



Eastern Baltic cod infected with *Contracaecum osculatum*: physiological mechanisms and the importance of monitoring infection loads

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DTU Aqua
National Institute of Aquatic Resources

PhD Thesis
Doctor in Philosophy

Eastern Baltic cod infected with *Contracaecum osculatum*: physiological mechanisms and the importance of monitoring infection loads

Marie Plambech Ryberg

Kgs. Lyngby, Danmark 2020



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*“For my kids, Vigga and Storm, my husband Kevin
and in memory of my grandma who passed away
during this PhD.”*

- M.P.R.

Preface

This PhD dissertation has been prepared to fulfill the requirements for the degree of Doctor of Philosophy at the Technical University of Denmark (DTU). The thesis consists of a synopsis and three individual studies (Papers in roman numerals **I-III**). The research conducted during this PhD project was carried out at the Section for Marine Living Resources under the National Institute of Aquatic Resources (DTU Aqua) between August 2017 and November 2020 under supervision of Jane W. Behrens and Peter V. Skov. The PhD was funded by European Maritime and Fisheries Foundation & Ministry of Environment and food (grant ID: 33113-B-16-071; "Effekt af sælrelateret leverorm på Østersøtorskens vækst og dødelighed" and 33113-B-17-110; TESLO) and DTU Aqua PhD school. In addition, funding was received from the Otto Mønsted Fond for participation in conferences.

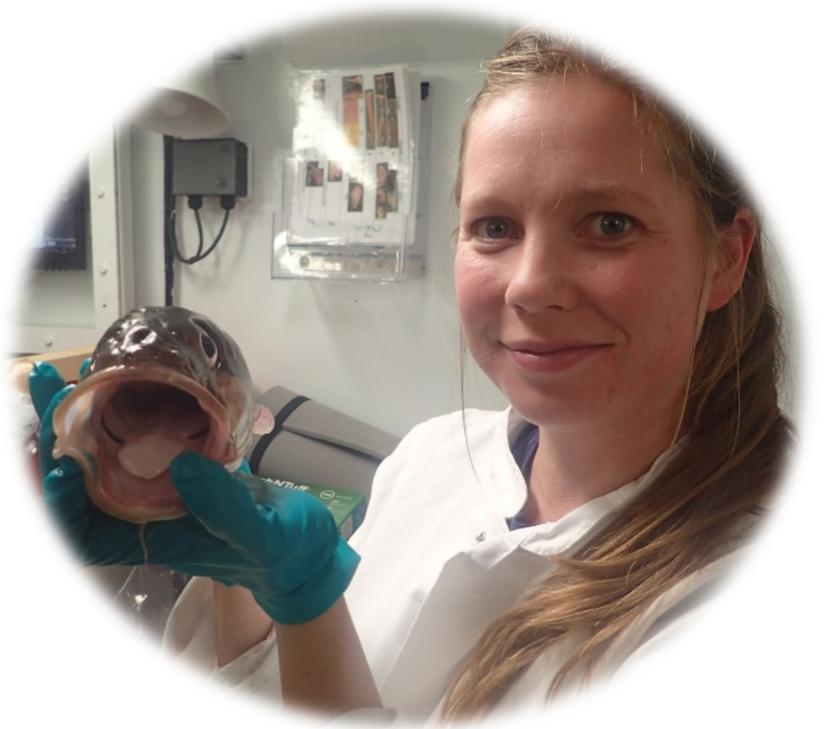
"Wars have been fought over it, revolutions have been spurred by it, national diets have been based on it, economies have depended on it, and the settlement of North America was driven by it. Cod, it turns out, is the reason Europeans set sail across the Atlantic, and it is the only reason they should."

(Kurlansky 1997)

Marie Plambech Ryberg

Marie Plambech Ryberg

Kgs. Lyngby, 5th of November 2020



List of publications presented in the thesis

Paper I	Ryberg, M. P., Skov, P. V, Vendramin, N., Buchmann, K., Nielsen, A., & Behrens, J. W. (2020). Physiological condition of Eastern Baltic cod, <i>Gadus morhua</i> , infected with the parasitic nematode <i>Contracaecum osculatum</i> . <i>Conservation Physiology</i> , 8(1), coaa093. https://doi.org/10.1093/conphys/coaa093
Paper II	Ryberg, M. P., Christen, A., Jørgensen, C., Neuenfeldt, S., Skov, P. V., & Behrens, J. W. (in prep.) Linking physiological mechanisms and growth processes: using bioenergetics modelling to study growth rate of Eastern Baltic cod, <i>Gadus morhua</i> , infected with a parasitic nematode, <i>Contracaecum osculatum</i>
Paper III	Ryberg, M. P., Huwer, B., Nielsen, A., Dierking, J., Buchmann, K., Sokolova, M., & Behrens, J. W. (2020) Parasite load of Eastern Baltic cod, <i>Gadus morhua</i> , assessed by the liver category method and the association between infection density and critical condition. Submitted, <i>Journal of International Parasitology</i> .

Other publications during PhD

Sguotti, C., Otto, S A., Frelat, R., Langbehn, T J., **Ryberg, M P.**, Lindegren, M., Durant, J M., Chr. Stenseth, N., and Möllmann, C. (2018). Catastrophic dynamics limit Atlantic cod recovery. *Proceedings of the Royal Society B: Biological Sciences*, 286, 1898.

ICES. (2018). Report of the Workshop on Evaluation of Input data to Eastern Baltic Cod Assessment, 23–25 January 2018, ICES HQ, Copenhagen, Denmark. ICES CM/ACOM: 36.68 pp.

ICES. (2019). Benchmark Workshop on Baltic Cod Stocks (WKBALTCOD2). ICES Scientific Reports. 1:9. 310 pp. DOI. <http://doi.org/10.17895/ices.pub.4984>

Conference presentations during PhD

Effekter af sæl-relateret leverorm på Østersø torskens sundhedstilstand.

Ryberg, M. P., 2019. Oral presentation (Danish), DHM 2019, Odense, Denmark

Effects of the seal-associated cods liver worm (*Contracaecum osculatum*) on the health status of Eastern Baltic cod.

Ryberg, M. P., 2019. Oral presentation (English), Baltic Sea Science Congress 2019, Stockholm, Sweden

Liver worm and cod health status and estimation of total parasite numbers in individual livers from visual inspections of the liver surface.

Ryberg, M. P., 2020. Oral presentation (English), Liver worm workshop, Kgs. Lyngby, Denmark

ICES working groups during PhD

Workshop on Evaluation of input data to Eastern Baltic Cod Assessment (WKIDEBCA)

ICES, Copenhagen, Denmark, 2018

Benchmark workshop on Baltic cod stock (WKBALTCOD2)

ICES, Copenhagen, Denmark, 2019

External stay during PhD

Collaboration with professor Kurt Buchmann on analyses of species identification of nematodes in livers of Eastern Baltic cod.

Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 2017, 2018

Ongoing collaboration with professor Christian Jørgensen on the bioenergetics model

Department of Biological Sciences, University of Bergen, Norway, February 2020

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First and foremost, I would like to thank my two supervisors Jane W. Behrens and Peter V. Skov who made it possible to conduct the most exciting and amazing research ever during the last three years. Jane, I owe you a big thanks for always believing in me and for making me believe in myself, providing invaluable advice, having indestructible patience with me and last but not least being my friend and academic mentor; without you I would not have managed to be where I am today in my career. A special thanks also goes to you Peter; for always taking the time to answer questions, for teaching me to be critical of my own results especially in terms of bridging between physiology and modelling and finally to encourage me to have faith in myself and never use the word “jegtros”.

I would also like to extend my sincere thanks to Stefan Neuenfeldt for all the highly valuable discussions we have had during this journey, for always taking the time to meet and for all your ideas and input for paper III and to Niels Gerner who inspired me to combine physiology and mathematics. Furthermore, a special thanks to all the co-authors: Anders Nielsen, for teaching me all the relevant statistical skills and for always taking your time; Basti Huwer – the map man – and my teacher in being detail oriented thanks for all your input during the whole process; Uwe Krumme and Jan Dierking thanks for providing me highly valuable input to paper III and for always being fast in responding to my mails; Kurt Buchmann thanks for passing on your passion for parasitology (parasites are amazing) and taking your time to teach me species identification of the nematodes; Asbjørn Christensen thanks for your patience with me during the modelling process, I have learned a lot from you; Niccolò Vendramin thanks for your input to paper I and for taking your time to help us vaccinate the first batch of cod – highly appreciated; Maria Sokolova thanks for your help in the beginning and for providing liver data and finally thanks to Christian Jørgensen for taking one week of to discuss and teach me bioenergetics modelling, I am looking forward to collaborate in the future.

A special thanks goes out to the people involved in catching the most precious cod in the world; the crew on Havfisken in 2017 and 2018 and especially you Hans for teaching me how to listen to rock music and for supporting me on Bornholm. Then I would also like to send a big thanks to Tine Iburg for being the best translator on veterinary terms and for inspiring me to study pathophysiology. Special thanks to Heidi Andreasen for being my friend, my coach and my hero during corona lockdown where you redesigned your kitchen into a laboratory and helped me with the final analysis.

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“Every task, goal, race

And year comes to

An end...

Therefore, make it

A habit to always

FINISH STRONG.”

