The sea louse Caligus elongatus (Caligidae). Genetic variation and host use by its two genotypes

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Front cover motive: Adult *Caligus elongatus* male attached with frontal filament on two-spotted goby's (*Gobiusculus flavescens*) dorsal fin

Abstract

Caligus elongatus (Caligidae: Siphonostomatoida) is a common ectoparasite of fish in the north Atlantic. Unlike the salmon louse (*Lepeophtheirus salmonis*) which is specific to salmonids, *C. elongatus* infects more than 80 fish species and is considered an unspecific generalist parasite. It is registered on most common fish species in Norway, including farmed fish like Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*) and lumpfish (*Cyclopterus lumpus*). Sudden infections with high intensities of adult *C. elongatus* on these farmed fish have been observed, without a preceding infection with chalimus larvae. Therefore, it is likely that these adult lice originate from wild fish outside the farms. We raise the question what role small-sized fish acting as intermediate hosts could play into the infections on farmed fish. It was recently discovered that *C. elongatus* actually consists of two (mtDNA) genotypes, genotype 1 and 2, which may be sibling species. This discovery necessitates renewed research into the ecology of the two *C. elongatus* variants, since much past work could have concerned a mix of these. Some recent studies provide indications of different host use, temporal occurrence and geographical distribution of the genotypes.

The aim of the present work was to examine the genetic variation, morphology and aspects of the ecology of the *C. elongatus* genotypes.

A likely intermediate host, the two-spotted goby (*Gobiusculus flavescens*), was sampled throughout a year to assess the infection dynamics of *C. elongatus* at a locality in western-Norway. Lice from these gobies, and additional ones from various sympatric hosts and from other locations from the north-east Atlantic, were genotyped. A novel primer assay based on the cytrochrome oxidase 1 (CO1) gene was tested. The CO1 gene was sequenced from 94 lice, and compared to reference sequences in GenBank.

The prevalence of *C. elongatus* on two-spotted gobies peaked in May (10%) and October (5%). Nearly all were attached stages, mostly chalimi. Adults developing on the gobies must leave them to find another host for reproduction. It is demonstrated that this phenomenon can be responsible for high densities of free adult *C. elongatus* in the water. Such lice may also infect farmed fish. All juvenile lice found on two-spotted gobies throughout the year was genotype 2. Adults from Atlantic cod were mostly genotype 2, while all adult *C. elongatus* from farmed Atlantic salmon were genotype 1. Chalimi from lumpfish were genotype 1. Novel genotyping assays for genotyping with PCR readily distinguished the genotypes. We found 21 nucleotide

positions defining the two genotypes based on the mtCO1 sequences. Morphometric comparison of major body proportions of copepodites and adult females from the two genotypes revealed significant differences: Genotype 1 *C. elongatus* were generally larger than genotype 2 and the cephalothorax shape of genotype 1 copepodids were more oblong than genotype 2. The present findings corroborate previous knowledge on the genotypes and their hosts, demonstrate the infection dynamics of genotype 2 on an intermediate host, and suggest morphological characters that should be examined further for their ability to distinguish these *C. elongatus* variants. The findings support the belief that the two genotypes could represent two species.

Acknowledgements

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Innholdsfortegnelse

Abbreviations

A	adenine
AA	amino acid
C	cytosine
CL	cephalothorax length
cm	centimetre
CW	cephalothorax width
dl	decilitre
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
e.g.	exempli gratia (“for example”)
et al.	et alia (“and others”)
FET	Fishers’ exact test
g	gram
G	guanine
i.e.	id est (“that is”)
IMR	Institute of Marine Research
km	kilometre
KW	Kruskal-Wallis test
l	litre
m	metre
MBS	Espegrend Marine Biological Station
ml	millilitre
mm	millimetre
mtCO1	mitochondrial cytochrome oxidase subunit 1
MW	Mann-Whitney U-test
N=	sample size
ng	nanogram
no.	number
nt	nucleotide
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid

SLRC	Sea Lice Research Centre
T	thymine
TL	total length
UV	ultraviolet
µl	microlitre
µm	micrometre

Terms

Abundance	Total number of individuals parasites of a species on/in a single host individual regardless of whether the host is infected (Bush *et al*. 1997)
Attached louse	*Caligus elongatus* attached to fish with frontal filament
Chalimus	The post-copepodid developmental stage of most siphonostomatoid fish parasites, characterised by possession of a frontal filament for attachment to the host (Boxshall & Halsey, 2004)
Copepodid	The postnaupliar phase in copepod development (Boxshall & Halsey, 2004)
Final host	The host on/in which a parasite reproduces sexually
Free louse	*Caligus elongatus* moving freely on a host, not attached with frontal filament
Intensity	The number of individual parasites of a species in/on a single infected host individual (Bush *et al*., 1997)
Intermediate host	A host different from the final host harbouring larval stages of a parasite.
Juvenile phase	Fish harbouring developing larvae of *Caligus elongatus*
Metacercaria	Encapsulated larval stadium of trematodes in second intermediate host, normally the infective stadium
Moult/ecdysis	To shed the outer exoskeleton.
Planktonic	Living free in the water masses without a host
Prevalence	The share of hosts in a sample or in a population which is infected. Normally given in percent.
Sea louse	Collective term of species in the family of Copepoda (order Siphanostomatoid

1. Introduction

The sea louse *Caligus elongatus* Nordmann, 1832 is a common ectoparasite on marine fish in the North Atlantic. The species belongs in family Caligidae (order Siphonostomatoida), which also harbours the salmon louse *Lepeophtheirus salmonis* Krøyer, 1837. *Caligus elongatus* is found as a parasite on over 80 fish species (Kabata, 1979), which suggests the species is unspecific with respect to hosts, compared to *L. salmonis* which mainly parasitizes salmonids (Tully & Nolan, 2002). Atlantic salmon (*Salmo* salar) was found infected with *C. elongatus* already in the early stages of salmon farming in Scotland and Norway in the 60's and 70's (Pike 1989; Bron, Sommerville, Wootten, & Rae, 1993; Wootten, Smith, & Needham, 1982).

1.1 Caligus elongatus in Norwegian aquaculture

Just over 30 years ago, *C. elongatus* was hardly mentioned in context with Atlantic salmon (Håstein & Poppe, 1986; Håstein, Lunder, & Poppe, 1989) and was considered a parasite occurring in such low intensities that it was not causing problems in salmon farms (Johannessen, 1990). However, *C. elongatus* has received more attention the last few years in Norwegian salmon aquaculture and seems to be an increasing problem, especially in the northern parts of the country (Imsland, Sagerup, Remen, KB-H, & Myklebust, 2019; Hemmingsen *et al.* 2020).

Caligus elongatus reduces the fish health and even few lice can cause reduced welfare, especially on small fish. Salmon infected with *C. elongatus* react with increased jumping activity, possibly to remove lice (Wootten *et al.,* 1982; Imsland *et al.,* 2019) and injuries on the salmon from collision with hard structures in the pens increase correspondingly. Reduced appetite is also a reported consequence of *C. elongatus* infections (Imsland *et al.,* 2019). The prevalence is highest during the autumn (September-November) when the sea water is warm, and decreases as the sea temperature decreases during late autumn/winter (Koren 2001). The intensity of *C. elongatus* infestations on farmed Atlantic salmon occasionally reach levels requiring the fish farmers to delouse their fish (Imsland *et al.,* 2019; Nodland, 2017), hence *C. elongatus* also bring economic consequences.

The lumpsucker (*Cyclopterus lumpus*) is a frequently used cleaner fish and is also a common host for *C. elongatus* (Imsland *et al.,* 2019). In extreme cases, lumpfish can be found infected with hundreds of *C. elongatus* and a large wild lumpfish was found infected with over 700 lice (Hansen & Øines, 2017). Other farmed fish species in Norway include rainbow trout

(*Oncorhyncus mykiss*), Atlantic cod (*Gadus morhua*), and flatfish like the Atlantic halibut (*Hippoglossus hippoglossus*). As a generalist, *C. elongatus* also infect these species. Atlantic cod is proven to be an attractive host species for the parasite (Øines & Heuch, 2006) and mass infestation on farmed cod resulting in wounds and reduced fish welfare has been reported (Nygaard, 2005). Atlantic halibut at around 500 g farmed in net pens were infected by *C. elongatus* with intensities reaching over a hundred lice per fish (Bergh, Nilsen, & Samuelsen, 2001). There have been reports of massive infestations on the salmon in cages practically over night (Revie, Gettingby, Treasurer, & Rae, 2002), leading to speculations that this louse species accompanied migratory wild fish species like saithe (*Pollachius virens*) or herring (*Clupea harengus*) (Wooten *et al.,* 1982; Bruno & Stone, 1990).

1.2 The Caligus elongatus life cycle

Caligus elongatus has a direct life cycle without any required intermediate host species. It consists of eight life stages with seven ecdyses (moults) and no preadult stage unlike *L. salmonis* (Piasecki, 1996) (Figure 1.1). The outer layer of the louse is a cuticle, consisting of chitin, cuticle proteins and lipids (Reynolds & Samuels, 1996). The first two stages after hatching from an egg string are planktonic nauplii. After approximately 38 day-degrees the nauplius II moults into a copepodid, which is also planktonic until it infects a fish (Piasecki & MacKinnon, 1995). The copepodid must infect a host in order to complete its life cycle. When it is close enough to sense a host, the copepodid jumps onto the host and grips the fish's skin with its second antennae and maxillipeds. Now the copepodid starts feeding and prepares for its first moult. Shortly before moulting the copepodid pushes out a preformed frontal filament from the frontal pocket and attaches it to its fish host, preferably a bony structure like a fish scale or a fin ray (Piasecki & MacKinnon, 1993). The louse remains attached to the host through a series of stages called chalimus stages until the louse moults into the adult stage and has developed a "suction cup cephalothorax" with a marginal membrane allowing the louse to move freely over the host surface. Unlike *L. salmonis,* which have two chalimus stages and two free living preadult stages before adult, *C. elongatus* has four chalimus stages and detaches from the filament attached by the copepodid first when reaching the adult stage. In contrast to *L. salmonis* which produces a new frontal filament between each ecdysis in the parasitic phase, *C. elongatus* keeps its filament, though adding a proximal extension lobe at each moult that can be used for stage determination (Piasecki & MacKinnon, 1993). The same is seen in other *Caligus* spp. (Gurney, 1934).

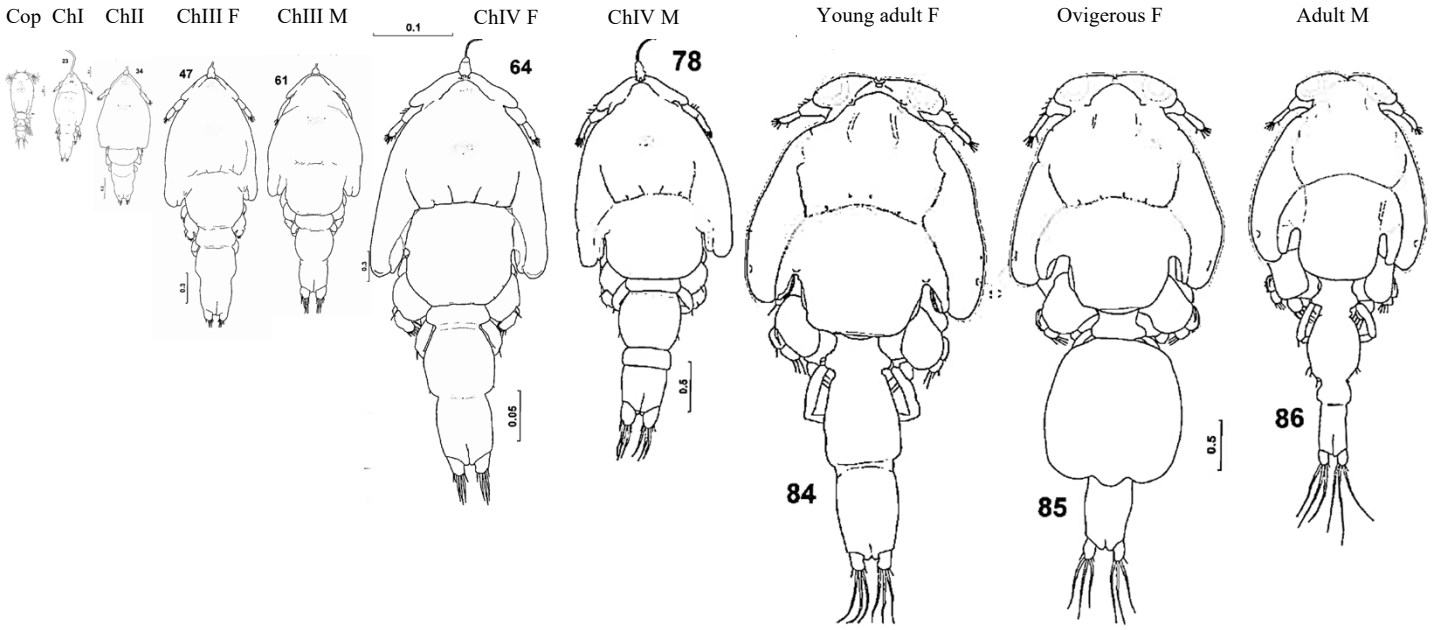


Figure 1.1. Assembly of *C. elongatus* developmental stages from infective copepodid to adults. Scale bars in mm. Cop= copepodite. Ch= chalimus. F= female. M=male. Original drawings from Piasecki (1996) modified and assembled by L. A. Hamre (SLRC).

1.3 A role for intermediate hosts?

The adult *C. elongatus* are very capable swimmers and are frequent findings in marine zooplankton samples (Lönnberg, 1889; Bassett-Smith & Surgeon, 1896; Björck 1916; Neilson, Perry, Scott, & Valeiro, 1987; Scott, 1900; Pike, 1989). Such observations have been made throughout the year. Adult *C. elongatus* can even constitute an important part of the diet of pelagic gadid fry (Neilson *et al.,* 1987). They occur in plankton samples from the surface and down to 125 m with a dominance of males and small immature females (Björck, 1916; Karlsbakk unpublished). The source of these free adults are likely larval lice developed on small and juvenile fish, leaving their first hosts, probably because they are unsuitable for the adult lice (Björck, 1916; Neilson *et al*., 1987). This phenomenon may explain the apparent sudden infection pressure posed by adult *C. elongatus* experienced by many farmers along the Norwegian west coast.

The source of the adult *C. elongatus* in sudden infections on farmed fish is unknown, but it could often be excluded that they developed on the farmed fish due to the absence of chalimi prior to the adult infections (Koren, 2001; Nygaard, 2005; Karlsbakk, Isaksen, & Hamre, 2009). A likely source is lice developing from copepodids to adults on other fish, but leave them. Chalimi occur on fish larvae and other small sized fish (Russell, 1933; Rosenthal, 1967; Tolonen & Karlsbakk, 2003; Jensen *et al.,* 2016) including pelagic juvenile gadids (Neilson *et*

al., 1987; Russell, 1933; Shotter, 1973; Karlsbakk & Nilsen, 1993; Karlsbakk, Otterlei, Høie, & Nylund, 2001). Heuch, Øines, Knutsen & Schram (2007) examined the prevalence of *C. elongatus* on various Norwegian marine fish species in southern Norway. They found sea trout and gadids, especially pollack, saithe, Atlantic cod and whiting to be infected with high prevalences of the parasite. They also detected chalimi on gobies, such as the black, sand and two-spotted gobies, in low prevalence.

In the Bergen area, two-spotted gobies are often seen infected late summer-autumn. This fish species and other gobies (*Pomatoschistus spp.*) are very abundant in shallow waters along the Norwegian west coast, and the density of e.g. two-spotted gobies is high especially in the summer months (Fosså, 1991). Due to a large host density leading to a high louse production, the small abundant gobies such as the two-spotted goby may therefore be important host species for *C. elongatus*, despite low prevalence and abundance. The adult lice leave their small-sized hosts and become free planktonic swimming in the water, searching for a final host for mating and reproduction. A similar role as intermediate host could be played by threespine stickleback in Maine in the northwest Atlantic (Jensen, Zydlewski, Barger, & Pietrak, 2016) and possibly by cod and haddock fry in the Grand Banks off Newfoundland or in northern Norway (Neilson *et al.,* 1987; Karlsbakk & Nilsen, 1993). This scenario involves a regular host shift for this caligid copepod, from a host for juveniles to a host for adults (reproduction). Such hosts are normally referred to as "intermediate" and "final" hosts. However, a problem here is that some hosts such as lumpsucker act both as a host to the larvae and for the adults. Therefore, the terms intermediate host or host for the juvenile phase can be used to designate the fish harbouring developing larvae and final host when they are infected with motile adult lice. Salmonids may act as hosts for the juvenile phase during autumn, but most of the year they harbour mainly adult lice (Bron *et al.,* 1993). Large gadids are normally infected by adults only, while cod fry are found to harbour juvenile *C. elongatus* (Karlsbakk *et al.,* 2009). Lumpfish is a species which clearly act as both nursery and final host, although apparently for one distinct genotype of the parasite (Øines & Heuch, 2007).

1.4 Genotypes

Studies of mitochondrial and nuclear genes of *C. elongatus* indicated the presence of two mitochondrial genotypes in Norway, called genotype 1 and 2 (Øines & Heuch, 2005; Øines & Schram, 2008). What Øines and Heuch called genotypes are strictly speaking genogroups. However, to avoid confusion and to keep it single, these two groups are in this study still called

genotype 1 and -2. These genotypes may even be sibling species, as the nucleotide variability between the groups are similar to that of other closely related caligid species, when comparing the mitochondrial sequences (Raupach *et al.,* 2015; Øines & Schram, 2008). They are now readily separated on the basis of their mitochondrial Cytochrome oxidase subunit 1 (mtCO1) gene, a gene commonly used to identify closely related species because of its high evolution rate (Raupach *et al.,* 2015).

The first reports of *C. elongatus* (previously also named *C. rapax*) are over 170 years old (Nordmann, 1832; Baird, 1850) and great numbers of publications on the parasite have been produced. The new discovery that this sea louse species can be divided into two genotypes and could be closely related sibling species complicates our knowledge. Much past information on morphology and development, ecology and host use concern unknown genotypes, so new studies are necessary. While both appears to be generalists, there are apparent host preferences and indications of differences in temporal occurrence (Øines & Heuch, 2005, 2007; Heuch *et al.,* 2007; Jensen *et al.,* 2016).

1.4.1 Genotyping assays

Barcoding using DNA sequences has the recent years become a popular way to identify organisms. Mitochondrial genes inherited from an organism's mother have high mutation rates and are therefore often used to detect genetic variations between closely related species or variants of species (Raupach *et al.,* 2015). Øines & Heuch (2005) discovered that *C. elongatus* occurred as two distinct genotypes with this barcoding system using the mentioned mtCO1 gene. Further investigation of the genetic differences between the genotypes with the additional nuclear 18S ribosomal RNA- and the mitochondrial 16S gene ribosomal RNA genes revealed similar variations in 16S, but not the nuclear gene (Øines & Schram, 2008).

Because the mtCO1 gene shows the highest variation in caligids, it is the most favourable gene to use when genotyping *C. elongatus*. Mitochondrial genes are also present in higher numbers in a single cell because of the high number of mitochondria, contrary to most nuclear genes, making them favourable to use when DNA concentration is low. When first describing the two genotypes, Øines & Heuch (2005) developed primers (WOBCOIF and WOBCOIR) to sequence the mtCO1 gene in caligid species. Later, a real-time PCR assay targeting mtCO1 with a primer pair (CetqwbF/R) and genotype specific probes (CEQ1 for genotype 1 and CEQ2 for genotype 2) was designed and used to genotype C. elongatus (Øines & Heuch, 2007). WOBCOI primers and CEQ probes have been used for sequencing and qPCR in other studies

(Agusti-Ridaura *et al.,* 2019; Jensen *et al.,* 2016). However, there are no primers available to genotype the lice with regular PCR. Further improvement on the genotype 2 probe CEQ2 was recommended (Øines & Heuch, 2007). Heidi Kongshaug at Sea Lice Research Centre (April 2020, Bergen) therefore developed novel primer assays in order to genotype PCR products using agarose gel electrophoresis. These primers have so far not been tested on a wide range of *C. elongatus* mtCO1 variants, nor are they published.

1.4.2 C. elongatus *genotypes – any non-genetic differences?*

Most studies on *C. elongatus* were done prior to the discovery of the two genotypes (Øines & Heuch, 2005). The significance of the two genotypes has not been robustly examined. There is a need for follow up studies using nuclear genetic markers, and the species problem could be examined with hybridization experiments testing the biological species concept. If proven to be two species, our present conception on morphology, host use, reproductive biology and ecology of "*C. elongatus*" may be erroneous, and need re-examination. Indeed, then the question arises which of the two genotypes that should be designated as *C. elongatus*.

After the discovery of the mitochondrial genotypes of *C. elongatus* a search for differences was done (Øines, 2007), including these aspects. Variations were noted in host preference, spatial and temporal distribution and even minor morphological differences on the appendages. These morphological characters were short processes on first leg, spine on fourth leg longer than the base of the following spine and the shape of sternal furca. They found genotype 1 lice to predominantly fit the two former characters while genotype 2 mostly did not. The third character did not vary between the genotypes (Øines & Schram, 2008).

Following the discovery of the two genotypes, researchers have in recent studies tended to genotype their *C. elongatus* (Øines & Heuch, 2005, 2007; Øines *et al.,* 2006; Jensen *et al.*, 2016; Pietrak *et al.,* 2019; Agusti-Ridaura *et al.,* 2019). An important consideration regarding host preferences is the lice stage, since *C. elongatus* is known to readily switch between host individuals. It has rarely been noted whether *C. elongatus* were attached with a frontal filament (attached) or motile adults (free) when sampled from the host fish for genotyping. If host preferences exist for *C. elongatus*, this could be most apparent on intermediate hosts. Previous results indicate that lumpfish is a key host for genotype 1, which also is the dominant type on farmed Atlantic salmon (Øines, Simonsen, Knutsen, & Heuch, 2006; Øines & Heuch, 2007). Genotype 2 seems to especially infect Atlantic cod, but other fish like sea trout, whiting (*Merlangius merlangus*) and gobies harboured this type. Only genotype 1 has been found as

chalimus on salmon, and challenge experiments with this genotype on salmon are successful and lead to the development of adult lice (Agusti-Ridaura *et al.,* 2019). Clearly, these host patterns have not been sufficiently examined.

1.4.3 Spatial and temporal distribution *of C. elongatus genotypes*

When the genotypes first were reported, there seemed to be no temporal difference in their appearance (Øines & Heuch, 2005). However, in a later study genotyping lice from various coastal hosts in south-east coast of Norway, Øines & Heuch (2007) found genotype 1 to be the only genotype present during spring and predominantly during summer, before a shift took place in the autumn where genotype 2 became dominant. The spring and summer lice were linked to mature lumpfish migrating from the Norwegian Sea to coastal areas to spawn, harbouring large numbers of genotype 1, which could have contributed to infestations on other fish species nearby. When most lumpfish migrated back to the Norwegian Sea to feed during the summer, the genotype 1 infestations on the examined coastal fish species decreased.

