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Instar growth and molt increments in *Lepeophtheirus salmonis* (Copepoda: Caligidae) chalimus larvae

Christiane Eichner [1], Lars Are Hamre *[,1], Frank Nilsen

SLCR—Sea Lice Research Center, Department of Biology, University of Bergen, Thormøhlensgt. 55, 5008 Bergen, Norway

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ABSTRACT

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasitic copepod causing severe problems to the fish farming industry and to wild salmonids. Morphologically, all stages in the life cycle of *L. salmonis* have been described in detail based on successive samples from host populations. However, the rate of development differs between males and females as well as between individuals. It has therefore been difficult to observe development within stages, and this has led to a longstanding misinterpretation of the number of chalimus stages. Here samples of chalimi obtained for 12 consecutive days were observed daily in incubators. Chalimus 1 was able to molt in incubators only when fully grown and close to molting, whereas chalimus 2 was able to molt at about 60% of total instar growth. Total length instar growth was about 35% in both chalimus 1 and chalimus 2 and about equal among males and females; the cephalothorax increased by about 12% and the posterior body by about 80%. Instar growth was probably the main factor that led to the former belief that *L. salmonis* had four chalimus stages. Relative total length increase at molting was at the same order of magnitude as instar growth, but total length of females increased significantly more than that of males at molting. Consequently, a sexual size dimorphism was established upon molting to chalimus 2 and males were about 10% smaller than females. While growth by molting was mainly caused by cephalothorax increase, instar growth was mainly due to increase of the posterior body. The cephalothorax/total length ratio decreased from beginning to end of the instar phase suggesting that it may be used as an instar age marker. Male and female chalimus 2 can almost uniquely be identified by cephalothorax length. Chalimus 1 lasted between 5 and 6 days for males and between 6 and 7 days for females at 10 °C. Chalimus 2 males lasted between 6 and 7 days and females between 7 and 8 days.

1. Introduction

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasitic copepod representing a significant threat to the fish farming industry [15,19,26] and to wild salmonids [5,6,12,22,24]. The parasitic stages of *L. salmonis* feed on mucus, skin and blood of salmonid fish. Prior to host attachment nauplia and copepodids feed on stored egg yolk only.

Growth in copepods like in other arthropods is considered to be restricted to molting. During molting, a new and larger exoskeleton is produced beneath the old cuticula that is replaced so that the animal can expand in size. The new exoskeleton is folded to allow increase in size after the animal emerges from the old cuticula. Molting is tightly controlled hormonally in crustaceans (e.g. reviewed in [11]). External factors such as food and temperature affect molting and may result in a modified intermolt period and/or modified molt increment in some species [11]. Ref. [2] defined the point of reserve saturation (PRS) as the time when molting can take place without additional food ingestion. This critical point is reached after one-third to one-half of the instar life span among first instar larvae in nine different decapod species [1].

Usually absolute molt increment (MI), the change of size from one molt to the next, is measured to describe the growth of crustaceans. However, growth may also take place without molting as described for adult *L. salmonis* females [7] and other arthropods [17,23].

The life cycle of the salmon louse has recently been revised and consists of 8 instars separated by molts: two naupliar stages, one infective copepodid stage, two chalimus stages attached by filament, 2 preadult stages and one adult stage [10]. Detailed morphological descriptions of the *L. salmonis* developmental stages have been given by [13] (Pacific louse) and [21] (Atlantic louse). Size measurements have been reported for all stages based on systematic samples from host populations. However, males develop quicker than females and reach the preadult 1 stage, which is the first stage when sex determination is possible, several days before the females [10,25]. At this point sexual dimorphism is evident [13]. In addition, the rate of development differs between individuals of the same sex resulting in a substantial difference between

* Corresponding author.
E-mail address: lars.hamre@bio.uib.no (L.A. Hamre).
[1] Both authors have contributed equally to the manuscript and are listed in alphabetical order.

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slow and quick developers [13]. It has therefore been difficult to assess the nature of growth in the attached *L. salmonis* chalimus stages. Here we present data based on molt observations in incubators to document that salmon lice not only expand in size when molting, but also display significant growth during the instar phase.

2. Materials and methods

2.1. Experimental design

In this study material obtained in Experiment 1 described in Ref. [10] is analyzed with emphasis on intra- and intermolt growth of *L. salmonis* chalimus instars. A detailed description of materials and methods is given in Ref. [10]. In short: a batch of Atlantic salmon (*Salmo salar*) was infected with copepodids at 10 °C full salinity seawater (34.5‰). Starting at day 6, daily samples of the louse population were obtained for 12 consecutive days. The sampled lice were photographed and placed in incubators. Prior to photography each louse was placed in a drop of seawater in a petri-dish and covered with a cover slip (18 × 18 mm). The chalimi were inspected daily for 4–6 days to check whether a molt had taken place as defined by presence of an exuvium in the incubator. Newly molted larvae were removed from the incubator and photographed again. All intact exuviae were also photographed. Some instars that did not molt during the period of observation were also photographed again in order to assess whether starvation affected instar length. An overview of the procedure is provided in Fig. 1.

In order to estimate the mean size of newly molted chalimus 1 a second experiment using the same experimental procedure as described above was conducted. For this experiment three fish were used and sampling started two days earlier than in the first experiment. Copepodids were sampled and placed in incubators for two consecutive days and monitored the two following days. Total length (TL) and cephalothorax length (CL) of the premolt copepodids and the corresponding post molt chalimus 1 were measured according to Ref. [10] (see also Fig. 1). Posterior body (PB) = TL − CL.

2.2. Instar growth and growth by molting

In order to assess instar growth, the mean size of young instars (newly molted, retrieved from incubators) and old instars (just before molting to the next stage) was calculated. The mean size of old instars prior to molting was estimated on the basis of larvae that molted in the incubators, whereas the mean size of young instars of the next stage was estimated on the basis of the corresponding post molt. Molt increment is the relative size increase (%) from premolt old instars to postmolt young instars of the next stage.