-Gary Ryan Blair-

Summary (English)

The Eastern Baltic cod stock, *Gadus morhua*, is in distress. Fish are in a historically poor individual nutritional state, natural mortality is high, and the large fish have disappeared while the stock biomass has declined substantially since the late 1980s. The stock is now considered on the verge of collapse. Several ecosystem factors have been suggested to drive these changes, including infections with the parasitic nematode *Contracaecum osculatum*, which is found in cod livers. Despite strong indications of reduced individual nutritional condition with increasing nematode infection load in cod, a knowledge gap exists in our understanding of the physiological consequences of the infections. Therefore, the main objective of this thesis was to investigate how infections with *C. osculatum* associate with the physiological and nutritional conditions in individual cod, and – where possible – to reveal causality between infection and Eastern Baltic cod health status. This was achieved through a series of laboratory experiments with live cod, and through data collection in the field. The results were subsequently integrated into bioenergetics and statistical models to shed further light on the impacts of high infection loads on cod health. Furthermore, the robustness of the liver category method, a newly implemented method to monitor the spatiotemporal development of infection load in cod livers at a pan-Baltic scale, was evaluated.

The results show that the impacts of nematode infections range from the level of the individual cod to the population. More specifically, high infection density is associated with an impaired physiological condition of the infected cod, and individuals with high infection densities reveal signs of suffering from severe liver disease. The bioenergetics model revealed that growth is lower in infected compared with non-infected individuals. Moreover, a strong negative relationship between condition and infection density was found, and the probability of cod being in a critical condition increased with increasing infection density. Finally, the assessment of the liver category method showed that the current approach is a good predictor of the total number of nematodes in the liver of Baltic cod; thus, it serves as a suitable tool for the continuous monitoring of infection loads in the Baltic region. Since growth, recruitment (reduced fecundity and skipped spawning), and natural mortality all are related to the body condition of cod, parasite-induced reduction in condition and impaired health of infected individuals are expected to negatively affect the productivity of the Eastern Baltic cod stock. The thesis highlights parasite infections as a potential important driver of the poor status of Eastern Baltic cod.

Resumé (Danish)

Bestanden af østlige Østersøtorske, *Gadus morhua*, er i dårlig forfatning. Ernæringstilstanden for individuelle fisk er historisk dårlig, biomassen er faldet betydeligt siden slutningen af 1980'erne, og bestanden betragtes nu som at være tæt på et decideret kollaps. Tilbagegangen i bestandsudviklingen skyldes flere forskellige ændringer i økosystemet, inklusive infektioner i torskens lever med den parasitiske nematode, *Contracaecum osculatum*. De fysiologiske konsekvenser af disse infektioner er stort set ukendte, på trods af, at stærke sammenhænge mellem nedsat ernæringstilstand og stigende infektionsgrad hos torsk har været dokumenteret før. Hovedformålet med denne PhD-afhandling var derfor at undersøge, hvordan infektioner med *C. osculatum* hænger sammen med de fysiologiske og ernæringsmæssige tilstande hos inficerede torsk, og – i videst muligt omfang – at undersøge både årsag og virkning mellem infektion og sundhedsstatus af den østlige Østersøtorske. Undersøgelserne inkluderede både eksperimentelle forsøg med levende torsk i laboratoriet, samt indsamling af data i felten. De opnåede resultater blev dernæst integreret i bioenergetiske og statistiske modeller, for at opnå bredere indsigt i konsekvenserne af høje infektioner med leverorm for torskens helbred.

Resultaterne viser, at infektionerne ikke kun påvirker den enkelte torsk, men også bestanden som helhed. Mere specifikt, så er der en sammenhæng mellem stigende infektionsgrad og nedsat fysiologisk tilstand hos torsk, og individer med meget høj infektionsgrad lider af en svær leversygdom. Resultaterne fra den bioenergetiske model viser desuden, at væksten er lavere hos inficerede torsk. Der eksisterede også en stærk negativ sammenhæng mellem ernæringstilstanden og infektionsgraden, og sandsynligheden for, at finde torsk i kritisk ernæringstilstand steg med stigende infektionsgrad. Endelig blev det bekræftet, at den eksisterende leverkategori-metode er god til at forudsige det samlede antal nematoder i lever fra torsk fra den østlige Østersø. Metoden anses derfor som et passende og værdifuldt værktøj til overvågningen af infektionsgrader i torsk i Østersøregionen. Eftersom både vækst, rekruttering (fekunditet og gydning) og naturlig dødelighed kan påvirkes af torskens ernæringstilstand, forventes det, at parasit-inducerede reduktioner i ernæringstilstand og sundhed vil påvirke bestandsproduktiviteten af den østlige Østersøtorske negativt henover tid. Infektioner med parasitter kan derfor spille en vigtig rolle i den forringede sundhedstilstand, som den østlige Østersøtorske oplever netop nu.

Chapter I

General Introduction

Setting the stage

The focus of this thesis is on the Eastern Baltic cod stock, *Gadus morhua*. This fish stock has substantially declined in biomass since the late 1980s and is now considered on the verge of collapse, with individuals exhibiting a historically poor nutritional state. In the scientific literature, it has been suggested that several ecosystem changes have given rise to the current distress of the Eastern Baltic cod, including infections with the parasitic nematode, *Contracaecum osculatum*, found in cod livers. Although increasing and high infection loads with this parasite are now being observed, the specific effects of this nematode remain largely uncertain. The overarching aim of this PhD thesis is to elucidate and describe associations between infection loads with *C. osculatum* and the physiological and nutritional conditions of the infected cod, as well as, where possible, to reveal the causality between infection and Eastern Baltic cod health status. Monitoring of fish health status is rare, but a method of monitoring the nematode burden in Baltic cod livers has recently been put forward, along with a decision to implement the method as part of the standard monitoring protocol on Baltic surveys in 2021. This is the so-called liver category method, where individual livers are assigned a category based on the number of nematodes visible on the surface of the organ, whereas nematodes hidden inside the organ are not accounted for. It has never been verified whether the assigned liver categories match infection load, i.e. do livers that are assigned different categories differ in their total number of nematodes? Therefore, an additional aim of the PhD project is to test the robustness of the liver category method, evaluating its applicability for providing valuable information to fisheries scientists.

In this thesis, I combine experiments with live cod with collection of field data and bioenergetics and statistical modelling approaches, integrating the results from the laboratory and the field. In the next sections, I set the scene for my thesis. First, I describe the effects of multiple stressors on fish stock dynamics and the past and present status of the Eastern Baltic cod, including stressors known to affect the current status of the stock. I then describe the physiology and performance of fish infected with parasites and discuss host manipulation and side effects. Next, I explain the lifecycle of *C. osculatum* and the pathology of infected cod livers. Finally, I present the three main objectives of this PhD project, the methodological considerations and the overall structure of the thesis.

Multiple stressors and their implications for marine fish stocks

Fish and fisheries provide a major source of food and livelihood for many people around the world (Bindoff *et al.*, 2019). However, fish stocks are exposed to various stressors arising from climate change and anthropogenic pressures, and these often have substantial effects on stock productivity and dynamics. The stressors bring about these outcomes by driving fluctuations in fish reproductive output, growth and survival (Hilborn and Walters, 1992; Halpern *et al.*, 2008; Hewitt *et al.*, 2016; Gordon *et al.*, 2018; Bindoff *et al.*, 2019; Dahlke *et al.*, 2020). Knowledge of the biological mechanisms behind the fluctuations is key for a better understanding of their causes and implications, as well as how they can lead to changes in stock productivity (Morgan *et al.*, 2018).

Stressors can have direct and/or indirect effects and may interact in unpredictable and complex ways (Watson *et al.*, 2020). Studies elucidating the mechanisms behind changes in stock dynamics range from correlations derived from field survey data that can identify important relationships but rarely the causalities behind them, to experimental work that may identify the biological mechanisms underlying the observed relationships (Krohn *et al.*, 1997; Metcalfe *et al.*, 2012). These studies have mainly focussed on climate-related factors, such as temperature, salinity and oxygen, or they have been related to fishery intensity (Halpern *et al.*, 2008; Hoegh-Guldberg and Bruno, 2010; Hewitt *et al.*, 2016; Reusch *et al.*, 2018; Dahlke *et al.*, 2020).

Potential effects of parasites are often ignored by fisheries scientists and ecologists, likely because of the notorious difficulties in quantifying their influences on the stock (Lloret *et al.*, 2012; Timi and Poulin, 2020). Parasites can cause adverse effects on their hosts and alter important variables in stock dynamics; these effects can occur at the level of the individual (e.g. physiological mechanisms, condition, growth and mortality; Barker *et al.*, 2002; Khan, 2005; McElroy and de Buron, 2014; Ferrer-Maza *et al.*, 2016; Hahn *et al.*, 2018) up to the population level (e.g. population growth, recruitment and mortality rates; Marcogliese, 2004; Link *et al.*, 2020; Timi and Poulin, 2020).

The past and the present status of Eastern Baltic cod

The Eastern Baltic cod represents an example illustrating how pressure from multiple stressors (including infection with parasites) can push a stock towards the verge of collapse. The Baltic cod stock comprises eastern and western stock. The Eastern cod stock has its main centre of distribution in the International Council for the Exploration of the Sea (ICES) subdivisions (SDs) 25 and 26, but fish from this stock are also found in SD27-SD32 (Eero *et al.*, 2012a); in contrast, the Western stock mainly resides in SD22–24 (Figure 1). Some mixing of the two stocks occurs in SD24 (Hemmer-Hansen *et al.*, 2018; ICES, 2019). Although the stocks are living side by side in two different areas, the Eastern Baltic cod stock has experienced more drastic changes in terms of stock dynamics over time.