Out of all genotyped *C. elongatus*, genotype 2 has only been recorded in the southern parts of Norway with the northernmost type 2 lice found at Frøya, Sør-Trøndelag (63°N). All *C. elongatus* genotyped north of this point in Norway, and from the Faroe Islands, Scotland and Canada/USA have been genotype 1 (Øines & Heuch, 2005, 2007; Øines *et al.,* 2006; Agusti-Ridaura *et al.,* 2019; Jensen *et al.,* 2016; Pietrak, Jensen, Zydewski, & Bricknell, 2019).

1.5 Aims

Øines (2007) expressed the need to closer study the molecular and morphological characters to correctly identify the *C. elongatus* genotypes and acquire a better understanding of their ecology. In this thesis, the aim was to examine the genetic variation, morphology and aspects of the ecology of the *C. elongatus* genotypes. A more detailed picture of the seasonality of *C. elongatus* was sought by examining an assumed important intermediate host, the two-spotted goby (Karlsbakk personal communication) throughout a year. By obtaining both genotypes and morphologically compare them, differences could be revealed. The more specific subaims were:

- Examine the nucleotide variation in the C. elongatus mtCO1 gene

- Validate a PCR based *C. elongatus* genotyping assay (developed by H. Kongshaug, SLRC) based on the mtCO1 gene.

- Reveal infection dynamics of *C. elongatus* genotypes on an assumed important intermediate host species, the two-spotted goby (*Gobiusculus flavescens*).

- Examine the *C. elongatus* genotypes' host associations focusing on chalimi.

- Examine the usefulness of some readily obtainable major length measurement and ratios in distinguishing the genotypes in different stages of development.

- Reveal whether Atlantic salmon (*Salmo salar*) can act as a host for *C. elongatus* genotype 2 chalimi.

2. Material and Methods

2.1 Locations

The two-spotted gobies exanimated in this study were mainly collected along the shoreline at Espegrend Marine Research Field Station (MBS), located in Hordaland south-west in Norway (60.268ºN, 5.221ºE). In addition to MBS, gobies were also collected at two other localities in Hordaland in October in order to get insight into the spatial variation in *C. elongatus* abundance on this host. These were in Svinavika at Misje (60.454ºN, 4.962ºE) and at Lepsøy (60.143ºN, 5.370ºE), respectively 24.8 km north-west and 15.8 km south-east of Espegrend. The Sea Lice Research Centre (SLRC) laboratory facilities at Høyteknologisenteret in Bergen were used to examine the two-spotted gobies. All locations, including those where additional *C. elongatus* specimens were obtained from other host species are shown in a map (figure 2.1), also differentiating host species. Details such as coordinates and sample times are given in table 2.1.

2.2 Methods for sampling Gobiusculus flavescens

From March 2020 to February 2021, two-spotted gobies were caught. Intervals varied with time of year, monthly was intended in the cold periods, and biweekly when infections appeared. A total of 22 samplings were conducted at MBS, actual intervals varied from 11 to 38 days. When sampling fish, the salinity and temperature were measured. The salinity and water temperature was measured at 1 m depth with a conductivity meter (*WT cond 3110*). Three methods were used to sample the fish (2.2.1-2.2.3)- fish traps, beach seine or modified nets. Regardless of the method, the sampled fish were transported in a closed container with sea water from the sampling site for about 30 minutes to be examined at the SLRC laboratory. An oxygen stone was put into the fish's water immediately after arrival.

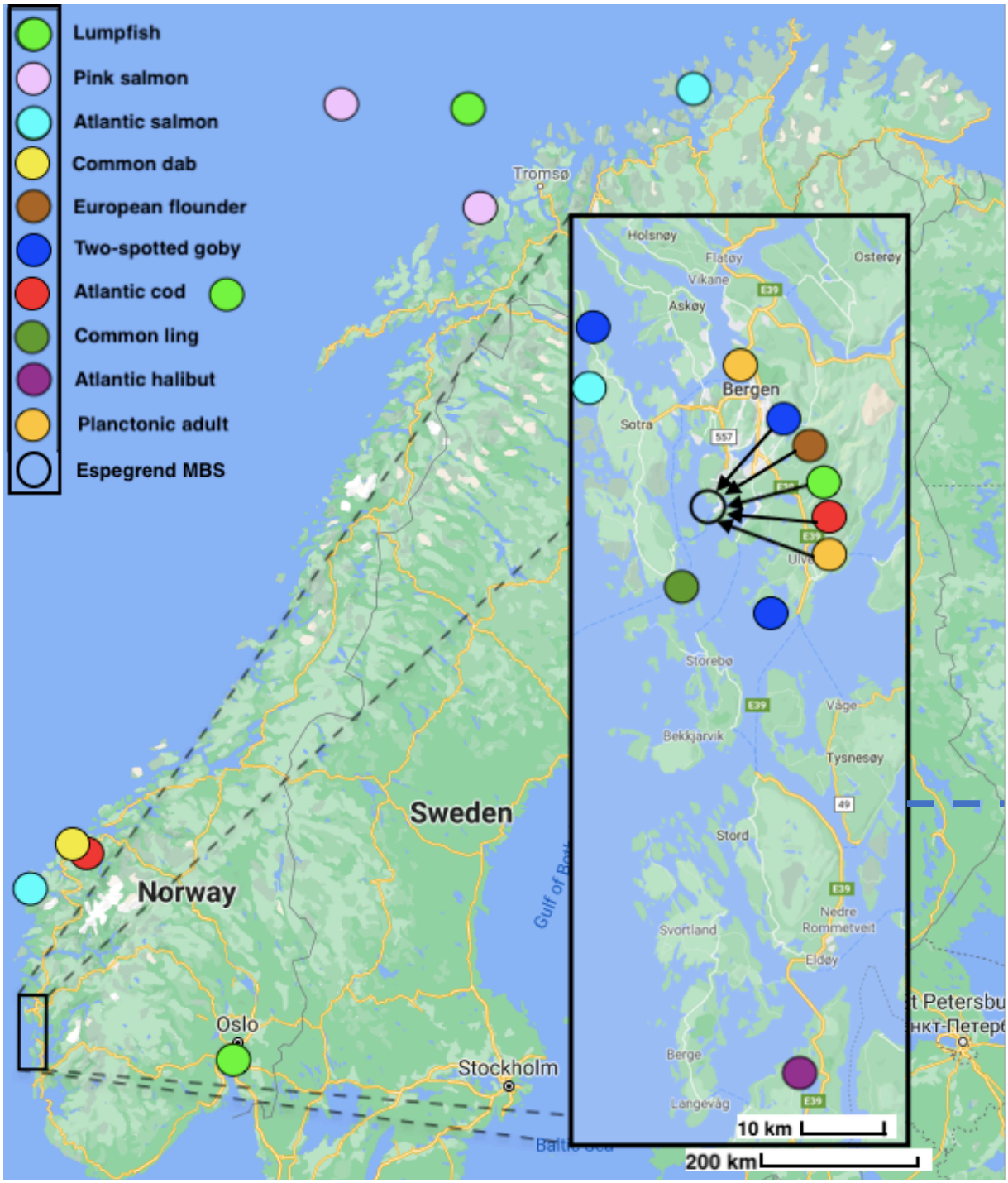


Figure 2.1. Overview over the geographical locations where fish with lice used in this thesis were caught. The colours of the markers indicate host fish species. Inside the magnified box, the coloured markers with arrows point to the transparent circle in which the fish were caught. Inside this circle lies also Espegrend Marine Research Field Station. The two dark blue markers without arrows are the locations of the reference sites of two-spotted goby sampling. From outside the map, a sample from farmed Atlantic halibut in the Faroe Islands was obtained. Map retrieved from Google (n.d).

Three fish traps, described by Breder (1960), were used at sampling 1-11. The traps had four plexiglass walls and a back part with nine holes at 35 mm in diameter covered with a 1 mm masked filter. The front part was installed with a pair of plexiglass "wings" (figure 2.2). The wings were slid into an inward track on the walls, leading to a 2 cm wide opening, thus being easy for the fish to enter yet difficult to escape. The wings were secured with strong rubber bands. Attached to the top wall was a rope with a marker buoy tied at the end. The traps were placed at the bottom among seaweed <1.4 m. This was mainly done in the evening, leaving the traps through the night. The following morning the traps were carefully pulled up to the water surface, though with the filter bottom still containing water. The wings were removed, and the fish were gently poured into a container filled with seawater taken from the sample spot to prevent water quality changes. Whenever we were unable to catch enough fish during the morning, the traps were put out again during the afternoon to achieve a larger total number of fish.

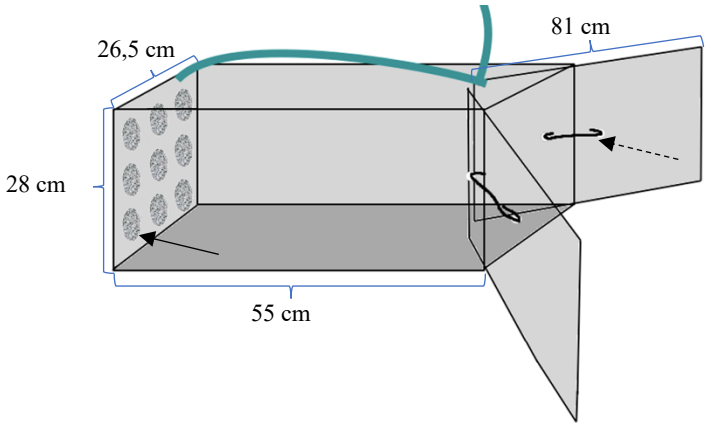


Figure 2.2. An illustration with measurements of a plexiglass fish trap used to catch two-spotted gobies. The dotted arrow points at rubber bands with metal hooks used to secure the wings. The full lined arrow points to one of the nine holes covered with a 1 mm filter mesh.

Modified nets were used during sampling number 8, 11 and the samplings at the two reference locations. The nets were used to sample fish the same morning they were to be examined. Two types of nets with the same principles were used (figure 2.3). One net had four ropes tied to a metal ring with a net sewed onto it and a small bucket was attached to the centre to collect the gobies (figure 3b). The other net was smaller with three ropes (figure 3a). One of two nets with led or a rock inside was immersed to the bottom. When a group of two-spotted gobies swam over the net, it was pulled upwards in a fast motion capturing the fish. Fish caught in the nets were gently put into a container filled with seawater from the sampling site.

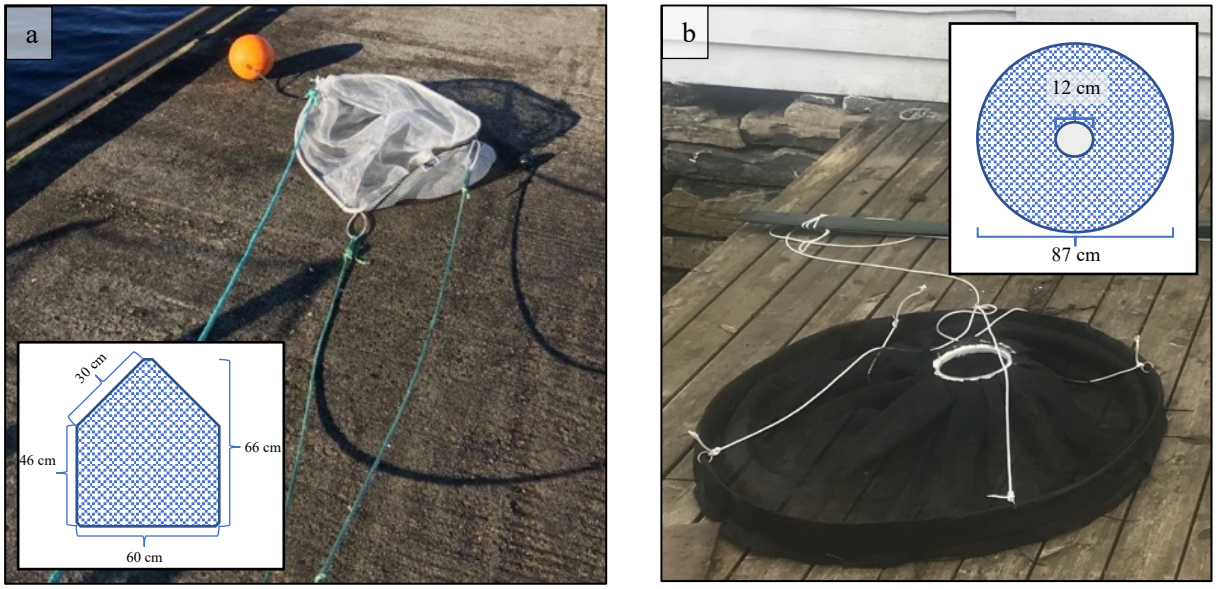


Figure 2.3. Two modified nets used to sample two-spotted gobies from shore. Measurements of the nets are given in the white boxes. **a**: net with nylon masks and three pulling ropes **b:** circular net with a bucked in the centre to keep fish in water.

Beach seine became the most frequently used sampling method, when the Covid restrictions allowed the staff at MBS to participate (boat). A beach seine owned by MBS was used (figure 2.4). It was transported to the sampling site by boat. One end of the seine was held back on land as the rest was slowly released in a U-shape until the other hauling rope ended on land. At this point an area of about 10x7 m was surrounded by the seine. The two hauling ropes on each side were gently pulled with equal speed to land whilst avoiding the upper floating line being dragged underwater. When reaching the split drag line, the foot rope was hauled along the beach floor to fully enclose the fish surrounded by the seine. The wings were systematically pulled to shore, leaving the area around a cod-end like described by Olsen (1975). Two-spotted gobies were then available for sampling. When gobies were in reach, a small hand net with 1.5 mm wide masks was used to gently catch and transport them to a container filled with fresh sea water.

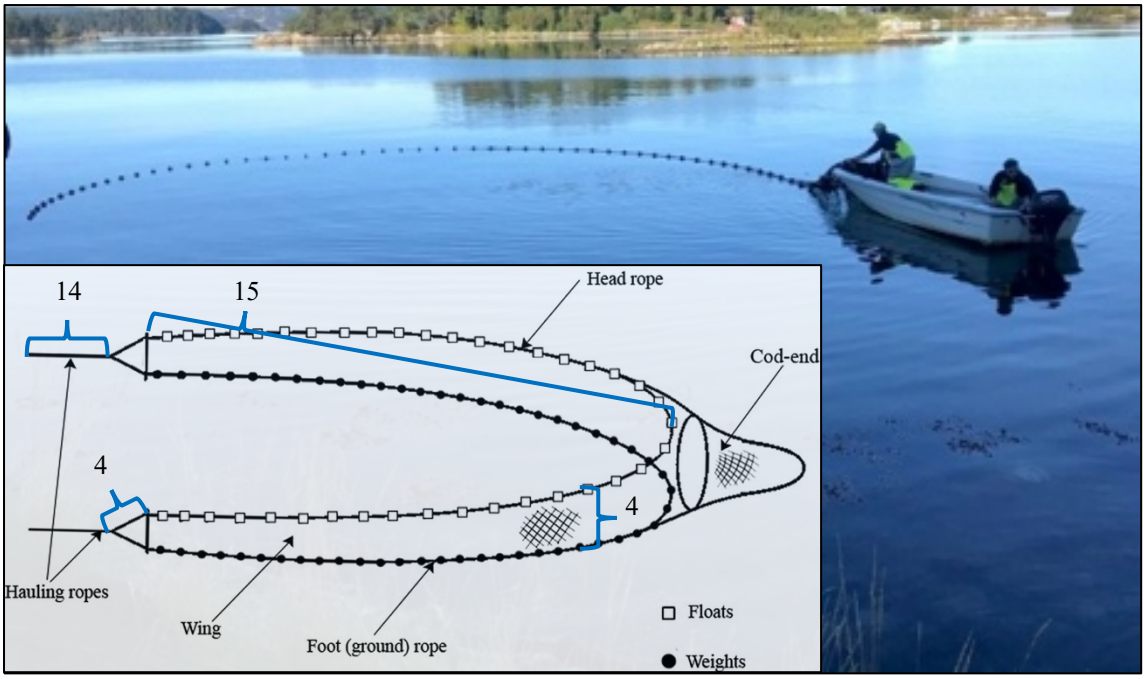


Figure 2.4. Picture of the beach seine in use to sample two-spotted gobies at Espegrend MBS. In the box down on the lower left is a figure retrieved from Gunawardena, Jutagate & Amarasinghe (2016) demonstrating the seine's structure. Measurements are given in meters.

2.3 Examination of Gobiusculus flavescens

Prior to examination, the fish were held at the SLRC laboratory in the transport container with aerated transport water. A group of 10-20 fish were moved with a fine meshed hand net into a 2 l plastic container filled with sea water. From there, groups of three fish were carefully put into anaesthetic water. The fish were anesthetized in a mixture of 1 ml metomidate hydrochloride (10mg/l) and 0.3 ml benzocaine (100mg/l) in 1 litre of fresh seawater for 2-3 minutes. When the fish's opercular activity was greatly reduced, they were placed with the koinet in a petri dish with fresh sea water just covering the fish. An *Olympus SZX12* microscope equipped with a trinocular head was used to examine the fish and images were obtained with a *Canon EOS 600D* camera attached to the microscope. Each two-spotted goby was examined with a magnification of ≥ 12.5x05, and handled by a bent forceps. The whole fish skin was closely inspected, including both sides of all fins.

When a louse was detected, pictures were taken using the Canon camera. Pictures were first taken of the louse in situ while attached to the fish, before the fin ray in which the louse's frontal filament was attached to, or the frontal filament itself was cut in order to free the louse. Pictures

of the freed louse were then taken as described by Eichner, Hamre & Nilsen (2015) with the louse placed on a microscope slide in a drop of sea water and enclosed with a cover glass. The louse was thereafter transferred to an 1.5 ml Eppendorf tube filled with 96 % ethanol and stored at -24 ºC before performing molecular analysis of the DNA.

During the examination other parasites were also observed, and their number was noted or estimated. Black spots representing metacercaria larvae of the trematode *Cryptocotyle lingua* were counted, while the number of *Gyrodactylus* sp. observable on skin and fins was estimated when intensities were high.

The total length was recorded, and the sex was determined following descriptions in Amundsen & Forsgren (2001). The transport water, the water in the 2 l plastic container and the anaesthetic water were filtered through a 90 µm mesh, for any detached lice. After examination, the unharmed gobies were collected in a large beaker with fresh sea water before releasing them in the sea at the end of the day. Damaged gobies were killed. A few fish of other species caught together with the two-spotted gobies were also examined and handled in the same way.

2.4 Louse measurements

Images of C. elongatus were uploaded individually to the image processing program *ImageJ* v. 1.53a. (https://imagej.nih.gov/ij/). After calibration based on images of rulers or objectmicrometers this program was used to measure selected parts of the lice (figure 2.5):

- Anterior end of cehphalothorax to centre between the eye lenses
- Centre between lenses to uropod end
- Cephalothorax length
- Cephalothorax width
- Distance lense-lense (centre left on left lense to centre left on right lense)

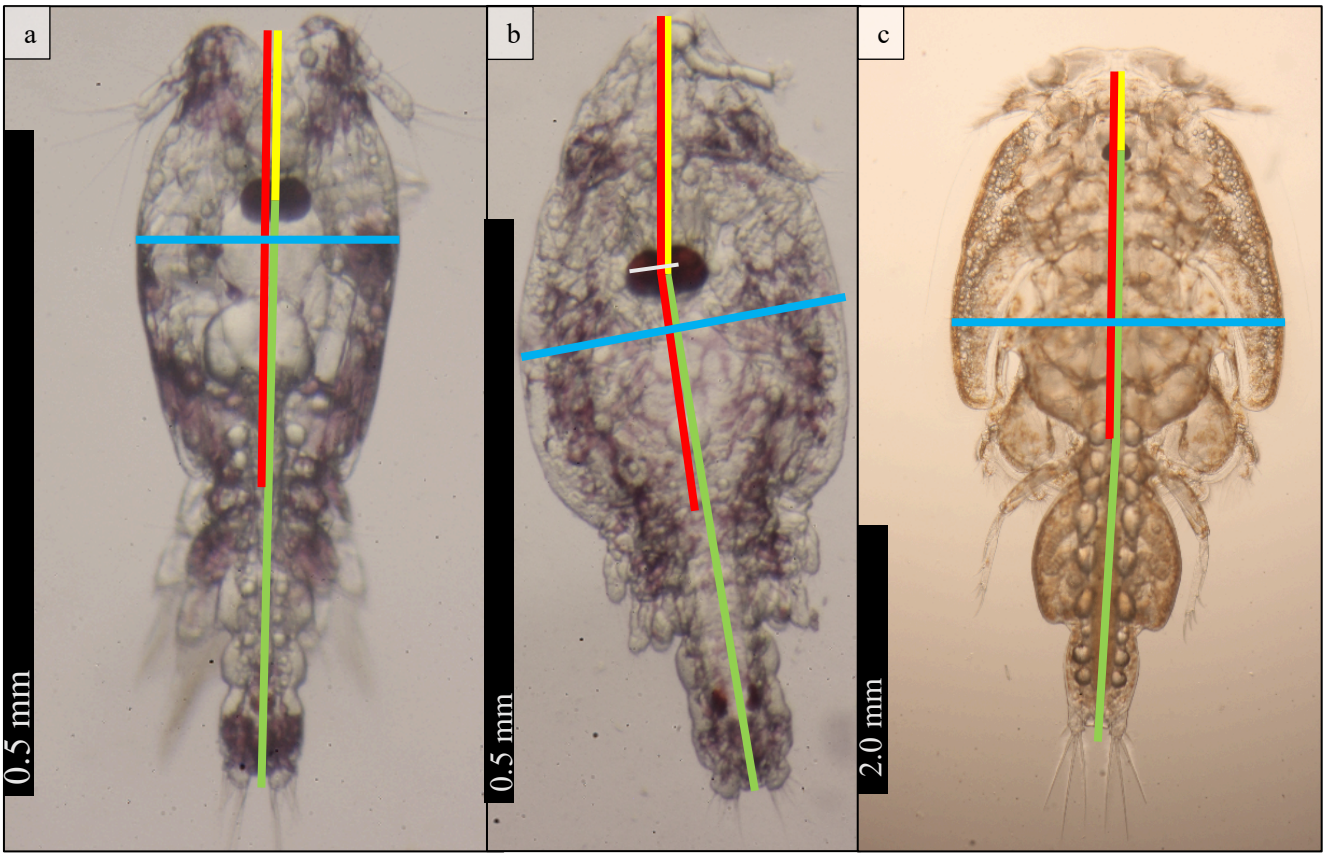


Figure 2.5. Examples of how the live *C. elongatus* were measured in *ImageJ*. Scale bars on each photo. The coloured lines demonstrate where measurements were taken. Yellow: anterior end of cephalothorax to centre of eyes. Green: centre of eyes to end of uropods. Blue: cephalothorax width. Red: cephalothorax length (unsegmented chalimus I in b which was measured below the bend of what is to become the cephalothorax). White in b: measurement of the eye width measured from centre left of the left lense to centre left on the right lense. **a**: copepodite. **b**: chalimus I. **c**: adult female.

The distance between the louse's lenses were measured when picture quality allowed it. As there is no segmentation in the chalimus I stage (Piasecki, 1996), the cephalothorax length measurement was set at the end of the "wing" of what looks like cephalothorax. When a louse was in the process of moulting growth, the actual louse and not the oldest outer chitin "skeleton" was measured. All of the copepodids measured in this study were hatched from eggstrings from females sampled from either lumpfish or Atlantic cod with maximum one day between (see 2.5). These lice hatched and developed under the same water conditions prior to a challenge experiment.