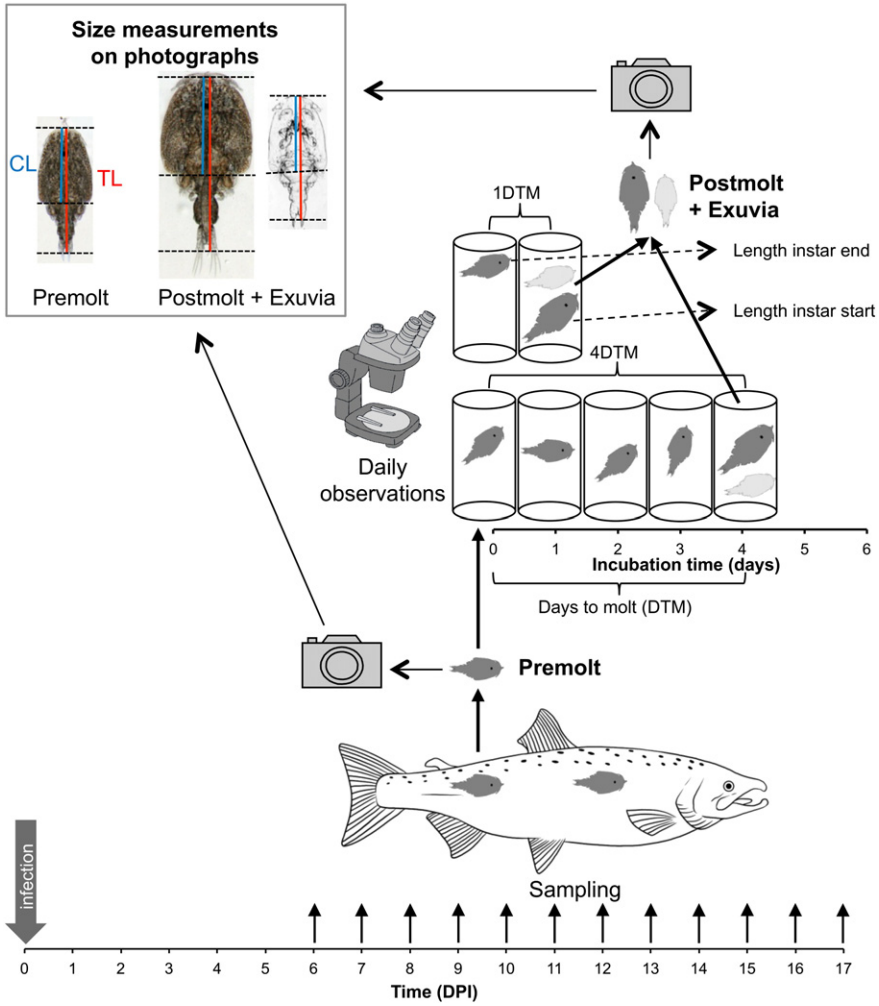

Fig. 1. Experimental setup. Lice were sampled for 12 consecutive days (premolt), photographed and then incubated in continuous flow through incubators until molting (postmolt) or at least 4 days. Incubation time until molting was recorded. DTM = days to molt. Molted lice and exuviae were photographed again. Number of days required to molt in the incubators was recorded. DPI = days post infection.

2.3. Time and instar size required to molt in incubators post sampling

Size of both premolts and postmolts of incubated chalimus 2 decreased with time required to molt in the incubators. Therefore only data from chalimus 2 that molted after one day were used to estimate size of fully grown chalimi and their respective postmolts (young instars of the next stage). This relationship was not evident among chalimus 1 instars and all individuals that molted were considered fully grown.

Only a few of the chalimus 2 that spent more than four days in the incubators molted. Hence, instars using 4 days to molt (DTM) were considered to represent the youngest instars that had obtained the capacity to molt when removed from the host (from here on termed molt capacity). Thus, the size difference between the very young instars and these were used to estimate the instar growth (%) required to reach molt capacity. Stage age is defined as days spent within a given stage.

2.4. Sex determination

Chalimus 2 larvae that molted to preadults in incubators were sex determined based on the corresponding post molt preadult 1. Young chalimus 2 could not be sex determined directly; however, sex could be inferred from the magnitude of molt length increase and cephalothorax length (see Results and Discussion).

2.5. Statistics

T-tests were used to test for sexual size dimorphism. One way ANOVA was used to test variations in pre and post molt length of chalimus larvae with respect to DTM. Spearman rank correlation was used to examine the relationship between cephalothorax length/total length ratio and time of sampling. The Shaprio–Wilk test was applied to evaluate distribution fitting. All statistical calculations were carried out using Statistica 10.0 (www.statsoft.com).

2.6. Histology preparation

For histological examination lice were fixed in phosphate buffered 4% paraformaldehyde (pH 7.4) for at least 24 h. Prior to embedding in Technovit (Technovit 7100, Heraeus Kulzer GmbH) according to the supplier's instructions, the animals were washed in PBS (pH 7.2), dehydrated in a graded ethanol series (50%, 70%, 96%), and preinfiltrated in Technocit/Ethanol (1:1) for 4 h. Embedded lice were sectioned by a Leica RM 2165 microtome (2 μm) and stained with toluidine blue (1% in 1% natriumborat) [8] for 1 min. The stained sections were mounted using Mountex (Histolab Products).

3. Results

3.1. Growth and development of chalimus 1

3.1.1. Growth by molting from copepodid to chalimus 1

Total length (TL), cephalothorax length (CL) and length of posterior body (PB) of premolt copepodids and corresponding young chalimus 1 emerging in incubators are given in Table 1. The difference between premolt (old) copepodids and postmolt (young) chalimus 1 size shows that TL increased by 26%, CL increased by 39% and that PB increased only 3% during molting (Table 2). Note that the molt increment given herein is defined by length increment caused only by molting not including potential intramolt growth as in many other studies.

Table 1
Premolt and postmolt size of chalimi. Mean total length (TL) and cephalothorax length (CL) (mm) at start and end of the instar life span of *L. salmonis* larvae. Cop = copepodid, Ch1 = chalimus 1, Ch2 = chalimus 2 and Pad1 = preadult 1, m = male and f = female. Start and end refers to the start and end of the instar life span, i.e. start = shortly after molting into a stage and end = shortly prior to molting into the next stage. Ch1 end and Ch2 start CL and TL values are estimated by the mean lengths of all chalimus 1 that molted; otherwise stage end and stage start values are mean length of premolt and postmolt larvae which molted within one day in the incubators. Mean TL ≥ 4 DTM = mean TL of younger chalimus instars that required 4 days or more to molt. SD = standard deviation, n = sample size, MIN = minimum, MAX = maximum in brackets. MI = molt increment (increment achieved by molt only), Ex = exuviae.

	Cop	Ch1		Ch2				Pad1	
	End[a]	Start[a]	End	m start	f start	m end	f end	m start	f start
Mean TL	0.82	1.03	1.37	1.80	2.06	2.47	2.71	3.12	3.66
(SD, n)	(0.05, 17)	(0.05, 14)	(0.04, 18)	(0.05, 9)	(0.06, 8)	(0.09, 16)	(0.10, 26)	(0.12, 17)	(0.13, 27)
(MIN, MAX)	(0.71, 0.88)	(0.93,1.13)	(1.30,1.45)	(1.73, 1.86)	(1.97, 2.11)	(2.34, 2.68)	(2.54, 2.94)	(2.86, 3.31)	(3.32, 3.87)
Mean TL ≥4 DTM			1.36			2.21	2.49	2.95	3.41
(SD, n)			(0.06, 6)			(0.06, 14)	(0.09, 12)	(0.23, 12)	(0.11, 11)
(MIN, MAX)			(1.30, 1.45)			(2.10, 2.28)	(2.37, 2.65)	(2.47, 3.33)	(3.25, 3.67)
Mean CL	0.51	0.71	0.80	1.22	1.39	1.37	1.52	1.85	2.23
(SD, n)	(0.02, 17)	(0.03, 14)	(0.03, 13)	(0.04, 9)	(0.03, 8)	(0.03, 16)	(0.04, 26)	(0.06, 17)	(0.07, 27)
(MIN, MAX)	(0.45,0.55)	(0.64, 0.77)	(0.76,0.86)	(1.17, 1.27)	(1.35, 1.43)	(1.32, 1.43)	(1.45, 1.60)	(1.76, 1.95)	(2.11, 2.40)
Mean CL ≥4 DTM			0.81			1.34	1.47	1.70	2.04
(SD, n)			(0.02, 6)			(0.04, 14)	(0.03, 12)	(0.13, 12)	(0.07, 11)
(MIN, MAX)			(0.77, 0.84)			(1.26, 1.39)	(1.41, 1.51)	(1.37, 1.83)	(1.91, 2.13)
Ex mean TL all	0.79		1.25			2.12	2.40		
(SD, n)	(0.04, 17)		(0.06, 34)			(0.09, 67)	(0.10, 70)		
(MIN, MAX)	(0.69,0.85)		(1.12, 1.40)			(1.83, 2.39)	(2.16, 2.64)		
Ex mean CL all	0.57		0.77			1.32	1.49		
(SD, n)	(0.02, 17)		(0.04, 36)			(0.06, 67)	(0.05, 69)		
(MIN, MAX)	(0.53, 0.59)		(0.67, 0.85)			(1.03, 1.41)	(1.30, 1.61)		
Ex mean TL 1 DTM						2.14	2.39		
(SD, n)						(0.10, 17)	(0.09, 26)		
(MIN, MAX)						(1.94, 2.39)	(2.16, 2.56)		
Ex mean TL ≥ 4 DTM						2.11	2.34		
(SD, n)						(0.11, 15)	(0.10, 8)		
(MIN, MAX)						(1.83, 2.25)	(2.23, 2.47)		
Ex mean CL 1 DTM						1.31	1.47		
(SD, n)						(0.08, 17)	(0.06, 26)		
(MIN, MAX)						(1.03, 1.40)	(1.30, 1.54)		
Ex mean CL ≥ 4 DTM						1.32	1.50		
(SD, n)						(0.07, 15)	(0.02, 7)		
(MIN, MAX)						(1.16, 1.41)	(1.46, 1.52)		

[a] Values obtained from the second experiment.