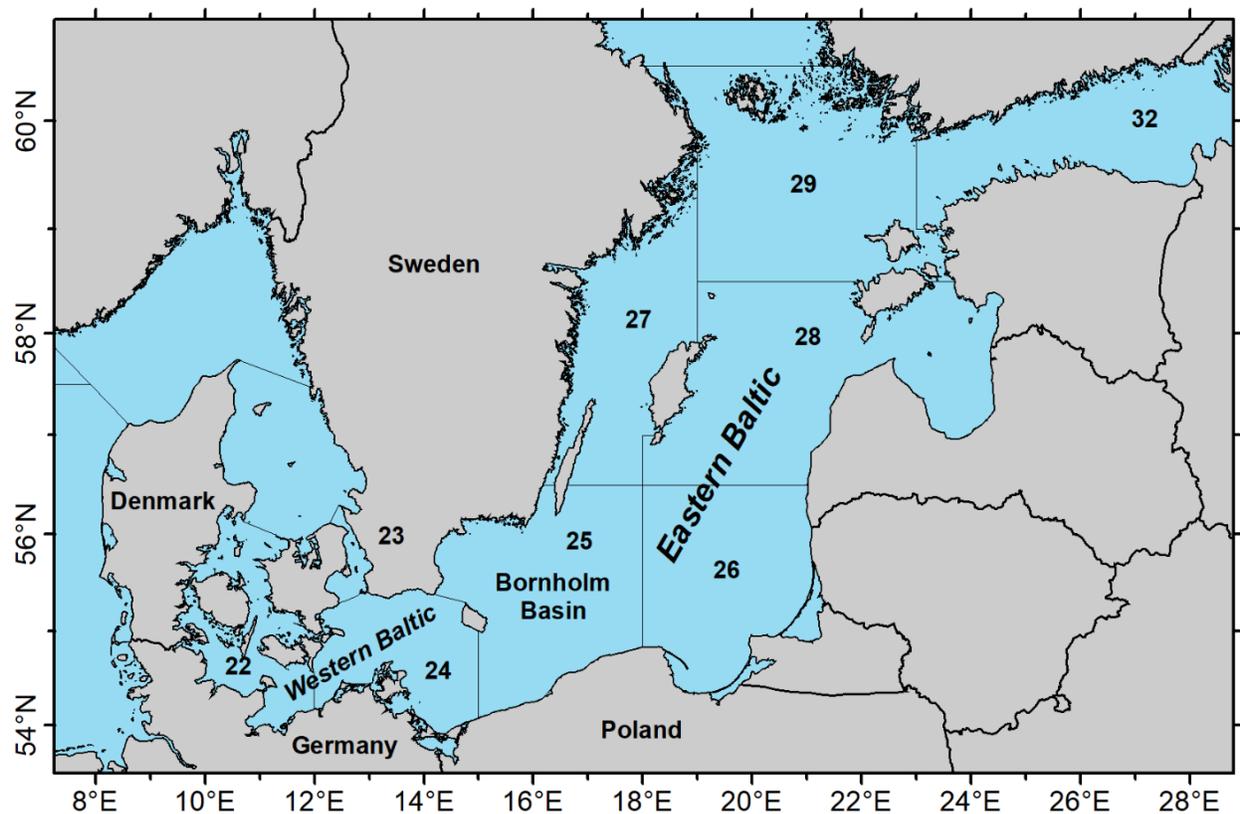


Figure 1. Map of the Baltic Sea showing the western and eastern parts, divided into subdivisions 22–29 and 32 as defined by the International Council for the Exploration of the Sea.

In the 20th century, Eastern Baltic cod was among the top five most economically important species in Baltic fisheries. Landings fluctuated throughout the century, with a peak in the mid-1980s, when approximately 400 000 tons of cod were caught in one year (Eero *et al.*, 2007, 2008). However, both biomass of cod above 35 cm and spawning stock biomass decreased drastically in the late 1980s, probably as a result of increased fishing effort, the introduction of gillnet fishery and decreased egg survival due to changes in the environmental conditions, resulting in years with poor recruitment (Eero *et al.*, 2012b). At the beginning of the 1990s and until late in the same decade, the proportion of old cod in the stock was still large, but body condition slowly started to decline (Eero *et al.*, 2012b). From the late 1990s, higher proportions of younger fish were found in the stock, and the overall age composition of the stock changed (ICES, 2008). In 2005, the spawning stock biomass was observed at the lowest level since the 1960s (70 000 tons), but it again started to increase, and in 2009, it reached 220 000 tons; this was defined as the level for recovery of the stock (Eero *et al.*, 2012b). High recruitment in the years up to 2009 was assumed to be responsible for this rapid recovery of Eastern Baltic cod, suggesting that the stock-recruitment relationship was one of the major drivers of stock dynamics in that period (Eero *et al.*, 2012b). Despite this apparent rapid recovery of the stock, a drastic decline in large individuals started to occur after 2010. Since then, things have been going downhill, and today, the stock is in distress, with no signs of recovery (Eero *et al.*, 2015; ICES, 2019).

The age and size composition of the stock have changed, and individual cod suffer from poor nutritional conditions (Eero *et al.*, 2015; Casini *et al.*, 2016; ICES, 2019), reduced potential fecundity (i.e. the number of vitellogenic oocytes) of low-condition individuals (Mion *et al.*, 2018), poor growth (Hüssy *et al.*, 2018; Brander, 2020; McQueen *et al.*, 2020; Neuenfeldt *et al.*, 2020) and disappearance of larger individuals (Neuenfeldt *et al.*, 2020). This has resulted in a reduced mean total length of the stock (Svedäng and Hornborg, 2014; Figure 2). Furthermore, maturation occurs at smaller sizes than observed previously (Köster *et al.*, 2016), suggesting that the spawning stock biomass of Eastern Baltic cod now consists of many small individuals in poor condition, which have lower potential fecundity than fewer but bigger individuals in good condition (Mion *et al.*, 2018). Finally, the natural mortality of cod in this stock is high and is considered to be higher than fishing mortality (ICES, 2019).

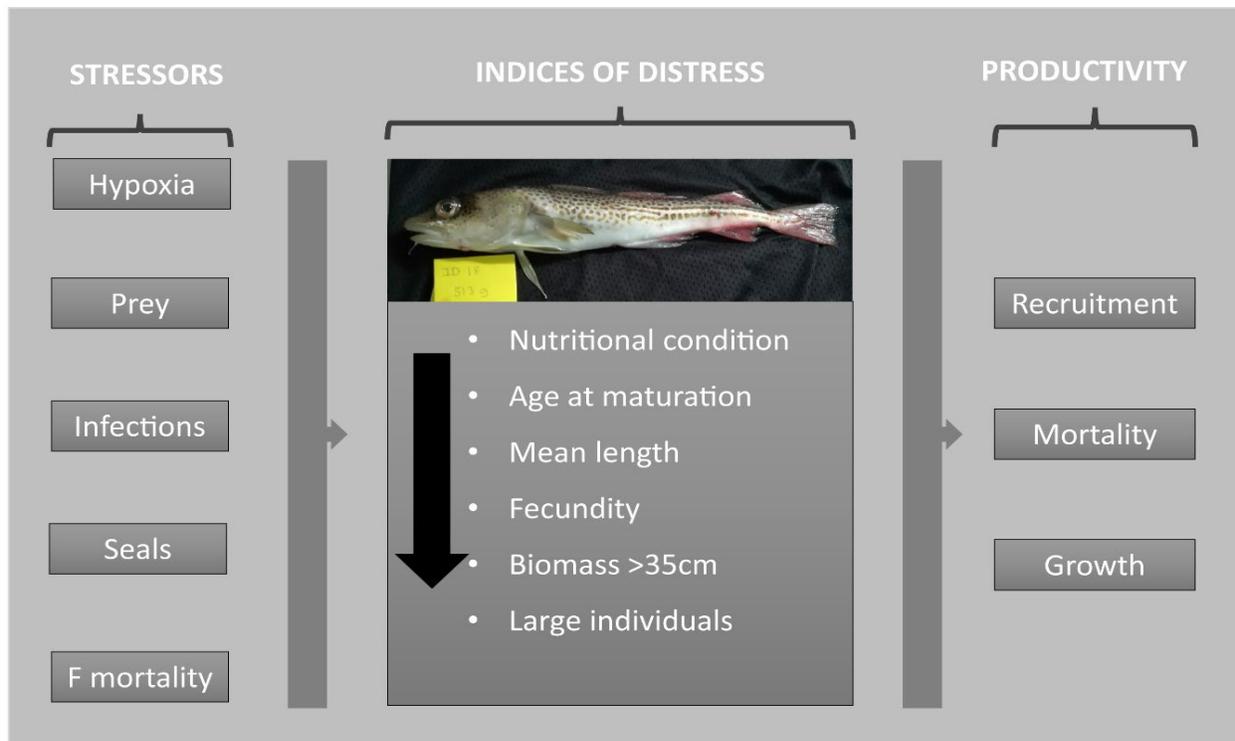


Figure 2. Schematic overview of the stressors described in the scientific literature suggesting the indices of distress that all tend to be decreasing presently. Some indices are shown and suggested to be linked to reduced productivity of the Eastern Baltic cod stock. See text for details and references.