2.5 Genotyping and sequencing

2.5.1 DNA extraction

All *C. elongatus* in this study, including the ones collected from the two-spotted gobies and additional ones selected from various hosts and locations, were DNA extracted (table 2.1). The

lice were stored at -20 ºC in Eppendorf tubes with 96 % ethanol. Prior to the DNA extraction, each louse was placed in a dry 1.5 ml Eppendorf tube by using a sterile teasing needle or scalpel. Adult females were cut, separating the cephalothorax from the abdomen, and the cephalothorax was used for DNA extraction. This was done to avoid potential amplification of DNA from sperm (spermatophores), which would not represent the sampled individual. Lice received in ethanol were dried in the opened Eppendorf tubes, if necessary, in a heating cabinet. This was in order to evaporate all leftover ethanol before starting the process of DNA extraction.

The E.Z.N.A.® Tissue DNA Kit (Omega BIO-TEK) was used to isolate the DNA from the lice. The steps in the E.Z.N.A.® Tissue DNA Spin Protocol were conducted, in short performing protease digestion, separation of soluble DNA from other insoluble materials, binding of DNA to a purification matrix, washing away non-DNA residues and elution of the DNA into a 1.5 ml Eppendorf tube. Following specifications and exceptions were done: After adding and mixing TL Buffer and Proteinase K to the dried louse, it was incubated in 55 ºC over night (12-18 hours). Also, the elution step was conducted one time, and with 50 µl Elution Buffer. The final solution with isolated DNA in the Eppendorf tube was stored at -20 ºC. A spectrophotometer (NanoDrop ® ND-1000) was used to measure the DNA concentration (ng/µl).

2.5.2 PCR

GoTaq® G2 master mix x 2 Cat # M743A set (Promega) was used to make a master mix for PCR. A 20 µl reaction was made in 0.2 ml PCR tubes with 10 µl GoTaq® G2 Hot Start Colorless Master Mix, 7 µl water, 1 µl forward primer, 1 µl reverse primer and 1 µl template. The final concentration of the primers was 0.5 μM. Three sets of primers targeting mitochondrial cytochrome C oxidase-1 (mtCO1) were used in PCR (table 2.2), in which two primer sets were specific for each *C. elongatus* genotype (developed by Heidi Kongshaug, Sealice Research Centre, spring 2020), whilst the third was designed to amplify both genotypes (modified by Egil Karlsbakk from primers created by Øines & Heuch (2005)). Kongshaug's novel primers were based on an alignment with 15 mtCO1 sequences from GenBank ® (https://www.ncbi.nlm.nih.gov/) available at that time and were designed for genotyping with PCR and visualization on agarose gel. The CeCOx primers were used in mtCO1 sequencing.

Table 2.1. Overview of all *Caligus elongatus* used in this study, with host, place and period of the year. Ho= Hordaland, Su= Sunnmøre, No= Nordland, Fi= Finnmark, Nf= Nordfjord. Tr= Troms

Host species, W/F	Sampling location (GPS coordinates N, E)	Dec-Feb (Winter)	Mar-May (Spring)	Jun-Aug (Summer)	Sep-Nov (Autumn)	Total from host species
Two-spotted goby *G. flavescens, W*	Espegrend, Ho (60.268, 5.221)	3	16	9	19	52
	Misje, Ho (60.454, 4.963)				4	
	Os, Ho (60.143, 5.370)				1	
Lumpfish *C. lumpus, W*	Raunefjorden, Ho (60.27, 5.21)				11	60
	Norwegian sea, off Tr (70.40, 17.08)			39		
	Oslofjord (59.68, 10.57)		1			
	Norwegian sea, off No (68.59, 11.68)			3		
	Norwegian sea, off No (70.40, 17.09)			6		
Atlantic cod *G. Morhua, W*	Borgundfjorden, Su (62.45, 6.32)		1		5	36
	Espegrend MBS, Ho (60.27, 5.21)				30	
Atlantic salmon *S. Salar, F*	Haverøy, Ho (60.40, 4.94)				12	16
	Hamnefjord, Fn (70.58, 23.03)				3	
	Saltkjel, Nf (61.95, 5.15)				1	
	Bergsøyan, Tr		1*			

Pink salmon	(69.43, 17.28)					2
O. gorbuscha, W	Norwegian sea, off Tr (70.42, 14.47)		1*			
Atlantic halibut	Vestvik, Ho (59.62, 5.41)				4	7
H. hippoglossus, F	Faroe Islands (62.10, -6.90)			3		
Common dab	Ellingsøyfjorden, Su					
L. Limanda, W	(62.48, 6.22)		10			10
European flounder	Espegrend, Ho			1		1
P. flesus, W	(60.27, 5.21)					
Common ling	Stegavågosen, Ho	1				1
M. molva, W	(60.16, 5.16)					
Sand goby	Espegrend, Ho		1			1
P. minutus, W	(60.2683, 5.221)					
Lice from water	Espegrend, Ho (60.26, 5.22)		1	1	2	5
	Bergen, Ho (60.40, 5.29)			1		
Total		4	30	2* 63	92	191

W=wild caught fish, F= farmed fish, * Sampled in May/June

Table 2.2. The primers used in PCR to amplify parts of the mtCO1 gene in Caligus elongatus. The CE1- and CE2-primers are new, specifically designed by Heidi Kongshaug (SLRC) for the amplification of genotype 1 and 2 respectively (i.e. genotyping).

Primer name	**Forward (F)/reverse (R)**	**Sequence 5'– 3'**	**Product size**
Ce1F	F	CCTATTACTGGGTGCGCCAGATATG	232
Ce1R	R	CGCCCCTAAAAGAGAAGAAATTCCT	
Ce2F	F	GGTACCATTATTATTAGGTGCACCGG	223
Ce2R	R	GGAAATTCCCGCTAAGTGAAGAGAGA	
CeCOxF	F	AGWGGATTTTGATCYGGGCT	602
CeCOxR	R	GGATCAAAAAAYCTGGTRTTTA	

Two lice from lumpfish and Atlantic cod were used as positive controls for genotype 1 and genotype 2, respectively. The PCR setups were similar when using the Ce- and CeCOx primers, with an annealing temperature of 58 °C and 50 ºC (table 2.3). The PCR products were stored at 4 ºC.

Table 2.3. The PCR thermoprofile used in this study.

Stage 1	Stage 2			Stage 3
X1	X35			X1
Denaturation	Denaturation	Annealing	Elongation	Elongation
95 ºC	95 ºC	58[a]/50[b] ºC	72 ºC	72 ºC
2 min	15 sek.	15 sek.	30 sek.	7 min (-> 4 ºC ∞)

[a] Annealing temperature for PCR using the Ce1- and Ce2 primers
[b] Annealing temperature for PCR using the CeCOx primers

2.5.3 Agarose gel electrophoresis

All PCR products were visualized with agarose gel electrophoresis. Bio Rad's *wide Mini-Sub Cell GT Cell* and *Mini-Sub Cell GT Cell* with matching gel trays were used. A 50 ml or 25 ml 1.5 % agarose (SeaKem ® LE Agarose) gel mixed with respectively 2 or 1 µl GelRed ™ with wells was prepared. 1x TAE Buffer was poured into the chamber, covering the gel and filling the gel wells. 6x µl loading dye was pipetted into wells in an ELISA tray to visualise progress of the electrophoresis. From the PCR products, 5 µl were mixed with the loading dye by pipetting up and down 8-10 times before loading 5 µl of the mixture into a gel well. *O'RangeRuler 100 bp DNA Ladder* (NEB) diluted 1:3 with 1x TAE buffer was used as a standard, ranging from 100 bp to 1500 bp. All PCR-products, including the positive and negative controls were run in agarose gel electrophoresis using 80 V for 25-45 minutes. The gels were documented in *Carestream GelLogic 212 PRO* with a UV light and camera connected to a computer to capture the results. Pictures showing positive bands and the molecular weight were saved as jpg. files.

Genotyping was based on the presence of product with the Ce1- or Ce2 primers, revealed by agarose gel electrophoresis. Subsequently, 47 lice from genotype 1 and 33 from genotype 2

were selected for mtCO1 gene sequencing, examining genetic variation. Sequencing was done on products amplified with the CeCOx primers. The obtained sequences were compared to three sequences deposited in GenBank, defining the genotypes. Lice with inconclusive genotyping results were always included in the mtCO1 sequencing.

2.5.4 PCR clean up and sequencing

PCR products from *C. elongatus* DNA were sequenced with the CeCOx primers, including a selection of lice from two-spotted goby, lumpfish, Atlantic cod, Atlantic salmon, pink salmon, common dab, Atlantic halibut, European flounder and one planktonic adult, as well as all lice with any ambiguity in the results from the genotype primers (double bands). All reaction components were kept at a low temperature with cooling racks at all time during the preparations for PCR clean up and sequencing. Excess primers and dNTPs in positive PCR samples were enzymatically inactivated (cleaned up) using 1 µl *ExoProstar 1-step* (Exonuclease I and Alkaline Phosphatase from illustra™) mixed with 2.5 µl PCR product at 37 ºC for 15 minutes before inactivating the enzymes at 80 ºC in 15 minutes. PCR products saturating the documentation system were subsequently diluted 1:5 with nuclease free water after the PCR clean up.

To prepare for the sequencing, 10 µl sequence reactions were made by adding 6.5 µl nuclease free water, 1 µl *Big Dye* with 4 fluorescently labelled dNTPs, 1 µl sequencing buffer, 0.5 µl primer (forward or reverse) and 1 µl PCR template to a 0.2 µl PCR tube. One sequence reaction was made for each mtCO1 primer, thus each positive band was sequenced in both directions. The sequence program used on the PCR machine was as follows: 96 ºC for 5 minutes, 25 cycles with 96 ºC for 10 seconds, 50 ºC for 5 seconds and 60 ºC for 4 minutes, and eventually a stage keeping the reactions at 4 ºC until the program was manually stopped. Before delivering the products to the sequencing facility, 10 µl was added to each PCR tube in order to achieve a desired volume of 20 µl.

2.6 Sequence analysis

Chromatograms and sequences were processed, assembled, and aligned in SnapGene ® v. 5.2.4. The chromatograms were edited manually to remove parts with bad sequence, unreliable ends and the primer sites. There were two sequences from each louse, one amplified with the forward CeCOx primer and one with the reverse. After editing, these chromatograms were assembled, giving a consensus sequence. All sequences were checked in BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) for similarity to other *C. elongatus* registered in

GenBank. Three sequences published in GenBank by Øines & Heuch (2005) were defined with genotypes (reference no. AY386273 for genotype 1, AY861365 and AY386272 for genotype 2). The genotype of each louse was then determined (again) based on identity with these reference sequences in GenBank. The sequences from this study's lice and all *C. elongatus* sequences from the same gene in GenBank were aligned in SnapGene. Probe site evaluations were conducted in AlignX.

A phylogenetic neighbour joining tree from an alignment with all unidentical sequences was created in Genious Prime ® v. 2021.1.1 (https://www.geneious.com) with the HKY distance model with a Bootstrap of 100 replicates. The lice's host species and free vs. attached state were marked behind every branch, also including all identical sequences. The mtCO1 sequences were also translated (invertebrate mitochondria code) in GeneDoc v. 2.6. 002, for possible amino acid (AA) substitutions.

2.6 Challenge experiment with genotype 2 on Atlantic salmon

2.6.1 Obtaining lice

A challenge experiment was conducted from 16th of October 2020 (12:00) to 28th of October 2020 (13:00). Adult *C. elongatus* from Atlantic cod were kindly donated from students during a parasitology course *BIO270* at 7th of October at University of Bergen. The fish were caught with trammel net by staffs at MBS prior to the course and transported to the university facilities in fresh seawater. When students were finished examining lice, the *C. elongatus* were placed in separate containers with fresh sea water and kept cold. Pictures of the lice and egg strings from adult females were taken before placing the egg strings in individual flow-through incubators with a 150 µ filter. The incubators were provided with fresh seawater constantly at a temperature varying from 9.8 ºC 7th of October to 9.3 ºC at the 15th. Adult females without egg strings were placed in incubators in case they would produce egg strings at a later time. 22 lice or pairs of egg strings were incubated and observed and daily to determine the timing of hatching and moulting in each incubator. Egg strings from 19 *C. elongatus* were collected from 14 Atlantic cods. From these, 12 had moulted into copepodids 9 days later at the day of the challenge experiment. While the egg strings hatched and developed into copepodids, the cephalothorax of the mother lice were DNA extracted and genotyped using the Ce primers in PCR and agarose gel electrophoresis to ensure only genotype 2 copepodids were included in the challenge experiment.

2.6.2 Estimating number of copepodids and preparing for the experiment

Estimating the number of copepodids was performed like in Hamre, Glover & Nilsen (2009). The genotype 2 copepodids were gently flushed into a 2 l jug containing 9 dl fresh sea water. A table spoon was used to mix the water in a random motion to create a random distribution of copepodids in the water. Whilst mixing, a 10 ml Bibbi pipette with the tip cut off was plunged into the water column and the water to collect subsamples. This was repeated until we had collected 38 ml water with copepodids in an Aslon 100ml measuring cylinder. The water inside the cylinder was poured onto a 90 µ zooplankton filter and the and the cylinder was flushed once to include all copepodids. A mixture of salt water and ethanol (70% alcohol with 9.2 g/l salt) was used to kill the copepodids before they were placed in a zooplankton counting chamber and counted in an Olympus SZX12 microscope. The subsample contained 4 copepodids/ml. From this, the total number of copepodids in the remaining 8.6 dl of water was estimated to be 215.

2.6.3 Implementation of the challenge experiment

Eight fish in a 1x1x0.5 m fish tank with 500 l seawater with a light regime of 12:12 were used during the experiment. The fish had a mean weight of 665 g and total length ranging from 37-39 cm. The water temperature at the time of initiation was 11.8 ºC and was kept at approximately 12 ºC throughout the experiment. The water level in the fish tank was lowered to about 15-20 cm prior to transferring the water with copepodids gently and gradually into the water near the tank wall. The experiment was ended 12 days later. Fish were anesthetized individually in 20 l seawater with a combination of 20 ml metomidate and 6 ml benzocaine l^{-1} for 2-3 minutes. Each fish's skin, including the fins and operculum, was thoroughly examined for chalimus with bright light both with and without a magnifying glass. The length and weight of the salmons were also noted.

2.7 Statistics

Statistical analyses were performed in Statistica version 13.3 (TIBCO Software Inc.). $P < 0.05$ were considered significant.

A Kruskal-Wallis (KW) and Fishers' exact test (FET) test was used to examine temporal or spatial changes in *C. elongatus* abundance. Two sample T-tests or sometimes Mann-Whitney U-tests (MW) were used to compare morphometric measurements. MW was used if variance differed significantly (Levene's test), violating this assumption of the T-test.

3. Results

3.1 Infection dynamics on two-spotted goby (*Gobiusculus flavescens*)

3.1.1 Caligus elongatus infecting two-spotted goby

Over-all 2 251 two-spotted gobies (*Gobiusculus flavescens*) were sampled (22 samples) from MBS and examined for *C. elongatus* at regular intervals over a period of 12 months. Mean sample size was 102 fish, ranging from 11-169 fish. The mean total length (TL) of two-spotted gobies ranged from 40-41.6 mm in the period March to June before decreasing during summer. The size increased again from December to February (figure 3.4). The salinity ranged from 25.8 to 31.7 ‰, with a mean at 28.7 ‰, and the temperature ranged from 4.5 °C in early spring to 18.6 °C during summer and the mean salinity was 28.7 ‰. A total of 41 two-spotted gobies were found infected with 1-3 (mean 1.1) *C. elongatus*. Overall prevalence and abundance were 1.9 % and 0.023, respectively. There were marked seasonal variations in prevalence and abundance. The prevalence was 0 % from March to mid-April, and increased significantly to a maximum prevalence of 10 % in May (FET, $p<0.001$) (figure 3.3). It then decreased to 0 % from July to late August before reaching a maximum during the autumn, again with a significant increase from June-August to a maximum of 5 % prevalence in mid-October (FET, $p<0.001$). Abundance followed prevalence ($KWH_{21,N=2252}=60.2$, $P<0.001$), since most infections consisted of single lice. A total of 44 *C. elongatus* were recovered. Of these were 16 chalimus I, 8 chalimus II, 9 chalimus III, 6 chalimus IV and 5 were young adults with the frontal filament

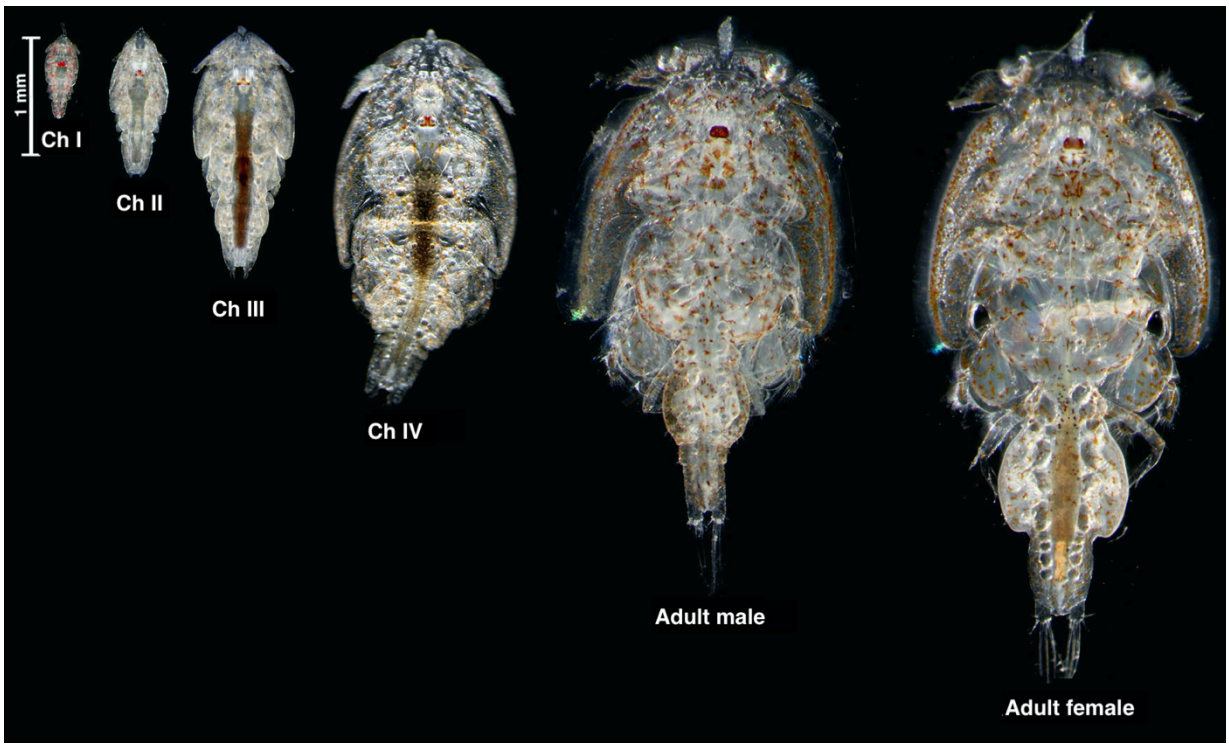


Figure 3.1. Montage of all recorded stages of *Caligus elongatus* found attached with the frontal filament to *Gobiusculus flavescens*. Ch= chalimus.

still attached to the fish (Figure 3.1). These were exclusively attached to the fins, mainly the dorsal fins (51.1 %) and the caudal fin (23.4 %) (figure 3.2).

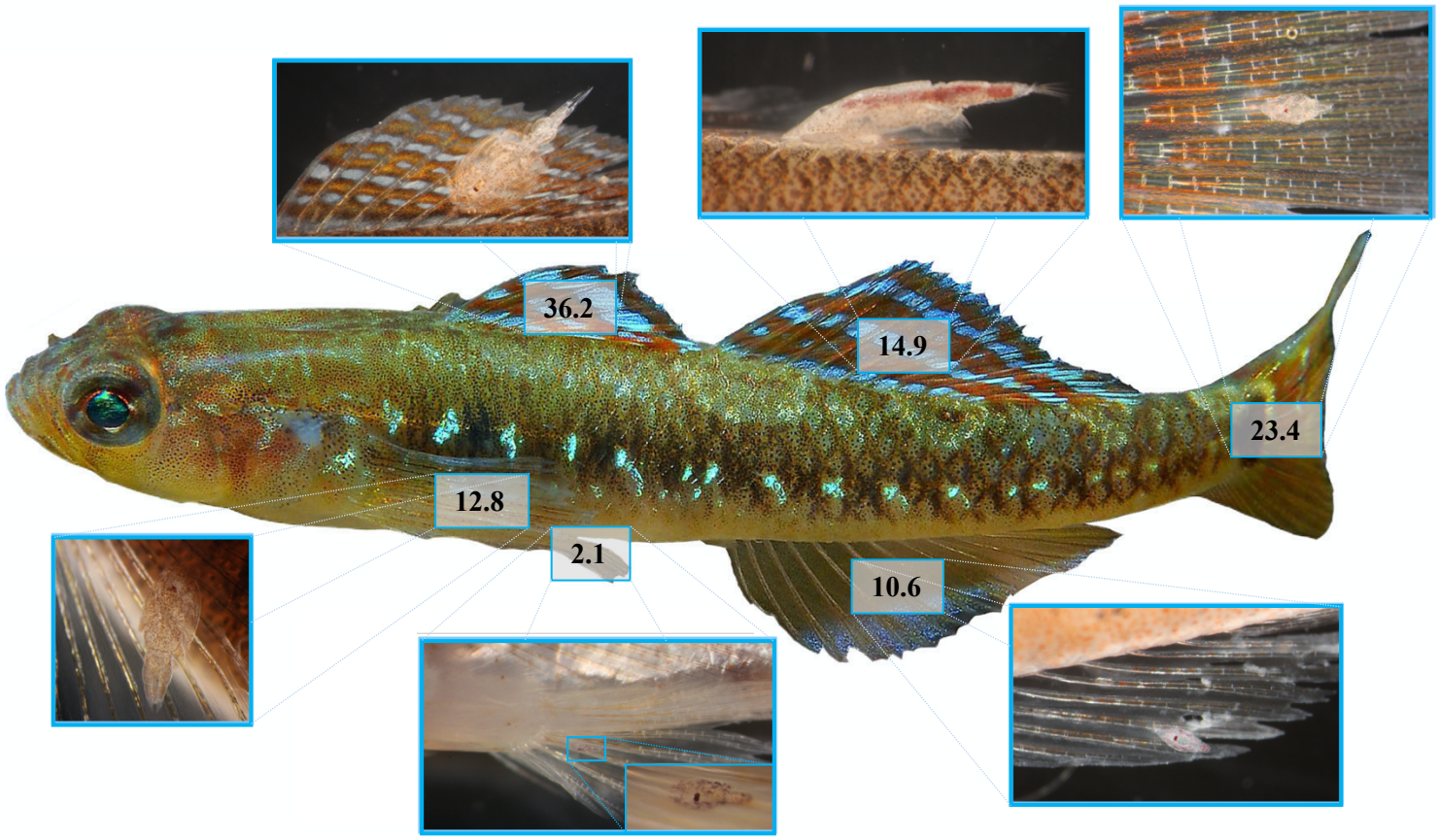


Figure 3.2. Attachment site distribution of 47 *C. elongatus* on two-spotted gobies (*G. flavescens*) given in percentage. The percentage on the breast fin is left and right fin combined. From each box are examples of lice found on that specific fin. Photo of the two-spotted goby retrieved and modified from Erling Svensen (https://www.artsdatabanken.no/Pages/237666/Tangkutling)

3.1.2 Production of adult C. elongatus from two-spotted gobies at MBS, an example

The number of adult *C. elongatus* released to the environment from two-spotted gobies can be roughly estimated if the host population density, sea temperature, and mean abundance of *C. elongatus* is known. For example, at 29th of October 2020 there was an estimated 349.2 fish meter shoreline^{-1}, giving 34 920 gobies per 100-meter shoreline which is the approximate length of the shore examined. The mean abundance of *C. elongatus* was 0,02 this day, giving a total of 698 *C. elongatus*. The temperature was 11.4 °C, and at this temperature the mean growth rate of *C. elongatus* is 0.28 stages day^{-1} on Atlantic salmon (unpublished development model, courtesy of Gine Myhre and Lars Hamre). With five parasitic stages on the host before adult, and assuming a similar rate of development on *G. flavescens*, about 39 ((0.28 stages $^{-1}$ day*698

lice)/5 stages) *C. elongatus* develops to adulthood on the two spotted gobies per day on this shore stretch.

