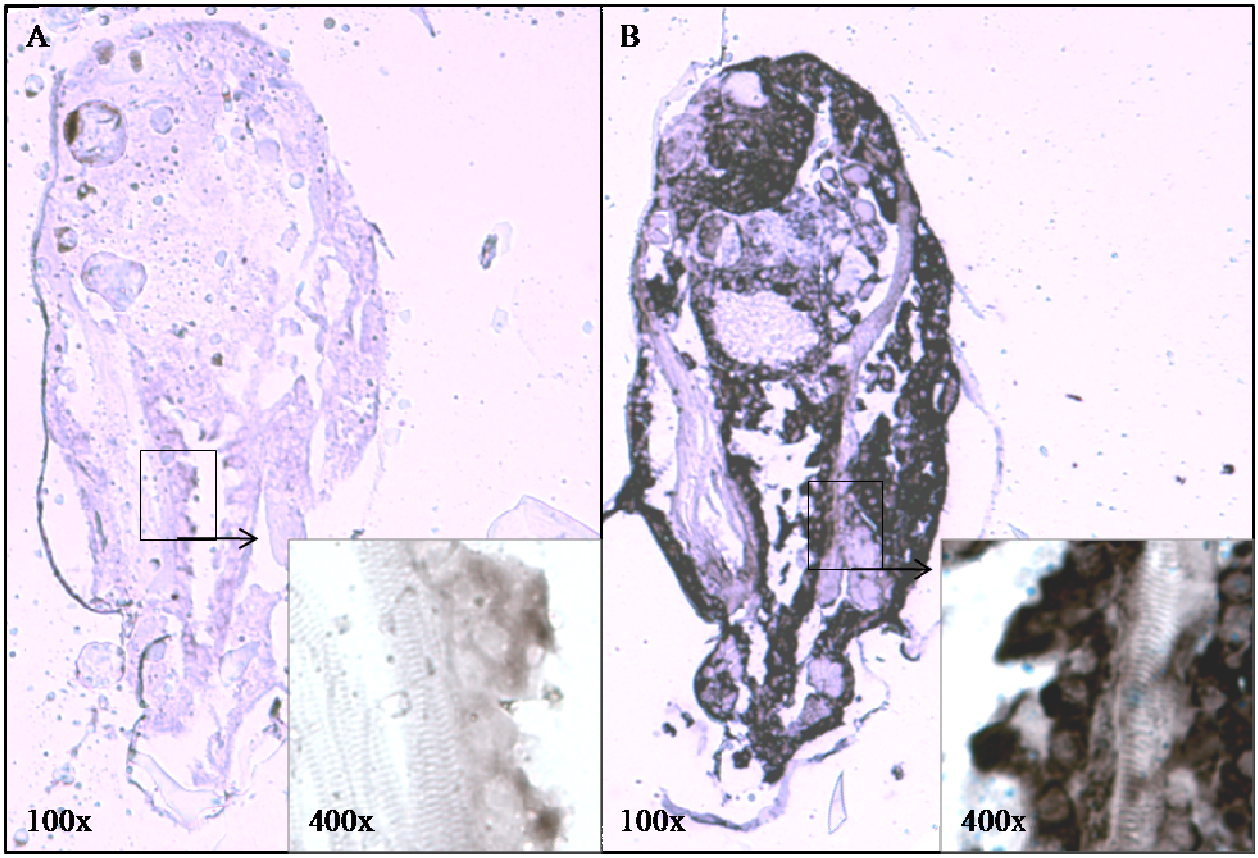

Figure XI. Sections of 6DPI copepodids hybridized with LsTryp2 antisense (A) and sense (B) probe. The signal in A is to weak to be considered as positive and the sense probe in B has marked the cytoplasm in a variety of cells all over the body, except muscle cells.

6 Discussion

In the present experiments we have demonstrated instar growth in *L.salmonis* copepodids after they infects Atlantic salmon host, thus supporting the findings of post molting growth in adult *L.salmonis* females (Eichner et al. 2008). Total body length, width, cephalothorax length and length of the posterior segments increased significantly when copepodids started the parasitic lifestyle. Infective copepodids from trial B had a significantly increase in the measured parameters, whilst the naïve free-living copepodids from trial A showed no or limited increase in body size. The size increase is preparing the copepodids for molting, as a massive proliferation of cells and the production of chalimus I cuticle takes place together with the observed increase in body size. These results are shared by all groups of the experiment and coincide with the findings of Johannessen (1978) which states that molting cannot occur without the parasitic relationship between the copepodid and its host.

The sample sizes of the last days in both trials were very low and cannot be regarded as representative, due to the low number and the fact that the molting process functions as a selection process leaving the disabled and weak individuals behind. With these considerations in mind, the growth of infective copepodids increase constantly until molting.

The resemblance between the young and the old infective group in trial B indicates that the nutritional status by means of yolk reserves does not affect development towards molt, body size or infection success. Both groups require approximately 70degree days on the host to prepare for the molt and there is no obvious trend differences based on the present study. This strongly indicates that host tissue is an essential source of nutrient for copepodids, and that the amount of yolk is of less importance for further development. Larval phases with such an abrupt change in nutrition consumption are often associated with low immunocompetence, vulnerability and sensitivity towards pathogens and stressors in many organisms (Lightner et al. 1983; Krol et al. 1990; Smith et al. 2003). In addition, parasitic organisms face the host immune system for the first time at this stage. Taken together, these factors points out the copepodid stage as a rather fragile state compared to the rest of the lifecycle, making them particularly interesting in the approach for an effective sea lice vaccine or other targeted treatment.

The lice strains in this study forms two distinct populations as significant differences occur between the two strains (table XV, appendix I). Hence, Oslofjord and Redlice copepodids constitute a research duplicate which increases the reliability of our results. The Redlice strain showed low variance as expected from an inbred strain whilst the Oslofjord copepodids had a more diverse distribution of the measured parameters.

Kvamme et al (2004) showed that *LsTryp1* is the most abundant trypsin in the *L.salmonis* gut. In contrast to the hypothesis of this study, free-living naïve copepodids reared in incubators appear to have detectable levels of *LsTryp1* mRNA in the cytoplasm of gut associated epithelial cells. Moreover, the levels of mRNA seem to decline since no detectable levels were found in naïve copepodids at the end of the study. Such findings could be explained by copepodids utilizing the remnants of the egg string material for nourishment, causing a temporary increased transcription of digestive enzymes which in turn declines when the substrate is consumed. Both a degradation of the egg string material and copepodids attached to it have been observed in the incubators, but whether the degradation is caused by copepodid feeding or decomposing by microorganisms is not clear. The attachment to the egg string appeared to be reversible and most individuals detached if disturbed by for instance water current. If copepodids in fact utilize dead organic material of maternal origin, it has to be regarded as limited to laboratory conditions as in nature, no contact should occur between copepodids and maternal egg string.

Another explanation for the pattern of *LsTryp1* transcription is that naïve copepodids produce mRNA for essential digestive enzymes prior to infection, in order to initiate external feeding immediately after first contact with a suitable host. The following decrease can be explained by the mRNA being translated into active enzymes and/or zymogenes thus limiting the amount of mRNA in the cytoplasm of gut associated epithelial cells. A similar strategy with production and storage of mRNA is described for ribosomal protein genes in the mosquito *Aedes aegypti* prior to blood feeding (Niu and Fallon 2000). Such a strategy would make the lice more fit as it allows a more rapid develop towards the chalimus I stage, and it refers to natural conditions as well as laboratory situations.

No transcription of *LsTryp2* genes were detected in this experiment. Signals could not be observed in neither infective nor free-living naïve copepodids, whilst strong specific signals

occur in adult females. This indicates that this method is not sensitive enough to detect the transcript levels of *LsTryp2* in copepodids. In cases of doubt whether a weak signal can regarded as positive or not, a comparison to a related section hybridized with the sense probe should be conducted. Such a comparison has no value in this experiment since the *LsTryp2* sense probe gave signals in the cytoplasm of a variety of cells; hence none of the weak signals that occurred in the *LsTryp2* hybridization could be regarded as positive.

The position of the *LsTryp2* probe on the *LsTryp2* gene was not identical to that of Kvamme et al. 2004 and the sense probe insert of this experiment overlaps an alternative open reading frame (ORF) on the negative strand of the *LsTryp2* gene. The ORF of interest were not present in the probes used by Kvamme et al (2004) and their hybridization with the sense probe resulted in no signal. The alternative ORF in the *LsTryp2* sense probe seems to be the main differences between the sense probes of these experiments, and we cannot exclude this as the factor that cause positive signals in the sense probe sections of this experiment. Open reading frames on the negative strain of *L. salmonis* genome have been described to encode putative proteins highly similar to functional proteins in other animals (Eichner et al. 2008). Hypothetically, the *LsTryp2* sense probe can function as an antisense probe for a protein transcript originating from the *LsTryp2* negative strain.

7 Conclusion

We have sampled and measured 2022 *L.salmonis* copepodids in length, width, cephalothorax length and length of the posterior segments. At the current experiment conditions, molting started 7DPI and the majority (86-100%) had completed molting at 10DPI. *In situ* hybridization and histological examinations have been used to study morphological changes and gene regulation. The results show that several processes that prepares the copepodid for molting is dependent of an active host-parasite relationship. Instar growth and cuticle production are factors that do not occur in copepodids without access to host tissue. Furthermore, yolk has minimal influence on these processes indicating a complete change of diet as the copepodid initiates the parasitic lifestyle. Free living copepodids have detectable transcript levels of LsTryp1 prior to infection but transcription is more prominent in infective copepodids, suggesting a transcription increase post infection. No detectable transcript levels of LsTryp2 were observed in the experiment.