Combined, all the changes described above led to failure of the analytical assessment of the stock in 2014, where the drastic decrease of large individuals and high percentage of individual cod in poor nutritional condition (i.e. Fulton condition factor < 0.8) especially led to inconsistency in the model output and unexplained trends in the stock data (Eero *et al.*, 2015). In 2019, for the first time in five years, ICES was able to undertake an analytical assessment, resulting in the advice of a zero catch quota for Eastern Baltic cod (ICES, 2019). As a result, a fishery ban in SD25 and SD26 was determined in both 2019 and 2020, and the current advice from ICES for 2021 is again a zero catch quota (EC, 2020; ICES, 2020).

Multiple stressors, besides high fishing mortality in the 1980s (Eero *et al.*, 2011), have been suggested to contribute to the poor state of Eastern Baltic cod. These include an increase in the severity and extent of hypoxic areas (Casini *et al.*, 2016; Brander, 2020), which can negatively influence the physiological performance (Plambech *et al.*, 2013) and feeding levels (Neuenfeldt *et al.*, 2020) of the fish; predation by seals (Scharff-Olsen *et al.*, 2019); and increased infection loads with the seal-associated parasite *C. osculatum* (Haarder *et al.*, 2014; Nadolna and Podolska, 2014; Sokolova *et al.*, 2018; Figure 2).

Such stressors have been shown to be associated with the reduced condition of Eastern Baltic cod. Hypoxia can either have a direct effect on the individual by inducing an energetic cost when cod dive into hypoxic areas (Plambech *et al.*, 2013) or an indirect effect in terms of reducing the quantity and/or quality of benthic prey, resulting in lowered growth potential of cod (Neuenfeldt *et al.*, 2020). More specifically, stomach content analysis revealed a regime shift in the diet composition of cod in the mid-1990s. Following this period, Eastern Baltic cod experienced an overall decreased food intake and a shift in the diet composition. Now, sprat, *Sprattus sprattus*, constitutes a larger part of the diet compared with the period before the regime shift but with lower average biomass (Neuenfeldt *et al.*, 2020). Finally, infection load with *C. osculatum* in Eastern Baltic cod has increased in the last decade, and high infection loads have been suggested to be associated with impaired nutritional condition of the fish (Horbowy *et al.*, 2016; Sokolova *et al.*, 2018).

Infections with *Contracaecum osculatum* in Eastern Baltic cod

Grey seal, *Halichoerus grypus*, is the main final host for *C. osculatum* in the Baltic Sea (Lunneryd *et al.*, 2015; Zuo *et al.*, 2018; Box 1: The lifecycle of *C. osculatum* and Figure 3). Thus, grey seal is a prerequisite for the occurrence of the parasite in this ecosystem, although other factors, such as abundance of infected prey (i.e. transport hosts), complexity of the food web and salinity, may also play a role (Sokolova *et al.*, 2018). A conservation success story is that of the Baltic grey seal. Intense hunting decreased seal numbers from 80 000–90 000 individuals at the beginning of the 20th century to approximately 4000 individuals in the late 1970s. The population remained low for many years because of sterility caused by high levels of organochlorines, especially polychlorinated biphenyls. Decreasing hunting pressure and levels of polychlorinated biphenyls since the early 1990s have led to gradual population recovery, and today, at least 30 000–40 000 grey seals are found in the Baltic region (Harding and Härkönen, 1999; HELCOM, 2018). The temporal fluctuations in the prevalence (i.e. the percentage of cod infected) and intensity (i.e. mean number of parasites per fish, including only infected individuals) of infections relate partly to the fluctuations in the number of grey seals in the Baltic Sea. More specifically, within the Bornholm Basin (SD25), the prevalence of *C. osculatum* in cod above 30 cm has increased from 22% in the early 1980s (Haarder *et al.*, 2014) to 100% in 2016 (Sokolova *et al.*, 2018); the prevalence is also high east of the Bornholm Basin and south of Gotland for similar-sized cod (90–100%; Sokolova *et al.*, 2018; Figure 1). In the same period, the intensity of infection increased from an average of 0.9 to 27 nematodes per cod (Haarder *et al.*, 2014; Sokolova *et al.*, 2018) in the Bornholm Basin, reaching highs of on average 29 and 40 parasites per cod south of Gotland and east of the Bornholm Basin, respectively (Sokolova *et al.*, 2018).

Further south in the Baltic Sea, in Polish waters, the prevalence also increased from below 5% in the period of 1987–1994 (Szostakowska *et al.*, 2005) up to 80% in 2014, depending on the length of the cod (Horbowy *et al.*, 2016). Notably, an east–west gradient in the prevalence and abundance of *C. osculatum* in Baltic cod is now evident, with the highest infection loads occurring in the low-salinity Central and Eastern Baltic areas, where the cod belong mainly to the Eastern Baltic stock (Sokolova *et al.*, 2018). One of the earliest studies on infections with nematodes in Baltic cod was based on cod caught in 1949 in the Gotland Basin and the eastern part of area SD26 (Petrushevsky and Shulman, 1955). In their study, the prevalence of *C. osculatum* in cod ranged between 53% and 97.5% depending on fish length, and individual cod contained up to 300 nematodes per liver (Petrushevsky and Shulman, 1955).

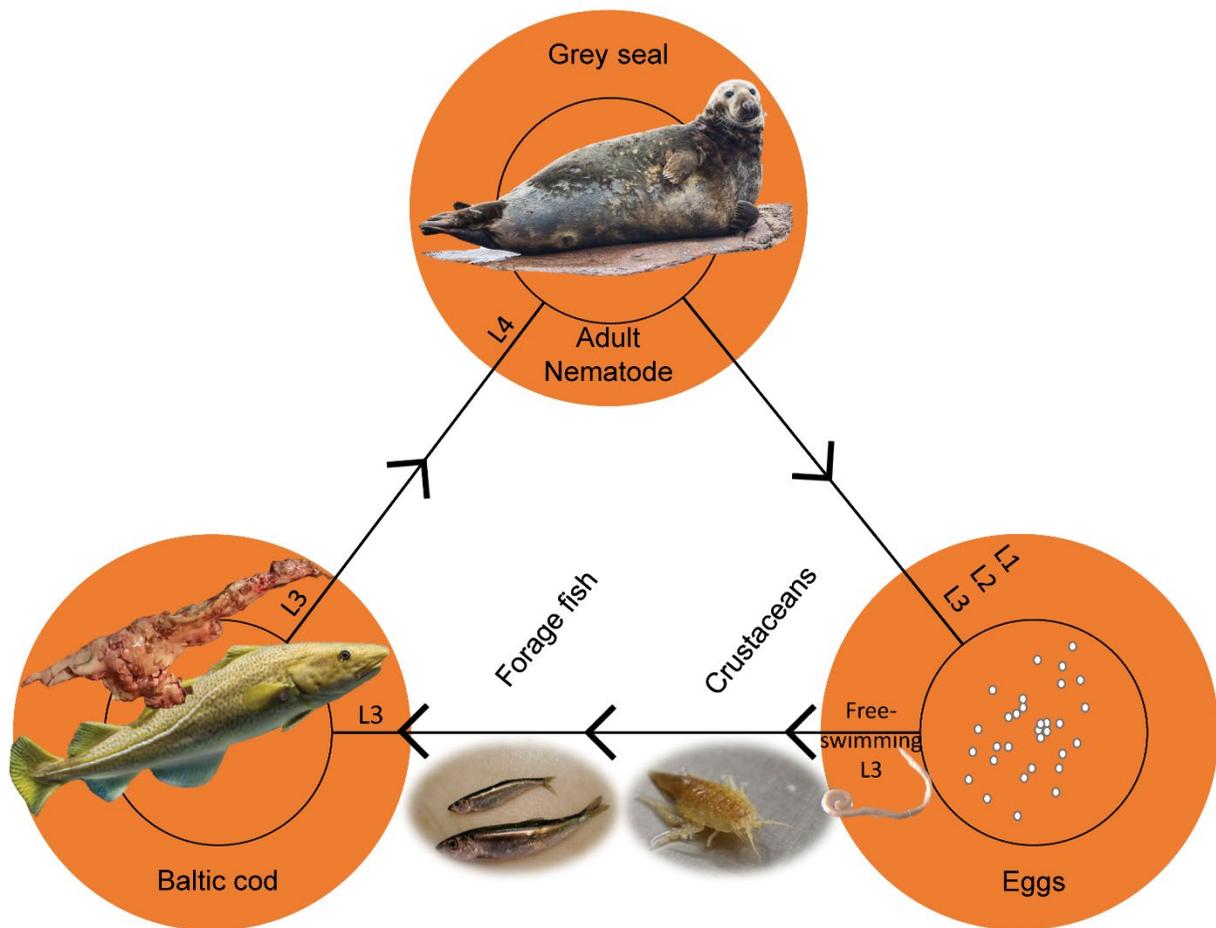


Figure 3. Schematic illustration of the lifecycle of *C. osculatum* in the Baltic Sea, including its main hosts. Eggs are laid by the adult parasites in the stomach of the grey seal and subsequently enter the ambient environment via the faeces of the seal. The eggs contain larvae stages L1–L3, and when the eggs hatch, free-living L3 larvae emerge; these are subsequently eaten by small crustaceans. Forage fish (e.g. sprat) then prey on the infected crustaceans, while cod prey on the infected forage fish. Finally, cod are eaten by the seal and the larvae become adult; reproduction occurs in the stomach of the seal. For more details, see Box 1.