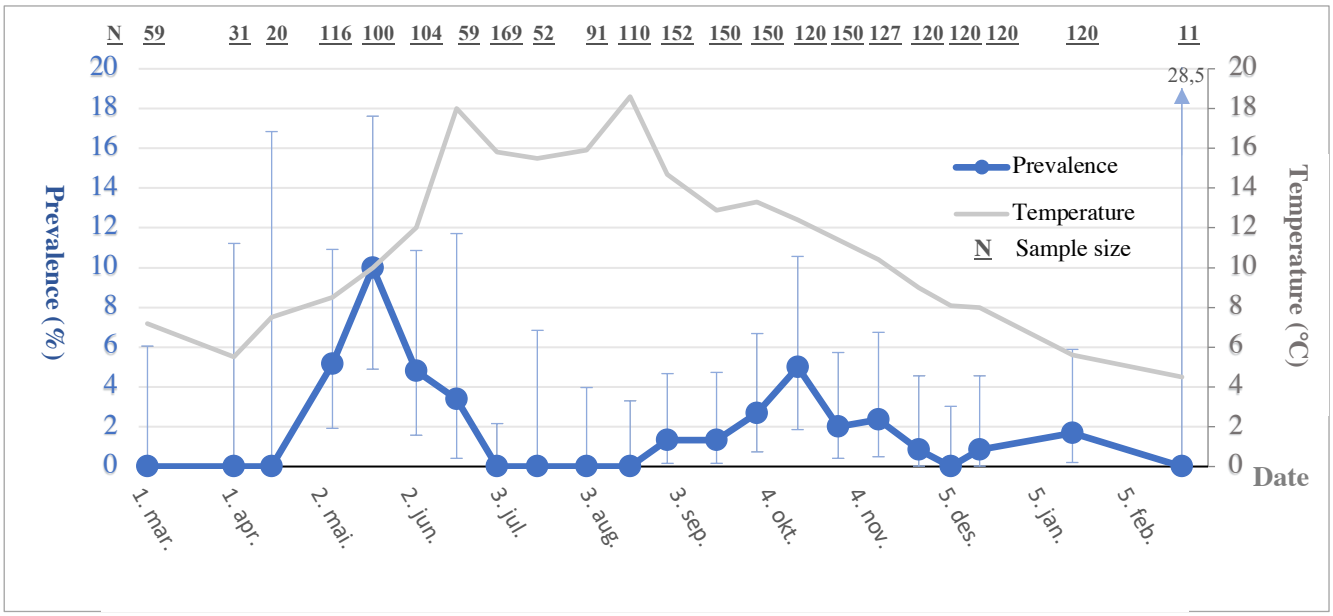


Figure 3.3. Prevalence of *C. elongatus* on two-spotted gobies at Espegrend, Hordaland from March 2020 to February 2021 (left y-axis), and seawater temperature at 1-1.5 m depth (right y-axis). Spreads indicate 95 % confidence intervals. The arrowed spread points to the samples' confidence interval. Underlined numbers over each indicator show the number of fish sampled that specific day.

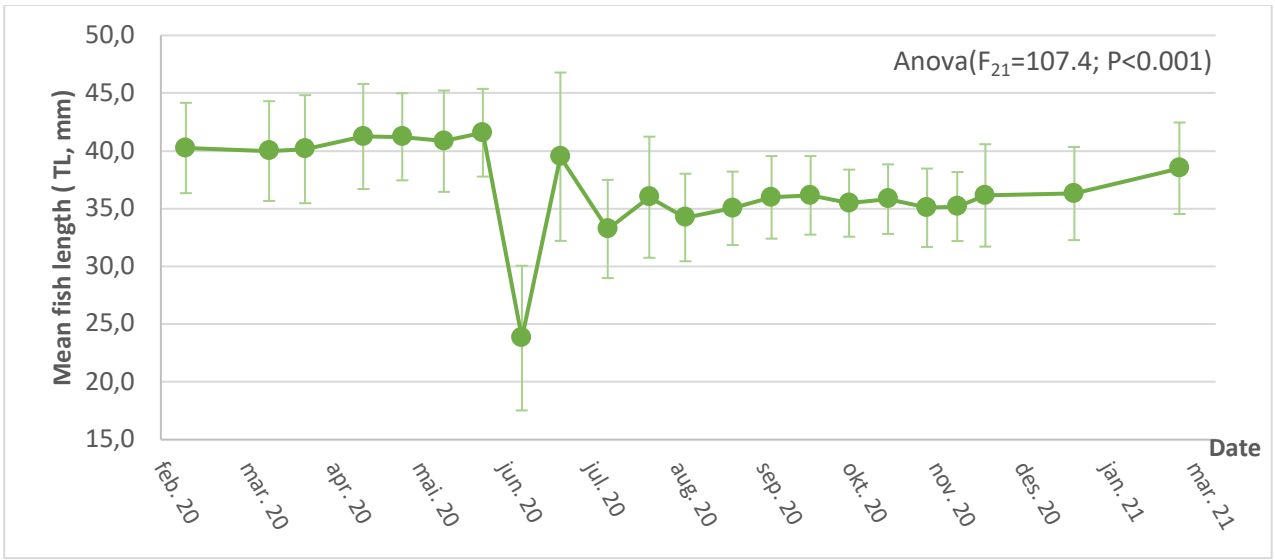


Figure 3.4. Mean length of two-spotted gobies (*G. flavescens*) from samples collected at Espegrend (Hordaland) in the period March 2020 to February 2021. Spreads indicate standard deviation (SD).

3.1.3 The reference locations

To examine if *C. elongatus* prevalence and abundance varied significantly spatially, gobies were sampled at two other locations in Hordaland and used as reference (table 3.1). At Misje, the prevalence of *C. elongatus* on two-spotted gobies was 3.1 % (N=4). The prevalence at Lepsøy was 1.3 % (N=1). The abundance of *C. elongatus* did not vary significantly between Espegrend and the two reference locations (ANOVA, $F=0.99$; $P<0.40$), nor did the fish size (ANOVA, $F=0.163$; $P<0.18$).

Table 3.1. Comparison of Espegrend samples in October 2020 with reference locations in Hordaland. N=sample size; TL=total length; SD=Standard deviation

	Date	Salinity (‰)	N	TL±SD	Prevalence (%)
Espegrend	15	29.5	120	36.2±3.4	5.0
Misje	19	30.3	131	36.2±5.3	3.1
Espegrend	29	29.3	150	35.5±2.9	2.0
Lepsøy	31	27.9	76	36.6±4.4	1.3

3.1.4 Other parasites infecting two-spotted gobies

Other than *C. elongatus,* the only ectoparasite found on the two-spotted gobies during sampling was the monogenean *Gyrodactylus sp*. The estimated prevalence and abundance of *Gyrodactylus sp*. varied greatly throughout seasons (figure 3.5). The prevalence was mostly > 60 % from December to mid-July with a mean abundance 8.0 in the same time period. The infestation of *Gyrodactylus sp*. was low in one sample in July consisting mainly of juvenile fish. Both prevalence and abundance dropped in August, remaining low until a gradual increase from November. The metacercaria stage of the digenean trematode *Cryptocotyle lingua*, causing black spots in the skin, was most abundant and had the highest prevalence in mature fish during summer.

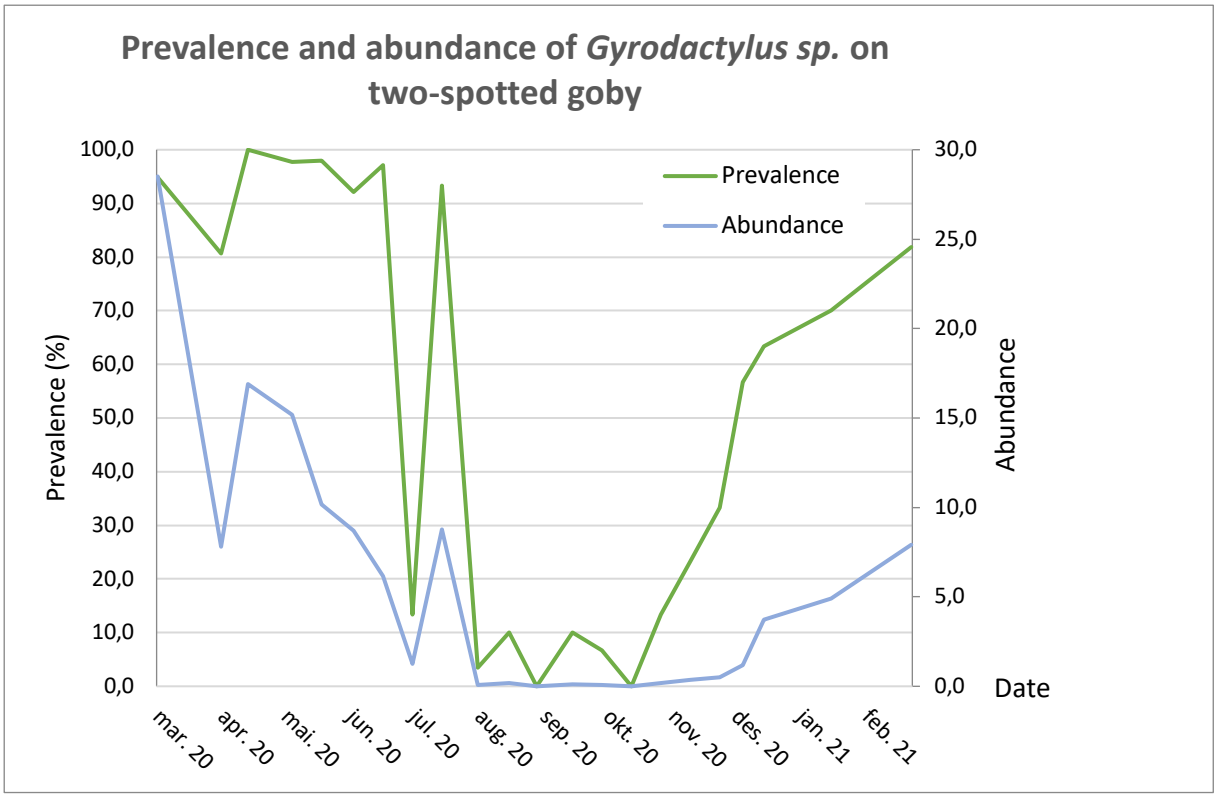


Figure 3.5. The prevalence and abundance of *Gyrodactylus sp*. on two-spotted gobies (*G. flavescens*) sampled regularly from March 2020 to February 2021 at MBS outside Bergen.

3.2 Caligus elongatus genotypes

Overall, DNA was extracted from 191 *C. elongatus* and all were successfully genotyped by using PCR and agarose gel electrophoresis, or by sequencing the mtCO1 gene and aligning them with reference sequences from GenBank and creating a neighbour joining tree.

3.2.1 Novel genotyping assays for PCR

Based on the genotyped mtCO1 sequences from Øines & Heuch (2005), specific primer sets targeting the mtCO1 gene of *C. elongatus* genotypes were developed by Heidi Kongshaug (SLRC, spring 2020) (table 2.2). The Ce primers amplified DNA from 190 of the 191 lice (figure 3.6). Occasionally, signals for both genotypes were seen from the same template. In these cases, the analyses were re-run, or nearly the whole CO1 sequenced with the CeCOx primers. The re-runs revealed a single genotype (i.e. likely contamination). In silico analysis of most sequences showed clearly that they belonged to one genotype, and primer mismatch was seen for the other. However, one louse did not show products with any of the Ce primer pairs. The CO1 sequence of that louse showed 93.7 % identity with the genotype 2 reference AY386272 and was named genotype 2b. When aligned with the reference and other CO1

sequences, the genotype 2b sequence matched with the site of Ce2R primer but not with the Ce2F primer in the 3' end (figure 3.8). Hence the Ce primers amplified CO1 fragments of 190/191 C- elongatus (99.5%). The false bands most likely occurred due to lab contamination. When combining the genotyping results from PCR and sequencing, a total of 84 *C. elongatus* were found to be genotype 1 and 107 were genotype 2.

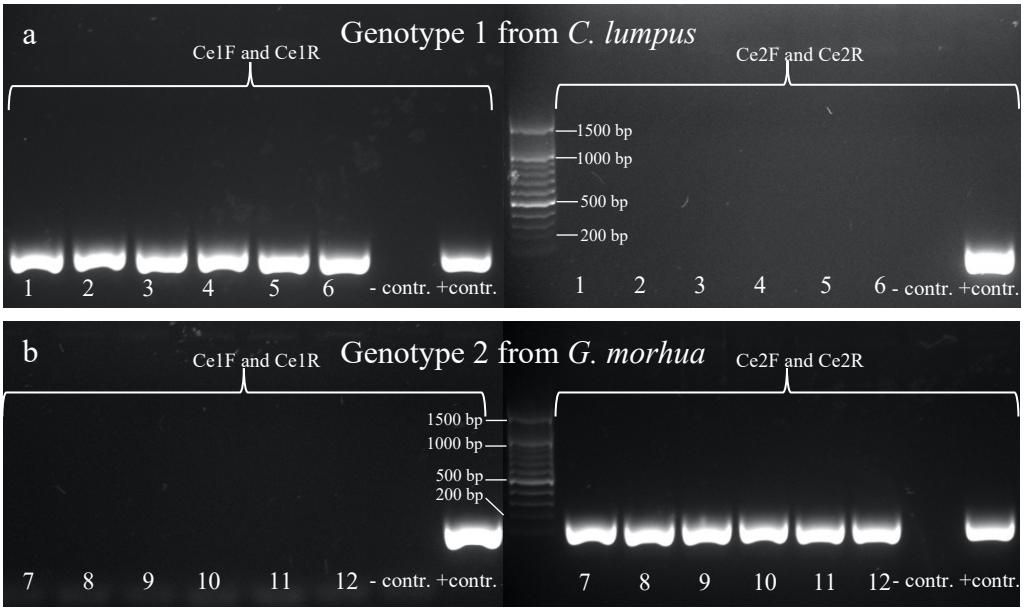


Figure 3.6. Genotyping results on agarose gel after using the Ce1 and Ce2 primers in PCR on *C. elongatus*. Ce1 primers are used on the standard's left, and the Ce2 primers are used on the right. There are six wells with PCR product plus two controls on each side. - contr.: negative control. + contr.: positive control. a: six lice (1-6) from lumpfish (*Cyclopterus lumpus*) reacted with genotype 1 primers and not genotype 2. b: six lice (7-12) from Atlantic cod (*Gadus morhua*) reacted with genotype 2 primers and not with genotype 1 primers.

3.2.2 Genetic diversity

Groups

In order to examine genetic variation in the CO1 gene in detail, a selection of lice from various hosts and geographic regions were sequenced. In addition, all lice with ambiguities in Ce genotyping or a lack of product with both Ce assays were included. This resulted in 94 *C. elongatus* mtCO1 sequences. These were analysed together with 15 sequences derived from GenBank, a total of 109 sequences/lice with an alignment 527 nt long. Among these 109 lice, there were 85 unique sequences where at least one nucleotide (nt) diverged from the other sequences. Based on these unique sequences, a neighbour joining tree was created, which clustered the sequences into two distinct groups, genotype 1 and genotype 2 (figure 3.7). This clustering matched the results from BLAST, based on match with reference sequences in

GenBank (AY386273 for genotype 1 and AY861365 or AY386272 for genotype 2). In the genogroups created by the neighbour joining tree, 58 lice were genotype 1 and 50 were genotype 2. Sequences from GenBank of adult *C. elongatus* from plankton samples from Helgoland were represented in both genogroups. An adult louse from the water inlet at IMR, Bergen (CE-HI-F1-160610), diverged from the two groups, but was closest to genotype 2 (93.7 % identity with genotype 2 reference in GenBank). This sequence is designated as genotype 2b in figure 3.7 and 3.8.

Nucleotide substitutions

Out of the 527 nt positions compared in the alignment with 109 mtCOI gene sequences, 21 positions were found unique to either genotype 1 or genotype 2. The nucleotides were identical within the genotypes and different between the genotypes in 20 positions (table 3.2). In one position (nt position 552 in reference sequence AY386273) genotype 1 lice had T/C whereas genotype 2 had A/G. One mtCO1 sequence derived from a genotype 1 *C. elongatus* from a flatfish in northwest Atlantic (Jensen, 2013) mismatched one of the 21 unique positions with G for A. The Ce primers were checked in the alignment to see how they matched with the sequences obtained in this study (figure 3.8). All but one louse matched both the forward and reverse primer of their genotype.

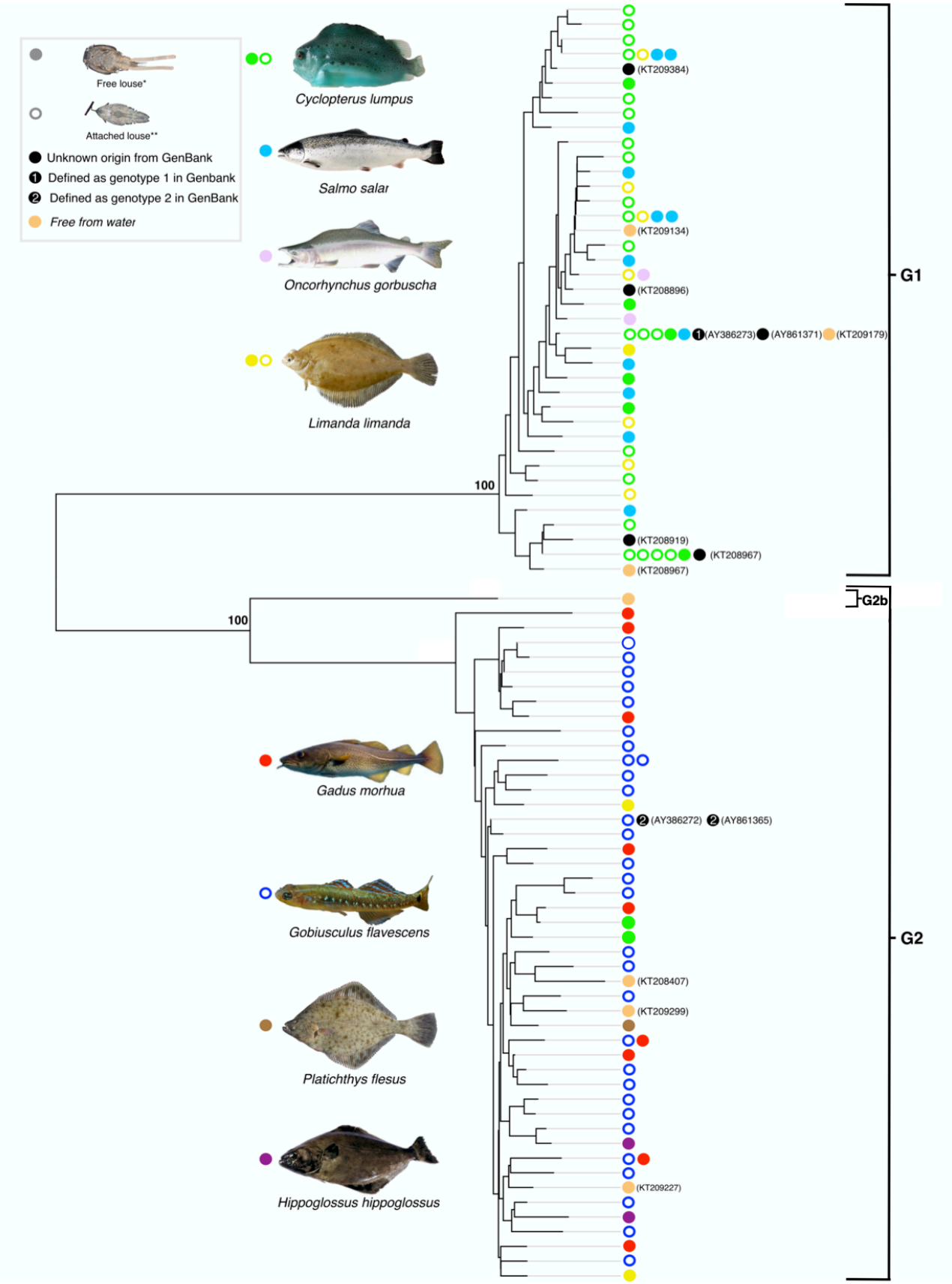


Figure 3.7. Neighbour joining tree based on Caligus elongatus CO1 sequences. Marker codes indicated in box at the top left and fish hosts indicated by colours in the figure. The accession numbers give (parenthesis) for sequences obtained from GenBank. Bootstrap support values are given over the two main branches, giving the percentage of times these clades appeared in the set of bootstrap replicate trees. G1= genotype 1, G2= genotype 2, G2b= genotype 2b. * Free louse is an adult without the frontal filament attached to a fish, **A louse is attached when the frontal filament is attached to a fish (also adults with frontal filament).

Table 3.2. Overview over mtCO1 sequence positions that appears to be diagnostic for the two *Caligus elongatus* CO1 genotypes in the Northeast Atlantic (alignment of 109 sequences). The nt positions are given with reference to GenBank sequence AY386273. The position of where a representative sequence from the north-western Atlantic (Jensen, 2013) mismatched is marked red.

AY386273 position	Genotype 1	Genotype 2
84	A	G
90	T	A
120	A*	T
183	A	G
195	G	A
243	G	A
255	T	C
267	G	A
277	C	T
285	A	T
408	G	A
420	T	C
459	C	T
471	T	C
483	G	A
517	C	T
519	T	A
525	C	T
528	C	T
537	C	T
552	Y	R

Y=C/T, R=A/G, * G in genotype 1 sequence provided in Jensen (2013) from the north-west Atlantic

Amino acid substitutions

All but one sequence from this study's lice were identical when translated into amino acids (AA). The exception is one louse in position 119 in the putative AA sequence reference AAR29054 (putative AA sequence for genotype 1 nt sequence reference AY386273). There

was an adenine-substitution for guanine, resulting in the amino acid serine replacing glycine. The position was included in sequences in both directions and unquestionably "A". This louse was found on a two-spotted goby mid-November in 2020 at a temperature and salinity of respectively 10.4 °C and 27.8 ‰. This putative AA substitution does not occur in any GenBank *C. elongatus* sequences. Another putative AA substitution was also seen in a GenBank sequence (accession number KT208919), with phenylalanine replacing leucine (AA position 155 in putative AA sequence reference AAR29054).