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Appendix I

Further results and statistical tables

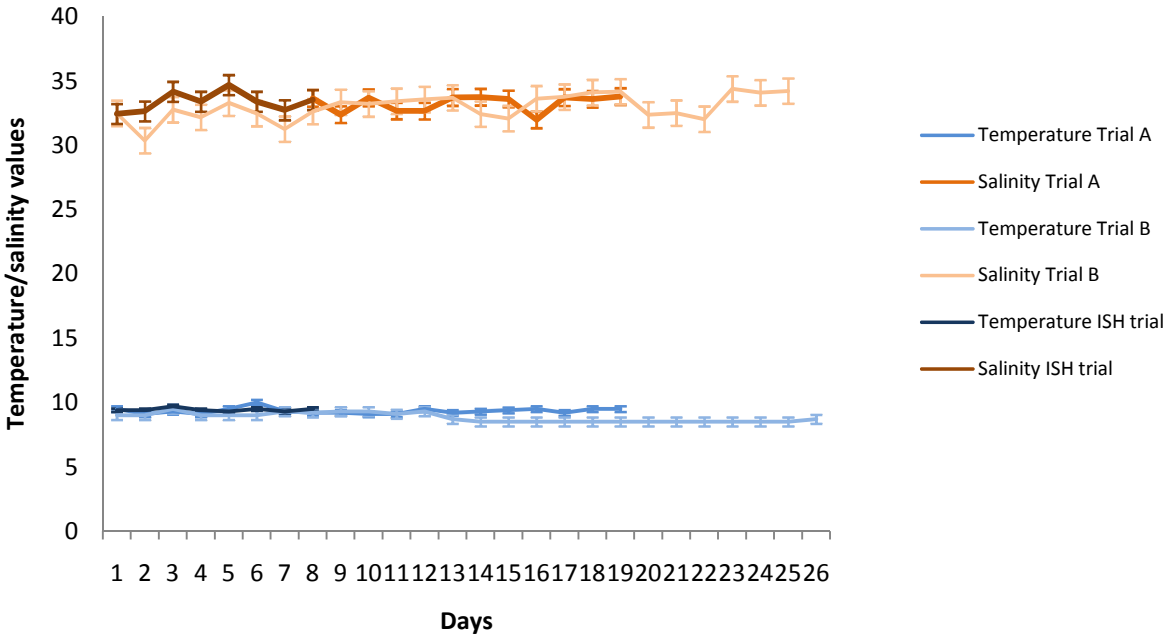

Figure XII. Temperature and salinity during trial A, trial B and ISH trial. Error bars denote standard deviation. Average temperature = 9.1 (± 0.4)°C Average salinity = 33.1 (± 0.9)‰

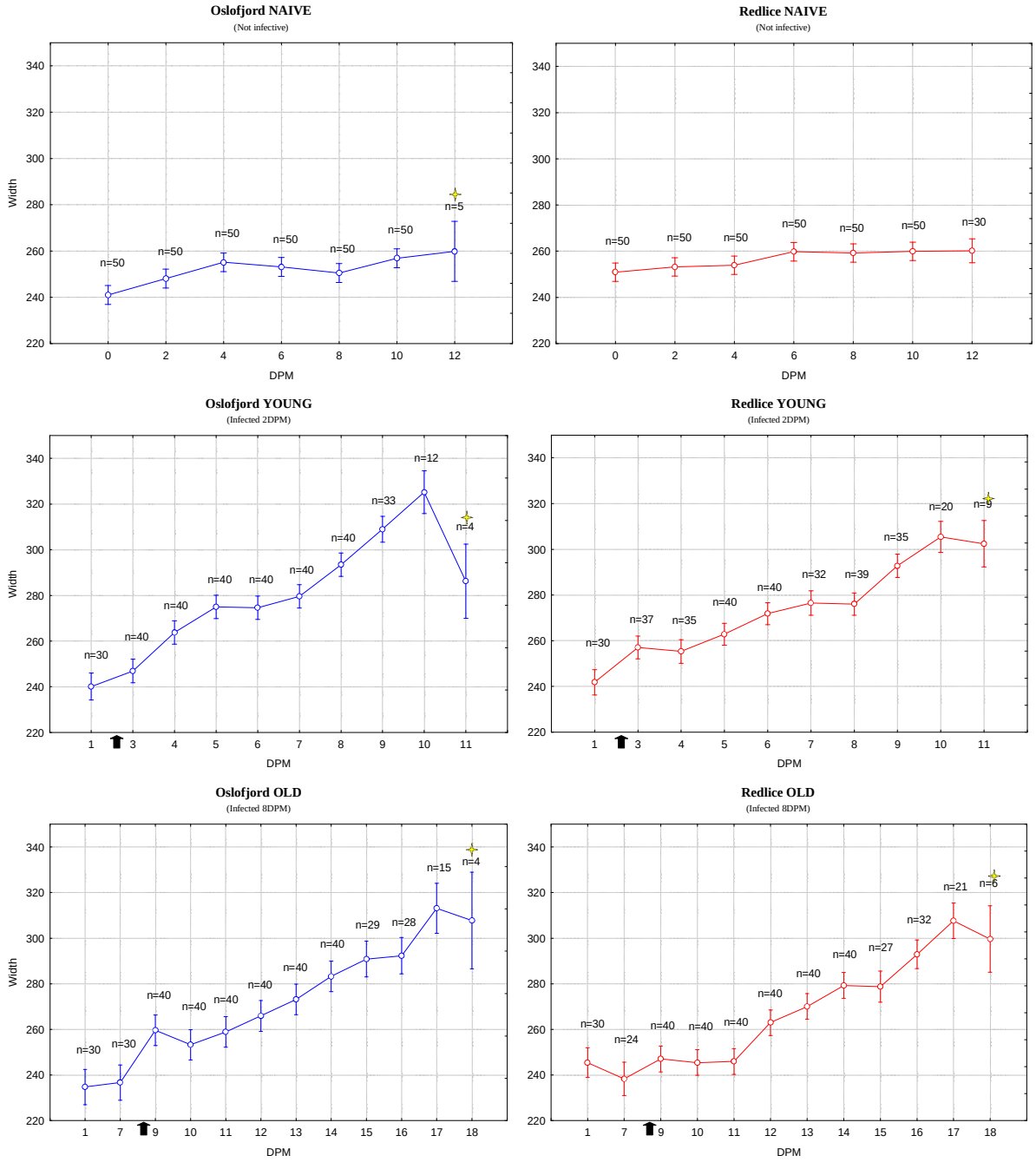

Figure XIII. Largest width (µm) at different timepoints of copepodids in trial A and B. Arrows indicate infection point. Vertical bars denote 0.95 confidence intervals. Number of measured individuals (n) is displayed above each sample point. Yellow markers indicate suboptimal sample size. ANOVA results are presented in table IV and P-values in table IX-X

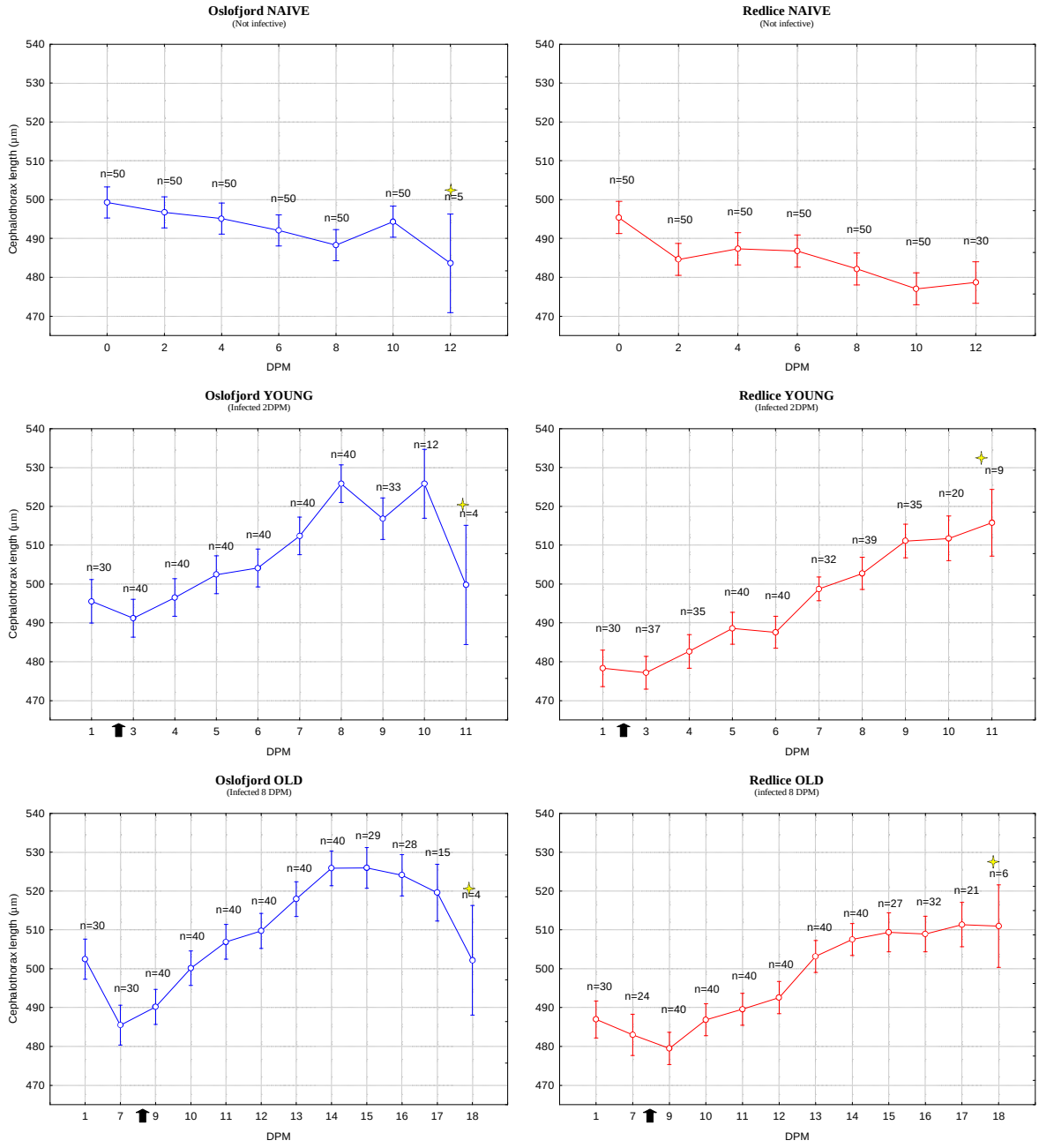

Figure XIV. Cephalothorax length (µm) at different time points of copepodids in trial A and B. Arrows indicate infection point. Vertical bars denote 0.95 confidence intervals. Number of measured individuals (n) is displayed above each sample point. Yellow markers indicate suboptimal sample size. ANOVA results are presented in table V and P-values in table XI-XII.