Box 1: Lifecycle of *Contracaecum osculatum*

C. osculatum belongs to the Anisakidae family (Nadolna and Podolska, 2014). The lifecycle of *C. osculatum* involves many different hosts (Figure 3), with seals being the final hosts (Koie and Fagerholm, 1995). In the Baltic Sea, the grey seal is the main final host (Lunneryd *et al.*, 2015; Zuo *et al.*, 2018). The adult male and female parasites copulate in the stomach of the seal; the female lay her eggs there, and the eggs are subsequently shed to the ambient water via the faeces of the seal. Free eggs can contain stage one (L1), two (L2) or three (L3) larvae depending on number of moults within the eggs, while stage four (L4) larvae only are found in the seal. The four larval stages are distinguished from each other by several morphological changes. The larvae may develop from L2 to L3 within an intermediate host, which has been suggested to be a crustacean. However, this has never been proven, and to date, no intermediate hosts have been documented in the lifecycle of *C. osculatum* in the Baltic Sea (Pawlak *et al.*, 2019). Instead, experimental studies have shown that two moults occur inside the egg, resulting in an L3-stage larva, which hatches from the egg and becomes a free-swimming larva (Koie and Fagerholm, 1995).

The free-living L3 larvae are eaten by a crustacean transport host (mainly copepods, or more rarely, gammarids; Koie and Fagerholm, 1995; Pawlak *et al.*, 2019). Forage fish eat infected crustaceans and act as the next transport host (Nadolna-Ałtyn *et al.*, 2018; Zuo *et al.*, 2016), while infected forage fish are subsequently eaten by larger predatory fish, such as cod (Zuo *et al.*, 2016). However, the larger fish may also become infected by eating infected crustaceans, although this remains speculation (Koie and Fagerholm, 1995). In cod, upon ingestion via infected prey items, the L3 larvae migrate to the liver. Aside from cod, herring, sand eel, flounder, salmon, smelt, burbot, Atlantic horse mackerel, tub gurnard and fourhorn and shorthorn sculpins have also been shown to carry L3 larvae, but at a very low prevalence and intensity of infections compared with those observed for cod (Szostakowska *et al.*, 2005; Wicaszek *et al.*, 2011; Rodjuk, 2014; Nadolna-Ałtyn *et al.*, 2017). When the grey seal obtains the L3 larvae by eating infected fish -especially cod- the parasite completes its lifecycle inside the seal's stomach by becoming first an L4 larva and subsequently an adult worm. From here, the cycle repeats.

Parasites in fish

Parasitism can be defined in many ways, but most commonly, the term covers a close association between two organisms, where the parasite feeds on the host (Rohde, 2002). Common to all parasites is that they are eukaryotes, but they can range from unicellular (microparasites) to multicellular organisms (macroparasites). Parasites in fish have been intensively studied, revealing a high diversity of taxonomic groupings (Marcogliese, 2002; Rohde, 2002). Some parasites, and especially aquatic parasites, have complex lifecycles where they rely on trophic transmission, that is, they depend on hosts from different trophic levels of the food web. From an evolutionary perspective, the goal of trophically transmitted parasites is to achieve successful transmission between the different trophic hosts to complete their lifecycle and reproduce, thereby maximising their fitness (Barker, 2009). To reach their final host, trophically transmitted parasites therefore use multiple intermediate and/or transport hosts as vehicles (Lafferty and Kuris, 2002). The final host of a parasite is the host harbouring the sexually mature stages of the parasite. Within the intermediate and transport hosts, the parasite is sexually immature, and developing stages of the parasite occur only in the intermediate host, while no development of the parasite occurs in the transport host (Rohde, 2002).

Trophically transmitted parasites need to optimise the chances of transmission between their hosts and may do this by causing changes in physiological mechanisms of the transport/intermediate host, which can subsequently affect the performance and the behaviour, resulting in reduced fitness of the host (McElroy and de Buron, 2014; Poulin and Maure, 2015; Binning *et al.*, 2017; Timi and Poulin, 2020). In support of this view, trophically transmitted parasites that rely on predation for transmission tend to indirectly increase mortality rates of their hosts compared with hosts infected with parasites using other transmission strategies (Robar *et al.*, 2010; Wilber *et al.*, 2020).

Parasites rarely cause direct mortality of the host because this would terminate the lifecycle of the parasite. Thus, adapting Arnold's (1983) performance paradigm to infections with parasites in fish makes it clear that many important physiological mechanisms can be affected, for example, enzyme activity, energy metabolism and cardiac function (Binning *et al.*, 2017). Yet, it is not trivial to disentangle whether physiological and/or behavioural changes of the host are direct results of manipulation by the parasite or simply side effects of the infection (Hafer and Milinski, 2016; Hafer-Hahmann, 2019). If the host undergoes phenotypic alteration as a result of genetic control of the parasite, and this alteration enhances the fitness of the parasite by successful transmission to its next host, then host manipulation suggestively takes place (Hafer and Milinski, 2016).

Parasites using trophic transmission are likely those that manipulate their hosts, although the physiological and/or behavioural changes of the host in relation to the parasite can also merely result from side effects of the infections (Hafer and Milinski, 2016). Along these lines, the energy drain hypothesis builds on the idea that the parasite does not cause a direct behavioural change of the host but instead extracts energy from it, which may lead to impaired physiology and subsequent reduced physical performance of the host (Lafferty and Shaw, 2013). If changes in the behaviour of the host arise from a shift in its trade-offs associated with being more prone to predation in favour of feeding, and these changes favour the parasite, then it is not host manipulation but a side effect of the infection (Lafferty and Shaw, 2013; Heil, 2016). This has been shown for stickleback, *Gasterosteus aculeatus*, infected with the trophically transmitted cestode, *Schistocephalus solidus*, where the behavioural changes have been suggested to be associated with an energy drain of the infections rather than active host manipulation by the parasite (Hafer and Milinski, 2016). *Infestation* with parasites refers to external parasites, while *infection* refers to endo-parasites. Infestation with parasites in fish has been associated with impaired cardiovascular performance, reduced swimming performance and increased metabolic rates (Östlund-Nilsson *et al.*, 2005; Binning *et al.*, 2013). Likewise, infections have been shown to be associated with impaired aerobic performance (Smith and Kramer, 1987; Kumaraguru *et al.*, 1995; Bruneaux *et al.*, 2017), for example, reduced swimming performance (Umberger *et al.*, 2013) and impaired cardiorespiratory function (Behrens *et al.*, 2014).

To disentangle whether physiological and behavioural changes of fish infected with parasites arise from host manipulation or side effects is especially difficult in wild fish stocks. However, because parasites are known to influence the physiological performance, behaviour and fitness of their hosts (Binning *et al.*, 2017), acting as stressors on life-history traits (Watson, 2013), more attention should be paid to the role of parasites in processes that can affect stock dynamics (Lloret *et al.*, 2012). Considering the above, it is imperative to broaden our understanding of the physiological mechanisms that cause the observed association between infections with *C. osculatum* and the poor nutritional condition and health status of Eastern Baltic cod.

Assessment of the health status of wild fish stocks

Monitoring of health status and parasites in wild fish is not common. This is mainly because the collection of such data requires expert knowledge, is often time consuming and cumbersome, difficult to conduct on board a ship and expensive (Lloret *et al.*, 2012). In many cases, body condition indices are used to reflect the pathological effects of parasites on fish because they represent one of the easiest parameters for assessment in wild fish stocks (Lloret *et al.*, 2012; Timi and Poulin, 2020). This is because poor nutritional condition is known to cause reduced reproductive potential (Lambert *et al.*, 2000; Mion *et al.*, 2018), impaired swimming performance (Martínez *et al.*, 2003; Lapointe *et al.*, 2006) and increased natural mortality (Dutil and Lambert, 2000). However, other condition indices can be used to assess and quantify individual health, for example, physiological condition that covers all physiological processes that at a higher level can lead to changes in behaviour and performance (Stevenson and Woods, 2006).

Obtaining accurate estimates of parasite-induced mortality in wild populations may be even more difficult than monitoring parasite loads. In an ideal world, mortality levels would be monitored before and after infections with parasites were discovered in a given population (Wilber *et al.*, 2020). However, this is rarely possible because fisheries scientists first start to investigate causes of mortality when mortality levels increase. Other types of relevant information are the use of historical data to shed light on previous fluctuations in a population (Deksne *et al.*, 2020) and/or integration of several approaches, such as laboratory experiments (i.e. physiological mechanisms of infections on an individual level), models linking the physical mechanisms on an individual level to stock level effects (Wilber *et al.*, 2020) and tests of predictions in natural situations (Watson, 2013).

In cases where the declining nutritional condition of fish in a particular stock has been observed, disentangling the causes behind this trend may not be easy. This is the case for Eastern Baltic cod. In an attempt to move a step closer to understanding what is driving this stock towards collapse, and based on the observed increase in liver parasite load, some Baltic Sea countries have voluntarily implemented the so-called liver category method in their monitoring surveys. The liver category method was inspired by severity grades used for the identification of externally visible diseases in some North Atlantic fish species (Bucke *et al.*, 1996). More specifically, using a scale from 0 to 4, the liver category method assigns a category to each inspected liver, depending on the number of visible nematodes on the surface of the organ, where categories 0, 1, 2, 3 and 4 correspond to counts of 0, 1–10, 11–20, 21–30 and >30 nematodes, respectively. With the effort of several/all Baltic countries, population-wide monitoring of the spatio-temporal development of parasite infection in cod livers would be obtained.