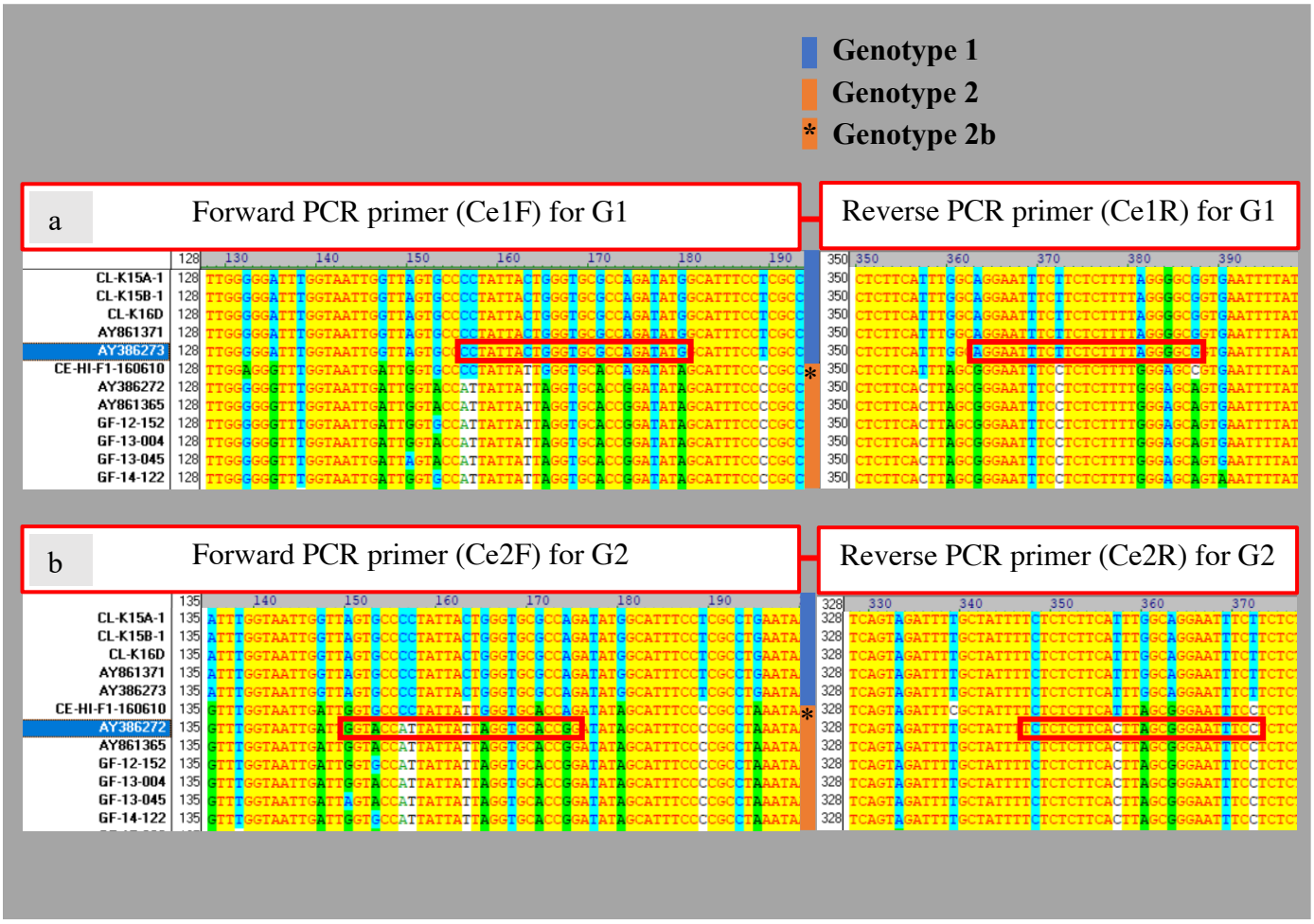


Figure 3.8. H. Kongshaug's PCR primers for genotyping *C. elongatus*. The positions they match are marked with red boxes in a representative sequence of the genotype. G1= genotype 1. G2= genotype 2. The sequences belonging to these are indicated by a colour bar along the 3' ends. The marked sequence names on the alignments left side are reference sequences genotyped by Øines & Heuch (2005). **a:** The genotype 1 reference is AY386273. **b:** the genotype 2 reference is AY386272. The positions indicated above is those of the alignment (AY386273positions -48).

3.2.3 Host associations of the genotypes

Free adults and attached *C. elongatus* from 10 host species were genotyped to investigate possible trends of the genotypes' host associations (figure 3.9). From two-spotted gobies sampled in the Bergen area, the *C. elongatus* were exclusively genotype 2 and all were attached

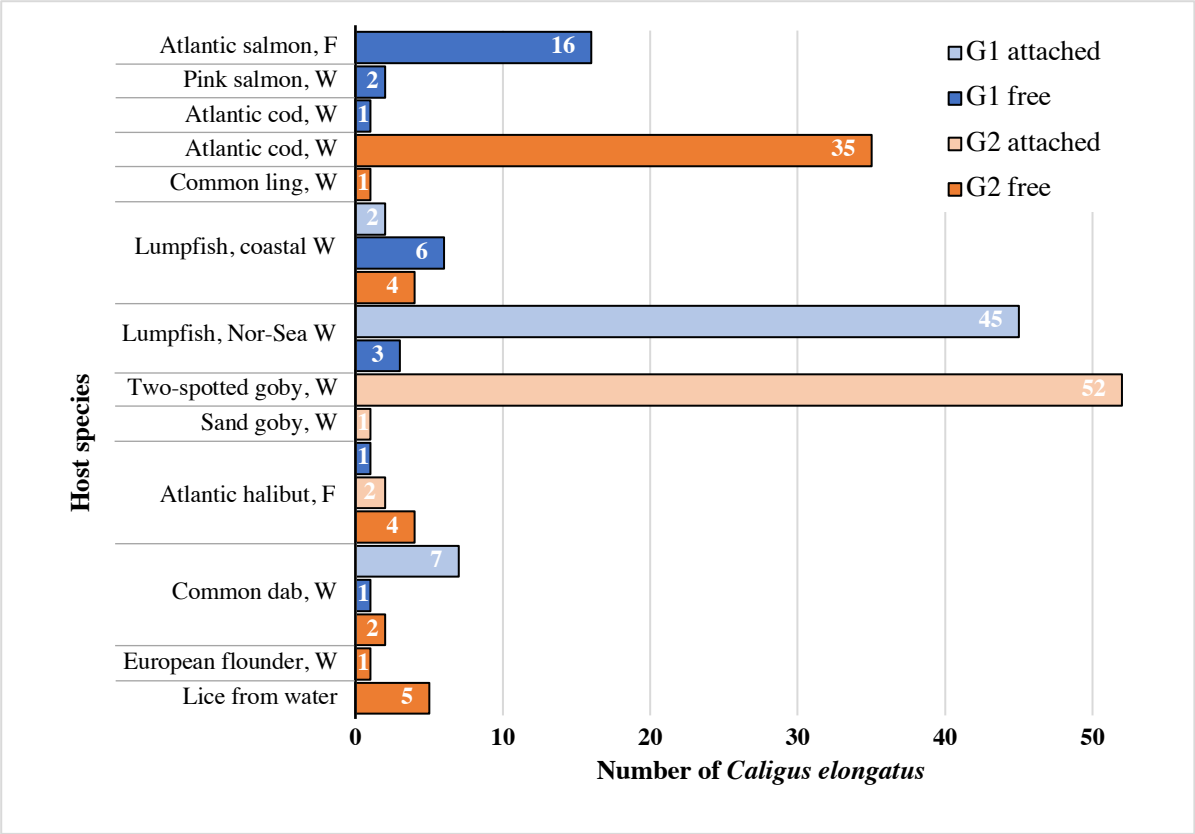


Figure 3.9. Overview over attached and free *C. elongatus* from ten host species divided in genotypes and free vs. attached. Free: adult louse found without frontal filament attached to the fish. Attached: any stage of development where the louse was found with its frontal filament attached to the fish, also including attached adults. Lice from lumpfish are separated into sampled in coastal areas or the Norwegian sea (Nor-Sea).

to the fish with frontal filament. All lice from farmed Atlantic salmon and wild pink salmon caught both north and south in Norway were genotype 1. These were all adults. The adult *C. elongatus* found on Atlantic cod were predominantly genotype 2 with the exception of a single genotype 1 louse from a cod caught in Borgundfjorden (mid-west in Norway) in the winter-spring fishery. From lumpfish, 56 out of 60 lice were genotype 1, including all attached lice and the ones found on lumpfish from the Norwegian sea. The lumpfish from Espegrend shared transport water- or were kept in a small pen together with cods, prior to being transported live to the university for use in laboratory classes. Hence, host switching was possible. The cod were infected with genotype 2 *C. elongatus*, and out of nine adult lice from these lumpfish, four were genotype 2. From a farm with 500 g halibuts heavily infected with *C. elongatus* at Vestvik on the Norwegian west coast (mentioned in Bergh *et al.*, 2001), all four adults genotyped were type 2. Additionally three *C. elongatus* found on farmed Atlantic halibut at the Faroe Islands were genotyped, of which two chalimi were genotype 2 and one adult was genotype 1. One

wild common dab caught at Sunnmøre at the west coast of Norway was infected with all 7 chalimi and 1 adult of genotype 1, and 2 adult genotype 2 lice. Four genotype 2 *C. elongatus* were found in the water of tanks, that had harboured live fish at MBS. A fifth free genotype 2 louse was found in a seawater tank at the Institute of Marine Research in Bergen and must have come in from the facility's inlet water.

3.2.4 Morphometric comparison of the genotypes

With the intention to compare morphological characters between *C. elongatus* genotypes, a total of 120 *C. elongatus* were measured of various lengths and ratios, where 14 lice were genotype 1 and 107 genotype 2. Of genotype 1, there were 7 copepodids, 2 chalimus IV, 1 adult male and 4 adult females and the genotype 2 lice were 13 copepodids, 16 chalimus I, 6 chalimus II, 13 chalimus III, 10 chalimus IV, 9 adult males and 35 adult females. Overall, the mean total length (TL) and cephalothorax length (CL) of genotype 1 lice tended to be larger than those of genotype 2 in all stages with both genotypes available for measuring (figure 3.12).

Copepodids

Genotype 1 copepodids were significantly longer than genotype 2 in both TL (MW, $Z_{7;13}$=3.57, P<0.001) and and CL ($T_{7;13}$=10.4, P<0.001) (figure 3.10 and 3.13). The length from the posterior end of cephalothorax to the end of uropods was also significantly longer for genotype 1 than genotype 2 ($T_{7;13}$=3.5, P<0.005). Other measurements and ratios where genotype 1 lice were significantly larger than genotype 2, was cephalothorax width (CW) ($T_{7;13}$=3.1, P<0.001) and CL/CW ($T_{7;13}$=4.7, P<0.001). The mean value on CL/CW was 2.0 and 1.7 for genotype 1 and genotype 2, respectively (figure 3.14). The location of the eye on cephalothorax, measured as percent length of CL from anterior end to the eyes, was moderately, but yet statistically significantly closer to the centre of cephalothorax in genotype 1 compared to genotype 2 (respectively 46.5 % and 38.1 %) (MW, $Z_{7;13}$=2.14, P<0.05).

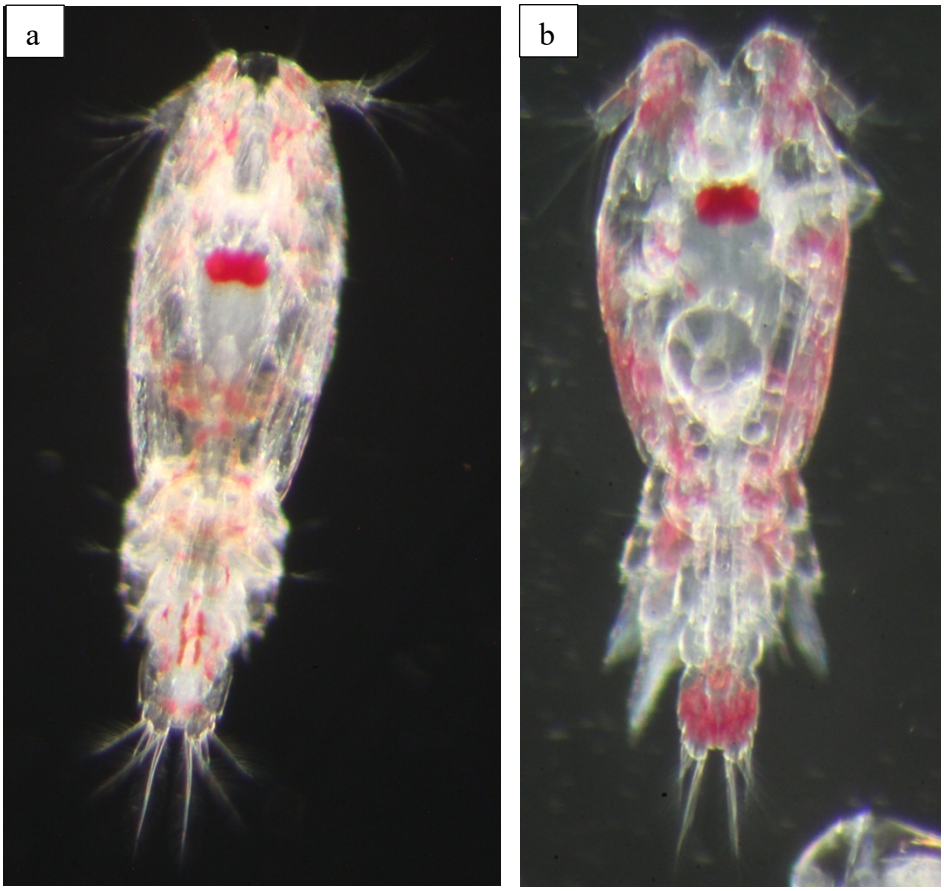


Figure 3.10. *Caligus elongatus* copepodids reared at the Sea Lice Research Centre. Total length (TL) is measured from the anterior end of cephalothorax to the uropods. **a**: Genotype 1 with mother caught on a lumpfish. TL= 0.641 mm. **b**: Genotype 2 with mother caught on an Atlantic cod. TL= 0.564 mm

Adult females

Despite few measured genotype 1 adult females, their total length ($T_{4;29}$=6.8, P<0.001) and the cephalothorax length ($T_{4;29}$=3.7, P<0.001) were significantly longer than genotype 2 (figure 3.11 and 3.15). Since the adult females from the two-spotted goby were recently moulted and possibly small for that reason (biazed), analyses were also done without goby lice. However, the results were similar. Cephalothorax was also significantly wider in adult genotype 1 females than in genotype 2 females ($T_{4;29}$=3.3, P<0.005). The distance between the eye lenses was also significantly larger among genotype 1 than genotype 2 lice ($T_{4;29}$=3.6, P<0.001), with a mean of 0.18 mm and 0.16 mm, respectively. Relative to total length, genotype 1 females were significantly wider than genotype 2 females ($T_{4;29}$=3.7, P<0.001) (figure 3.14) but the shape of cephalothorax (CL/CW ratio) did not differ ($T_{4;29}$=0.9, P>0.05). The body posterior to the cephalothorax was significantly longer in genotype 1 than genotype 2, both when including

young adults found on two-spotted gobies ($T_{4;29}$=1.2, P<0.001) and when these were excluded in the statistical analysis ($T_{4;29}$=6.5, P<0.001).

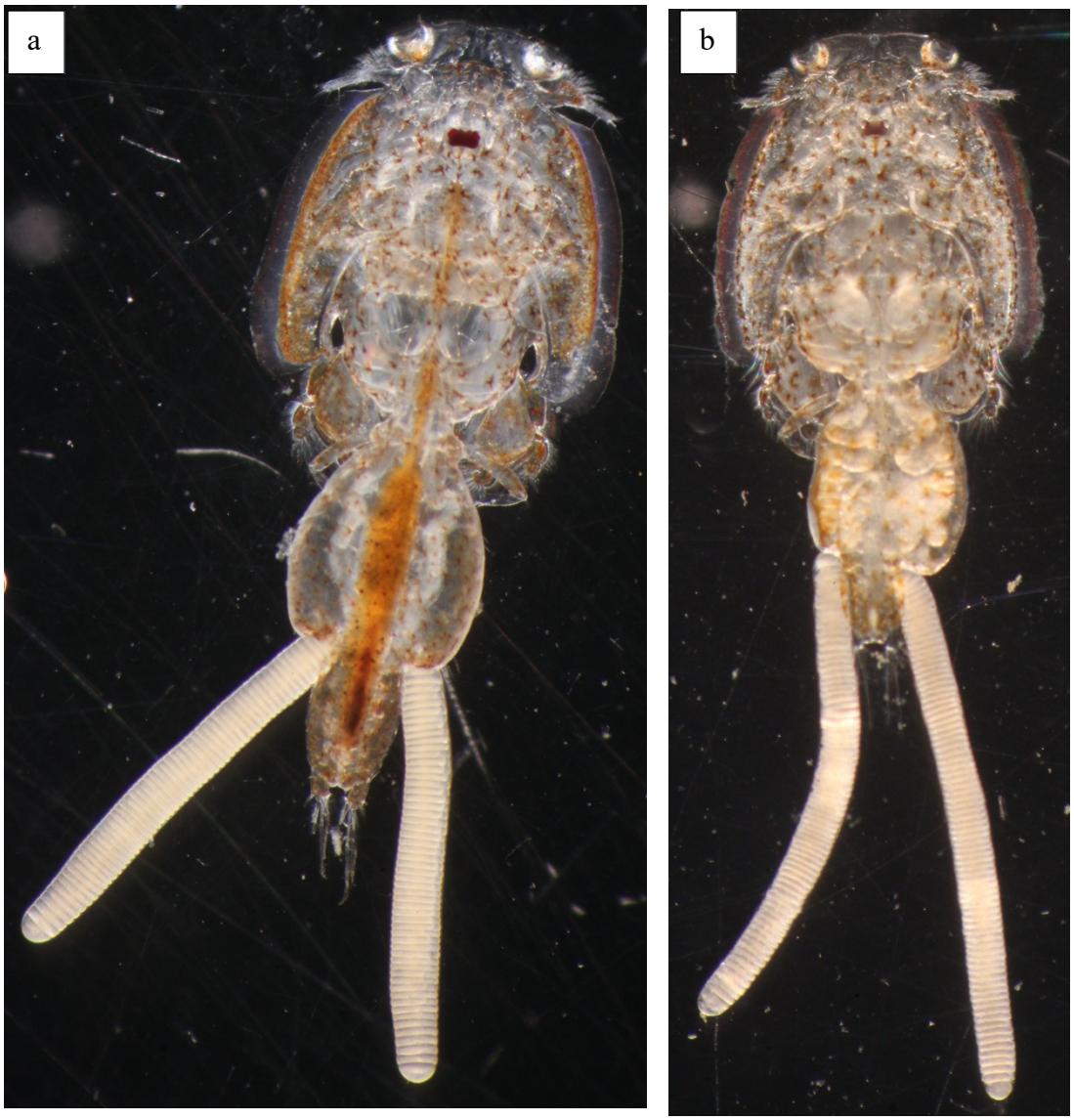


Figure 3.11. *Caligus elongatus* ovigerous females caught on wild fish. Total length (TL) is measured from the anterior end of cephalothorax to the uropods. **a**: Genotype 1 caught on a lumpfish. TL= 5.64 mm. **b**: Genotype 2 caught on an Atlantic cod. TL= 4.53 mm.

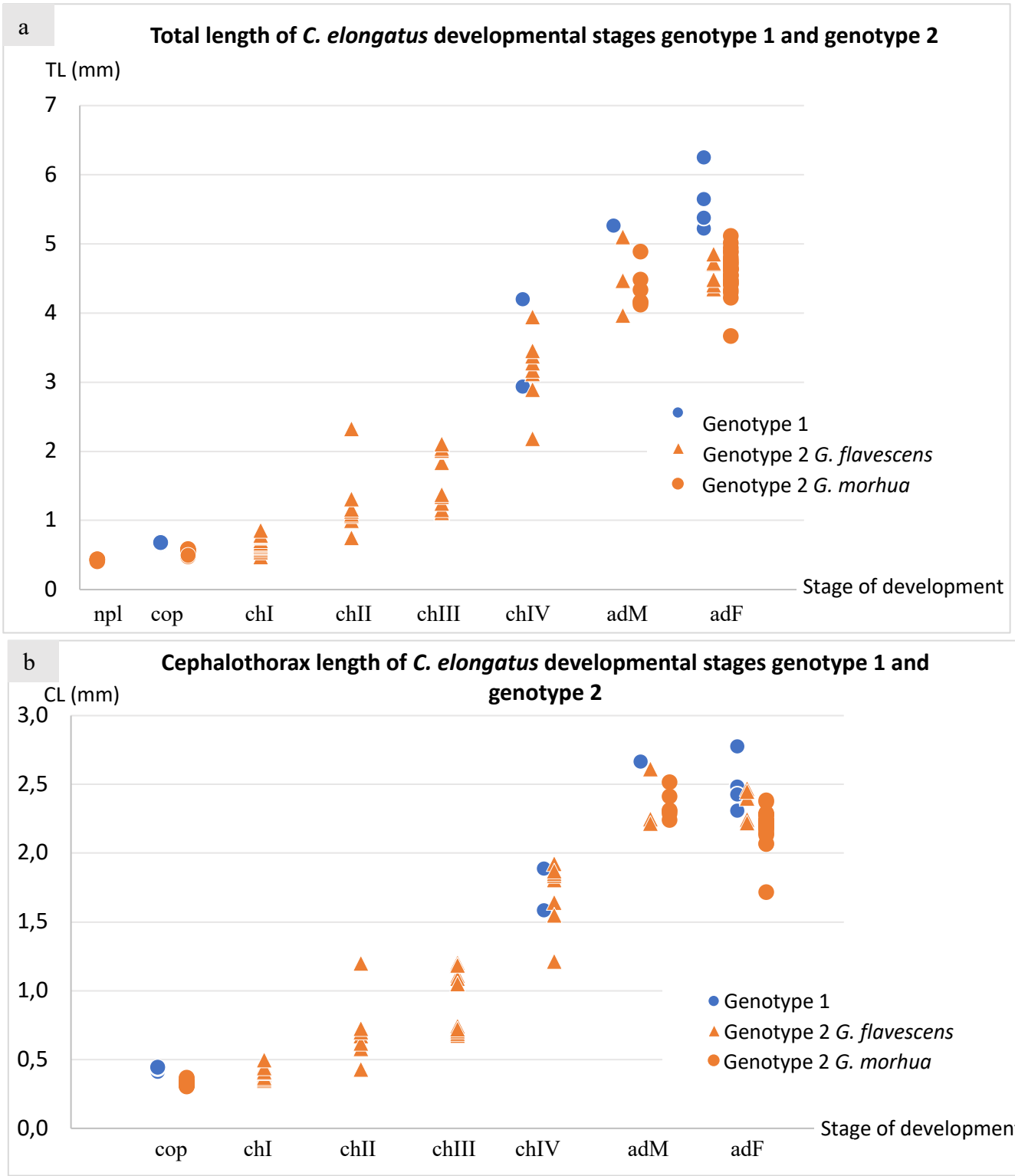


Figure 3.12. Measurements of *C. elongatus* genotype 1 and genotype 2 (from *G. flavescens* and not from *G. flavescens*) gouped in developmental stages. **a**: Total length (TL) measured from anterior end of cephalothorax to end of the uropods. **b**: Cephalothorax length (CL) measured from anterior end of cephalothorax to its most posterior part. cop= copepodite, ch= chalimus, adM= adult male, adF= adult female. *G. flavescens*= two-spotted goby, *G. morhua*= Atlantic cod.