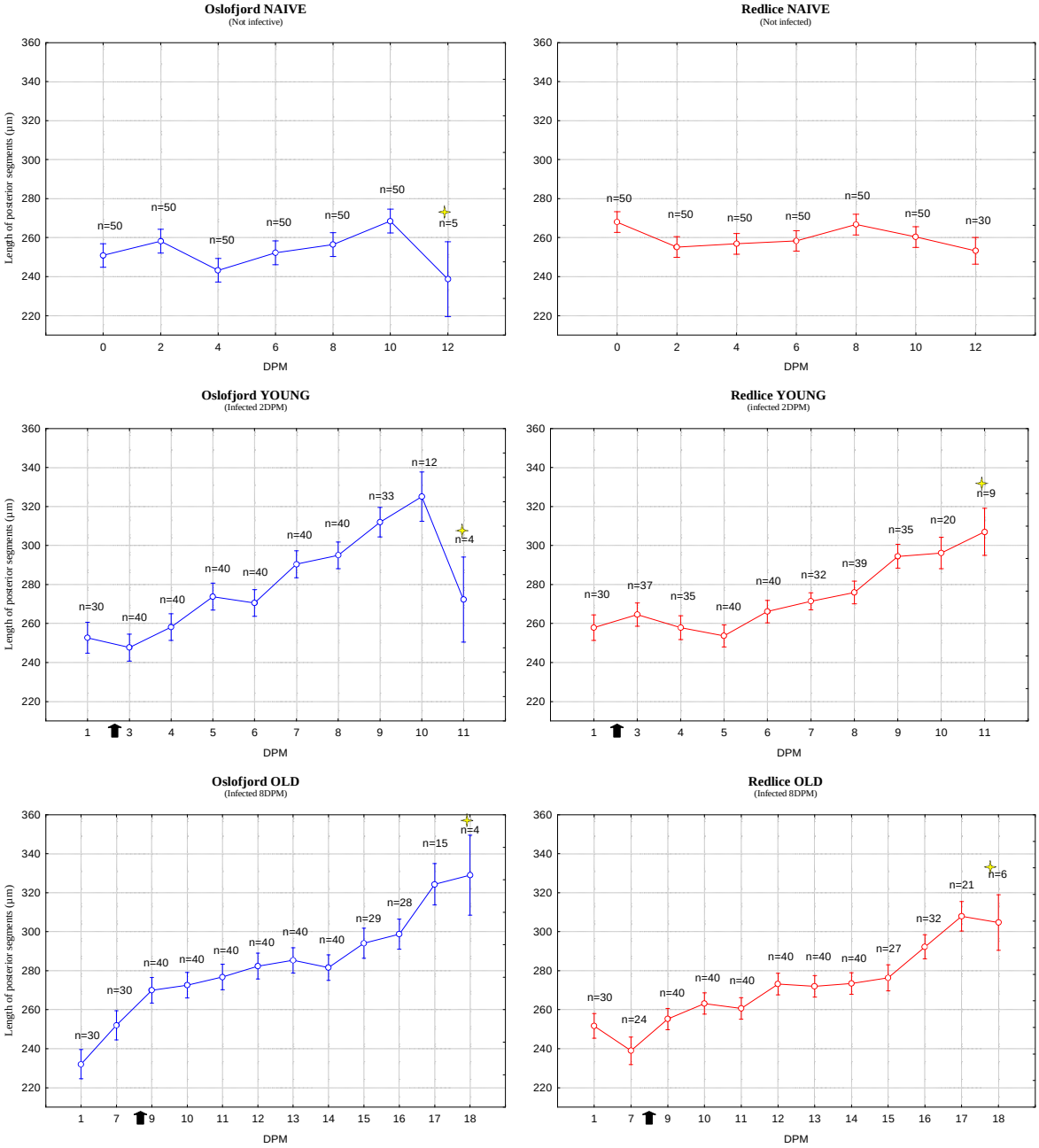

Figure XV. Posterior segments length (µm) at different time points of copepodids in trial A and B. Arrows indicate infection point. Vertical bars denote 0.95 confidence intervals. Number of measured individuals (n) is displayed above each sample point. Yellow markers indicate suboptimal sample size. ANOVA results are presented in table VI and P-values in table XIII-XIV.

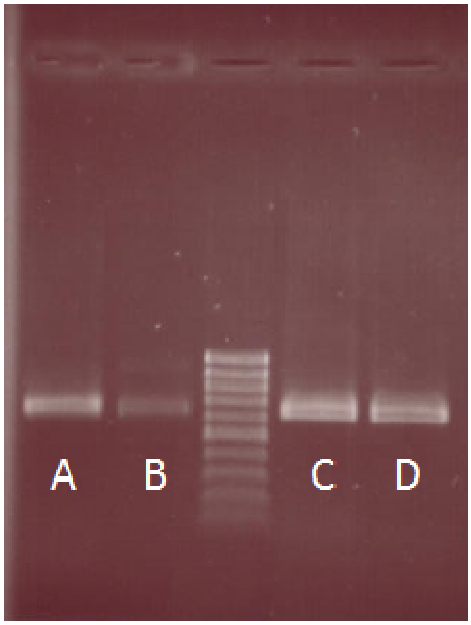

Figure XVI. Verification of the PCR product in1% agarose gel. The bands are single and orientated next to the ladder band of 600bp. **A**.LsTryp2 antisense. **B**.LsTryp2 sense. **C**.LsTryp1 antisense. **D**.LsTryp1 sense.

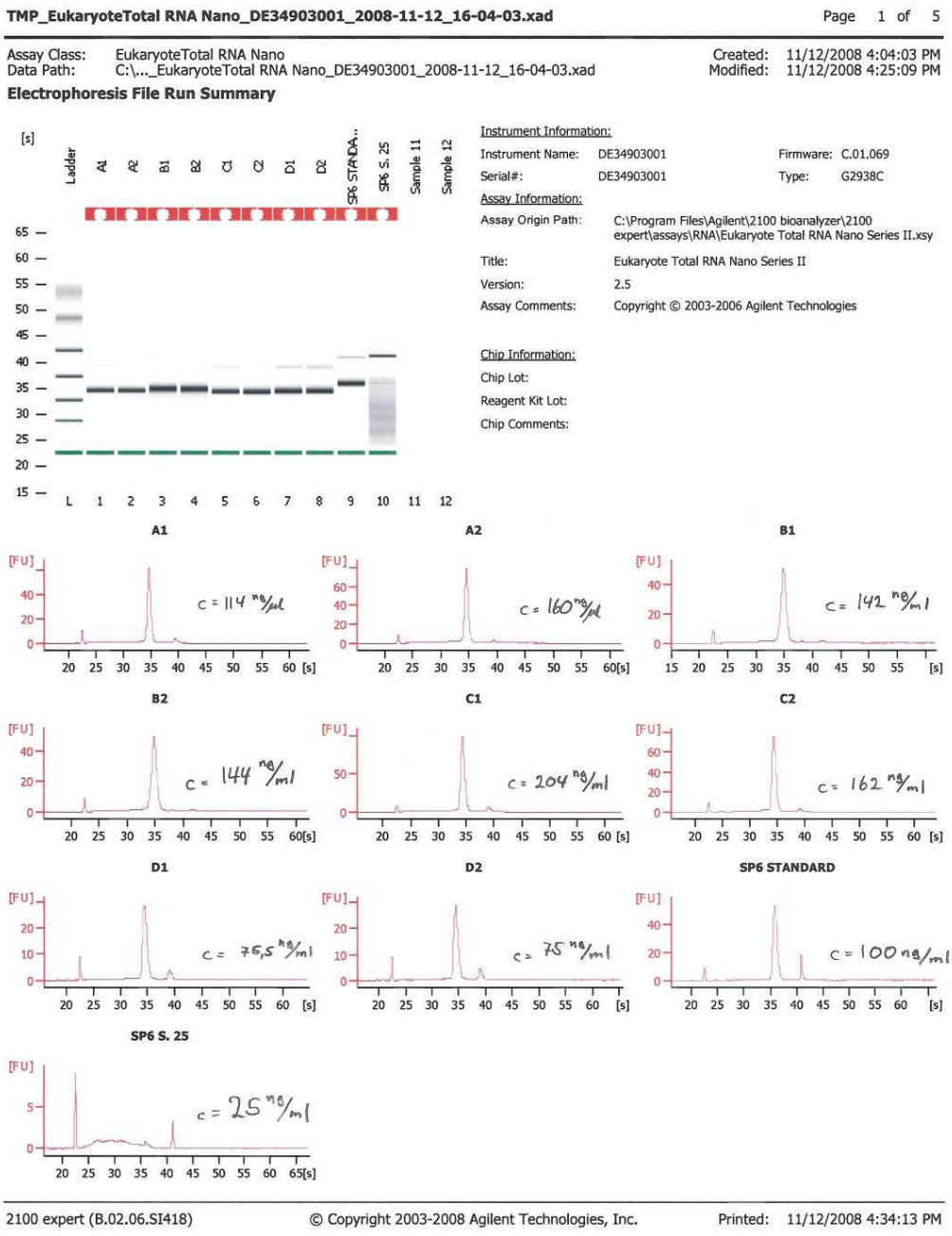

Figure XVII. Verification of the probe quality and concentration by nano assay. All probes are free of contamination and concentration varies from 75ng/ml to 204 ng/ml. . **A1-2**.LsTryp2 antisense. **B1-2**.LsTryp2 sense. **C1-2**.LsTryp1 antisense. **D1-2**.LsTryp1 sense.

Analysis of variance (ANOVA)

Table III. Test result from one-way ANOVA run on total length per days past molting (DPM).

	Naive Redlice				
Effect	SS	Df	Ms	F	P
Intercept	177631802	1	177631802	247563	<0,001
DPM	28101	6	4685	6.5	<0,001
Error	231759	323	718		

	Young Redlice				
Effect	SS	Df	Ms	F	P
Intercept	161378752	1	161378752	352553,9	<0,001
DPM	227248	9	25250	55,2	<0,001
Error	158379	346	458		

	Old Redlice				
Effect	SS	Df	Ms	F	P
Intercept	168610773	1	168610773	348052	<0,001
DPM	266474	11	24225	50	<0,001
Error	178274	368	484		

	Naive Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	85280215	1	85280215	99695,04	<0,001
DPM	22836	6	3806	4,45	<0,001
Error	254912	298	855		

	Young Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	113455700	1	113455700	165938	<0,001
DPM	347898	9	38655	56,5	<0,001
Error	211270	309	684		

	Old Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	149872143	1	149872143	207021,7	<0,001
DPM	346733	11	31521	43,5	<0,001
Error	262792	363	724		

Table IV. Test result from one-way ANOVA run on greatest width per days past molting (DPM).

	Naive Redlice				
Effect	SS	Df	Ms	F	P
Intercept	21065836	1	21065836	102633.8	<0.001
DPM	4337	6	723	3.5	0.002
Error	66297	323	205		

	Young Redlice				
Effect	SS	Df	Ms	F	P
Intercept	20409232	1	20409232	86826.40	<0.001
DPM	96233	9	10693	45.49	<0.001
Error	81330	346	235		

	Old Redlice				
Effect	SS	Df	Ms	F	P
Intercept	20343854	1	20343854	62035.52	<0.001
DPM	155549	11	14141		<0.001
Error	120681	368	328		

	Naive Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	9729182	1	9729182	45143.55	<0.001
DPM	8579	6	1430	63	<0.001
Error	64224	298	216		

	Young Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	14270476	1	14270476	52368.91	<0.001
DPM	156409	9	17379	63.78	<0.001
Error	84202	309	272		

	Old Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	17693954	1	17693954	38188.23	<0.001
DPM	156220	11	14202	30.65	<0.001
Error	168191	363	463		

Table V. Test result from one-way ANOVA run on Cephalothorax length per days past molting (DPM).

	Naive Redlice				
Effect	SS	Df	Ms	F	P
Intercept	75038239	1	75038239	339582,6	<0.001
DPM	10606	6	1768	8,0	<0.001
Error	71374	323	221		

	Young Redlice				
Effect	SS	Df	Ms	F	P
Intercept	66641312	1	66641312	388495,4	<0.001
DPM	50513	9	5613	32,7	<0.001
Error	59352	346	172		

	Old Redlice				
Effect	SS	Df	Ms	F	P
Intercept	70213941	1	70213941	399916,7	<0.001
DPM	46756	11	4251	24,2	<0.001
Error	64610	368	176		

	Naive Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	37182191	1	37182191	178665,2	<0.001
DPM	4179	6	696	3,3	0.003
Error	62017	298	208		

	Young Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	14270476	1	14270476	52368,91	<0.001
DPM	156409	9	17379	63,78	<0.001
Error	84202	309	272		

	Old Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	61802826	1	61802826	299621	<0.001
DPM	66578	11	6053	29,3	<0.001
Error	74876	363	206		

Table VI. Test result from one-way ANOVA run on length of posterior segments per days post molting (DPM).