Table 2
Molt size increments and instar growth of *L. salmonis* during the chalimus phase of the life cycle. TL = total length, CL = cepalothorax length, PB = posterior body length. cop = copepodid, ch = chalimus, pread = preadult, IG = instar growth, ch1-ch2 = molt size increment between old ch1 larvae and young ch2 larvae, ch2-pread1 = molt size increment between old ch2 larvae and young pread1 larvae.

			Males			Females		
	cop-ch1	ch1 IG	ch1-ch2	ch2 IG	ch2-pread1	ch1-ch2	ch2 IG	ch2-pread1
TL (%)	26	33	31	37	26	50	32	35
CL (%)	39	13	53	12	35	74	9	47
PB (%)	3	78	2	90	15	18	78	20

3.1.2. Molt capacity

The first chalimus 1 was observed and sampled from the host 5 days post infection (DPI). At 10 DPI the first chalimus 2 was observed, and within 12 DPI most of the chalimus 1 had molted into chalimus 2. Only a few chalimus 1 were sampled at 12 DPI and later (Fig. 2). Instars collected the first three sampling days were not able to molt in incubators with the exception of one instar sampled at 8 DPI. The majority of chalimus 1 able to molt were sampled at 9–11 DPI (Fig. 2). Since most of the chalimus 1 molted on the host between 9 and 12 DPI it was evident that only instars sampled close to molting were able to molt in incubators.

3.1.3. Instar growth

Instar growth was observed in chalimus 1 larvae. TL increased by 33%, CL increased by 13% and PB increased by 78% during the instar phase (Table 2). Instar growth was allometric; the CL/TL

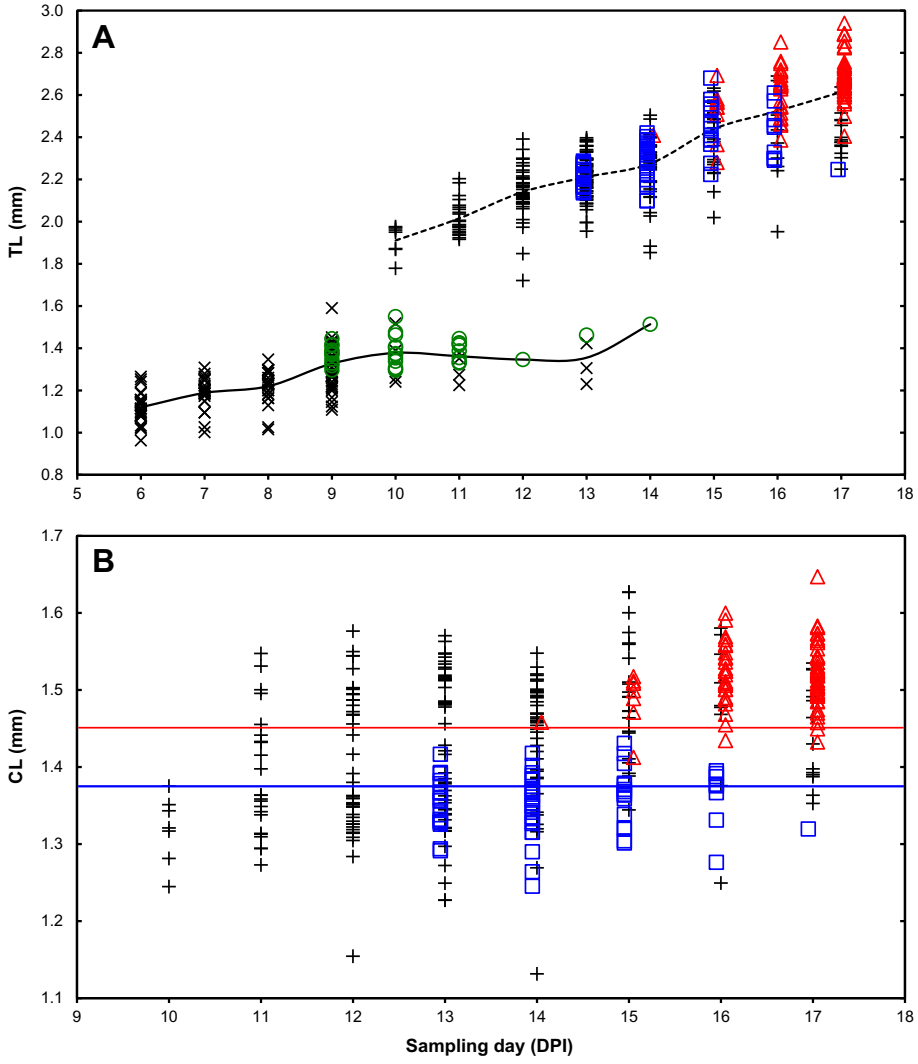

Fig. 2. Sizes of *Lepeophtheirus salmonis* chalimus larvae at sampling. Chalimi were stocked in incubators and observed once a day for up to 6 days post sampling. x = chalimus 1 that did not molt during the period of observation, green open circles = chalimus 1 that molted, + = chalimus 2 that did not molt, blue open squares = chalimus 2 males that molted, red open triangles = chalimus 2 females that molted. Full trend line reflects the mean size of sampled chalimus 1; dotted trend line reflects mean size of sampled chalimus 2 DPI = days post infection. Note that sampling was stopped after 17 DPI before all female chalimus 2 had molted to the preadult stage. A) Total length (TL) at sampling, (B) chalimus 2 cephalothorax length (CL) at sampling. Blue line (CL = 1.375) represents the size of the largest individual (i.e. male, see text) found at day 10 and upper red line (CL = 1.451) represents the size of the smallest of the fully grown females (i.e. that molted within one day post sampling). These data are part of the dataset shown in Fig. 2 in Ref. [10].

Table 3
Cephalothorax length/total length (CL/TL) compared to former studies. Drawings in Refs. [13] and [21] were scanned and measured as described for photographed lice in this study. ch = chalimus, EX = exuvium.

This study	cop end	ch1 start	ch1 end	ch2 start	ch2 end	pad m start	pad f start
CL/TL	0.63	0.69	0.59[a]	0.68[a]	0.56[a]	0.59	0.61
CL/TL EX	0.72		0.62[a]		0.62[a]		ns

Literature	cop	Former ch1	Former ch2	Former ch3	Former ch4	pad m	pad f
[13]	0.63	0.68	0.57	0.67	0.61	0.60	0.66
[21]	0.60, 0.65	0.67 (young) 0.66 (old)	0.60	0.77 (young) 0.66 (old)	0.58	0.58	0.62

ratio changed from 0.69 for young instars to 0.59 for old instars (see also Table 3).