Yet, so far, the method has not been standardised among countries, as some countries doing this voluntarily apply the five described categories, whereas others use only four, with livers with >20 nematodes in the highest liver category 3. To ensure standardisation of future liver categorisations, DTU Aqua hosted a workshop in 2019 for all institutes with Baltic monitoring surveys; the purpose of this workshop was to agree on the most suitable liver category protocol and discuss whether this method should be made mandatory in the Baltic Sea survey protocol. Based on the outcome and initiative from the workshop participants, the ICES Working Group for Baltic International Fish Surveys (WGBIFS) has decided to make it a mandatory part of the monitoring protocol to assign liver category to individual cod in the Baltic Sea from 2021 (WGBIFS, personal communication).

Pathophysiology of *C. osculatum* in Eastern Baltic cod

Prevalence and intensity of infections with *C. osculatum* increase with length (and thereby age) of Eastern Baltic cod, revealing that this nematode accumulates over time as cod eat infected prey items (Myjak *et al.*, 1994; Haarder *et al.*, 2014; Nadolna and Podolska, 2014; Horbowy *et al.*, 2016; Zuo *et al.*, 2016). Parasites are often aggregated in their hosts, where some individuals are highly infected and some avoid infections by the parasite. This type of distribution by the parasite is best described by a negative binomial distribution (Rohde, 2002), and it has been shown to occur for *C. osculatum* in Eastern Baltic cod (Sokolova *et al.*, 2018; Mohamed *et al.*, 2020). An density-dependent pathology, where the most heavily infected (i.e. individuals on the right side of the negative binomial distribution) are more likely to die (Wilber *et al.*, 2020), has been suggested; however, this has never been proven for this host-parasite system (Horbowy *et al.*, 2016). However, there likely exists a critical level of parasite load where the infections have a negative impact on the health of the infected cod that ultimately may lead to mortality.

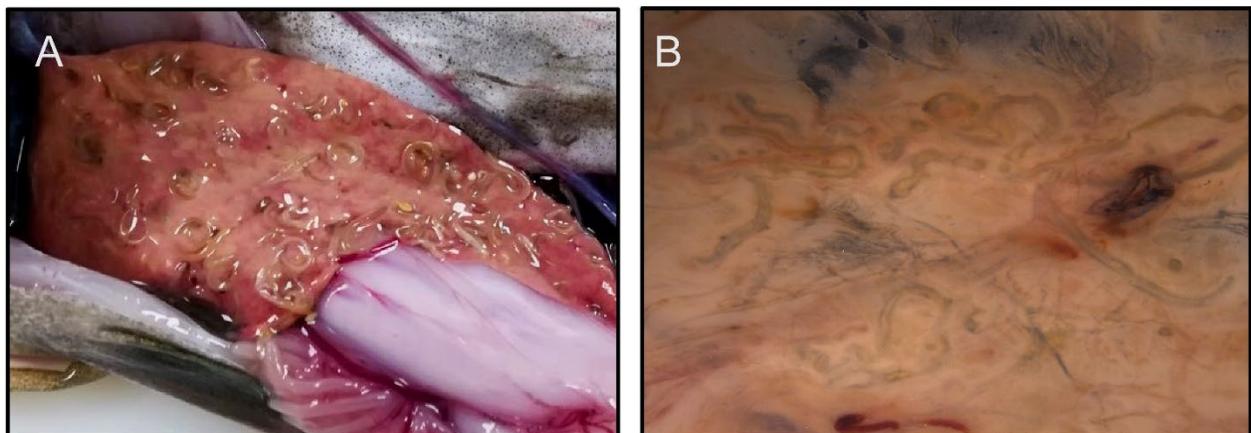


Figure 4. A) Infections with *C. osculatum* in an Eastern Baltic cod liver (photo: Marie P. Ryberg). B) Several *C. osculatum* found close to the major blood vessels in the liver (photo: Heidi Andreassen).

In cod, *C. osculatum* migrates to the liver after ingestion, where it either lays close to the surface of the liver or penetrates the liver parenchyma (Nadolna and Podolska, 2014; Figure 4A). Within the liver, many nematodes are often seen close to the blood vessels of the organ (own observations, Figure 4B). The lengths of nematodes in cod livers can range from 3 to 27 mm (Mohamed *et al.*, 2020; Figure 5), with the difference in size potentially reflecting that the nematodes grow inside the liver tissue, indicating that the nematodes gain energy from the cod. Notably, it is difficult to document the growth of parasitic nematodes in their hosts; however, the discovery of host cells has been found in the intestine of another nematode, *Thynnaasscaris adunca*, suggesting the growth of this parasite in its host (Larsen, 1980). So far, the growth of *C. osculatum* in cod livers has not been examined, although histological observations revealed glycogen in the intestine of live nematodes, suggesting an active metabolism of the nematode that requires energy from the host (own unpublished observations).

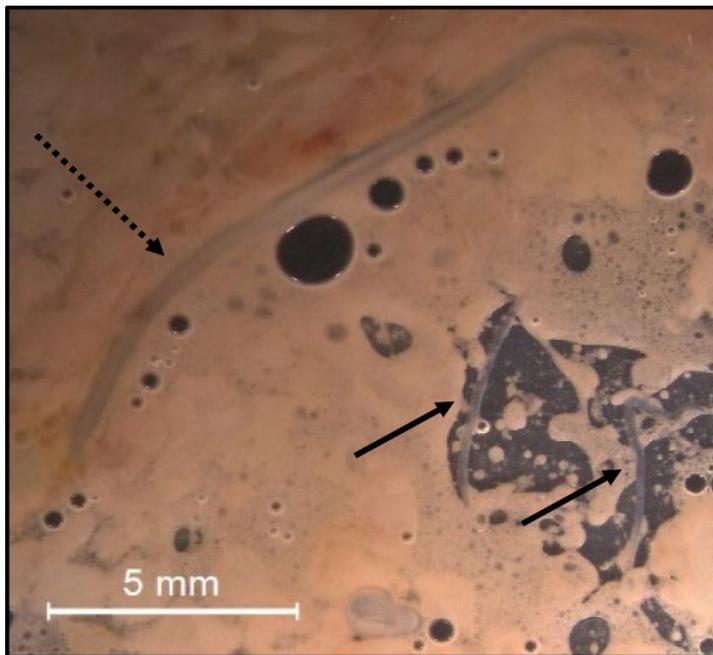


Figure 5. Examples of different sizes of *C. osculatum* in a liver from Eastern Baltic cod. Dashed arrow: large nematode (>1 cm); solid arrows: small nematodes (<1 cm; photo: Heidi Andreassen).

In the liver, as a host response to prevent damage to the liver tissue by the parasite (Buchmann, 2012), *C. osculatum* becomes encapsulated (Buchmann and Mehrdana, 2016; Zuo *et al.*, 2018). This response is an inflammatory reaction that is mainly caused by macrophages (Zuo *et al.*, 2017). Histological examinations have revealed that not only do infections with *C. osculatum* cause an inflammatory response surrounding the nematodes (Figure 6A), but the nematode movement also causes large tracks in the liver tissue, creating tunnels with cellular debris and haemorrhages, sometimes surrounded by granulomatous inflammation (Chondromatidou, 2020; Figure 6B). These findings point towards substantial damage to the liver tissue by *C. osculatum* and the destruction of functional liver cells.

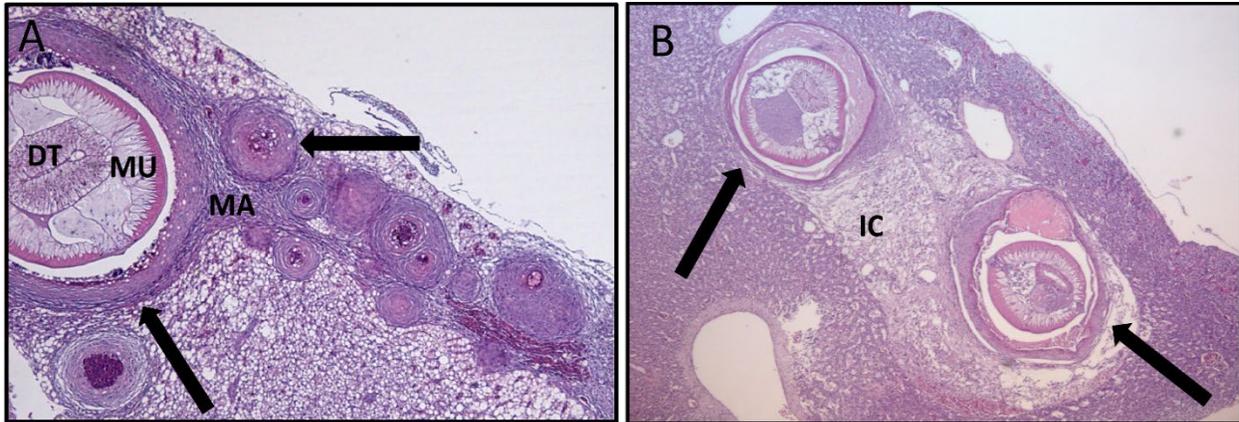


Figure 6. Liver tissues of Eastern Baltic cod infected with *C. osculatum* (anatomical view; DT: digestive tract, MU: subcuticular muscles) revealing A) extensive formation of granulomas (black arrows) often connected by an inflammatory infiltrate dominated by macrophages (MA) and fibrosis, and B) a tract lesion with oedema between two granulomas (black arrows) containing a nematode (perhaps it is the same nematode represented in each end of a long granuloma), lack of hepatocytes and infiltration of inflammatory cells (IC) representing a lesion most likely caused by movement of the nematodes (photos: Chondromatidou, 2020).