	Naive Redlice				
Effect	SS	Df	Ms	F	P
Intercept	21569615	1	21569615	59745,43	<0.001
DPM	8604	6	1434	3,97	<0.001
Error	116611	323	361		

	Young Redlice				
Effect	SS	Df	Ms	F	P
Intercept	20459830	1	20459830	60523,92	<0.001
DPM	70246	9	7805	23,09	<0.001
Error	116964	346	338		

	Old Redlice				
Effect	SS	Df	Ms	F	P
Intercept	21062418	1	21062418	66887,06	<0.001
DPM	102449	11	9314	29,58	<0.001
Error	115881	368	315		

	Naive Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	9770232	1	9770232	20559,74	<0.001
DPM	19196	6	3199	6,73	<0.001
Error	141613	298	475		

	Young Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	14309650	1	14309650	29175,07	<0.001
DPM	156702	9	17411	35,50	<0.001
Error	151557	309	490		

	Old Oslofjord				
Effect	SS	Df	Ms	F	P
Intercept	19121736	1	19121736	43821,50	<0.001
DPM	153015	11	13910	31,88	<0.001
Error	158397	363	436		

Student-Newman-Keuls test

Table VII. P-values from Student-Newmans-Keuls tests, testing for differences in total length of Redlice copepodids between days post molting (DPM). Values for Old Redlice1DPM is not displayed.

Naive Redlice

	DPM	1	2	3	4	5	6	7
1	0		0,001	0,002	0,001	0,004	0,000	0,000
2	2	0,001		0,814	0,953	0,753	0,316	0,117
3	4	0,002	0,814		0,952	0,728	0,431	0,119
4	6	0,001	0,953	0,952		0,484	0,564	0,153
5	8	0,004	0,753	0,728	0,484		0,267	0,035
6	10	0,000	0,316	0,431	0,564	0,267		0,328
7	12	0,000	0,117	0,119	0,153	0,035	0,328	

Young Redlice

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,376	0,735	0,622	0,021	0,000	0,000	0,000	0,000	0,000
2	3	0,376		0,989	0,966	0,139	0,000	0,000	0,000	0,000	0,000
3	4	0,735	0,989		0,924	0,045	0,000	0,000	0,000	0,000	0,000
4	5	0,622	0,966	0,924		0,089	0,000	0,000	0,000	0,000	0,000
5	6	0,021	0,139	0,045	0,089		0,004	0,000	0,000	0,000	0,000
6	7	0,000	0,000	0,000	0,000	0,004		0,148	0,000	0,000	0,000
7	8	0,000	0,000	0,000	0,000	0,000	0,148		0,000	0,000	0,000
8	9	0,000	0,000	0,000	0,000	0,000	0,000	0,000		0,458	0,006
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,458		0,021
10	11	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,006	0,021	

Old Redlice

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,059	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,059		0,057	0,099	0,000	0,000	0,000	0,000	0,000	0,000	0,000
3	10	0,000	0,057		0,997	0,035	0,000	0,000	0,000	0,000	0,000	0,000
4	11	0,000	0,099	0,997		0,013	0,000	0,000	0,000	0,000	0,000	0,000
5	12	0,000	0,000	0,035	0,013		0,099	0,032	0,007	0,000	0,000	0,000
6	13	0,000	0,000	0,000	0,000	0,099		0,386	0,259	0,000	0,000	0,000
7	14	0,000	0,000	0,000	0,000	0,032	0,386		0,482	0,006	0,000	0,000
8	15	0,000	0,000	0,000	0,000	0,007	0,259	0,482		0,017	0,000	0,000
9	16	0,000	0,000	0,000	0,000	0,000	0,000	0,006	0,017		0,017	0,026
10	17	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,017		0,601
11	18	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,026	0,601	

Table VIII. P-values from Student-Newmans-Keuls tests, testing for differences in total length of Oslofjord copepodids between days post molting (DPM). Values for Old Oslofjord 1DPM is not displayed.

Naive Oslofjord

	DPM	1	2	3	4	5	6	7
1	0		0,791	0,317	0,542	0,317	0,623	0,003
2	2	0,791		0,277	0,549	0,414	0,507	0,002
3	4	0,317	0,277		0,512	0,757	0,088	0,064
4	6	0,542	0,549	0,512		0,957	0,274	0,033
5	8	0,317	0,414	0,757	0,957		0,216	0,051
6	10	0,623	0,507	0,088	0,274	0,216		0,000
7	12	0,003	0,002	0,064	0,033	0,051	0,000	

Young Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,334	0,323	0,007	0,006	0,000	0,000	0,000	0,000	0,006
2	3	0,334		0,123	0,000	0,000	0,000	0,000	0,000	0,000	0,000
3	4	0,323	0,123		0,082	0,058	0,000	0,000	0,000	0,000	0,035
4	5	0,007	0,000	0,082		0,924	0,003	0,000	0,000	0,000	0,960
5	6	0,006	0,000	0,058	0,924		0,006	0,000	0,000	0,000	0,860
6	7	0,000	0,000	0,000	0,003	0,006		0,032	0,007	0,000	0,006
7	8	0,000	0,000	0,000	0,000	0,000	0,032		0,373	0,001	0,000
8	9	0,000	0,000	0,000	0,000	0,000	0,007	0,373		0,007	0,000
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,001	0,007		0,000
10	11	0,006	0,000	0,035	0,960	0,860	0,006	0,000	0,000	0,000	

Old Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,016	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,016		0,136	0,012	0,001	0,000	0,000	0,000	0,000	0,000	0,000
3	10	0,000	0,136		0,170	0,047	0,001	0,000	0,000	0,000	0,000	0,000
4	11	0,000	0,012	0,170		0,321	0,050	0,014	0,000	0,000	0,000	0,000
5	12	0,000	0,001	0,047	0,321		0,177	0,111	0,006	0,003	0,000	0,000
6	13	0,000	0,000	0,001	0,050	0,177		0,513	0,134	0,107	0,000	0,011
7	14	0,000	0,000	0,000	0,014	0,111	0,513		0,207	0,242	0,001	0,051
8	15	0,000	0,000	0,000	0,000	0,006	0,134	0,207		0,729	0,034	0,393
9	16	0,000	0,000	0,000	0,000	0,003	0,107	0,242	0,729		0,047	0,339
10	17	0,000	0,000	0,000	0,000	0,000	0,000	0,001	0,034	0,047		0,159
11	18	0,000	0,000	0,000	0,000	0,000	0,011	0,051	0,393	0,339	0,159	

Table IX. P-values from Student-Newmans-Keuls tests, testing for differences in greatest width of Redlice copepodids between days post molting (DPM). Values for Old Redlice1DPM is not displayed

Naive Redlice

	DPM	1	2	3	4	5	6	7
1	0		0,455	0,581	0,026	0,029	0,032	0,034
2	2	0,455		0,806	0,122	0,108	0,160	0,182
3	4	0,581	0,806		0,123	0,077	0,185	0,227
4	6	0,026	0,122	0,123		0,851	0,961	0,991
5	8	0,029	0,108	0,077	0,851		0,970	0,989
6	10	0,032	0,160	0,185	0,961	0,970		0,939
7	12	0,034	0,182	0,227	0,991	0,989	0,939	

Young Redlice

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,001	0,001	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	3	0,001		0,687	0,161	0,001	0,000	0,000	0,000	0,000	0,000
3	4	0,001	0,687		0,168	0,000	0,000	0,000	0,000	0,000	0,000
4	5	0,000	0,161	0,168		0,030	0,007	0,004	0,000	0,000	0,000
5	6	0,000	0,001	0,000	0,030		0,541	0,315	0,000	0,000	0,000
6	7	0,000	0,000	0,000	0,007	0,541		0,958	0,000	0,000	0,000
7	8	0,000	0,000	0,000	0,004	0,315	0,958		0,000	0,000	0,000
8	9	0,000	0,000	0,000	0,000	0,000	0,000	0,000		0,006	0,020
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,006		0,468
10	11	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,020	0,468	

Old Redlice

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,460	0,363	0,470	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,460		0,951	0,833	0,003	0,000	0,000	0,000	0,000	0,000	0,000
3	10	0,363	0,951		0,928	0,005	0,000	0,000	0,000	0,000	0,000	0,000
4	11	0,470	0,833	0,928		0,004	0,000	0,000	0,000	0,000	0,000	0,000
5	12	0,000	0,003	0,005	0,004		0,178	0,011	0,008	0,000	0,000	0,000
6	13	0,000	0,000	0,000	0,000	0,178		0,188	0,098	0,000	0,000	0,000
7	14	0,000	0,000	0,000	0,000	0,011	0,188		0,927	0,010	0,000	0,000
8	15	0,000	0,000	0,000	0,000	0,008	0,098	0,927		0,020	0,000	0,000
9	16	0,000	0,000	0,000	0,000	0,000	0,000	0,010	0,020		0,015	0,202
10	17	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,015		0,130
11	18	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,202	0,130	

Table X. P-values from Student-Newmans-Keuls tests, testing for differences in greatest width of Oslofjord copepodids between days post molting (DPM). Values for Old Oslofjord 1DPM is not displayed.

Naive Oslofjord

	DPM	1	2	3	4	5	6	7
1	0		0,111	0,013	0,033	0,084	0,005	0,000
2	2	0,111		0,394	0,498	0,594	0,279	0,087
3	4	0,013	0,394		0,655	0,551	0,689	0,533
4	6	0,033	0,498	0,655		0,553	0,673	0,427
5	8	0,084	0,594	0,551	0,553		0,473	0,216
6	10	0,005	0,279	0,689	0,673	0,473		0,504
7	12	0,000	0,087	0,533	0,427	0,216	0,504	

Young Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,212	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	3	0,212		0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,000
3	4	0,000	0,002		0,100	0,047	0,020	0,000	0,000	0,000	0,000
4	5	0,000	0,000	0,100		0,948	0,397	0,004	0,000	0,000	0,099
5	6	0,000	0,000	0,047	0,948		0,632	0,005	0,000	0,000	0,146
6	7	0,000	0,000	0,020	0,397	0,632		0,030	0,000	0,000	0,226
7	8	0,000	0,000	0,000	0,004	0,005	0,030		0,004	0,000	0,185
8	9	0,000	0,000	0,000	0,000	0,000	0,000	0,004		0,003	0,000
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,003		0,000
10	11	0,000	0,000	0,000	0,099	0,146	0,226	0,185	0,000	0,000	

Old Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,004	0,015	0,003	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,004		0,619	0,917	0,355	0,117	0,003	0,000	0,000	0,000	0,000
3	10	0,015	0,619		0,407	0,246	0,029	0,000	0,000	0,000	0,000	0,000
4	11	0,003	0,917	0,407		0,559	0,158	0,003	0,000	0,000	0,000	0,000
5	12	0,000	0,355	0,246	0,559		0,291	0,030	0,001	0,001	0,000	0,000
6	13	0,000	0,117	0,029	0,158	0,291		0,138	0,025	0,026	0,000	0,000
7	14	0,000	0,003	0,000	0,003	0,030	0,138		0,265	0,383	0,000	0,002
8	15	0,000	0,000	0,000	0,000	0,001	0,025	0,265		0,836	0,006	0,037
9	16	0,000	0,000	0,000	0,000	0,001	0,026	0,383	0,836		0,007	0,024
10	17	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,006	0,007		0,430
11	18	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,037	0,024	0,430	