3.1.4. Molt increment and sex dimorphism

Sizes of premolt (old) chalimus 1 and postmolt (young) chalimus 2 are given inTable 1. Size was equal among chalimus 1 sampled at 9, 10 and 11 DPI; however, TL and CL of the corresponding post molt chalimus 2 emerging in incubators increased significantly with time of sampling (ANOVA $F_{TL} = 12.24$, $p < 0.0002$, $F_{CL} = 8.24$, $p < 0.002$) (see Fig. 3A). Two populations of young chalimus 2 larvae appeared in the incubators: post molt chalimus 2 that emerged from chalimus 1 sampled at 9 DPI displayed a mean molt increment in CL of 53% and all had CLs smaller than 1.30 mm. All (except one) post molts of chalimus 1 sampled at 11 DPI had CLs larger than 1.35 mm and displayed a significantly higher mean molt increment in CL of 74% (t-test $t = -9.47$, $p < 0.000001$). Among those sampled at 10 DPI the occurrence of post molts with CLs above and below 1.30 mm was about 50/50. This shows that two size groups of chalimus 2 emerged at molting; the larger group displayed a significantly higher molt size increment than the other and appeared later than the smaller group. This was also reflected in Fig. 2B showing that the first chalimus 2 appeared on the host at 10 DPI and that a new and larger chalimus 2 size group appeared at 11 DPI. From this it is evident that female chalimus 1 appeared later than males and displayed a larger molt size increment. It can therefore be assumed that among chalimus 1 sampled at 9 DPI only males were close to molting and able to molt in incubators (i.e. had obtained molt capacity). At 10 DPI both males and females had obtained molt capacity and at 11 DPI predominantly females remained. In sum this means that males molted between 9 and 11 DPI on the host while females molted between 10 and 12 DPI.

3.1.5. Delayed molting in incubators

As much as 68% of the sampled chalimus 1 required three or four days to molt in the incubators. The incubated larvae sampled at 9 DPI molted at 11–13 DPI and those sampled at 11 DPI molted at 12–14 DPI. Thus, molting was delayed for chalimus 1 in incubators compared to those on the host (see above). There was no size difference between instars that molted after one day and those that required four days to molt. Instars with TL shorter than 1.29 mm did not molt.

3.1.6. Exuviae

TL of shed exuviae were significantly longer (21%) than TL of young chalimus 1 instars (t-test, $t = -11.3$, $p < 0.00001$), but shorter (9%) than the length of old instars (t-test, $t = -8.26$, $p < 0.00001$), i.e. shed exuviae appeared to be shorter than the animals themselves. CL of exuviae was on the other hand only 8% longer than the CL of young

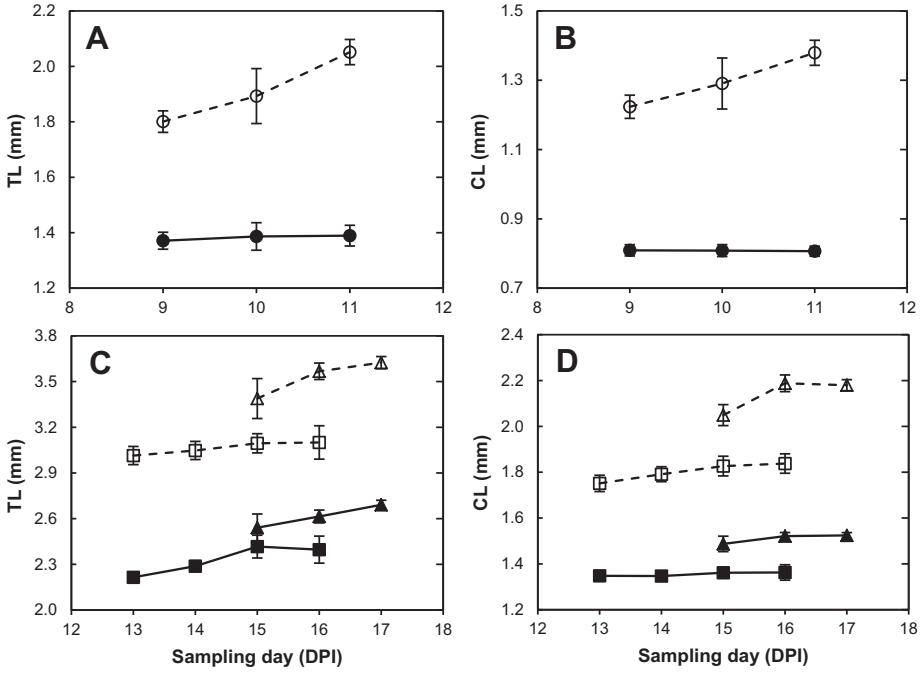

Fig. 3. Mean length of *L. salmonis* chalimi before and after molting in incubators. Length of both premolt and postmolt animals are plotted at the time point they were sampled from the fish. Means were not calculated if fewer than 3 of the lice sampled at a given day molted in the incubators. The graph shows when the majority of chalimi with molt capacity were present on the host (i.e. larvae that were able to complete a molt when maintained in incubators). Magnitude of molt increment is indicated by the difference between full and dotted lines. Filled symbols = mean length premolt chalimi, empty symbols = mean length postmolt larvae. A, B: chalimus 1. C, D: chalimus 2 males = squares, chalimus 2 females = triangles. TL = total length, CL = cephalothorax length, DPI = days post infection. Bars denote 95% confidence intervals. Note that bars in several cases are covered by the symbols. DPI = days post infection with copepodids.

instars (CL_{ch1}: $t = -3.81$, $p < 0.0004$), thus instar cuticle size increase was evident mainly in the posterior body. The mean CL/TL ratio of shed exuviae was 0.62.

In most cases shed exuviae from chalimus 1 were found with the frontal filament attached to the exuvium. Morphometric data for instars and exuviae are shown in Table 1.

3.2. Growth and development of chalimus 2

The sex of chalimus 2 emerging in incubators from chalimus 1 sampled at 9–11 DPI was inferred from sampling day, cephalothorax length and molt length increment (see Results above and Discussion below). From this it can be assumed that lice sampled at 9 DPI were males and all (except one) chalimus 2 emerging from instars sampled at 11 DPI were females.

3.2.1. Growth by molting from chalimus 1 to chalimus 2

The TL, CL and PB of these young male and female chalimus 2 are given in Table 1. The mean TL molt increment from chalimus 1 to chalimus 2 was 31% for males and 50% for females. CL increased by 53% and 74% for males and females respectively, while the posterior body increased by 2% for males and 18% for females (Table 2).

3.2.2. Molt capacity and instar growth

Chalimus 2 males with molt capacity were sampled from the host between 13 and 17 DPI, and females predominantly later than 15 DPI (except one at 14 DPI, Fig. 2). Hence, males developed molt capacity three days after first appearance on the host, females four days (Fig. 2). Males and females with TLs shorter than 2.27 and 2.54 mm respectively did not molt.

Chalimi that had a capacity to molt after removal from the host molted normally 1–4 days after introduction to the incubators. Only a few of the larvae observed for more than four days molted. As the overall age and mean length of the chalimus 2 population increased on the fish host (Fig. 2A), the mean number of days required to molt (DTM) in the incubators decreased with time of sampling (Fig. 4) (ANOVA, $F_{females} = 18.0$, $p < 0.00001$, $F_{males} = 13.7$, $p < 0.00001$). Chalimus 2 that molted shortly after sampling were significantly longer both pre- and post-molting than instars that required longer time to molt (Fig. 5) ($ANOVA_{Premolt}$, $F = 12{,}8$ $p < 0.00001$, $ANOVA_{Postmolt}$, $F = 9.0$ $p < 0.00002$). Males and females that required 4 days or more to molt ($DTM \geq 4$) had reached 61% and 66% respectively of total instar length growth when sampled from the fish (Table 1). Evidently, chalimus 2 was able to molt when removed from the host before reaching maximum instar size, and only chalimi that required one day to molt (1 DTM) could be regarded as fully grown instars. The sizes of old (premolt) chalimus 2 (1 DTM) males and females are given in Table 1. In males, TL instar growth was 37%, CL increased by 12% and PB by 90%. In females TL increased by 32%, CL by 9% and PB by 78%.