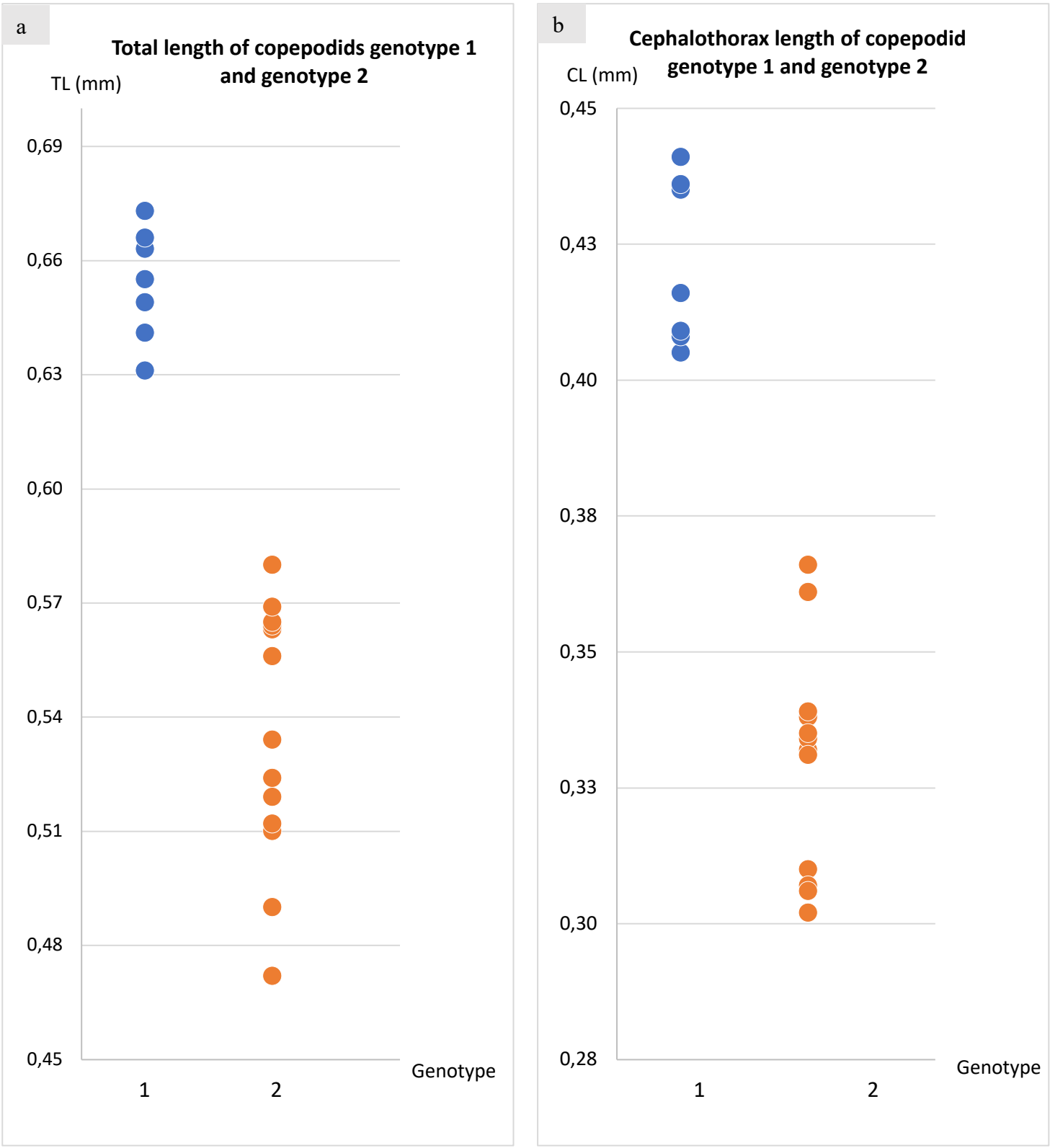


Figure 3.13. Size of *C. elongatus* genotype 1 and genotype 2 copepodids. **a**: total length (TL) measured from the anterior end to end of uropods. **b**: cephalothorax length (CL) measured from the anterior end to the most posterior part of cephalothorax.

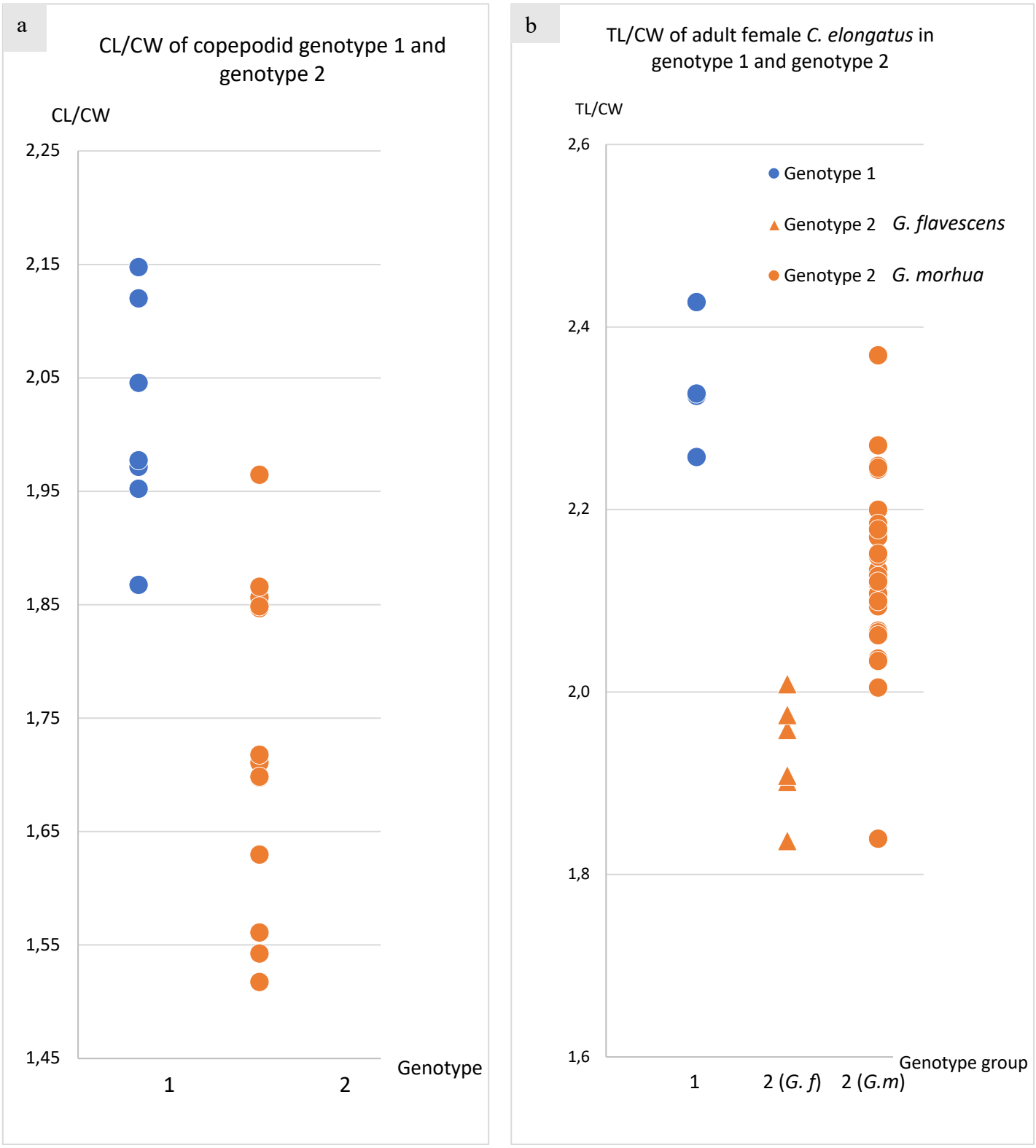
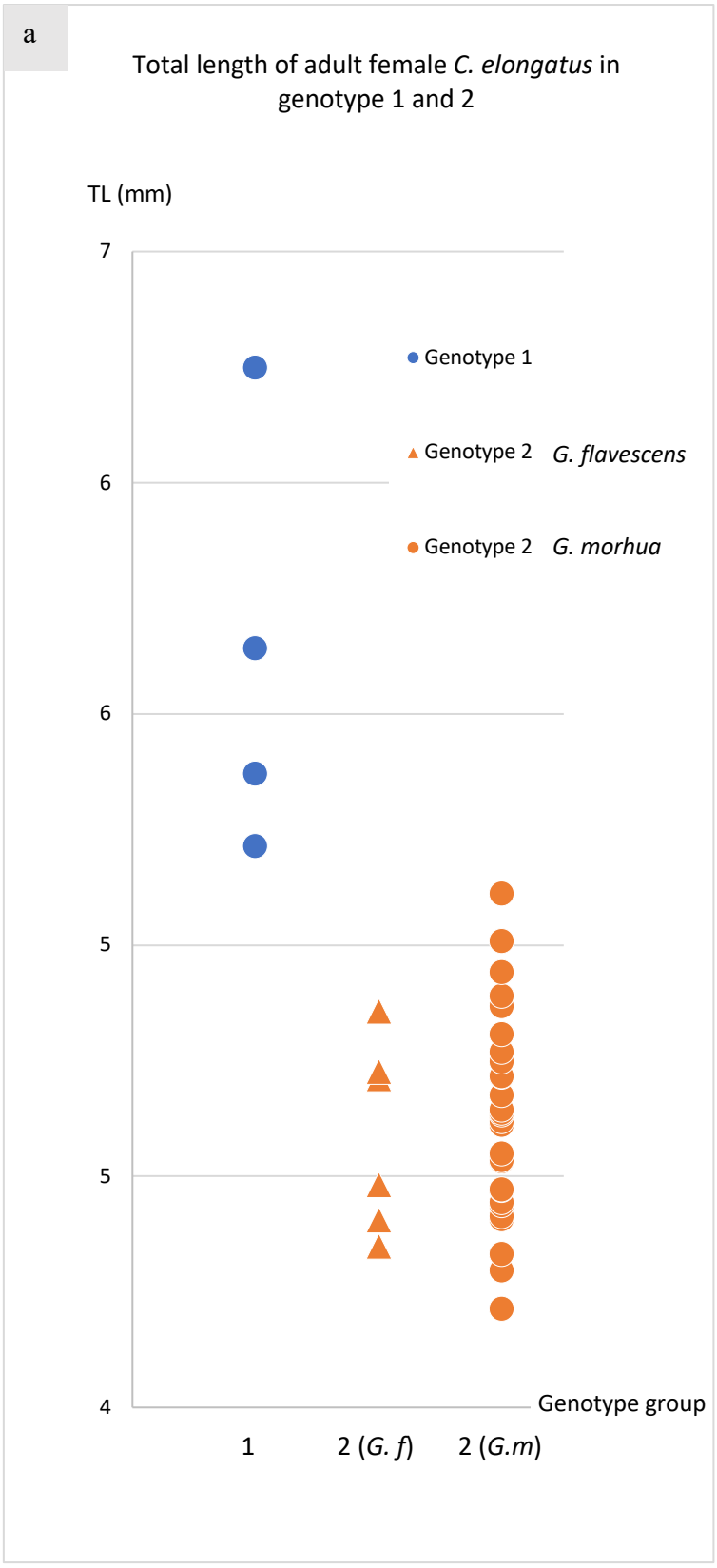


Figure 3.14. Body proportions of *C. elongatus* genotype 1 and genotype 2 **a:** Ratio between copepodids cephalothorax length (CL) and with (CW): CL/CW. **b**: Ratio on of adult females between total length (TL) and width (CW): TL/CW. *G. f*= two-spotted goby (*G. flavescens*), *G. m*= Atlantic cod (*G. morhua*)



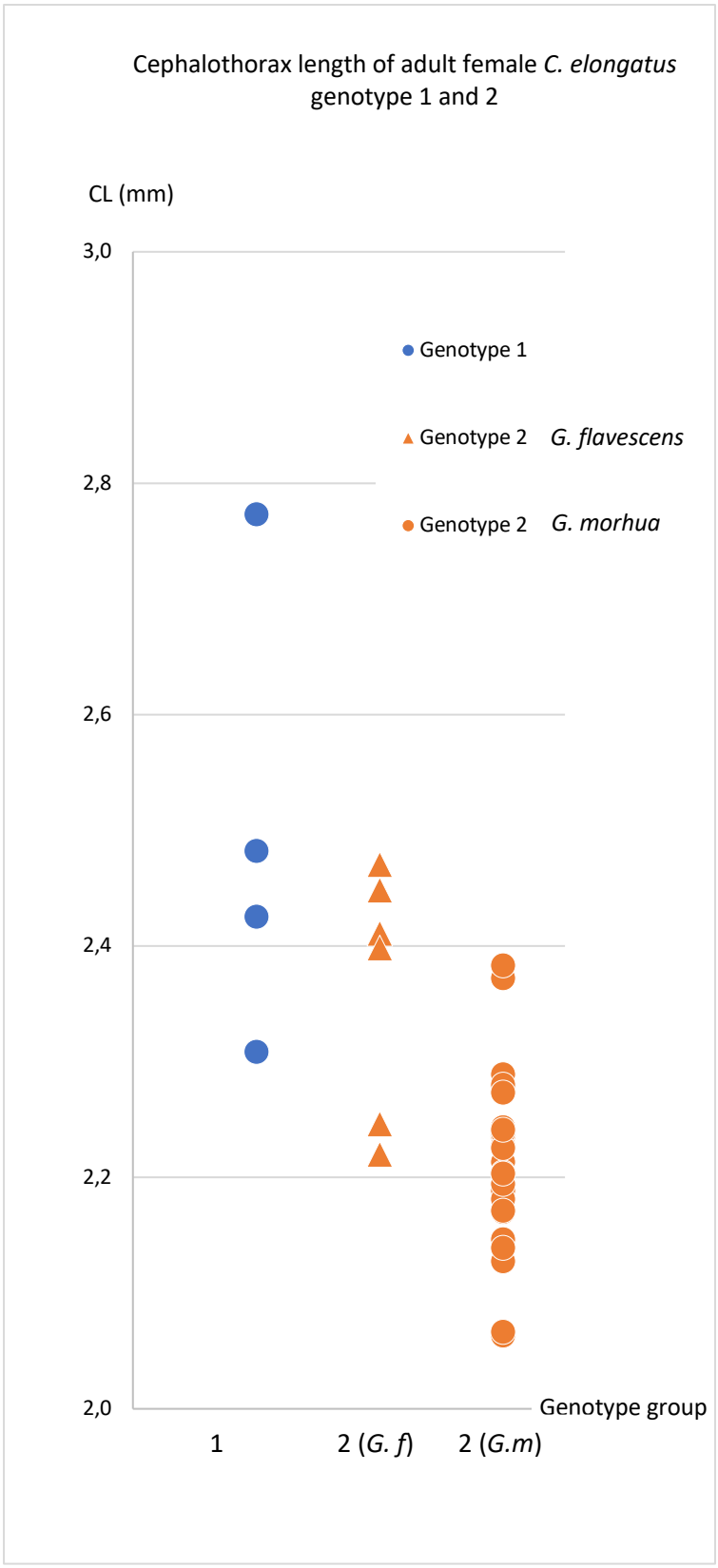


Figure 3.15. Measurements of adult female *C. elongatus* grouped in genotype 1 and genotype 2 from *G. flavescens* or not from *G. flavescens*. **a**: total length (TL) measured from the anterior end to end of uropods. **b:** cephalothorax length (CL) measured from the anterior end to the most posterior part of cephalothorax. *G. f*= two-spotted goby (*G. flavescens*), *G. m*= Atlantic cod (*G. morhua*)

3.3 Challenge experiment

A challenge experiment with both genotype 1 and genotype 2 copepodites on Atlantic salmon and gobies was planned. This could not be conducted. A pilot experiment with genotype 2 only was performed. Of the 215 genotype 2 copepodids that were introduced to a tank with eight Atlantic salmon, none were recovered at sampling 12 days post infection (based on several other experiments with genotype 1, recovery of chalimus 3-4 is expected at this time (Piasecki & MacKinnon, 1995; L. Hamre unpublished).

4. Discussion

4.1 Origin of *Caligus elongatus* on farmed fish

Farmed fish species in Norway are regularly infected with *C. elongatus* and can in varying degrees host juvenile stages of the louse. Salmonids are infected with both juvenile and adult *C. elongatus*. Bron *et al*. (1993) registered high prevalences of attached *C. elongatus* on Atlantic salmon farmed in Scotland. Atlantic salmon held in a small inshore cage in Ireland were infected with juvenile lice which developed into adults (Tully, 1989). Sea trout and rainbow trout in southern Norway may be infected with chalimi, however predominantly they carry adults (Schram *et al*. 1998; Øines & Heuch, 2005). Infections by *C. elongatus* on farmed Atlantic halibut have also occurred. Juvenile halibut from Nordland have been found infected with chalimi and adults (Karlsbakk unpublished), as have some juveniles from the Faroe Islands in this study. Farmed Atlantic cod may also be exposed to this sea louse. Chalimi are rare findings on adult cod (Heuch *et al*., 2011) but are however commonly observed on wild caught juveniles (Neilson *et al*., 1987; Karlsbakk & Nilsen, 1993; Eydal, Helgason, Kristmundson, & Bambir, 2005). Sudden infections with adult *C. elongatus* on farmed salmon (Imsland *et al*. 2019; Koren, 2001; Nodland, 2017), cod (Nygaard, 2005; Karlsbakk *et al*., 2008, 2009) or halibut (Bergh, *et al*. 2001) are however observed. This phenomenon can usually not be explained by chalimi infections, as high intensities of chalimi did not precede the appearance of adults.

A hypothesis explaining these sudden infestations of adult *C. elongatus* on farmed fish, is the transfer of adult lice from wild fish (Wooten *et al*., 1982). Leaving the host would be hazardous for most ectoparasites, however, this seems not to be the case with *C. elongatus*, which may be found free swimming as adults (Lönnberg, 1889, Neilson *et al*., 1987). Bruno *et al*. (1990) found *C. elongatus* transferring regularly from wild saithe outside sea cages to farmed Atlantic salmon inside the pens and this behaviour has also been seen in experiments where lice obtained from lumpfish and saithe transferred between different host species (Øines *et al*., 2006). The phenomenon has also been shown in related species. Pacific herring (*Clupea pallasii*) hosts *Caligus clemensi,* another generalist caligid closely related to *C. elongatus*, and act as a source of lice colonizing farmed salmon in British Colombia, Canada (Goodwin, 2021).

4.2 On which hosts do free *C. elongatus* grow up?

4.2.1 Lumpfish?

Lumpfish, with an estimated population of 150 million individuals in the Norwegian Sea (Durif 2020), is an important host species for *C. elongatus* and harbours both juvenile and adult lice (Boxshall, 1974; Øines *et al.,* 2006). Heuch *et al*. (2007) found high intensities of chalimi on lumpfish caught in the North Sea, thus indicating transmission of the parasite in the oceanic feeding areas. Øines *et al*. (2006) showed in an experiment that adult *C. elongatus* originally found on lumpfish had about the same preference for cod, and some lice switched host species during the experiment. Lumpfish migrating from oceanic waters to coastal areas during spring to spawn (Blacker, 1983) may thus transport abundances of *C. elongatus* that may later leave their hosts as adults in coastal waters (Øines *et al*. 2006). In addition to the role as outside reservoir freeing adult *C. elongatus* to the water that subsequently can infect farmed fish, the lumpfish is also frequently used as a cleaner fish in salmon farming (Imsland *et al.,* 2018). Lumpfish held in the pens may be a both an intermediate host for attached lice and a host for the adult phase in which the adult lice have direct access to farmed salmon.

4.2.2 Detached from farmed fish? Escaping salmon lice treatments?

In Norway, especially in the northern parts of the country, salmon farmers have experienced infections with high intensities of *C. elongatus* on both the salmon and lumpfish (Myklebust, 2017; Paulsen, 2018). Fish in open net pens may possibly be a source of adult lice to the environment or to other farms. Farmed fish with high intensities of *C. elongatus* are occasionally deloused with medicament- or non-medicament treatments (Imsland *et al.,* 2019), and Myklebust (2017) observed extreme infections by *C. elongatus* adults on Atlantic salmon runts, in pens receiving slice treatments. The lice left the treated fish, were swimming in large numbers in the water, and aggregated on the salmon individuals that had not eaten the slice-feed (Myklebust, 2017; Paulsen, 2018). The same phenomenon can probably occur during salmon lice treatments, that *C. elongatus* leaves the fish rather than being harmed by the drug. This may facilitate the release of adults to the environment, an input to the pool of free-swimming adult C. elongatus of unknown importance. Adult *C. elongatus* are common findings in plankton samples (Björck 1916; Neilson *et al*. 1987; Raupach *et al*. 2015), which supports the host transfer hypothesis. Adult lice going pelagic after leaving their intermediate hosts can explain the sudden appearance of *C. elongatus* adults in farmed fish without a prior chalimus abundance.

4.2.3 Small-sized intermediate hosts

C. elongatus chalimi are frequently observed on both juvenile fish and other small sized fish that live in open waters or along the shore in high abundances (Russell 1933; Rosenthal 1967; Neilson *et al*. 1987; Karlsbakk *et al*. 1993). Adults are rarely seen, so these may leave these small fish hosts where their possibility for mating is very low. Such small fish may therefore represent an important source for planktonic adults, that eventually may infect a fish host where they mate and the females produce eggs. This would then be a two-host life cycle, respectively with an intermediate and a final host. Several researchers have reported juvenile fish infected with predominantly attached developmental stages. Shotter (1973) found whiting in the Irish Sea to be infected with *C. elongatus* mainly as postlarvae, and only with copepodids or chalimi. In the Norwegian Sea off Vesterålen and Troms, 0-group haddock and Atlantic cod were also infected with chalimi of *C. elongatus* in July-August 1993 and 1995 (Karlsbakk & Nilsen, 1993; Karlsbakk unpublished). The same appears to have been observed on 0-group cod in Icelandic waters (Eydal et al. 2005), and off Newfoundland (Neilson *et al.,* 1987) (chalimi designated as *Caligus* sp.). In the Georges bank area off Cape cod, USA, the abundance of chalimi decreased as the fish grew larger, in the length range 13-58 mm. In the same area, up to 17.5% of the stomachs of 0-group cod contained adult *C. elongatus*, thus being a significant part of the diet. These adults likely originated from the chalimi on the 0-group gadids (Neilson *et al.,* 1987). That such small fish are infected with only juvenile lice indicates that the lice leave their host when becoming adults.