Table XI. P-values from Student-Newmans-Keuls tests, testing for differences in Cephalothorax length of Redlice copepodids between days post molting (DPM). Values for Old Redlice1DPM is not displayed

Naive Redlice

	DPM	1	2	3	4	5	6	7
1	0		0,003	0,010	0,015	0,000	0,000	0,000
2	2	0,003		0,648	0,490	0,427	0,071	0,138
3	4	0,010	0,648		0,844	0,333	0,012	0,042
4	6	0,015	0,490	0,844		0,298	0,015	0,047
5	8	0,000	0,427	0,333	0,298		0,230	0,268
6	10	0,000	0,071	0,012	0,015	0,230		0,597
7	12	0,000	0,138	0,042	0,047	0,268	0,597	

Young Redlice

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,755	0,221	0,019	0,024	0,000	0,000	0,000	0,000	0,000
2	3	0,755		0,273	0,011	0,018	0,000	0,000	0,000	0,000	0,000
3	4	0,221	0,273		0,213	0,165	0,000	0,000	0,000	0,000	0,000
4	5	0,019	0,011	0,213		0,773	0,004	0,000	0,000	0,000	0,000
5	6	0,024	0,018	0,165	0,773		0,005	0,000	0,000	0,000	0,000
6	7	0,000	0,000	0,000	0,004	0,005		0,264	0,002	0,001	0,000
7	8	0,000	0,000	0,000	0,000	0,000	0,264		0,019	0,030	0,001
8	9	0,000	0,000	0,000	0,000	0,000	0,002	0,019		0,853	0,386
9	10	0,000	0,000	0,000	0,000	0,000	0,001	0,030	0,853		0,258
10	11	0,000	0,000	0,000	0,000	0,000	0,000	0,001	0,386	0,258	

Old Redlice

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,366	0,311	0,314	0,093	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,366		0,133	0,066	0,009	0,000	0,000	0,000	0,000	0,000	0,000
3	10	0,311	0,133		0,761	0,453	0,000	0,000	0,000	0,000	0,000	0,000
4	11	0,314	0,066	0,761		0,441	0,001	0,000	0,000	0,000	0,000	0,000
5	12	0,093	0,009	0,453	0,441		0,006	0,000	0,000	0,000	0,000	0,000
6	13	0,000	0,000	0,000	0,001	0,006		0,256	0,367	0,291	0,272	0,251
7	14	0,000	0,000	0,000	0,000	0,000	0,256		0,878	0,716	0,858	0,807
8	15	0,000	0,000	0,000	0,000	0,000	0,367	0,878		0,903	0,867	0,681
9	16	0,000	0,000	0,000	0,000	0,000	0,291	0,716	0,903		0,922	0,855
10	17	0,000	0,000	0,000	0,000	0,000	0,272	0,858	0,867	0,922		0,922
11	18	0,000	0,000	0,000	0,000	0,000	0,251	0,807	0,681	0,855	0,922	

Table XII. P-values from Student-Newmans-Keuls tests, testing for differences in Cephalothorax length of Oslofjord copepodids between days post molting (DPM). Values for Old Oslofjord 1DPM is not displayed.

Naive Oslofjord

	DPM	1	2	3	4	5	6	7
1	0		0,552	0,605	0,465	0,118	0,665	0,006
2	2	0,552		0,718	0,716	0,303	0,849	0,032
3	4	0,605	0,718		0,766	0,398	0,854	0,063
4	6	0,465	0,716	0,766		0,384	0,609	0,126
5	8	0,118	0,303	0,398	0,384		0,350	0,283
6	10	0,665	0,849	0,854	0,609	0,350		0,067
7	12	0,006	0,032	0,063	0,126	0,283	0,067	

Young Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,212	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	3	0,212		0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,000
3	4	0,000	0,002		0,100	0,047	0,020	0,000	0,000	0,000	0,000
4	5	0,000	0,000	0,100		0,948	0,397	0,004	0,000	0,000	0,099
5	6	0,000	0,000	0,047	0,948		0,632	0,005	0,000	0,000	0,146
6	7	0,000	0,000	0,020	0,397	0,632		0,030	0,000	0,000	0,226
7	8	0,000	0,000	0,000	0,004	0,005	0,030		0,004	0,000	0,185
8	9	0,000	0,000	0,000	0,000	0,000	0,000	0,004		0,003	0,000
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,003		0,000
10	11	0,000	0,000	0,000	0,099	0,146	0,226	0,185	0,000	0,000	

Old Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,302	0,004	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,001
2	9	0,302		0,029	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,023
3	10	0,004	0,029		0,446	0,217	0,001	0,000	0,000	0,000	0,000	0,654
4	11	0,000	0,002	0,446		0,535	0,041	0,000	0,001	0,002	0,027	0,553
5	12	0,000	0,000	0,217	0,535		0,072	0,004	0,005	0,009	0,077	0,346
6	13	0,000	0,000	0,001	0,041	0,072		0,302	0,396	0,368	0,716	0,005
7	14	0,000	0,000	0,000	0,000	0,004	0,302		0,984	0,694	0,353	0,000
8	15	0,000	0,000	0,000	0,001	0,005	0,396	0,984		0,910	0,501	0,000
9	16	0,000	0,000	0,000	0,002	0,009	0,368	0,694	0,910		0,325	0,000
10	17	0,000	0,000	0,000	0,027	0,077	0,716	0,353	0,501	0,325		0,002
11	18	0,001	0,023	0,654	0,553	0,346	0,005	0,000	0,000	0,000	0,002	

Table XIII. P-values from Student-Newmans-Keuls tests, testing for differences in posterior segments length of Redlice copepodids between days post molting (DPM). Values for Old Redlice1DPM is not displayed

Naive Redlice

	DPM	1	2	3	4	5	6	7
1	0		0,017	0,042	0,072	0,761	0,137	0,004
2	2	0,017		0,679	0,717	0,030	0,563	0,619
3	4	0,042	0,679		0,717	0,062	0,650	0,633
4	6	0,072	0,717	0,717		0,085	0,601	0,579
5	8	0,761	0,030	0,062	0,085		0,109	0,009
6	10	0,137	0,563	0,650	0,601	0,109		0,375
7	12	0,004	0,619	0,633	0,579	0,009	0,375	

Young Redlice

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,372	0,995	0,402	0,347	0,052	0,004	0,000	0,000	0,000
2	3	0,372		0,182	0,129	0,752	0,356	0,104	0,000	0,000	0,000
3	4	0,995	0,182		0,675	0,225	0,034	0,003	0,000	0,000	0,000
4	5	0,402	0,129	0,675		0,092	0,005	0,000	0,000	0,000	0,000
5	6	0,347	0,752	0,225	0,092		0,291	0,122	0,000	0,000	0,000
6	7	0,052	0,356	0,034	0,005	0,291		0,366	0,000	0,000	0,000
7	8	0,004	0,104	0,003	0,000	0,122	0,366		0,000	0,000	0,000
8	9	0,000	0,000	0,000	0,000	0,000	0,000	0,000		0,729	0,033
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,729		0,031
10	11	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,033	0,031	

Old Redlice

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,005	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,005		0,272	0,289	0,005	0,006	0,006	0,001	0,000	0,000	0,000
3	10	0,000	0,272		0,630	0,130	0,086	0,191	0,080	0,000	0,000	0,000
4	11	0,000	0,289	0,630		0,074	0,071	0,097	0,029	0,000	0,000	0,000
5	12	0,000	0,005	0,130	0,074		0,830	0,952	0,811	0,001	0,000	0,000
6	13	0,000	0,006	0,086	0,071	0,830		0,959	0,839	0,001	0,000	0,000
7	14	0,000	0,006	0,191	0,097	0,952	0,959		0,578	0,001	0,000	0,000
8	15	0,000	0,001	0,080	0,029	0,811	0,839	0,578		0,002	0,000	0,000
9	16	0,000	0,000	0,000	0,000	0,001	0,001	0,001	0,002		0,007	0,015
10	17	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,007		0,539
11	18	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,015	0,539	

Table XIV. P-values from Student-Newmans-Keuls tests, testing for differences in posterior segments length of Oslofjord copepodids between days post molting (DPM). Values for Old Oslofjord 1DPM is not displayed

Naive Oslofjord

	DPM	1	2	3	4	5	6	7
1	0		0,681	0,249	0,835	0,670	0,057	0,156
2	2	0,681		0,156	0,637	0,794	0,117	0,037
3	4	0,249	0,156		0,361	0,185	0,002	0,492
4	6	0,835	0,637	0,361		0,519	0,064	0,170
5	8	0,670	0,794	0,185	0,519		0,161	0,055
6	10	0,057	0,117	0,002	0,064	0,161		0,000
7	12	0,156	0,037	0,492	0,170	0,055	0,000	

Young Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10
1	1		0,485	0,460	0,033	0,040	0,000	0,000	0,000	0,000	0,038
2	3	0,485		0,321	0,005	0,009	0,000	0,000	0,000	0,000	0,007
3	4	0,460	0,321		0,142	0,090	0,000	0,000	0,000	0,000	0,130
4	5	0,033	0,005	0,142		0,899	0,024	0,011	0,000	0,000	0,838
5	6	0,040	0,009	0,090	0,899		0,035	0,008	0,000	0,000	0,814
6	7	0,000	0,000	0,000	0,024	0,035		0,530	0,009	0,000	0,036
7	8	0,000	0,000	0,000	0,011	0,008	0,530		0,020	0,000	0,011
8	9	0,000	0,000	0,000	0,000	0,000	0,009	0,020		0,073	0,000
9	10	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,073		0,000
10	11	0,038	0,007	0,130	0,838	0,814	0,036	0,011	0,000	0,000	

Old Oslofjord

	DPM	1	2	3	4	5	6	7	8	9	10	11
1	7		0,007	0,005	0,001	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	9	0,007		0,694	0,559	0,334	0,186	0,298	0,005	0,000	0,000	0,000
3	10	0,005	0,694		0,526	0,453	0,304	0,364	0,015	0,002	0,000	0,000
4	11	0,001	0,559	0,526		0,678	0,569	0,471	0,068	0,012	0,000	0,000
5	12	0,000	0,334	0,453	0,678		0,653	0,905	0,179	0,064	0,000	0,000
6	13	0,000	0,186	0,304	0,569	0,653		0,837	0,186	0,106	0,000	0,000
7	14	0,000	0,298	0,364	0,471	0,905	0,837		0,232	0,071	0,000	0,000
8	15	0,000	0,005	0,015	0,068	0,179	0,186	0,232		0,482	0,000	0,000
9	16	0,000	0,000	0,002	0,012	0,064	0,106	0,071	0,482		0,000	0,000
10	17	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000		0,475
11	18	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,475	

Table XV. P-values from Student-Newmans-Keuls tests, testing for difference in total length between infective groups of 6DPI and naïve groups of 8DPM.

	Group	1 751.58	2 779.86	3 782.54	4 745.09	5 821.49	6 809.66
1	Naive Redlice		0,000	0,000	0,283	0,000	0,000
2	Young Redlice	0,000		0,642	0,000	0,000	0,000
3	Old Redlice	0,000	0,642		0,000	0,000	0,000
4	Naive Oslofjord	0,283	0,000	0,000		0,000	0,000
5	Young Oslofjord	0,000	0,000	0,000	0,000		0,040
6	Old Oslofjord	0,000	0,000	0,000	0,000	0,040	

Table XVI. P-values from Tukey HSD tests, testing for difference in total length between infective groups of 6DPI and naïve groups of 8DPM.in total length between groups at 6dpi or 8dpm.