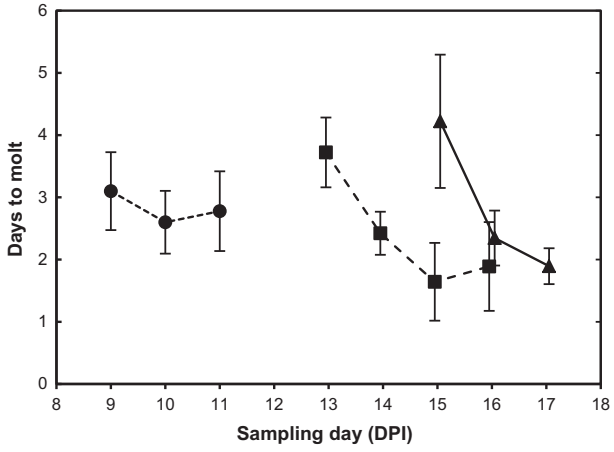

Fig. 4. Time required to molt in incubators for *L. salmonis* chalimus larvae vs sampling day. Means were not calculated if fewer than 3 lice sampled at a given day molted in incubators. The majority of chalimus 1 (circles) that were able to molt in the incubators were collected from fish at 9–11 days post infection (DPI), chalimus 2 males at 13–16 DPI (squares) and chalimus 2 females at15–17 DPI (triangles). Note that sampling ended before all chalimi on fish had molted into preadults. Bars denote 95% confidence intervals.

3.2.3. Growth by molting from chalimus 2 to preadult 1

Mean TL molt increment from chalimus 2 to preadult 1 was 26% for males and 35% for females. CL increased by 35% for males and 47% for females, and the posterior body increased by 15% for males and 20% for females (Table 2).

3.2.4. Molting in incubators vs molting on fish

Only 18% of the chalimus 2 that molted in incubators required three or more days to molt. Molting appeared to occur simultaneously in incubators and on the fish; however, due to scarcity of data on emergence of preadult 1 on the host we were not able to evaluate this satisfactorily.

3.2.5. CL as sex identifier, CL/TL as stage age marker

Males and females with molt capacity could be almost uniquely recognized by CL as demonstrated by its bimodal distribution (Fig. 6A). Among all the sampled chalimus 2 larvae, also including the larvae that did not molt, an almost equivalent bimodality in CL was evident (Fig. 6B). The median length of the chalimus 2 cephalothorax was 1.44 mm. All females identified by the corresponding postmolt preadult 1, except one, fell within the 50% longest CLs and all the males fall within the 50% shortest CLs.

Both young and old instars displayed a sexual size dimorphism with a male/female TL ratio of 0.9. Body proportions (CL/TL ratio) were identical between males and females of equal stage age throughout the instar period. The CL/TL ratio decreased from 0.68 for young individuals to 0.56 for old individuals (Tables 1 and 3). This is also demonstrated in Fig. 7 showing how the CL/TL ratio changed as the louse population grew older with time of sampling (see also photographs in Fig. 8).

3.2.6. Exuviae

Shed exuviae of both males and females were about 18% longer (TL) than the body of young chalimi, and about 13% shorter than old chalimi (only chalimus 2 with DTM = 1 included, all t-tests $p < 0.05$). TL of exuviae shed from chalimus 2 that recently had obtained molt capacity ($DTM = 4$) was only marginally shorter than the bodies they were shed from (female: 2%, male: 5%). CL of exuviae was 8% and 7% longer than the size of young males and females respectively (CL_{Male}: $t = -3.4$, $p < 0.002$, CL_{Female}: $t = -4.25$, $p < 0.0002$). Thus similar to chalimus 1, instar cuticle growth/stretching of chalimus 2 was evident mainly in the posterior body. CL/TL ratio of shed exuviae was 0.62. Frontal filaments attached to shed chalimus 2 exuviae were not observed. Morphometric data for exuviae are shown in Table 1.

3.3. Death rate and effect of starvation in incubators

The highest death rate of chalimus 1 in incubators was observed among young larvae collected during the first three days where between 24% and 37% of the larvae died. Chalimus 1 sampled later had mortality rates of about 10%. For chalimus 2 the highest death rate was observed among larvae sampled day 11 (24%), otherwise between 9 and 14% of the sampled chalimus 2 died. Dead animals often had observable damage caused by handling and the highest death rate was found among the young and more fragile larvae. Most of the chalimi that did not molt remained alive until the end of observation suggesting that larval death was in general caused by handling, and not starvation.

Starvation had no effect on the size of incubated chalimi that did not molt during the period of observation ($n = 95$). However, young chalimus 2 emerging in incubators were about 5% smaller than young chalimus 2 appearing on the host, hence the mean CL

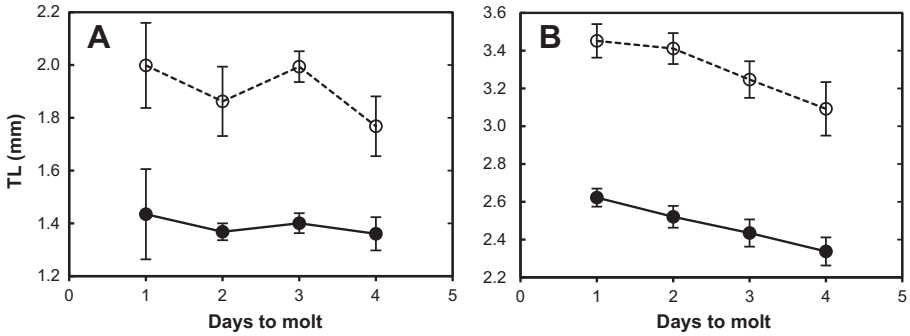

Fig. 5. Mean total length (TL) of premolt and postmolt *L. salmonis* chalimus 1 (A) and chalimus 2 larvae (B) vs. time required to molt in incubators. Filled circles = length of instars prior to molting (premolt length), open circles = length of the emerging instars post molting (post molt length). Bars denote 95% confidence intervals.

separating the sexes was smaller among chalimi emerging in incubators (CL ~ 1.31 mm). Since incubated chalimi were inspected once every 24 h, instars had been starving for on average 12 h (DTM = 1) before molting compared to those molting on the host. Likewise, the first chalimus 2 had been feeding/growing on average for 12 h post molting when sampled from the host.

3.4. Histology

Sections of chalimus 1 directly after molting from copepodid (5 DPI) display a straight and somewhat fuzzy cuticle closely connected to subcuticular tissue (Fig. 9A, B). The same can be seen in chalimus 2 directly after molting from chalimus 1 (15 DPI, Fig. 9E, F). In chalimus 1 (14 DPI) directly before molting to chalimus 2, the old outer cuticle is loose and overlying a highly folded epidermis with a new cuticle in both the cephalothorax and the genital segment (Fig. 9C, D).