In a study of sea lice of wild fish in Cobscook Bay, Maine, high prevalence with *C. elongatus* was observed on the highly abundant threespine stickleback (*Gasterosteus aculetaus*), (Jensen *et al.,* 2016). Over 95 % of the lice on these sticklebacks were chalimi, and free-swimming adult lice were found in thew tanks used to transport the fish. This pattern seems very similar to that observed here with two spotted gobies (see below). Again, the observations by Jensen et al. (2016) support the hypothesis that small fish in coastal shallow waters can act as intermediate hosts "releasing" considerable numbers of adult *C. elongatus*. In Norway, gobies (i.e. two-spotted gobies, sand gobies and black gobies) have been found to harbour *C. elongatus* in shallow waters (Heuch *et al*. 2007). It has been observed that gobies captured and reared in aquaria develop *C. elongatus* infections (i.e. infected when captured), and the prevalence of macroscopically visible chalimus-stages can exceed 10%, even approach 20% on large gobies late summer-early autumn in Krossfjorden, near Bergen (Karlsbakk personal communication, SCUBA observations). Although being a highly abundant fish along the shallow waters on the

Norwegian West coast (Fosså, 1991), the role of the two-spotted goby as a host for *C. elongatus* has not been examined until now.

The predominance of juvenile stages seen throughout a year on the two-spotted gobies in this study suggests that the gobies lose the lice when these become adults. Hence the gobies may act as intermediate hosts, and this can be a similar *C. elongatus* lifecycle as involving sticklebacks in Cobscook Bay (Jensen et al. 2016).. The lice from gobies were exclusively attached with frontal filaments, also including adult stages. None of the young adult females had egg strings, but egg strings have been observed on female lice on these gobies (E. Karlsbakk personal communication; Figure 4.1). A relatively low prevalence was observed in the current study, similar to that observed by Heuch *et al*. (2007). However, a modest prevalence can still amount to high *C. elongatus* densities in shallow waters, as two-spotted goby densities can reach over 100 individuals meter $^{-3}$ (Fosså 1989). A rough estimate of the mean daily production of adult lice from the gobies was calculated based on a sample taken late October 2020. Although the prevalence was only 2%, considering the high host density and louse development rate, the estimates suggested that 39 adult lice developed on the two-spotted gobies per day per 100 meters of shore. This number is most likely an underestimate of the lice production, because:

i) the beach seine significantly underestimates the number of two-spotted gobies as they escape the beach seine due to for instance macroalgae and rocks (Olsen, 1975; Fosså, 1991).

ii) other gobies may also harbour lice (Heuch & Øines, 2007)

iii) abundance may at times be higher. It thus appears that a considerable amount of adult *C. elongatus* is released after developing on gobies along the coast of southern Norway. These free-swimming or planktonic adult *C. elongatus* can infest any suitable fish host in the area. In the Espegrend area adult cod was probably a main recipient, since they do not harbour chalimus stages and often hunting gobies in shallow waters (Fosså, 1991). It is thus likely that the production of *C. elongatus* from small fish could be responsible for invasions with adult lice seen in farms (Imsland *et al.,* 2019) from late spring to early autumn when the abundance of two-spotted gobies is at its highest (Fosså, 1991).

Figure 4.1. Mature female two-spotted goby infected with ovigerous adult *C. elongatus*. The pair of pale coloured egg strings (arrow) indicate that the louse is not fertilized. Screenshot from video (June 2017) kindly shared by Magne Tessem.

4.3 The two-spotted goby

There is little information regarding infection dynamics of *C. elongatus* on two-spotted gobies. Øines *et al*. (2005) and Zander *et al*. (2005) recorded the louse on two-spotted gobies in south Norway and Helgoland, respectively. Only the latter calculated a prevalence (4 %) but these studies did not specify the time of sampling. However, Heuch *et al*. (2007) studied the temporal infection dynamics of *C. elongatus* on wild coastal fish outside Arendal in southern Norway, including two-spotted gobies. In the spring/early summer, 27 gobies were caught with a beach seine, and none were infected with the louse. In September, however, about 170 two-spotted gobies had a prevalence of just under 6 %. In the present study we recorded a similar prevalence from two-spotted gobies at Espegrend outside Bergen during autumn, peaking at 5 % in October. This was however not the highest prevalence recorded, which was 10 % in May. The spring and autumn peaks were when the water temperatures were 10 and 12.4 °C, respectively. The prevalence during the winter was generally low, which was expected as the *C. elongatus* life cycle is slower in cold temperatures (Koren, 2001). Also, the abundance of gobies in the catches clearly decreased throughout the winter as the fish expectedly migrated to deeper waters

(Olsen, 1975). There was a generation shift in the goby population during summer, with a massive influx of juvenile fish. That likely affected (reduced) the prevalence, albeit goby abundance was very high.

In order to examine if the prevalence values seen on the gobies at Espegrend was representative for a larger geographic area, two other (reference) locations were also studied. This sampling became delayed and happened in October, rather than in September as intended, because a high prevalence was expected then (see Paulsen, 2018). The prevalence of *C. elongatus* on two spotted gobies at the reference sites did not differ significantly from Espegrend. It therefore seems possible that the patterns are regional. However, there are various factors that could influence (host density, salinity, hydrodynamics) (see Paulsen, 2018), that needs to be better understood.

Recent molecular analyses of *C. elongatus* have revealed two genotypes – genotype 1 and genotype 2 (Øines *et al*. 2005; 2008). The genotypes differ in their host use and temporal occurrence and even in some minor morphological characters, and could represent two species (Øines et al. 2008). This may be a factor explaining the infection dynamics of *C. elongatus*, but studies predating the discovery of this genetic heterogeneity may have concerned both variants. In the present study, all *C. elongatus* were genotyped. Throughout one year of sampling, lice obtained from two-spotted gobies in this study were all genotype 2. Zander (2005) did not genotype the *C. elongatus* on two-spotted gobies in Helgoland, Øines *et al*. (2005; 2008) only genotyped a total of 11 lice from this fish host (genotype 2). Hence, our study is the first to reveal the genotype-2 infection dynamics. The two peaks could reflect two generations on the gobies, the peak in May producing adult lice infecting some other fish (e.g. cod) final host, where adult females gave rise to a second generation during autumn, and likely one in winter. The similar life cycle pattern observed by Jensen et al. (2016) on sticklebacks in Cobscook Bay, Maine, however, represented genotype^{-1}. There, a June and a winter (November-) peak was seen. The patterns seen by Koren (2001) on Atlantic salmon in Northern Norway likely concerned genotype-1, since Genotype-2 lice has not been found north of Frøya, and all lice from salmonids in the north have been found to be type-1. He observed (Fig. Y) a peak in chalimus in October on the salmon, adult lice dominating the rest of the year. There was no spring (May) peak.

4.4 Hosts of *C. elongatus* genotypes and their infection dynamics

Former studies have registered host species of *C. elongatus* (Parker 1969; Kabata 1979; 1992; Boxshall 1974; Zander 2005; Øines *et al*. 2005, 2007; Heuch *et al*. 2007; Jensen 2016). A few of these have genotyped the lice and are listed with an overview in table 4.1. However, the overall data on genotypes on host species is sparse and it is rarely pointed out in studies whether the fish were hosts for attached lice or freely moving adults. Adults may be able to utilize a wide range of hosts since they can move around to feed and thus avoid the build-up of potential harmful local immune responses, or they may be found attached to fish that are not true hosts from which they can feed and produce eggs, rather they only represent random encounters and thus transient infections. The juvenile phase of *C. elongatus* requires a longer and more intimate contact with its hosts, including the ability to counter immune responses and digest host tissue from the same location during the entire development to adult. These are important aspects that may potentially change our views on *C. elongatus* host preferences since the hosts for the juvenile phase must be more compatible, and thus its host specificities will be most reflected in these developmental stages.

Caligus elongatus is currently considered to be a generalist with regard to the hosts they utilize. The realization that there are two genotypes that potentially represent two species add the possibility that there indeed is a degree of host specificity in the juvenile-phase. Among the five host species included in this study, none were found infected with both genotypes of attached chalimi. It should although be noted that sample size was low except from two-spotted goby which had only genotype 2 chalimi, and lumpfish where predominantly genotype 1 chalimi was found.

4.3.1 Gobies

In this study, the over two thousand two-spotted gobies sampled on the Norwegian west coast exclusively hosted genotype 2 *C. elongatus* throughout a whole year. Studies sampling two-spotted gobies outside Arendal on the south-east coast of Norway and attained the same results; all *C. elongatus* infecting these gobies were genotype 2 (Øines & Heuch, 2005, 2007). In the current and Øines' studies, other sympatric host species harboured genotype 1 lice at the same time, including ovigerous adult females as seen on lumpfish in the present study. Thus, our results indicate that genotype 1 copepodids do not infect two-spotted gobies. This may be true also for other gobies such as *Pomatoschistus spp*. and the black goby, also harbouring genotype 2 (Øines & Heuch, 2007; present study). The sand goby (*P. minutus*), for instance, thrives on

sandy bottom in contrary to the two-spotted goby, and may have the potential to cover the role as an intermediate host in such habitats where the two-spotted goby does not live. However, Øines & Heuch (2007) found a transparent goby (*Aphia minuta*) infected with genotype 1, an indication that there is no specificity at the family level - Gobiidae. However, besides the two-spotted goby, few gobies and their *C. elongatus* have been examined, so their role as hosts or genotype repertoire of the lice are uncertain.

Elsewhere, *C. elongatus* infestations has been found on two-spotted goby which had a 4 % prevalence in Helgoland, Germany (Zander, 2005). The genotype involved is unknown, but Raupach *et al.* (2015) found adult lice of both genotypes in Helgoland plankton samples. The distribution of two-spotted goby in Scandinavia ranges from the Swedish mid-east coast to Vesterålen in northern Norway (Whitehead, Bauchot, Hureau, Nielsen, & Tortonese, 1984). It would be interesting to investigate the role of two-spotted gobies in the *C. elongatus* life cycles in more northern areas. Ultimately, as in Finnmark, other intermediate hosts could be involved, but so far only genotype 1 has been detected in the north.

4.3.4 Gadoids

A large number of adult *C. elongatus* from various adult gadoids have now been genotyped and they may host both genotypes (Øines, 2007). The adult lice from Atlantic cod in this study were also both genotypes, though with just one genotype 1 out of 35 lice. This was however also the only louse from cod sampled during spring. A pattern with a seasonal shift from genotype 1 to 2 during the summer has been observed (Øines & Heuch, 2007). In that study, the majority of *C. elongatus* (> 90 % from Atlantic cod and pollack and > 70 % from saithe) found on wild gadoids southeast in Norway were genotype 1 in the spring, whereas over 90 % from the same host species were genotype 2 during autumn (Øines & Heuch, 2007).

However, in the gadids, particularly evident in cod, haddock and whiting, there seems to be a difference in the suitability as host with life stage. Juveniles are frequent hosts to chalimi and may harbour high intensities (Karlsbakk & Nilsen, 1993; Neilson *et al.,* 1987; Shotter, 1973). Adults nearly exclusively harbour adult lice. The genotype developing as chalimi on juvenile cod in Norway has so far not been established. Heuch *et al.* (2011) collected 6 *C. elongatus* chalimi from wild and farmed cod from Norway (Ålesund to Finnmark). These rare findings (343 cod examined) represented 5 genotype 1 and one genotype 2. As evidenced by Neilson *et al.* (1987), *C. elongatus* leave the juvenile gadids when becoming adults. In the west Norwegian

fjords, the large numbers of genotype 2 lice developing on gobies may eventually settle on gadids as adults.

In experiments allowing adult *C. elongatus* of both genotypes to choose host, Atlantic cod was a preferred host, even over lumpfish (Øines *et al.,* 2006). In Rogaland, western Norway, farmed Atlantic cod were heavily infected with adult *C. elongatus* in October, the lice causing haemorrhage and ulcers on the heads and necks (Nygaard, 2005). Hence, if farming of Atlantic cod in Norway increase, *C. elongatus* has the potential to cause problems (Bjørn *et al.,* 2021). It is important to find out which genotype infects young and old cod, and the risks they pose.

4.3.2 Lumpfish

In this study, all *C. elongatus* genotyped, collected from lumpfish in the Norwegian Sea (Karlsbakk & Nilsen, 1993), were genotype 1. The prevalence on these lumpfish was 24 % and mainly with juvenile lice (Karlsbakk, Nilsen, & Hodneland, 1994). Lumpfish migrating from oceanic to coastal areas in the spring to spawn, transporting abundances of *C. elongatus* (Heuch & Øines, 2007). Chalimi on lumpfish from the Norwegian southwest coast outside Bergen were also all genotype 1. However, four out of ten free adults collected from lumpfish caught near the shore in August were genotype 2. This infection pattern matches prior studies where immature lumpfish caught in the North Sea consistently were infected with genotype 1, whereas lumpfish caught near shore during the autumn after spawning can be infested with both genotypes (Øines *et al.* 2007). This includes both genotype 1 and 2 attached stages. An explanation for this could be that *C. elongatus* genotype 1, although not being a host specific parasite, generally favours the lumpfish as host in both the juvenile phase and adult phase before other species (Øines *et al.* 2006). Hatching egg strings could release nauplii that lead to an infection pressure with genotype 1 copepodids, which could possibly explain why genotype 1 was the most abundant genotype found on other fish species during this season (Øines & Heuch, 2007). After the spawning season in the spring, female lumpfish leave the coast and return to the Norwegian Sea to feed (Durif, 2020), male lumpfish stay and protects eggs and young, and the juveniles live in the kelp forests for about a year (Myrseth, 1971). These should then be exposed to genotype 2 copepodids before leaving for their oceanic phase (Holst, 1993). Hence, the apparent rarity of genotype 2 on lumpfish from the ocean is puzzling.

4.3.3 Salmonids

The infection dynamics on sea trout off the southeast coast of Norway followed the general seasonal pattern of the genotypes on other fish species sampled in the same area: a clear

dominance of genotype 1 infecting the fish in the spring, while genotype 2 is predominant during autumn (Øines & Heuch, 2007). Both wild and farmed Atlantic salmon are commonly infected by *Caligus elongatus* (Berland, 1993) but for the time being, only *C. elongatus* from farmed salmon have been genotyped. Even though genotype 1 lice have been the most recorded, adult lice of both genotypes have been found infecting Atlantic salmon (Øines & Heuch, 2005, 2007; present study). However, there are no records of genotype 2 chalimi on Atlantic salmon (Øines & Heuch, 2005, 2007; Agusti-Ridaura *et al.,* 2019) and the present study includes only free genotype 1 adults from this species. In challenge experiments, genotype 1 develops through chalimus stages on farmed salmon, confirming that *C. elongatus* chalimi on farmed salmon may also be genotype 1. Challenge experiments are needed to examine the role of salmon as a host to the juvenile phase of genotype 2 *C. elongatus*. This was intended here, but the inclusion of positive controls was a problem at the time. Our pilot challenge experiment without a positive control host (eight Atlantic salmon with 215 genotype 2 copepodids) did not result in attached lice after 12 days. The experiment was performed in the exact same way as > 30 previous experiments with genotype 1 *C. elongatus*, based on which approximately 20-30 % of the copepodids were expected to attach and develop to chalimi (Hamre personal communication). This represents an observation suggesting that genotype 2 does not readily infect this host. New experiments should be performed using copepodids of both genotypes, and with the present results from screening of the two-spotted goby, that host species could be used as the susceptible (positive control) host for genotype 2.

4.3.5 Flatfish

Flatfish aquaculture in Norway has included sole (*S. solea*), Atlantic halibut, Turbot (*S. maximus*) and European plaice (*P. platessa*) but flatfish farming is currently predominantly of halibut. Several flatfish species are hosts for *C. elongatus,* and in the past high intensities (>100 halibut^{-1}) with adult lice have caused skin lesions in Atlantic halibut kept in pens (Nilsen in Bergh *et al.,* 2001). A low number of lice have been genotyped from flatfish, and this study is the first to examine the *C. elongatus* genotypes occurring on Atlantic halibut. Both genotypes were registered on Atlantic halibut and common dab including two and seven genotype 2 chalimi, respectively. From the mentioned summer outbreak southwest in Norway on 500g halibut (Bergh *et al.,* 2001), four adult lice were genotyped, and all were genotype 2. In that case, two-spotted gobies are among the possible intermediate hosts from which the adult lice originated. However, chalimi were collected from Atlantic halibut juveniles reared in tanks at the Faroe Islands. Some of these chalimi from were analysed and found to be genotype 2, the

first record of this genotype from the Faroes. Genotype 1 chalimi on common dab were also confirmed here, in addition to free adults of genotype 2 which has not previously been reported from this host species. Other flatfish species, brill and plaice from the Arendal region, southern Norway were infected by genotype 2, while a dab in the same area harboured a genotype 1 louse (Øines & Heuch, 2005, 2007). Few lice have so far been genotyped from flatfish, so there is no clear patterns in host use. Pleuronectids appears to be susceptible to copepodids of both genotypes, so the temporal and spatial distribution of the parasites as well as the hosts can be responsible for the abundance of these *C. elongatus* variants on these hosts.

4.5 Morphometric comparison *C. elongatus* genotype 1 and 2

There are currently no good morphological distinguishing characters for the *C. elongatus* genotypes. The non-random distribution, ecological differences and the Co1 gene divergence all suggests that these genotypes could represent two cryptic species (Raupach *et al.,* 2015; Øines, 2007; present study). Øines *et al*. (2008) examined morphological differences between the genotypes based on the length of processes on the first leg, the length of the fourth leg's spine and on the shape of the sternal furca. Most genotype 1 adults had short processes on the first leg, and a long pine on fourth leg, but these characters were not genotype specific (diagnostic). The sternal furca did not contribute to distinguishing the genotypes from each other. It would be beneficial to reveal diagnostic phenotypic differences, that could allow fast identification. The validation of any characters must account for intra-genotype morphometric variation also with season, host or geographic area, so this is a large undertaking. Here, some major readily obtainable measurements of the lice were compared, which resulted in interesting findings that needs corroboration in follow-up studies.

Regardless the stages of development examined, the total length and cephalothorax length were greater on genotype 1 lice than genotype 2, most evidently in the copepodids and adult females. The copepodid cephalothorax was also significantly more oblong (greater CL/CW value) in genotype 1 than genotype 2 whose cephalothorax were rounder in the shape. The placing of the eyes on cephalothorax was also different between the genotypes, as genotype 1 copepodids' eyes were more centred in the cephalothorax while genotype 2 generally had the eyes placed more anterior. The latter statistics could be affected by preformed filaments pushing the eyes more posterior, and it should be examined to what extent frontal filament development influenced cephalothorax shape and eye location. Our results suggest that *C. elongatus* has morphometrical differences distinctive to each genotype. These measurements are promising.

The next step must be to substantiate these results and examine the influence of host and environmental variables. Also, an investigation of the morphological and morphometric ratios between the two genotypes in controlled experiments could become possible, perhaps with cod juveniles as host.

4.5.1 Molecular divergence between *C. elongatus* genotype 1 and 2

It is readily accepted that *C. elongatus* can be divided into two mitochondrial genotypes. Phylogenetic analysis of the mitochondrial CO1- and 16s genes, and the nuclear rRNA 18s gene have formerly been performed (Øines & Schram, 2008). The level of heterogeneity in the mitochondrial genes found between the *C. elongatus* genotypes is similar to the heterogeneity between other closely related caligid species. Thus, it has been suggested that the genotypes in reality are sibling species (Raupach *et al*. 2015; Øines & Schram, 2008).

In the current study, the mtCO1 gene of over 100 lice from different hosts and geographic areas were sequenced. Several nucleotide positions (N=21) apparently defining the genotypes were found. The one deviant louse with lowest percent identity in this study matched the genotype 2 nucleotides in the defining positions and was therefore categorized genotype 2b. That louse came from deep waters near Bergen, and deep-water *C. elongatus* were probably poorly represented in the present study and should be given attention. One mtCO1 gene sequence is available from the northwest Atlantic (Jensen, 2013). That genotype 1 sequence had two unique substitutions in our defining positions for genotype 1 based on the northeast Atlantic sequences, perhaps a signature for the NW Atlantic *C. elongatus*.

The recently designed Ce primer assays for PCR based identification of the *C. elongatus* genotypes were mostly successful and turned out to be a good tool for quickly genotyping of this louse. Even with low DNA concentrations bands were obtained on the agarose gel revealing the genotype. In some other cases where the genotyping failed, the samples were very old, perhaps initially formalin fixed, and DNA quality very bad. On the other hand, several chalimi stored in 70% ethanol from 1993 were successfully typed.

However, the genotype 2b louse was not genotyped with the assay and was typed based on the nearly full mtCO1 sequence. This case was the only where primer mismatch (last base 3'-end) was the cause. This could indicate that other deviating lice exist, perhaps necessitating

adjustments to the CE primers. Based on our now much better understanding of the in-genotype genetic variation, it is also possible to design new genotyping primers.

Table 4.1. An overview including all studies (May -21) on genotyped *C. elongatus* and their hosts. Fish were wild if not noted otherwise (F=farmed and OF= wild outside farm). n= number of lice. ad= adult louse, ch= chalimus and co= copepodite when specified in the studies. Numbers are solus when the stage of development is unknown.

Host species (farmed or wild)	**Sampling site**	**G1**	**G2**	**Reference**
Lumpfish (31)	Skagerrak area	31 ad		1
Saithe (30)	Skagerrak area	1 ad	29 ad	1
Atlantic cod (*G. morhua*)	Skagerrak area	26	77	2
Atlantic horse mackerel (*T. trachurus*)	Skagerrak area		1	2
Atlantic mackerel (*S. scombrus*)	Skagerrak area	2	1	2
Black goby (*G. niger*)	Skagerrak area		1	2
Brill (*S. rhombus*)	Skagerrak area		6	2
Corkwing wrasse (*S. melops*)	Skagerrak area		1	2
Dab (*L. limanda*)	Skagerrak area	1		2
Garpike (*B. belone*)	Skagerrak area	1		2
Goldsinny wrasse (*C. rupestris*)	Skagerrak area		1	2
Grey gurnard (*E. gurnardus*)	Skagerrak area	3	16	2
Herring (C. *harengus*)	Skagerrak area	5	1	2
Lumpfish (*C. lumpus*)	Skagerrak area	118	3	2
Plaice (*P. platessa*)	Skagerrak area		4	2
Pollack (*P. pollachius*)	Skagerrak area	27	66	2
Saithe (*P. virens*)	Skagerrak area	35	69	2
Sand goby (P. *minutus*)	Skagerrak area		1	2
Sea trout (*S. trutta*)	Skagerrak area	19	38	2
Transparent goby (*A. minuta*)	Skagerrak area	1		2
Two-spotted goby (*G. flavescens*)	Skagerrak area		8	2
Whiting (*M. merlangus*)	Skagerrak area	4	29	2
Lumpfish (*C. lumpus*)	North Sea pelagic	26		2
Atlantic salmon (*S. salar*) (F)	Sørøya (70.65°N, 23.15°E)	14		2
Atlantic cod (*G. morhua*) (OF)	Sørøya (70.65°N, 23.15°E)	4		2
Saithe (*P. virens*) (OF)	Sørøya (70.65°N, 23.15°E)	11		2
Atlantic salmon (*S. salar*) (F)	Værlandet (61°N, 04°E)	1	3	2
Atlantic salmon (*S. salar*) (F)	Faroe Islands (62°N, 07°W)	10		2

Atlantic salmon (*S. salar*) (F)	Canada (45°N, 66°W)*	34*	2
Atlantic salmon (*S. salar*) (F)	Scotland (56°N, 05°W)	30	2
Atlantic cod (*G. morhua*) (F)	Karmøy (59°N, 05°E)	3	2
Saithe (*P. virens*)	Kalvåg, Bremanger	20 ad	3
Lumpfish (*C. lumpus*) (ch n=22)	Maine, USA*	22 ch	4
Threespine stickleback (*G. aculeatus*) (ch n=380)	Maine, USA*	380 ch	4
Blackspotted stickleback (*G. wheatlandi*) (9)	Maine, USA*	39 ch	4
Ninespine stickleback (*P. pungitius*)	Maine, USA*	1 ch	4
Winter flounder (*P. americanus*) (	Maine, USA*	16 ch	4
Rainbow smelt (*O. mordax*)	Maine, USA*	1 ch	4
Longhorn sculpin (*M. octodecemspinosus*)	Maine, USA*	2 ch	4
Mummichog (*F. heteroclitus*)	Maine, USA*	1 ch	4
Red hake (*U. chuss*)	Maine, USA*	4 ch	4
Atlantic tomcod (*M. tomcod*)	Maine, USA*	1 ch	4
Threespine stickleback (*G. aculeatus*)	Maine, USA*	71*	5

1= Øines *et al*. 2006. 2= Øines & Heuch (2007). 3= Agusti-Ridaura *et al*. (2019). 4= Jensen *et al*. (2016). 5= Pietrak *et al*. (2019). * Lice sampled from Northwest Atlantic.