So far, studies dealing with *C. osculatum* infections in Eastern Baltic cod have used the total number of nematodes in the liver (i.e. intensity of infection) as an indication of the severity of infection. It is, however, questionable whether the intensity of infection is the best metric to use when dealing with the severity of the infection in relation to potential effects on the physiology and health status of the infected fish. This is based on the line of thought that a 20 g liver containing 100 nematodes is arguably more influenced by the nematodes compared with a 200 g liver containing the same number of nematodes. Consequently, using the number of nematodes per gram of liver (i.e. infection density), instead of the number of nematodes per liver, likely provides a better proxy of the severity of the infection. The liver of the fish, like livers in other taxa, is responsible for many vital processes, and impairment of the functionality of this organ is expected to influence the health status of (at least heavily) infected cod (Box 2: Liver physiology of teleosts).

The muscle mass of cod and lipid content of the liver have been found to be negatively associated with intensity of parasite infection (Petrushevsky and Shulman, 1955; Mohamed *et al.*, 2020), and a recent study has shown that immune-related genes are downregulated in livers with heavy infection loads, probably as a sign of local immune suppression in the organ (Marnis *et al.*, 2020). Despite these findings, little is known about potential changes in the physiological mechanisms and morphological features of Eastern Baltic cod in relation to infections with *C. osculatum*. Thus, based on the performance paradigm, morphological and physiological changes can lead to changes in the performance, behaviour and fitness of the host (Figure 7).

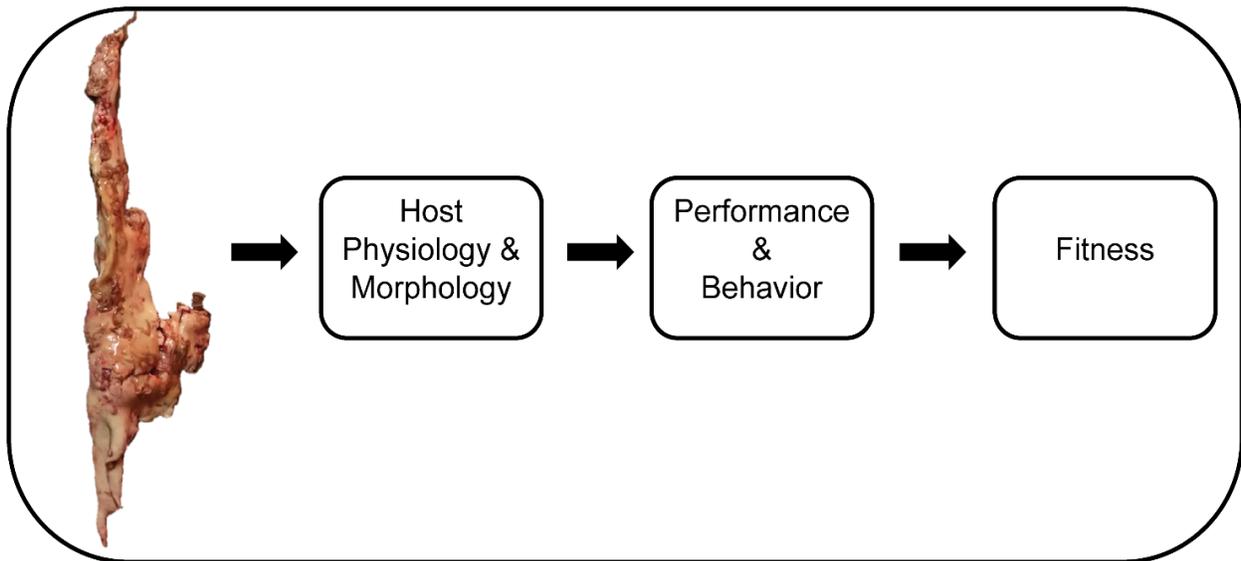


Figure 7. Schematic illustration of the performance paradigm reflecting how infections with *C. osculatum* can lead to changes in physiological mechanisms and morphological features that affect performance and behaviour, which may lead to behavioural fitness-related changes of the Eastern Baltic cod. Adapted from Binning *et al.* (2017).

Box 2: Liver physiology of teleosts

In both mammals and bony fishes (teleosts), the liver is a major gluconeogenic organ with many important functions responsible for maintaining whole-body homeostasis. All ingested nutrients (i.e. glucose, fatty acids and amino acids) are absorbed into the bloodstream, and from there, transported to the liver through the portal vein circulation system. In non-starving individuals, glucose can be stored as glycogen and/or converted into fatty acids in the liver. In times of starvation or during energy requiring activities, the liver is responsible for providing metabolic fuels (Rui, 2014; Kietzmann, 2017).

In teleosts, the liver is involved in a multitude of other functions, such as production of bile acids, egg yolk protein, vitellin and insulin-like growth factor-I, and it is a major site of leptin expression, which plays a role in regulation of feeding and metabolism. For example, bile produced in the liver and gall bladder are moved by the bile ducts into the small intestine where it is involved in digestion of food (Roberts, 2012). Moreover, the liver produces fibrinogen and albumin (albumin being the main protein of the blood), where fibrinogens are responsible for blood clotting, whereas albumin has numerous functions, such as maintaining colloid osmotic pressure and ensuring blood pH buffering, in addition to being a source of amino acids and a carrier for transportation of hormones (Hinton *et al.*, 2017).

Damage to the liver tissue can result in reduced functionality of the organ. This can lead to severe effects on the metabolism and reduce the production of vital elements of the individual (Roberts, 2012).

Objectives of the thesis

Referring to the information provided in the general introduction of this thesis, the main objectives and hypotheses of this PhD project are as follows:

- I) To identify, describe and quantify potential pathophysiological effects of *C. osculatum* infection on the physiological condition of individual Eastern Baltic cod (**paper I**).
Hypothesis I) There is an energetic cost related to infection with *C. osculatum* in Eastern Baltic cod.
Hypothesis II) The function of the liver is impaired due to tissue damage caused by *C. osculatum* infection.
Hypothesis III) Eastern Baltic cod infected with *C. osculatum* suffer from severe liver disease.
- II) To gain a mechanistic understanding of how infection with *C. osculatum* can affect the growth of cod by implementing the knowledge derived under objective I into an existing bioenergetics model for cod (**paper II**).
- III) To test the robustness and applicability of the 'liver category' method that will be implemented in the Baltic International Trawl Surveys (BITS) monitoring program in 2021 as a means of following the spatio-temporal development in infection load at the population level (**paper III**).
- IV) To examine the link between infections and critical individual condition of cod to assess whether infections are associated with an increase in natural mortality (**paper III**).

To meet the four objectives, I have used a multidisciplinary approach combining field and experimental data and bioenergetics and statistical modelling.

Methodological considerations

Experimental approach

The experimental work had a dual purpose; to gain a physiological mechanistic understanding of the Baltic cod infections and to obtain data for use in the parameterisation of the bioenergetics model to study the growth of infected cod (**papers I and II**). Experimental protocols involving different aspects of physiology were used to study changes in the following parameters: metabolism, body and energy composition, plasma protein levels and the sizes of internal organs (**paper I**). These parameters were chosen because they were considered the most relevant for examining the physiological condition of infected Eastern Baltic cod. Thus, there are some parts of the experimental work that could have been carried out differently. For example, the compression method that was used to analyse livers for the presence of nematodes in the present work (Ryberg *et al.*, 2020) is likely underestimating the total number of nematodes. A digestion method involving an artificial stomach juice (pepsin and hydrochloric acid solution; Nadolna-Altyn *et al.*, 2017) may have provided a more accurate determination of the number of nematodes in the liver. However, because the compression method was used in the first analyses in this PhD work, this method was employed throughout to ensure consistency in all the analyses of nematodes in livers in the project.

In the experimental work, it may have been more relevant to use a swim-flume respirometry approach rather than the intermittent-flow respirometry approach, used in the present work that involved an exhaustive chase protocol to obtain data on the maximum metabolic rate of infected cod. The swim-flume respirometry method would also have provided critical information concerning the swimming ability of individuals in relation to infection density and intraspecific variation in metabolic rate would have been more easily detected, as the current within the swim-flume respirometer can be controlled during the swimming trial (Norin and Clark, 2016). During the chase protocol in the present study, it turned out to be difficult to chase the fish in sufficient time to measure the maximum metabolic rate. It was necessary to reduce the chasing time from 5–10 minutes, as has been applied in previous studies on cod (Powell *et al.*, 2011; Tirsgaard *et al.*, 2015), to 2 minutes, since longer chasing resulted in premature fatigue. However, swim-flume respirometry is far more time consuming and does not allow for a high throughput of individuals. Furthermore, flume respirometers are expensive and more cumbersome to house than static respirometers used in the exhaustive chase protocol (Norin and Clark, 2016). Thus, using the chasing protocol to assess changes in the maximum metabolic rate of Eastern Baltic cod in relation to different infection densities might have resulted in poor estimations of the maximum metabolic rate in the present study. One could speculate that the low-infected individuals were chased for too short a time and the highly infected individuals for too a long time, to only rely on aerobic metabolism.