4. Discussion

4.1. Instar growth

This is the first study providing detailed data on instar growth for a caligid copepod. Substantial instar growth corresponding to about 50% of total growth of *L. salmonis* chalimi was demonstrated by comparing the sizes of instars immediately after entering and before leaving a stage. The major part of instar growth was due to an increase in posterior segments and to a lesser extent in the cephalothorax, whereas the main cephalothorax increase took place at molting. The relatively high shrinkage of exuviae shed by fully developed instars showed that a somewhat elastic cuticle was inflated by the growing larvae inside. Thus, the previous misconception on the number of chalimus stages in the *L. salmonis* life cycle could, in addition to a minor sexual size dimorphism, mainly be explained by a substantial instar growth inflating the cuticle and obscuring the segmental boundaries as suggested by Ref. [18]. Our data show that sexual size dimorphism accounts for 10% of the instar size variation, whereas instar growth constitutes about 35%. This is in contrast to Ref. [16] who argued that sexual size dimorphism was the main reason for the previous misinterpretation of instar numbers in *Lepeophtheirus* spp.

TL and CL of shed exuviae were about 18% and 8% longer respectively than TL and CL of young instars. This suggests that an actual intramolt cuticle increase by either stretching or growth takes place, predominantly in the posterior body, but also in the cephalothorax. Cuticle growth in the posterior body has been described for adult *L. salmonis* [7] and several other caligid species [3,20]. In *Lernaeocera branchialis* intramolt growth could be explained partly by straightening of folds in the cuticle, even though this accounted for less than half of length increase [23]. Interestingly, in contrast to the highly folded cuticle observed in the posterior body of young *Lernaeocera* [23] or folds in newly molted adult *L. salmonis* females [7], comparable folding was not observed in the new cuticle of the posterior body in chalimi directly after molting (see Fig. 9). Hence, in the present study it was not possible to point at any particular mechanism of cuticle growth in the posterior body of *L. salmonis* chalimi, nor was it possible to ascertain to which extent a true cuticle growth took place or if the size increase was merely due to stretching of an elastic cuticle.

Starvation may have had an influence on size estimates of chalimi molting in incubators. Incubators were inspected once a day. Chalimi molting within one day spent on average 12 h in starvation off the host prior to molting, which is about 7–8% of the total instar life span. Thus, size estimates of both young and old chalimi used to calculate instar growth were somewhat underestimated. However, in the present experimental design young and old instars were equally underestimated, and the estimates of relative instar growth (%) and relative molt increment (%) are probably adequate.

4.2. Molt capacity

Chalimus 1 were able to molt in incubators only when sampled at full size close to molting. The number of days required to molt was independent of size and molting was delayed by one or two days in incubators compared to development on fish. For chalimus 2, the number of days required to molt in incubators decreased as age and size of the sampled chalimi increased (Fig. 4). In contrast to chalimus 1, chalimus 2 were able to molt in incubators when sampled before they were fully grown, demonstrating that chalimus 2 obtained a capacity to molt before reaching their optimal size for molting. Similar observations have been made in studies of free living crustaceans and termed Point of Reserve Saturation (PRS) [2]. However, we find it more appropriate to use the term "molt capacity" as this term incorporates both the developmental and nutritional states of instars. Molt capacity was reached at 64% of mean total instar length growth for chalimus 2 assuming that those individuals using the longest time to molt represented the youngest chalimus 2. The first chalimus 2 developed molt capacity three days after the first occurrence on the fish (i.e. at an instar age of approximately three days). These were probably male lice with a total instar duration of five to six days (see Discussion below), suggesting that the earliest chalimus 2 males obtained a molt capacity when reaching about 50% of total instar life span. Altogether this suggests that chalimus 2 had passed a threshold of molt cycle regulation when reaching about 50–60% of total instar growth, as observed in many other crustaceans ([1] and citations therein). This was not observed in chalimus 1. The data show that molting to the chalimus 2 stage took place within a relatively narrow time frame and that molt capacity was reached close to molting for the vast majority of chalimus 1. A substantial part of the chalimus 1 males obtained molt capacity at 9 DPI, one day before the first chalimus 2 males appeared on the host (10 DPI) (Fig. 2B). Likewise, the first chalimus 1 females obtained molt capacity at 10 DPI one day before the first chalimus 2 females were observed on the host at 11 DPI (Fig. 2B). At 12 DPI most of the chalimus 1 had molted to chalimus 2 on the host

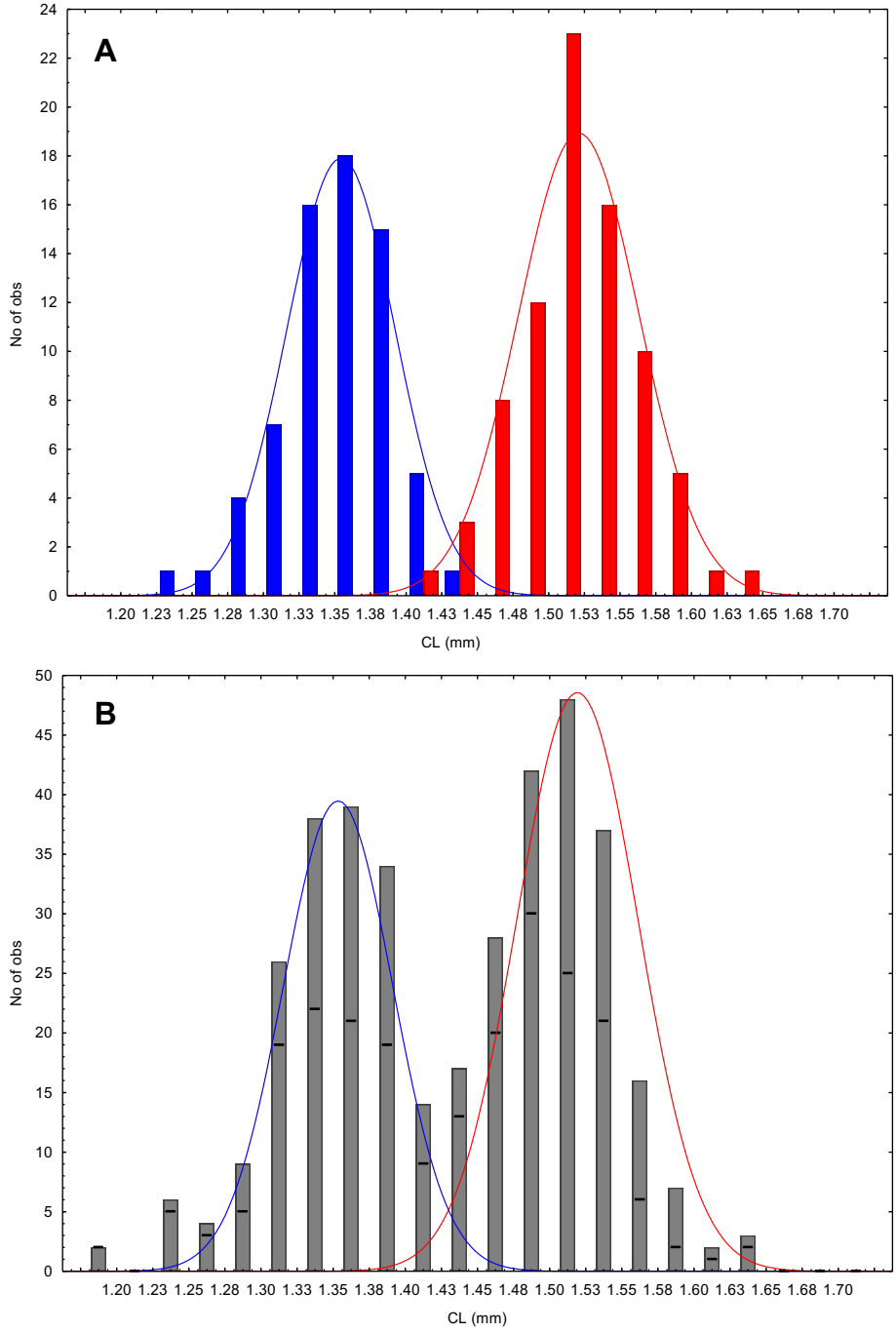

Fig. 6. Length frequency distribution of *L. salmonis* cephalothorax length (CL). A) CL of confirmed male and female chalimus 2 (sex assigned by the corresponding post molt preadult 1). Blue and red curves represent normal distributions fitted to data for males and females respectively. Distributions were not significantly different from normal distributions (Shapiro–Wilks test, $p_{male} = 0.44$ and $p_{female} = 0.90$). (B) CL frequency distribution including all chalimus 2 collected from fish. Short line in the bar (-) indicates the frequency distribution of exclusively chalimus 2 that did not molt. Blue and red curves are superimposed normal distribution curves that were fitted to the confirmed males and females in Figure A.