4.6 Assessment of the methods

4.6.1 Fish sampling

The two-spotted gobies in this study were collected during a full year of sampling. As the year progressed and the activity and distribution of the gobies changed, the sampling gears had to be changed in order to get fish. Fish traps, beach seine and modified nets were used, each method with strengths and weaknesses. In all methods there was a possibility for (particularly) adult *C. elongatus* to detach when the fish came in contact with the sampling gear, but this was minimized in each sampling method.

The fish traps were used the first eleven samplings. This was the only passive sampling method and was also most gentle to the gobies. A likely advantage with the traps is the minimal contact between the gobies and the trap walls, which reduces the risk of losing any parasites. They are relatively easy to place on rocky bottom and among seaweed but sampling with these traps

requires time to attain a sufficient number of fish. The number of two-spotted gobies caught in the traps varied much, with weather and apparently with the activity of the gobies. When trap capture proved to be too time consuming, more efficient gears were used instead.

A beach seine was the most effective in catching two-spotted gobies. In the use of a beach seine, there is a higher risk of clustering of the fish or the fish rubbing against the seine which can cause detachment of *C. elongatus* (Heuch *et al.* 2007). This risk was accounted for by pulling the net slowly and focus on catching gobies that did not touch the seine. The modified nets tested act similarly to the beach seine regarding contact with the fish and risk of lice detachment. Other disadvantages with these nets are a low reach and the need for a shoal of gobies to swim over the net in order to catch them, but unlike the beach seine which needs to be placed in an area with no large rocks and an even bottom, the nets were additionally suitable for any bottom, making them appropriate for their purposes.

4.6.2 Molecular analyses

Because mitochondria are only inherited from the mother in diploid organisms, using the mitochondrial CO1 gene thereby excludes the male evolution. However, sperm contain numerous mitochondria, so if e.g. a genotype 1 female received spermatophores from a genotype 2 male, then this possibly could lead to some female lice scoring both as genotype 1 and 2 with the Ce assay. The possibility was counteracted by removing the genital segment from all adult female lice, analysing those separately.

The sequences produced from the *C. elongatus* in this study were the major basis of sequence analysis, assessment of the novel genotyping primers and the finding of nucleotide substitutions defining the two genotypes. It was therefore important that the sequences were correctly read and that single substitutions were investigated on a false basis. This source of error was however eliminated by sequencing the mtCO1 gene with both forward and reverse primers in order to reading the chromatograms when there were single substitutions interfering with any defining positions. As the chromatograms were not available for the reference sequences in GenBank or the one sequence published by Jensen (2013) this controlling measure could not be performed here.

Feilkilde: human errors/contamination on lab?

4.6.3 Morphometrical analysis

Measuring sea lice with image analysis is a relatively recently developed method (Hamre *et al*. 2013; Eichner *et al*. 2015). When measurements of *C. elongatus* developmental stages were presented by Piasecki (1996), the wooden slide method (Humes & Gooding, 1964) was used to "fixate" the specimens prior to measuring with a scaled eyepiece. The wooden slide method involves applying lactic acid which temporarily contracts the copepods before allegedly restoring their original size (Humes & Gooding, 1964). The method used in the present study with photographing the lice is less time consuming than the latter, and it does not require application of other substances than saline water, the louse's natural element. The image processing program ImageJ allows subjective and precise measurements to be taken.

Since the only morphological characters compared between the genotypes are three minor appendages (Øines & Schram, 2008), we chose to investigate whether there are more easily detectable differences between the genotypes. The total length of caligids can be affected by pressure put onto the louse by the cover glass if too little water was applied, as the body may be stretched (L. Hamre personal communication). The female genital segment also grows after reaching the adult stage, making it a body part with varying length depending on the age (Hamre *et al*. 2013), which is the reason adult females found on two-spotted gobies were handled as a separate group from other adult females found on final hosts. Hence, total length is not an optimal measurement to use in this case. The cephalothorax, however, is a more rigid structure on *C. elongatus* which does not grow dramatically during each developmental stage (Hamre *et al*., 2013) and is therefore a reasonable body part to compare between the genotypes. The location of the eyes on cephalothorax could in the copepodid stage be pressed posteriorly by the presence of a preformed frontal filament in the frontal organ (E. Karlsbakk personal communication) and may therefore be affected by other factors than genetics/genotypes.

Excluding *C. elongatus* from two-spotted gobies, adult lice and their offspring measured in the current study were found on fish (genotype 1 on lumpfish and genotype 2 on cod) caught in the same area approximately at the same time (autumn). We do not know if the lice developed on other fish than these hosts. The fish infected were however male lumpfish guarding their eggs on the bottom and were therefore stationary, and the cods were caught in an area with approximately 20 m maximum depth. The lice on these fish were in similar water conditions if they infected their host for a long time.

4.7 Caligus elongatus – two genotypes or sibling species?

Even though *C. elongatus* has been found as two major genotypes (genogroups), it has been suggested ever since this discovery that these variants could be sibling species (Raupach *et al.,* 2015), however, more evidence is needed to confirm this (Øines, 2007; Øines *et al.,* 2008). The genotypes are based on their differences in the mitochondrial genes CO1 and 16S (Øines & Heuch, 2005; Øines *et al.,* 2008). The molecular heterogenicity between the *C. elongatus* varieties found in these genes are similar to other closely related crustacean species (Øines *et al.,* 2008). The results in the present study support this differentiation between the groups and additionally found 21 nucleotide positions within the mtCO1 gene together defining the genotypes. However, nuclear DNA characters differentiating the mitochondrial genotypes have so far not been found (Øines *et al.,* 2008), which is an expected finding if the genogroups represents true separate species. Prior to this study, the host preferences of genotype 1 and 2 have been investigated on wild and farmed fish species in the northeast Atlantic, mainly southern Norway. The genotypes of adult lice found on some species indicated a difference in host preference between the types (Øines *et al.* 2006; Øines & Heuch, 2007). The findings in our results where no fish species harboured juvenile lice of both genotypes strengthens the support for the case that *C. elongatus* may be more host specific, with different preferences between the genotypes, than first assumed. The two small morphological characters investigated by Øines *et al.* (2008) indicated a phenotypic variance between genotype 1 and 2 but were not all bound to the types. Our morphometric measurements do indicate a size and ratio difference between the genotyped groups. Even though the results in Øines *et al.* (2008) and the results obtained in the current study are not defining distinct phenotypes of each genotype, some morphological differences appear to be presented when group of lice from each genotype are compared.

Combined, the data on different aspects of the differentiation between *C. elongatus* variants indicate the presence of two sibling species, like previously suggested (Øines & Heuch, 2007; Raupach *et al.* 2015). However, more research is needed to confirm this, and the next step may be to examine the genotypes' ability to fulfil the biological species concept (Mayr, 1942).

4.8 Other parasites

When examining the two-spotted gobies for *C. elongatus,* infections with another ectoparasite, *Gyrodactylus* sp. was noted. This could represent *Gyrodactylus flavescensis*, originally described from this host from the exact same locality (Huyse, Malmberg, & Volckaert 2004).

They found this species, the only *Gyrodactylus* species known from this host species, on the gill arches in addition to the fins. The present abundance estimates based just on the external surfaces are likely underestimates, specimens on the gills or in the mouth went unregistered. Abundance was highest during spring and was found with highest prevalence about the same time as this study did.

Another parasite discovered on the skin of two-spotted gobies was the metacercaria larvae of *Cryptocotyle lingua,* which occur in skin cysts surrounded by pigment appearing as black spots.. The gobies are infected by free swimming cercaria larvae released from infected periwinkles (*Littorina littorina*). Final hosts are mostly fish-eating birds (Sindermann & Farrin, 1962). This parasite has previously been registered on two-spotted gobies in Germany (Zander, 2003). Our observation of *C. lingua* on two-spotted goby is the first in Norway.

Concluding remarks and future research

Conclusions

The infection dynamics of *C. elongatus* chalimi on a goby species common in Norway (two-spotted goby) varied throughout a year, where the prevalence reached a spring- and autumn maximum of respectively 10 % and 5 %. Since the two-spotted goby appears in high abundances along the Norwegian coast, great numbers of adult lice developed on these gobies can leave their host to the environment and infect nearby fish.

Genotyped *C. elongatus* obtained from different host species indicated that each genotype has its own host preferences. All juvenile lice found on two-spotted gobies and adults from Atlantic cod were mostly genotype 2, while all adult *C. elongatus* from farmed Atlantic salmon were genotype 1 and lice genotyped from lumpfish were predominantly genotype 1. There were no fish species recorded with both genotypes of attached stages.

We tested novel genotyping assays designed for genotyping *C. elongatus* with PCR and they were mainly successful in distinguishing the genotypes of all stages from chalimus I to adult. The over 100 mtCO1 sequences with both genotypes obtained in this study were used to find 21 nucleotide positions defining the genotypes.

Morphometric assessments comparing various lengths and ratios of lice from the genotypes resulted in significant differences between the groups: genotype 1 *C. elongatus* were generally larger than genotype 2 and the cephalothorax shape of genotype 1 copepodids were more oblong than genotype 2 which had a more oval shape of the cephalothorax.

Future research

It is possible that much of what is known about *C. elongatus* is incorrect or only half truths due to the unawareness of the two genotypes prior to this discovery made in 2005. Future research should focus on further investigating the genetics/genotypes of *C. elongatus* obtained from geographical locations other than northeast Atlantic and from small-sized and juvenile intermediate hosts harbouring juvenile stages of the lice in northern Norway where farmed Atlantic salmon are heavily infected with the parasite. The fact that we now have found an intermediate host fitted for the of genotype 2 *C. elongatus*, the two-spotted goby, allows researchers to rare and experiment with the juvenile stages of this genotype in controlled laboratory environments. An experiment trying to infect genotype 2 copepodids should be performed in a larger scale with more lice with their parents reared in controlled conditions. Testing if the genotypes are species based on the biological species concept should also be carried out.

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Appendix

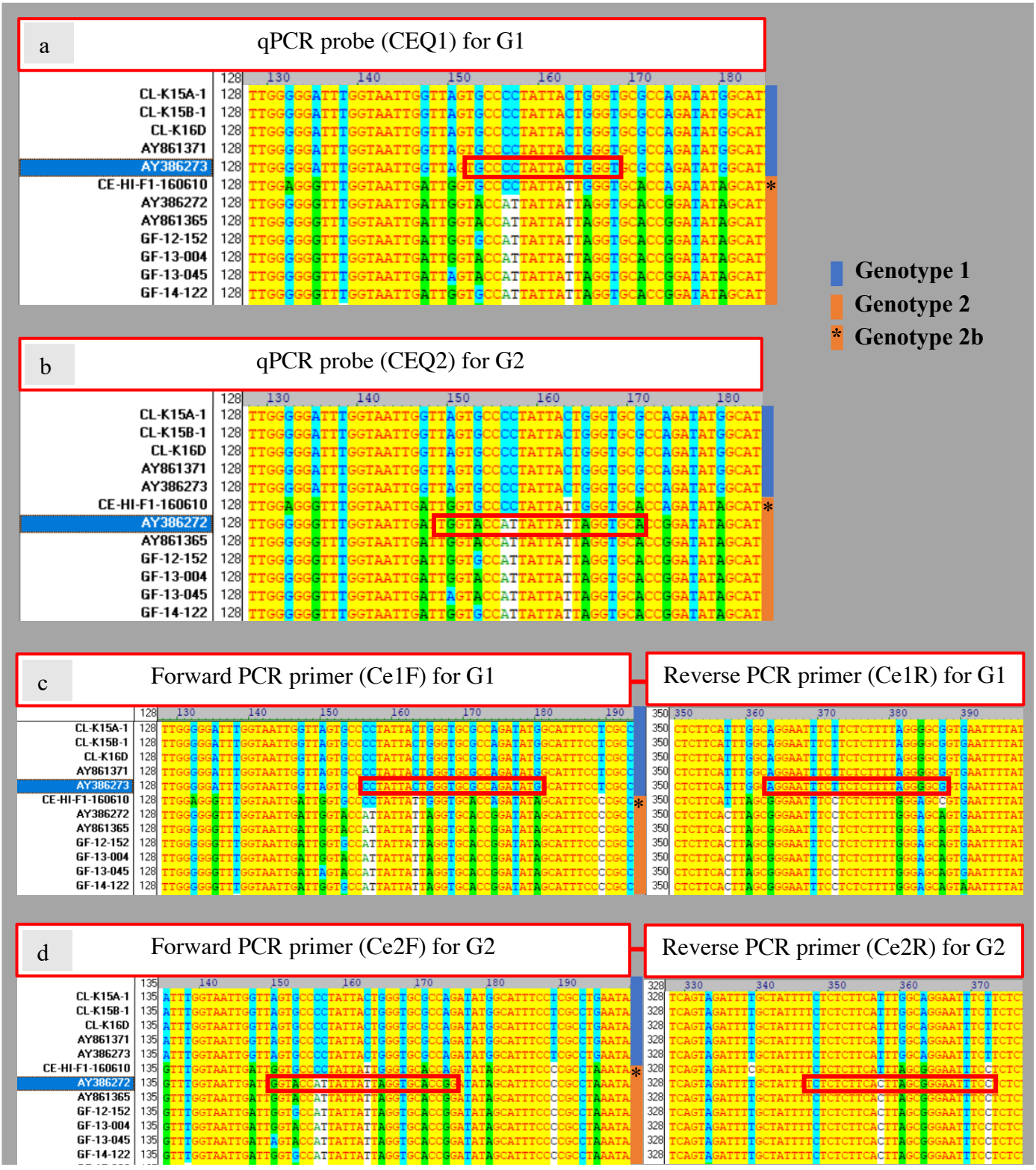

Figure 3.8. The qPCR probes developed by Øines *et al*. (2007) and H. Kongshaug's PCR primers for genotyping *C. elongatus*. The positions they match are marked with red boxes in a representative sequence of the genotype. G1= genotype 1. G2= genotype 2. The sequences belonging to these are indicated by a colour bar along the 3' ends. The marked sequence names on the alignments left side are reference sequences genotyped by Øines & Heuch (2005), The genotype 1 reference is AY386273, the genotype 2 reference is AY386272. The positions indicated above is those of the alignment. (AY386273positions -48)

Table 2.4. COI-gene sequences retrieved from GenBank ® used in this study.

Accession number	Location	Louse info	Reference
AY386272[b]	---	---	Øines. Submitted (09-SEP-2003) Section for Fish Health, National Veterinary Institute, Ullevaalsveien 68, Oslo 0033, Norway
AY861365[b]	Probably Arendal (58823'N 08844'E), men kan være andre steder...	From fish	Øines *et al*. (2005)
AY386273[a]	---	---	Øines. Submitted (09-SEP-2003) Section for Fish Health, National Veterinary Institute, Ullevaalsveien 68, Oslo 0033, Norway
AY861371	Probably Arendal (58823'N 08844'E), men kan være andre steder...	From fish	Øines *et al*. (2005)
KT209179	Helgoland, North Sea (54.186, 7.900)	Adult Plankton trawl 2 m deep	Raupach *et al*. (2015). collected by Rebekka Schueller.
KT208407	Helgoland, North Sea (54.186, 7.900)	Adult Plankton trawl 2 m deep	Raupach *et al*. (2015). collected by Rebekka Schueller.
KT208896	German Bight (53.953, 8.593)	Chalimus 2 m deep	Raupach *et al*. (2015). Collected by Silke Laakmann
KT208919	North Sea (56.628, 5.301)	Adult 59 m deep	Raupach *et al*. (2015). Collected by thomas Knebelsberger
KT208967	Helgoland, North Sea (54.186, 7.900)	Adult Plankton trawl 2 m deep	Raupach *et al*. (2015). Collected by Rebekka Schueller.
KT209080	North Sea (58.744, 2.564)	Adult 111 m deep	Raupach *et al*. (2015). Collected by thomas Knebelsberger
KT209134	Helgoland, North Sea (54.186, 7.900)	Adult Plankton trawl 2 m deep	Raupach *et al*. (2015). Collected by Rebekka Schueller.
KT209227	Helgoland, North Sea (54.186, 7.900)	Adult Plankton trawl 2 m deep	Raupach *et al*. (2015). Collected by Rebekka Schueller.

KT209299	Helgoland, North Sea (54.186, 7.900)	Adult Plankton trawl 2 m deep	Raupach *et al*. (2015). Collected by Rebekka Schueller.
KT209384	North Sea (58.744, 2.564)	Adult 111 m deep	Raupach *et al*.. (2015). Collected by Thomas Knebelsberger
EF452647	---	---	Hayward, C.J *et al*. Caligus warlandi n. sp. (Siphonostomatoida: Caligidae) from farmed marine fishes off Port Lincoln, South Australia. Submitted (25-FEB-2007) South Australian Research and Development Institute, GPO Box 397, Adelaide, SA 5001, Australia

[a] defined as genotype 1 in GenBank

[b] defined as genotype 2 in GenBank

---=information not given

Table 4.1. An overview including all studies (May -21) on genotyped *C. elongatus* and their hosts. Fish were wild if not noted otherwise (F=farmed and OF= wild outside farm). n= number of lice. ad= adult louse, ch= chalimus and co= copepodite when specified in the studies. Numbers are solus or marked as «uk» when the stage of development is unknown.

Host species (farmed or wild)	Sampling site	G1	G2	Reference
Lumpfish (31)	Skagerrak	31 ad		Øines *et al*. 2006
Saithe (30)	Flødevigen/Tjuvholmen	1 ad	29 ad	
Atlantic cod (*G. morhua*)	Arendal (n=535) Skagerrak coastal area (n=30) ch n= 121 ad n=721	26	77	Øines *et al*. (2007)
Atlantic horse mackerel (*T. trachurus*)			1	
Atlantic mackerel (*S. scombrus*)		2	1	
Black goby (*G. niger*)			1	
Brill (*S. rhombus*)			6	
Corkwing wrasse (*S. melops*)			1	
Dab (*L. limanda*)		1		
Garpike (*B. belone*)		1		
Goldsinny wrasse (*C. rupestris*)			1	
Grey gurnard (*E. gurnardus*)		3	16	
Herring (C. *harengus*)		5	1	
Lumpfish (*C. lumpus*)		118	3	
Plaice (*P. platessa*)			4	
Pollack (*P. pollachius*)		27	66	
Saithe (*P. virens*)		35	69	
Sand goby (P. *minutus*)			1	
Sea trout (*S. trutta*)		19	38	
Transparent goby (*A. minuta*) Two-spotted goby (*G. flavescens*)		1	8	
Whiting (*M. merlangus*)		4	29	
Lumpfish (*C. lumpus*)	North Sea pelagic	26		
Atlantic salmon (*S. salar*) (F) Atlantic cod (*G. morhua*) (OF) Saithe (*P. virens*) (OF)	Sørøya (70.65°N, 23.15°E)	14 4 11		
Atlantic salmon (*S. salar*) (n=29) (F) Rainbow trout (*O. mykiss*) (n=23) (F) Saithe (*P. virens*) (n=70)	Frøya (63°N, 08°E)	89	33	
Atlantic salmon (*S. salar*) (n=12) (F) Atlantic cod (*G. morhua*) (n=4) (OF)	Hidra (58°N, 06°E)	4	12	
Atlantic salmon (*S. salar*) (F)	Værlandet (61°N, 04°E)	1	3	
Atlantic salmon (*S. salar*) (F)	Faroe Islands (62°N, 07°W)	10		
Atlantic salmon (*S. salar*) (F)	Canada (45°N, 66°W)*	34*		

Atlantic salmon (*S. salar*) (F)	Scotland (56°N, 05°W)	30		
Atlantic cod (*G. morhua*) (F)	Karmøy (59°N, 05°E)		3	
Atlantic cod (*G. morhua*)	Arendal (58823'N 08844'E) Frøya (63840'N 08840'E) (G1) Sulesund (62823'N 06809'E) (G1) Sandefjord (59805'N 10814'E) (G2) Jøssøya (63816'N 08821'E) (G2) Eastern Canada (G1)* Faroe Islands (G1)		2 ad, 1 ch	Øines et al (2005)
Atlantic salmon (*S. salar*)		7 ad	2 ad	
Sea trout (*S. trutta*) (7 ad, 4 ch)		6	5	
Lumpfish (*C. lumpus)* Rainbow trout (*O. mykiss*)		8 ad		
		5 ad, 1 ch		
Pollack (*P. pollachius*)		5 ad	7 ad	
Saithe (*P. virens*)		4	11	
Atlantic herring (*C. harengus*)		3 ad	1 ad	
Whiting (*M. merlangus*)			2 ad	
Corcwing wrasse (*S. melops*)			1 ad	
Two-spotted goby			2ad, 1ch	
Goldsinny-wrasse (*C. rupestris*)			1 ad	
Atlantic horse mackerel (*T. Trachurus*)			1 ad	
Grey gurnard (*C. gurnardus*)			2 ad	
Brill (*S. rhombus*)			1 ad	
Atlantic mackerel (*S. scombrus*)			1 ad	
Atlantic salmon (*S. salar*) (F) Saithe (*P. virens*)	Aukra area (farm) Austrheim area (farm) Kalvåg, Bremanger	20 ad		Agusti-Ridaura et al (2019)
Lumpfish (*C. lumpus*) (ch n=22) Threespine stickleback (*G. aculeatus*) (ch n=380) Blackspotted stickleback (*G. wheatlandi*) Winter flounder (*P. americanus*) Mummichog (*F. heteroclitus*) Red hake (*U. chuss*) Atlantic tomcod (*M. tomcod*) Rainbow smelt (*O. mordax*) Ninespine stickleback (*P. pungitius*) Longhorn sculpin (*M. octodecemspinosus*)	Maine, USA*	2 ch* 487 ch* 15 ad* 4 uk*		Jensen *et al.* (2016)
Threespine stickleback (*G. aculeatus*)	Maine, USA*	71*		Pietrak *et al.* (2019)