	Group	1 751.58	2 779.86	3 782.54	4 745.09	5 821.49	6 809.66
1	Naive Redlice		0,000	0,000	0,891	0,000	0,000
2	Young Redlice	0,000		0,997	0,000	0,000	0,000
3	Old Redlice	0,000	0,997		0,000	0,000	0,000
4	Naive Oslofjord	0,891	0,000	0,000		0,000	0,000
5	Young Oslofjord	0,000	0,000	0,000	0,000		0,311
6	Old Oslofjord	0,000	0,000	0,000	0,000	0,311	

Table XVII. Test results for Kolmogorov-Smirnov test for normality. All parameters but width are normal distributed (P > 0.05).

Total Length			
Group	n	Max D	**P**
Redlice Naive	330	0,032212	p > .20
Redlice Young	356	0,043857	p > .20
Redlice Old	380	0,043012	p > .20
Oslofjord Naive	305	0,028705	p > .20
Oslofjord Young	319	0,043288	p > .20
Oslofjord Old	375	0,029881	p > .20

Width			
Group	n	Max D	**P**
Redlice Naive	330	0,041595	p > .20
Redlice Young	356	0,060246	p < ,20
Redlice Old	380	0,089461	p < ,01
Oslofjord Naive	305	0,045533	p > .20
Oslofjord Young	319	0,060640	p < ,20
Oslofjord Old	375	0,090939	p < ,01

Cephalothorax Length			
Group	n	Max D	**P**
Redlice Naive	330	0,039544	p > .20
Redlice Young	356	0,037906	p > .20
Redlice Old	380	0,060258	p < ,15
Oslofjord Naive	305	0,027391	p > .20
Oslofjord Young	319	0,035185	p > .20
Oslofjord Old	375	0,039443	p > .20

Length of posterior segments			
Group	n	Max D	**P**
Redlice Naive	330	0,046229	p > .20
Redlice Young	356	0,047878	p > .20
Redlice Old	380	0,063047	p < ,10
Oslofjord Naive	305	0,047872	p > .20
Oslofjord Young	319	0,042013	p > .20
Oslofjord Old	375	0,036619	p > .20

Table XVIII. Results from the Levine`s F-test testing for homogeneity of variance in the total length results. Even though significant differences occur (P<0,05) the F values are below 5.2 and regarded as acceptable.

Group	Ms effect	Ms error	**F**	**P**
Oslofjord Naive	1494,111	284,2176	5,256926	0,000036
Oslofjord Young	349,3136	241,8188	1,444526	0,168205
Oslofjord Old	531,0413	212,5476	2,498458	0,004895
Rødlus Naive	865,2345	259,6852	3,331859	0,003375
Rødlus Young	427,5198	163,4395	2,615768	0,006334
Rødlus Old	144,2561	179,0559	0,805648	0,634475

Appendix II

Recipies and sequences

Table XIX. Karnovsky fixative recipe (Karnovsky 1965). The basic media, buffer and fixative are prepared separately and mixed upon use.

Quantity	Substance	Function
6,75g	NaCl	Basic media
0,105g	KCl	Basic media
0,09g	$CaCl_2$	Basic media
0,015g	$NaHCO_3$	Basic media
1000ml	Double distilled H_2O	Basic media
1,5g	Glucose	Basic media
1,84g	$NaH_2PO_4 \cdot H_2O$	Buffer
8,41g	$Na_2HPO_4 \cdot 2H_2O$	Buffer
10ml	25% glutaraldehyd	Fix
10ml	10% paraformaldehyd	Fix
4g	Sukrose	-

Table XX. LsTryp1 and LsTryp2 sequences. The probe location is outlined

LsTryp1Forward primer TGTTCCTAGTTGTTGCAGCCAATGC -607bp

1 tccttatcca tcaaatccaa tcttatttta cagacatgat gaaattcgtc tgtacccttt
61 ***tgttcctagt tgttgcagcc aatgc****tgttc cccctcagat caaatactct gagagtttca*
121 *tgaaagtcaa gtccatgaga cacagattcg gtggaagaat cgtcggagga gaagaagttg*
181 *aacccaactc tattcccttc caaatttcat tccaaactac aggaggattc cacttctgtg*
241 *gtgcttctgt catggacaag gacactatca tcactgctgc tcattgctgt gacagcttct*
301 *acccatcgga agtccaagtt gttgctggtg aacacgatct cttttctact agtggagatg*
361 *aacaaaagat tgctgtatct gatatcacat accacgagaa gtttgcatcc catggaacca*
421 *actatgacgt ttgtctttta aaattgaagt catccttgca cttcaacgag taagaactct*
481 *atcatgccat tcctgtgaca atcgttaatt tttattttct taataggaaa gtcaagccta*
541 *ttgctctccc agaaaaggac caagaattca tcggtgatgt tgttgtttcc ggatggggaa*
601 *ctatctcctc cagtggtcca ccttctccag ttcttaaagc tgttacagtc caagttgttt*
661 *ctgatga*agg tgagactcat aatcatattc atatacgatg tttatgaatt tacataagat
721 attttatatt agactgtagc gatgcctact atggatctat tgatgagacc atgatctgtg
781 ctgctgcccc tggaaaagac tcctgccaag gcgactctgg tggcccattg gctcaagacg
841 gaaccctcgt tggcattgtt tcctggggat atggttgtgc tgctcctgga tacccaggtg
901 tctacggcaa ggtctccaag ttcattgact ggattgctga acaccaatag ataaattttg
961 atttcatttg tcaattattt gaaagttttt gaaaatatat ccaccca

LsTryp2 Forward primer GAAATGATGAAATTCTTGGGTGCCC -588bp

1 catagcagta agttactcaa gattattagt tcaaaataat atttcaactt taatatttca
61 *ta****gaaatgat gaaattcttg ggtgccc****ttt tgttcttggc tgctgccgcc catgctgctc*
121 *ctagaatgaa acaaagtgga agaattgtcg gaggagatga agttgagcca aactcaattc*
181 *ccttccaaat ttctttccaa actattacag gattccactt ttgtggtggc tctgtcatgg*
241 *acaaggatac cattattact gctgctcatt gctgtgatgg cttcagagcc gaagacgttc*
301 *aaatcattgc tgctgaacac gatctcttct ctataagtgg agatgaacaa aaggttggtg*
361 *tatcaaaaat cacctaccac gagaagtttg gatcccatgg caccaactat gacgtttgtc*
421 *ttttgaaatt gaagtcctcc ttggacctca acgagaaagt caagccagtt gctctcccag*
481 *aaaaggacca agaattcact ggtgatgttg ttgtgtctgg atggggaact atcgcctcca*
541 *atggcccatc ttctccagtc cttagatcag ttaccgtcca cgttgtttct gatgaggact*
601 *gtagcgatgc ctactttgga tctactgatg agaccatgat ctgtgctgct* gctccaggaa
661 aggactcctg ccaaggtgac tctggtggtc cattggctca agatggaacc ttagttggta
721 ttgtttcttg gggatatggt tgtgctgctc ctggataccc aggtgtctac ggcaaggtct
781 ccaagtttat tgactggatc gctgaaacaa aataatttat taggttattg attttgaact
841 tttgaaatat aatattttca aaaaaaaaaa aaaaaaaaaa aaa

Table XXI. Specific primers for LsTryp1 and LsTryp2. Manufactured by Invitrogen.

Tryp1F	TGTTCCTAGTTGTTGCAGCCAATGC
Tryp1F-SP6	GATTTAGGTGACACTATAGTGTTCCTAGTTGTTGCAGCCAATGC
Tryp 1R	GCAGCAGCACAGATCATGGTCTCA
Tryp 1R-SP6	GATTTAGGTGACACTATAGGCAGCAGCACAGATCATGGTCTCA
Tryp2F	GAAATGATGAAATTCTTGGGTGCCC
Tryp2F-SP6	GATTTAGGTGACACTATAGGAAATGATGAAATTCTTGGGTGCCC
Tryp2R	AGCAGCAGCACAGATCATGGTCTCA
Tryp2R-SP6	GATTTAGGTGACACTATAGAGCAGCAGCACAGATCATGGTCTCA

Table XXII. Polymerase chain reaction recipe.

Volume (µl)	Substance
10	Flexi b
4	Mg_2^+ (25 mM)
4	dNTP (1,25 mM)
1	FW-primer
1	RW-primer
0,4	Gold Taq
3	Template
26,6	H_2O

Table XXIII. Recipe for synthesis of ISH probes.

Volume (µl)	Substance
9	PCR product
4	Nuclease free water
2	DIG label mix
2	Buffer
2	Polymerase
1	Protector RNAase inhib

Appendix III

Histology protocol

UTTDRAG FRA DRIFTSPROSEDYRE FOR HISTOLOGI

MOLEKYLÆRBIOLOGISK LAB, HAVFORSKNINGSINSTITUTTET

3.7.3 Fremstilling av parafininnleirede preparater til lysmikroskopiering.

I) Instrumenter, utstyr, kjemikalier og oppskrifter – se vedlegg..

II) Journalføring / merking av histologiprøver

Histologijournalene der man skal føre inn vevsprøver som skal innleires i parafin står i bokhyllen på histologilaboratoriet.

TRINN	ARBEIDSBESKRIVELSE
1	Finn frem histologijournal, kulepenn, sprittusj og prøveglasset med vevet som skal journalføres.
2	Finn frem det neste ledige kronologiske journalnummeret i boken. Eksempel; F-3/00 (Histologiprøve nr 3 i år 2000) neste blir da F-4/00 osv. Bokstaven F. foran prøvenummeret betyr fisk nr.
3	Noter etter journalnummeret i boken opplysninger som førsøksnavn, fiskeart, dato for prøvetak og eventuelt andre opplysninger som er gitt.
4	Merk prøveglasset med vevsbiten tydelig med journalnummeret (sprit tusj).

3.7.3.1 Dehydrering / Parafin innfiltrering

Innfiltreringen må bare utføres i godkjente avtrekkskap for histologisk arbeid.

Bruk alltid nitril hansker.

Histokinetten gir helautomatisk infiltrering av formalinfiksert vev med parafin/Histovoks.