(Table 1 in [10]) while many of the incubated chalimus 1 were delayed and molted at 13–14 DPI. Chalimus 1 were by far more difficult to handle than the larger chalimus 2, and molting was evidently postponed due to handling and incubation by one or two days compared to the chalimi residing on the host.

4.3. Sexual size dimorphism and instar age

Sex of chalimus 2 could not be determined directly by distinct morphological features as in preadults and adults [13]. However, it was possible to sex determine old chalimus 2 males and females based on the corresponding preadults emerging in incubators. Their CL frequency diagrams were almost overlapping the bimodal CL frequency diagram of chalimus 2 that did not molt (Fig. 6), suggesting that CL displayed only a moderate increase during the instar phase and, as demonstrated by its distinct bimodal distribution, represented a morphological trait enabling sex determination of chalimus 2 instars. According to Fig. 2B it can be assumed that CL < 1.37 mm = male and CL > 1.44 = female. Between these values is a window where males and females may overlap in size, in particular CL of old males and younger females. However, information on stage age can be inferred from the CL/TL ratio (Fig. 7). The ratio is sex independent, mean ratio for young chalimus 2 was

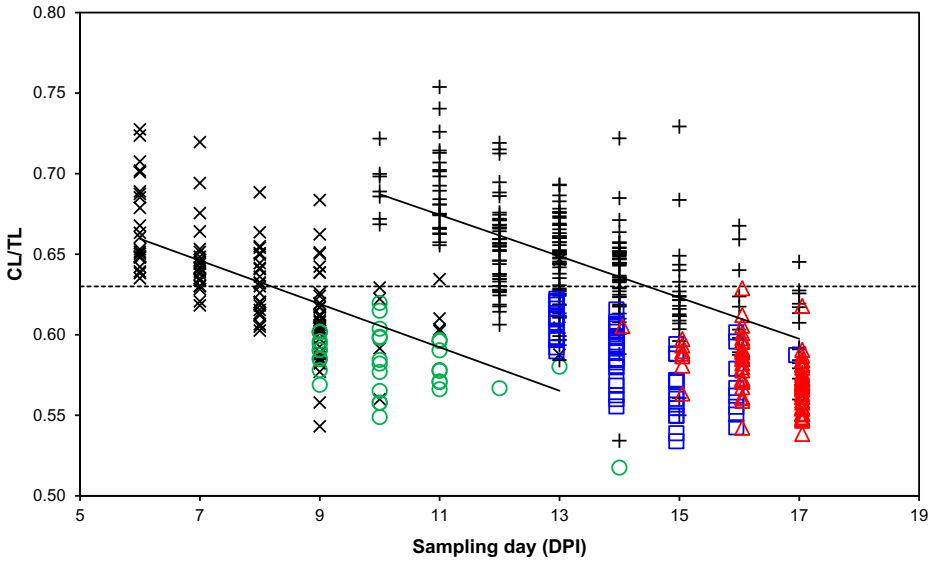

Fig. 7. CL/TL ratio of *L. salmonis* chalimus larvae. The CL/TL ratio decreased significantly with day of sampling (Spearman $R_{chalimus\ 1} = -0{,}76$, $p < 0.001$, Spearman $R_{chalimus\ 2} = -0.72$, $p < 0.001$). TL = total length, CL = cephalothorax length. Green circle = chalimus 1 with molt capacity, blue square = male chalimus 2 with molt capacity, red triangle = female chalimus 2 with molt capacity. The CL/TL ratio above which no larvae molted (0.63) is marked with a dotted line.

about 0.68 and decreased with increasing age to about 0.56 for old individuals. All instars with molt capacity had a CL/TL ratio below 0.63, and among these all the confirmed males belonged to the 49% smallest individuals and all confirmed females belonged to the 51% largest individuals.

Chalimus 2 females were about 10% longer than males. The sexual size dimorphism was observed among the fully developed chalimus 2 (DTM = 1) and among younger chalimus 2 that recently had acquired molt capacity (DTM = 4) (Table 1). Size estimates of old chalimus 1 and young chalimus 2 show that sexual size dimorphism could not be observed among late chalimus 1, and that the size difference arose solely as a result of a larger female than male molt size increment when molting to chalimus 2 and not as a result of differential chalimus 1 instar growth.

Chalimus 2 males appeared one day before the females and, hence at any given sampling point the mean male stage age was higher than the mean female stage age. For males and females of equal stage age sexual size dimorphism remained constant during the instar life.

4.4. Sex determination of young chalimus 2

The young male chalimus 2, represented by the first chalimus 2 that appeared on the fish at 10 DPI, had CLs ranging from 1.25 to 1.38 mm.

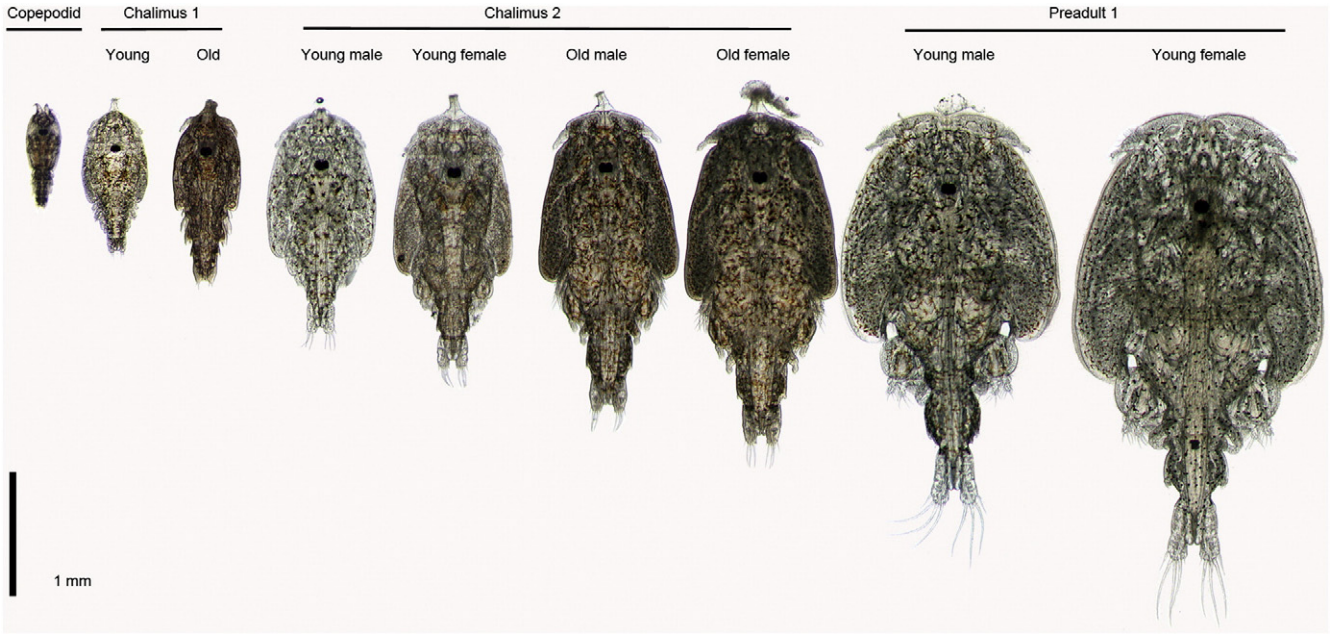

Fig. 8. Photographs showing the development of *Lepeophtheirus salmonis* from copepodid to the preadult 1 stage. Chalimus 1 = Ch1, chalimus 2 = Ch2.