TRINN	ARBEIDSBESKRIVELSE
1	Formalinfiksert vev overføres fra journalmerkete prøveglass til støpekassetter (WLAS-1060) som merkes med prøvenes journalnr. Rester av fikseringsmidlet behandles som spesialavfall.
2	Merk støpekassettene med *blyant* da tusj er spritløselig.
3	Lukk støpekassettene *godt.*

4	Slå på histokinetten (“Start”). Trykk på hevefunksjonen (“Normal raise rotor”). Støpekassettene legges i histokinettens metallkurv som plasseres i histokinettens karusell i posisjonen *før* bad 1 (Over bad 12) da karusellen alltid går en posisjon frem når histokinetten startes.
5	Kontroller at kassettene blir dekket av infiltrasjons-væskene i de 12 badene, om nødvendig etterfyll med formalin, etanol, xylen eller histowax. Når det er behov for utskifting av løsningene (eventuelt blakking og fargenedslag) behandles løsningene i bad 1, 2, 9,10,11 og 12 som risikoavfall. Løsningene i de andre badene tømmes ut i vask i avtrekkskap, skyll etter med rennende vann.
6	Sjekk at utgangsplasseringen på displayhjulet er korrekt, rett over start. Senk histokinetten (“Normal raise rotor”). Nå som histokinetten er startet vil karusellfunksjonen automatisk føre metallkurven med støpekassettene gjennom 12 bad med infiltrasjonsløsninger. (Bad 1. 4% fosfatbufret formalin 1 time) Bad 2. 50 % etanol 1 time Bad 3. 70 % etanol 1 time Bad 4. 80 % etanol 1 time Bad 5. 96 % etanol 1 time Bad 6. 96 % etanol 1 time Bad 7. 100 % etanol 1 time Bad 8. 100 % etanol 1 time Bad 9. Xylen, hist 2 timer Bad 10. Xylen, hist 2 timer Bad 11. Parafin/Histowax 56-58^{o} C 2 timer Bad 12. Parafin/Histowax 56-58^{o} C 2 timer +

3.7.3.2 Parafininnleiring

For instrumenter, utstyr og kjemikalier, se vedlegg.

Innleiringsmaskinen og pinsettvarmeren slås på en halv time før støpingen skal skje.
Om nødvendig etterfyll med histowax i pelletform i innleiringsmaskinen.
Husk å slå av støpemaskinen og pinsettvarmeren etter bruk.
Arbeid utføres i avtrekkskap, bruk nitril engangshansker.

TRINN	ARBEIDSBESKRIVELSE
1	Når histokinetten er ferdig med syklusen lyser det rødt på histokinettens frontpanel og prøvene står i flytende parafin/histowax (56-58^{o}C), bad 12.
2	Karusellen heves ("Normal raise rotor") og støpekassetten legges på varmeplaten til innleiringsmaskinen. Unngå å søle parafin ved overføringen. Lukk histokinetten ("Normal raise rotor") og slå av maskinen ("Start").
3	En metallstøpeform fylles med flytende parafin (Histowax 56-58^{o}C) fra innleiringsmaskinens dispenser.
4	Vevet plasseres i støpeformen med ønsket snittflate *ned.*
5	Metallformen plasseres på kjøleelement som oppbevares i frys. Med en oppvarmet pinsett presses vevsbiten forsiktig mot bunnen av metallformen til vevet fester seg til metallet. Arbeid raskt da vevet vil feste seg på pinsetten straks den kjølner.
6	Den merkete delen av støpekassetten fra infiltrasjonsprosessen plasseres over metallformen med vevsbiten slik at journalnummeret vises. Formen fylles opp med flytende parafin. Støpekassetten bør fylles helt opp med flytende parafin for å unngå løsriving under vevssnittingen. Dersom det går for lang tid før det andre parafinlaget blir lagt på det første kan det lett oppstå bruddflater, arbeid derfor hurtig.
7	Kjøleelementet med metallformen settes deretter i fryser (- 20^{o}C) i 5-10 minutter, da løsner parafinblokken lett fra metallformen.

3.7.3.3 Semisnitting

For instrumenter og utstyr, se vedlegg.

Arbeid utføres ved lab. Benk.

Nedenfor beskrives semisnitting av vev innleiret i parafin med mikrotom (Reichart-Jung Biocut 2035).

TRINN	ARBEIDSBESKRIVELSE
1	Engangskniven er meget kvass, så benytt alltid pensel ved fjerning av parafin fra knivseggen. Håndter kniven med varsomhet.
2	Parafinblokken legges på kjøleelement en tid før snitting. Kald parafin er lettere å arbeide med. OBS! Ved for lav temperatur over lengre tid kan parafinen og vevsbiten sprekke.
3	Overflødig parafin fjernes rundt parafinblokken med en bordkniv.
4	Parafinblokken festes i hurtigklemmen for parafinblokker vertikalt på rotasjonsmikrotomen.
5	Parafinblokken føres nå forsiktig mot engangskniven som er festet i et eget knivholderfeste.
6	Blokken kan føres fremover mot kniven eventuelt bakover bort fra kniven manuelt eller halvautomatisk med sveivene på venstre og høyre side av mikrotomen.
7	Bak hurtigklemmen ligger et orienterbart feste. Ved å vri på skruene rundt dette festet kan man få orientert den ønskete snittflaten mot kniven.
8	Når man skal snitte er det viktig at kniven er vinklet 4 grader i forhold til parafinblokken. Denne vinkelen stiller man med ved å regulere festet for knivholderen der det er avmerket en skala fra 1-10 grader.
9	Når man har fått blottlagt den ønskete vevsflaten ved forsiktig grovsnitting gjennom parafinen (venstre sveiv) stiller man halvautomatikken på 2–4 μm. Ta 2-3 prøvesnitt med denne snitt-tykkelsen, disse kastes da det ofte er resonans i snittene.
10	Nå er det tid for å ta det første vevssnittet, høyre sveiv tas en omdreining. Det bør nå ligge ett vevssnitt på kniven. Dersom det er *vanskelig* å få snitt hjelper det å puste på vevsflaten eventuelt fukte den med litt vann.

11	Med en tynn pensel vipper man den siden av vevssnittet som har ligget mot kniven ned på et objektglass med en film av kaldt springvann. Det kalde vannet får vevssnittet til å strekke seg.
12	Snittet legges forsiktig ned i et vannbad (46-48^{o}C) med destillert vann for å strekke seg ytterligere.
13	Vevssnittet fanges nå opp fra vannbadet med et objektglass m/skrivefelt som føres 90 grader gjennom vannoverflaten mot parafinsnittet slik at det fester seg til glasset.
14	Ta ut parafinblokken av hurtigklemmen og merk objektglasset med *blyant.* Gi objektglasset identisk journalnummer med parafinblokken.
15	Parafinblokken lagres i egnet arkivsystem. Parafinblokken lagres i henhold til journalmerkingen.
16	Det merkede (journalnr) objektglasset med vevssnittet plasseres i en objektglassholder.
17	Objektglassholderen plasseres i varmeskap ved 58^{o}C i 30 minutter. Vevssnittet vil feste seg til objektglasset og tåle videre behandling uten å falle av. Vevssnittet er nå klart til farging.
18	Etter avsluttet snitting fjernes kniven fra knivholderen av sikkerhetsmessige årsaker. Mikrotomen og området rundt den støvsuges for løse parafinrester. Resterende parafinrester fjernes fra mikrotomen med 70% etanol på en stor pensel. Tørk over etterpå med papir. NB!! Snitt som beveger seg fritt i rommet gjør gulvet såpeglatt!! Det er derfor viktig å bruke håndstøvsugeren for å få fjernet alle overflødige snitt under og etter snitting.

3.7.3.4 Rutinefarging

I) Hydrering

Parafinen i det histologiske snittet (objektglasset med semisnittet vevsmateriale) erstattes med vann gjennom flere infiltrasjonsbad (Labtec eller Assistent).
Arbeid utføres i avtrekk nr B på rom 119, bruk engangs nitril hansker.

TRINN	ARBEIDSBESKRIVELSE
1	Hydreringen utføres manuelt ved at objektglassholderen med det histologiske snittet føres gjennom flere infiltreringsbad.
2	Xylen (Bad 1/Bad 2) vasker bort siste rest av parafin i vevssnittet.
3	Etanol (Bad 3-Bad 7) vasker bort xylen fra vevssnittet.

I a) Fast oppsett hydreringsbad

BAD	LØSNING	TID (minutter)
1	Xylen – hist	10
2	Xylen – hist	10
3	100% etanol	5
4	100% etanol	5
5	96% etanol	5
6	80% etanol	5
7	50% etanol	5
8	Springvann	5

Når det er behov for å skifte løsningene i bad 1, 2,3,4 og 5 skal løsningene behandles som spesialavfall. Løsningene i de andre badene kan tømmes ut i vask i avtrekk, skyll etter med vann.

II) Standard rutine farge; HES - farge

Hematoxylin farger blant annet de basofile delene av cellene dvs. kjernen blå.

Erytrosin farger blant annet de acidofile (eosinofile) delene av cellene, dvs. cytoplasma rødlig. I tillegg har erythrosin en sterkere affinitet til muskelfibre som gjør at erythrocyttene (røde blodceller) får en spesiell klar rød farge.

Saffron er en gul plantefarge med spesiell affinitet til bindevev.

Fargeprosessen utføres i avtrekkskap, og med engangs nitril hansker.

Vei inn stoffene i veiebenken, ikke ha på ventilasjon da fargestoffene er i pudderform og dermed veldig "flyktige". Bruk nitril engangshansker og munnbind. Intet søl med fargestoff må forekomme.

Husk å rydde og vaske vekten og benkeplaten i veiebenken etter bruk.

Se også kapittelet Vevsfarging.

II a) Fast oppsett fargebad

Ved å føre objektglassholderen med vevssnittet for hånd gjennom

Farge / infiltrasjonsbadene (Bad 9-Bad 20) blir vevssnittet HES-farget. Snittene blir dehydrert og vann blir erstattet med xylen.

BAD	LØSNING	TID (minutter)
9	Haematoxylin	2,5
10	Springvann	4
11	1% Erythrosin vandig, pH = 6,5	1,5
12	Springvann	1
13	96% etanol	1
14	100% etanol	1
15	100% etanol	1
16	Alkoholisk saffron	20 sekunder
17	100% etanol	1
18	100% etanol	1
19	Xylen-hist	5
20	Xylen-hist	5

Når det er behov for å skifte løsningene i bad 19 og 20 behandles disse som spesialavfall.

Løsningene i de andre badene tømmes i vask i avtrekksskap, etterskyll med rennende vann.

II b) Tillaging av fargeløsning, HES-farge se vedlegg.

3.7.3.5 Dekking / montering/ arkivering

For utstyr se vedlegg.

Alt arbeid utføres i avtrekkskap og med engangs nitril hansker

Vevssnittet fra fargeprosedyren holdes fuktig i xylen (Bad 20).

TRINN	ARBEIDSBESKRIVELSE
1	Med en pinsett løftes objektglasset ut av objektglassholderen og plasseres på en ren bomullsfille med vevssnittet opp.
2	En dråpe med limen Histokitt fra en limtube plasseres på ett dekkglass som hvelves over vevssnittet (Histokitt, syntetisk lim med samme brytningsindeks som lys, inneholder xylen).
3	Dekkglasset gis et *lett trykk* med pinsetten slik at all luft mellom det og objektglasset forsvinner.
4	Legg objektglasset på en preparatmappe uten lokk.
5	Plasser et messinglodd, 20-50 g, oppå dekkglasset. Se til at preparatmappen ligger i water, da unngår en at loddet begynner å bevege seg.
6	Etter en til to dager avdamping av løsemiddel (xylen) i avtrekk er det histologiske preparatet klart til bli vurdert av en histolog.
7	Etter at snittet er ferdig vurdert lagres det i egnet arkivsystem. Snittene lagres fortløpende i henhold til journalmerkingen.

3.7.4 Fremstilling av resininnleirede preparater til lysmikroskopiering.

I) Instrumenter, utstyr, kjemikalier og oppskrifter – se vedlegg.

II) Journalføring / Merking

Hver fremføring av vevsprøver til resin føres i lab. journal. Her noteres forsøksnavn, fiskeart, dato for fremføring, prøvenummer samt fulgt prosedyre. Alt som skjer med prøvene under fremføring noteres.
Viktig informasjon om vevsprøve følger vevet i alle stadier, fra fiksering til objektglass med snitt. Informasjon om vevsprøve kan bl a være forsøksnavn, navn på forsøksansvarlig, fiskeart, type vev, dato for prøvetaking, snittykkelse osv. All merking i forbindelse med prøven skal skje med gråblyant.

Ulike løsninger og glassutstyr som er i bruk skal merkes med innhold, dato og navn på bruker. Det skal aldri være tvil om hvilke kjemikalier en til en hver tid behandler, det gjelder også for annet lab. personell som i en gitt situasjon må overta andres arbeid.

3.7.4.1 Dehydrering

Alt arbeid utføres i avtrekk og med engangshansker på histologilaboratoriet.

TRINN	ARBEIDSBESKRIVELSE
1	Ta vevet ut av fikseringen og over i merkede prøverør med tett kork. Eventuelle rester av fikseringsløsningen tas vare på som spesialavfall.
2	Prosentene oppgitt for etanolløsninger regnes ut fra: 96% etanol m/ 2% metylisobutylketon er det samme som 95% etanol.
3	Vevet skal gjennomgå følgende dehydrering: 70% etanol 1 time 70% etanol natten over 90% etanol 1 time 90% etanol 1 time 95% etanol 1 time Forholdet mellom vevsmengde og dehydreringsvæske må minimum være 1 : 10.

4	La vevet stå på omrøring, uten magnet. Omrøringen må være kraftig nok til å sikre god utskiftning mellom løsning og vev, men ikke så kraftig at vevsprøven blir skadet. Alle oppgitte tider i dehydreringen er minimumstider, store mengder vev krever lengre tid.

3.7.4.2 Infiltrering

Alt arbeid utføres i avtrekk og med engangshansker på histologilaboratoriet.

TRINN	ARBEIDSBESKRIVELSE
1	Resinkit består av tre deler: basic resin hardener 1/activator (pulver) hardener 2 (væske) Blandingsforholdene mellom de ulike komponentene vil variere for de ulike kit. Følg instruksene som er i det enkelte kit.
2	Alle typer aktivert resin, dvs gitt blanding av basic resin og hardener 1 / activator, oppbevares i mørk glassflaske i kjøleskap. Ved bruk skal løsningene holde romtemperatur. Brukt aktivert resin blir oppbevart som spesialavfall. Husk å merke flaskene godt.
3	Nylaget aktivert resin blir *etter* bruk filtrert og lagret som aktivert resin *X1*. Aktivert resin X1 blir etter bruk filtrert og blandet 1:1 med 95% etanol, løsningen lagres som aktivert resin *X2*.
3	Væskenivået ved infiltrering skal være minimum 10 x vevsmengde.
4	Gjennomfør pre–infiltrering : Aktivert resin X2 ……. 2 timer – natten over Økt mengde vev gir økt tid. Husk tilstrekkelig omrøring. Brukt aktivert resin X2 blir oppbevart som spesialavfall. Herdet resin kan kastes som vanlig avfall.
5	Gjennomfør infiltrering : Aktivert resin X1 …….. natten over Nylaget aktivert resin …… natten over Sørg for tilstrekkelig omrøring vha røreverk. Omrøringen må ikke

	fysisk skade vevsprøvene.

3.7.4.3 Innleiring / Polymerisering

Alt arbeid utføres i avtrekk og med engangshansker på histologilaboratoriet.

TRINN	ARBEIDSBESKRIVELSE
1	Plasser Historesin mold trays (mykt teflonbrett med brønner) på is. Legg vevet i brønnene, pass på orienteringen av vevet. Skriv prøvenummer ved brønnen med vannfast tusj. Lag gjerne to paralleller av hver vevsprøve.
2	Bland nylaget aktivert resin og hardener 2, i et begerglass. Tilsett løsningen med engangs pasteurpipette til brønnene på Historesin mold trayet. Brønnene skal være ca. halvfulle. Fortsett å ha Historesin mold trayene på is til den første eksoterme reaksjonen er over, ca 15 – 30 min.
3	Etter endt tid på is plasseres Historesin mold trayene i avtrekkskap i romtemperatur og dekkes av hardplastplater. Polymeriseringen tar 2 – 5 timer eller natten over.
4	Herdet resin kan kastes som vanlig avfall.

3.7.4.4 Montering av blokkholder

I) Montering av blokkholder

Alt arbeid utføres i avtrekkskap og med engangshansker.

TRINN	ARBEIDSBESKRIVELSE
1	Skriv prøvenummer, og eventuelt andre viktige opplysninger, med gråblyant på alle Historesin mold tray adapters (blokkholdere). Sett blokkholderne i avtrekket ved siden av Historesin mold trayene.
2	Ta av hardplastplatene som dekker de polymeriserte vevsprøvene i Historesin mold trayene.
3	Bland raskt sammen Mounting medium, dvs pudder- og væskekomponent, i et forhold gitt av produsenten i et stort engangs veieskip. Hell straks et lag av Mounting medium over det ferdig polymeriserte vevet i brønnene. Plasser Historesin mold tray adapter, med korrekt merking, i brønnene.

4	La Mounting medium herde i ca 15 minutter. Løsne de ferdige blokkene fra Historesin mold trayene. Historesin mold trayene rengjøres etter bruk og blir benyttet flere ganger. Ferdig innleirede vevsprøver lagres for snitting.

II) Lagring av innleiret vev før snitting

TRINN	ARBEIDSBESKRIVELSE
1	Blokker med ferdig innleiret vev lagres i en fuktig atmosfære. Fuktig atmosfære oppnås ved vannholdig løsning i bunnen av en stor plastboks med tett lokk. Vevsblokkene skal ikke være i berøring med den vannholdige løsningen.
2	Lag løsningen: 70% glyserin : 30% dest vann Fyll ca 1 cm opp i en stor plastboks med tett lokk. Plastboksen skal stå på lab. benk og i romtemperatur.
3	Sett merkede 1 l begerglass opp i plastboksen. Legg resin-blokkene i begerglassene i passende grupperinger. Eksempelvis : Glass A – blokker til snitting, dato X Glass B – blokker i reserve, dato X
4	Husk og alltid ha på et tett lokk.

3.7.4.5 Snitting

I) Snitting av vev med mikrotom 1 (Leica RM 2155)

TRINN	ARBEIDSBESKRIVELSE
1	Blokkholderen med den resin innleirede vevsprøven festes i preparatklemmen på frontsiden av mikrotomen.
2	Med hardmetall - kniven oppgitt i utstyrslisten kan en snitte vev fra 0,5–15 μm på oppgitt mikrotom. Bruk gjerne en slitt kniv til grovsnitting (4-15 μm), og en feilfri kniv til finsnittene (0,5– 4 μm) som en ønsker å analysere.
3	Snitthastigheten vil variere med mengden av og hardheten på vevet, generelt : Økt vevsmengde krever lavere hastighet. Hardt vev krever lavere hastighet.
4	Still clearence angel til 4 – 6 grader for oppgitt mikrotom og kniv.
5	Still mikrotomen på ønsket snittykkelse og snitt automatisk eller manuelt. Ved snitteproblemer, særlig interferensproblemer, hjelper det å snitte manuelt og med lav hastighet.

6	Snitt fjernes fra kniven vha penselkost og pinsett(er). Snittene føres fra kniven til vannbad for strekking. To varianter av vannbad blir brukt, sistnevnte er særlig gunstig for store snittflater : Glasskar med rent vann plassert på varmeplate, temperatur +60 ^{o}C. Glasskar med vann tilsatt noen dråper amoniakk som holder romtemperatur.
7	Ta opp strukket vevssnitt fra vannbadet med et objektglass. Objektglasset bør ha skrivefelt og være merket med prøvenummer og eventuelt andre viktige opplysninger om snittet, eks snitt tykkelse.
8	Tørk vevssnittet på varmeplate, ca +100 ^{o}C. Snittene er nå klare til farging.
9	Vevssnitt som ikke blir brukt ryddes vekk vha en bordstøvsuger etter endt ”snitteøkt”.

II) Lagring av innleiret vev og snitt etter snitting

TRINN	ARBEIDSBESKRIVELSE
1	Ufargede vevssnitt lagres i objektglassmappe med lokk eller i kassetter for objektglass.
2	Husk at god og forståelig merking er viktig. Merkingen bør inneholde vevstype eller forsøkstittel samt dato og ansvarsperson.
3	Blokker med resininnleiret vev som er ferdig snittet lagres i plastposer, med lukkerand, i kjøleskap. Plastposene merkes utenpå med forsøksnavn, ansvarsperson, dato og status.

3.7.4.6 Rutinefarging

Rutinefarging av resinsnitt gjøres med 2% toluidinblått med 1% borax.
De mest benyttede vevsfargene for parafinsnitt kan også benyttes for resinsnitt, med eventuelle justeringer, se kapittelet Spesialfarging.
Alt arbeid med fargeløsningene gjøres i avtrekk og med engangshansker i avtrekk.

TRINN	ARBEIDSBESKRIVELSE
1	Legg objektglass med vevssnitt som skal farges i en objektglassmappe uten lokk.
2	Bruk foldefilter til å filtrere et passe volum av toluidinblåttløsningen til en glasskolbe.

3	Tilsett filtrert fargeløsning til objektgass med ufarget snitt, vha engangs pasteur-pipette. Når vevet har fått passe farge skylles overflødig fargeløsning vekk med rennende varmt springvann direkte på snittet, da fjernes også uønsket bakgunnsfarge.
4	Innfargingstid avhenger av vevssnittets tykkelse. Tykkere snitt medfører mindre fargetid. Ved standardsnitt på 1 μm er fargetiden 15 – 30 sekunder.
5	Etter farging tørkes vevssnittene på varmeplate ved ca 60 ^{o}C.

3.7.4.7 Montering / dekking

I) Montering

Alt arbeid utføres i avtrekkskap og med engangshansker på histologilaboratoriet.

TRINN	ARBEIDSBESKRIVELSE
1	Legg objektglass med ferdig fargede vevssnitt i en objektglassmappe uten lokk.
2	Drypp 1–2 dråper monteringsvæske, Histokitt Assistent, på objektglasset eller dekkglasset.
3	Legg et dekkglass av passende størrelse over vevssnittet og press monteringsmiddelet forsiktig utover.
4	Plasser et messinglodd, 20 –50 g, oppå dekkglasset slik at eventuelle luftbobler presses ut. Pass på at objektglassmappene ligger i water slik at loddet ikke beveger seg. La dette stå i avtrekkskap natten over for tørking og avdunsting av løsemiddel (xylen).

II) Lagring av ferdige snitt

TRINN	ARBEIDSBESKRIVELSE
1	Ferdige vevssnitt lagres i kassetter for objektglass eller egnet arkivsystem for objektglass.
2	Husk at god og forståelig merking er viktig. Merkingen bør inneholde vevstype eller forsøkstittel samt dato og ansvarsperson.