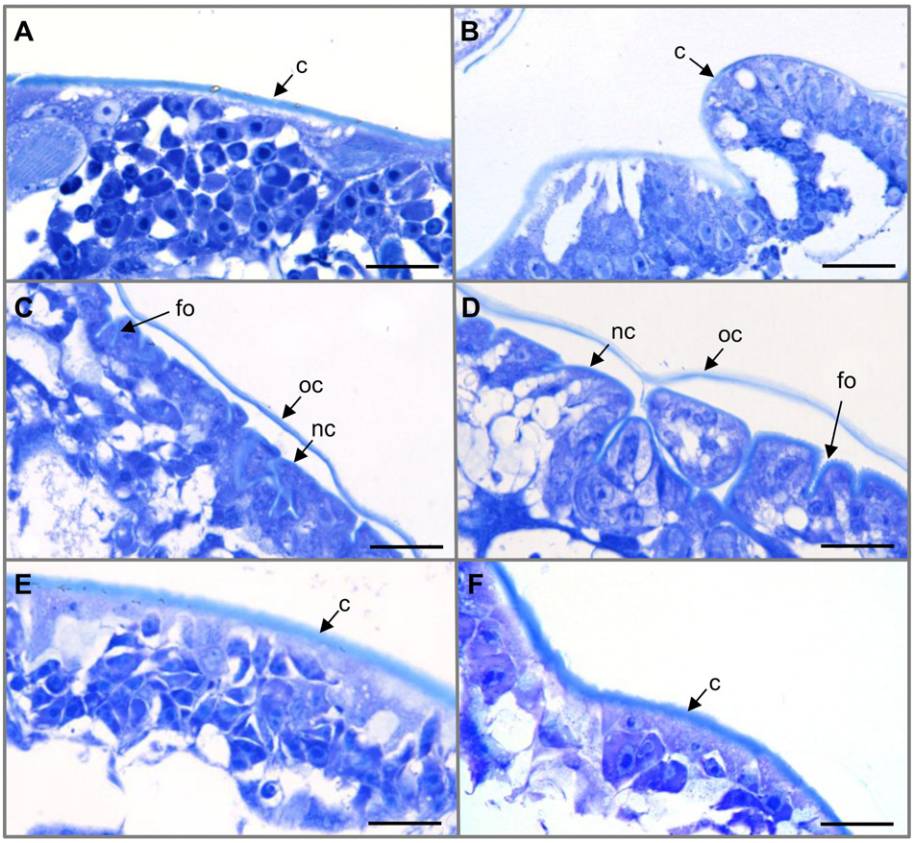

Fig. 9. Toluidine blue stained sections of *L. salmonis*. Left panel are horizontal plane sections from cephalothorax, right panel are horizontal plane sections from genital segment. A, B: Young chalimus 1 (5 DPI, directly after molting from copepodid); C, D: old chalimus 1 (14 DPI, directly before molting to chalimus 2) and E, F: young chalimus 2 (15 DPI, directly after molting from chalimus 1); c = cuticle, nc = new cuticle, oc = old cuticle, fo = fold. Scale bar = 20 μm.

Observations of a new and larger size group of chalimus 2 at 11 DPI with CLs above 1.4 mm (Fig. 2) strongly suggest that females appeared about one day later than males since a CL growth rate of this magnitude is highly unlikely. Preadult 1 females appeared two days later than males [10] and chalimus 2 females obtained molt capacity two days later than males (Fig. 2). They also displayed a significantly higher relative molt length increase than males when molting to preadult 1 (Fig. 1 and Table 2). Thus, observing that the first chalimus 1 to obtain molt capacity molted into smaller chalimus 2 than equally sized individuals sampled later (Fig. 3) further supports the idea that the first chalimus 1 with molt capacity at 9 DPI were males and the majority of chalimi with molt capacity at 11 DPI or later were females.

Among the instars that obtained molt capacity at 9 DPI there were no corresponding premolt chalimus 2 with CL above 1.27, this identifies all the newly molted instars as males with substantial confidence (Fig. 6). Among those that obtained molt capacity at 11 DPI there was one individual with CL = 1.28 mm, the rest were between 1.35 and 1.47 mm suggesting that all but one were females (Fig. 6). At 10 DPI there were about 50/50 males and females (data not shown).

The present observations are further supported by descriptions of "young" and "old" chalimus 3 larvae by Ref. [21]. Here, the size of chalimus 3 termed "young" and "old" was strikingly similar to our estimates of young chalimus 2 male and female size (Table 1), and the length ratio between "young"/"old" chalimi was 0.9. The CL/TL ratio measured from the drawings of "young" and "old" chalimus 3 (Fig. 4 in [21]) was 0.67, thus demonstrating that the depicted instars were both young. In light of the present study the "young" chalimus 3 larvae described were likely young chalimus 2 males with un-inflated cuticulas and the later chalimus 3 larvae with un-inflated cuticles identified as "old" were likely young chalimus 2 females. See Table 3 for comparison of data from Schram's study [21], Johnson and Albright [13] and this study.

4.5. Instar life span

The chalimus phase lasted on average 12 days for males and 14 days for females [10]. By including CL measurements in the present study it was possible to identify young chalimus 2 males and females. A rough estimate of the point in time where 50% of chalimus 1 had molted to chalimus 2 would be between 10 and 11 DPI for males and 11–12 DPI for females. The time span between first observation of chalimus 1 (5 DPI) and first observation of chalimus 2 on the fish (10 DPI) was 5 days, demonstrating the developmental rate of the quickest developing males. Hence, the chalimus 1 stage lasted 5–6 days for males and 6–7 days for females at 10 °C. Chalimus 2 males lasted 6–7 days and females 7–8 days. A differential life span for male and female chalimi was also demonstrated by Ref. [25].

4.6. Frontal filament

Several authors provide evidence suggesting that the frontal filament of the chalimus 1 larvae is replaced by a new filament upon molting to the chalimus 2 stage [4,9,14]. Our observations of the shed chalimus 1 exuviae with the frontal filament attached are in accordance with these observations (see Fig. 4 in Ref. [10]). We did not see frontal filaments attached to the shed chalimus 2 exuviae. However, we could observe a structure similar to a preformed filament anteriorly in the premolt chalimus 2 larvae. This structure was neither visible among the very young chalimus 2 nor among the very young preadults. Thus, in sum the observations of Refs. [3,14,9], and this study show that a total of 5 separate filaments are formed during the *L. salmonis* life cycle, i.e. the filaments produced prior to molting by the copepodid, chalimus 1, chalimus 2, preadult 1 and preadult 2.

4.7. Concluding remarks

The present study provides detailed information regarding the growth and development of *L. salmonis* during the chalimus stages. The significant instar growth explains to a large extent why the life cycle of *L. salmonis* was described with four chalimus stages and not two as recently shown. The results obtained here are important for future studies on salmon louse since it is now possible to evaluate instar age and to identify male and female chalimus 2. By this better and more precise samples for functional studies can be obtained.

Acknowledgments

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