Nevertheless, the measurements of changes in the standard metabolic rate in relation to different infection densities were not biased by using static respirometers, as intermittent-flow respirometry is highly efficient in obtaining this type of measurements (Steffensen, 1989; Chabot *et al.*, 2016).

It is mandatory to consider the concept of the three Rs (3Rs), introduced and defined by Russell and Burch in 1959, in experimental work that involves animals. The 3Rs cover replacement, reduction and refinement, and the overall aim of implementing the 3Rs is to improve the treatment of the research animal and to assure a high quality of scientific output (Tannenbaum and Bennett, 2015). In this section, a description of each of the 3Rs is provided, together with considerations concerning the experimental work carried out during this PhD project (**paper I**).

Replacement is defined as ‘the substitution for conscious living higher animals of insentient material’ (Russell and Burch, 1959). In the present study, when possible, field data were obtained from cod caught during monitoring surveys. However, it was not possible to replace the use of live cod in the PhD project with *in vitro* methods, as one of the major goals was specifically to examine the physiological mechanisms of infections with *C. osculatum* in Eastern Baltic cod.

Reduction refers to methods that reduce the number of experimental animals without compromising the strength of the statistics and the consistency of the study. One difficulty when working with wild Eastern Baltic cod infected with *C. osculatum* is that the distribution of *C. osculatum* in individual cod is unknown until after the experiment is done, the fish euthanised and its liver examined. This inevitably increases the number of individuals used in the experimental work to obtain sufficient variability in the infection load of cod. To address reduction in the experimental work, the optimal solution was to use artificially infected cod. Artificial infection of cod was not part of this PhD project, but it was carried out by collaborators at Copenhagen University (Kurt Buchmann and Shaozhi Zuo). The goal was to define four groups of infection levels as follows: low, medium, high and a control (non-infected) to assure a broad range of parasite loads in the study and to examine whether individuals in poor condition at infection time are more susceptible to infections with *C. osculatum* compared with individuals in good nutritional condition. Unfortunately, the artificial infections failed, and wild, naturally infected Eastern Baltic cod were used as experimental animals. Thus, to ensure a broad range of infection densities of wild cod used in the experimental work, a pilot study was conducted in 2017 to obtain information on infection load for given lengths of the cod. This included an assignment of the liver category method and length measurements of 87 cod from the specific area where it was planned to obtain wild cod for the experimental work. The goal of the pilot study was to gain information about the probability of finding cod in the two highest liver categories in relation to the length of the fish.

The probability analysis based on the liver category and length data revealed that it was important to include as many cod above 39 cm as possible (i.e. those assumed to be most heavily infected) to ensure cod with different infection densities would be included in the experimental work (Figure 8).

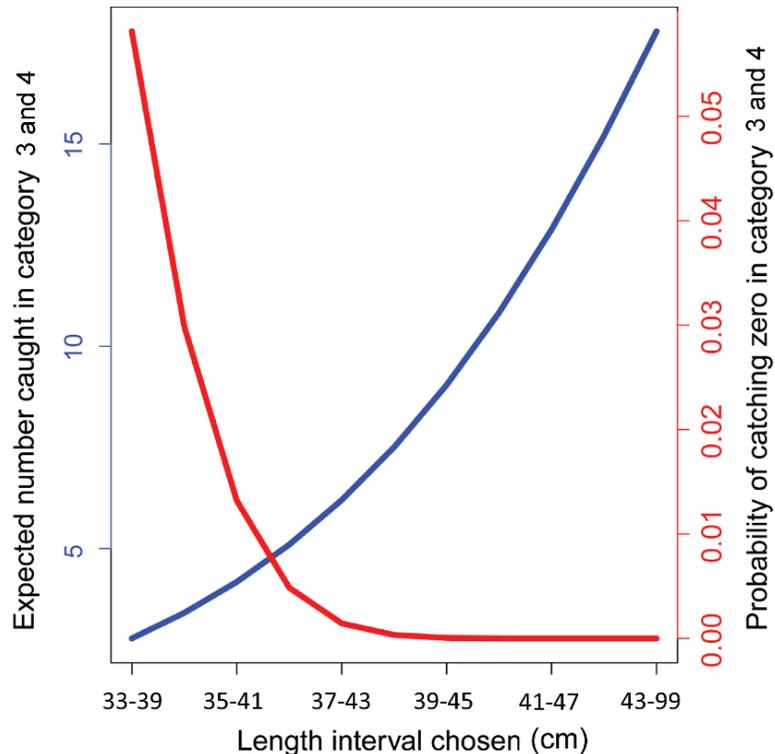


Figure 8. Expected number of cod caught with liver categories 3 and 4 (blue line) and the probability of catching zero cod in categories 3 and 4 (red line) for different length intervals of the cod. Both lines are based on data from 87 Eastern Baltic cod caught in October 2017 as part of the pilot study used to reduce the number of live cod included in the experimental work and to ensure sufficient variability in parasite load of the experimental wild cod.

Refinement covers methods that minimise the distress and pain of the experimental animal to achieve the best animal welfare without compromising the quality of data. Daily surveillance included examinations of water quality, fish behaviour, signs of disease, water flow and mortality. Moreover, as mentioned the chasing time of the fish used in the exhaustive chase protocol was reduced to minimise distress to the fish.

The bioenergetic modelling approach

A bioenergetics modelling approach was used to assess changes in the growth rate of Eastern Baltic cod infected with *C. osculatum*. This was done by implementing results from the experimental work (**paper I**) into a bioenergetic model (**paper II**). With this type of model, it was possible to dig deeper into the mechanisms behind the potential effects of infections on the growth rate of Eastern Baltic cod.

The bioenergetic modelling approach involves an energy-balanced equation that accounts for energy intake and energy allocation (Deslauriers *et al.*, 2017). One of the first bioenergetics models for fish, as we know it today, was defined by Kitchell *et al.* (1977); these researchers applied a bioenergetics model to simulate the growth of yellow perch, *Perca flavescens*, and walleye, *Stizostedion vitreum*, after inspiration from previous work on energy partitioning in fish (references in Deslauriers *et al.*, 2017). The early bioenergetics models were typically used to gain a mechanistic understanding of factors driving growth or to quantify the effects of predators on their prey (Deslauriers *et al.*, 2017). Not surprisingly, present-day bioenergetics models are much more advanced and address many perspectives of physiology, ecology, aquaculture and fisheries management, while also involving life-history traits (Thompson and Beauchamp, 2016; Deslauriers *et al.*, 2017).

The statistical modelling approach

To meet the third and fourth objectives of the PhD project, a database was compiled based on field data of livers assigned a liver category via data from research cruises from DTU Aqua, GEOMAR (Germany) and Thünen Institute (Germany) and own data. Subsequently, the total number of nematodes in the livers was counted for 642 cod, resulting in both information on the liver category and total number of nematodes. Then, a statistical modelling approach was used to develop an estimation tool including data from the liver database. With this, the robustness of the current disease monitoring tool used for Eastern Baltic cod, the liver category method, was assessed (section 1.6), and predictions of the total number of nematodes based on the assigned liver categories were provided. In a perfect scenario, the final objective of this PhD would have been to provide an estimate of natural mortality caused by infection with *C. osculatum*. However, it turned out not to be trivial to separate the effects of *C. osculatum* from other stressors that the fish had experienced during their lifetime in the wild. As a consequence, and in an attempt to obtain the best estimate of how infections can be associated with the natural mortality of cod, the probability of cod having a critical condition factor in relation to different infection loads was calculated.

Structure of the thesis

In summary, this PhD project integrates experimental work, field data collection and bioenergetics and statistical modelling. Together, these approaches have resulted in three papers that cover individual and population-level effects of infection with the parasite *C. osculatum* in Eastern Baltic cod:

Paper I: Published article describing the physiological condition of infected cod.

Paper II: Manuscript in preparation presenting a bioenergetics modelling approach aimed at revealing the mechanisms of infections on cod growth and condition.

Paper III: Manuscript submitted to the *International Journal for Parasitology*, deriving how a simple and cost-effective tool can be used to monitor the infection load of cod and providing important information on the probability of cod having a critical condition factor in relation to different infection densities.

Chapter II

Paper I: Physiological condition of Eastern Baltic cod, *Gadus morhua*, infected with the parasitic nematode *Contracaecum osculatum*

Physiological condition of Eastern Baltic cod, *Gadus morhua*, infected with the parasitic nematode *Contracaecum osculatum*

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Establishing relationships between parasite infection and physiological condition of the host can be difficult and therefore are often neglected when describing factors causing population declines. Using the parasite–host system between the parasitic nematode *Contracaecum osculatum* and the Eastern Baltic cod *Gadus morhua*, we here shed new light on how parasite load may relate to the physiological condition of a transport host. The Eastern Baltic cod is in distress, with declining nutritional conditions, disappearance of the larger fish, high natural mortality and no signs of recovery of the population. During the latest decade, high infection levels with *C. osculatum* have been observed in fish in the central and southern parts of the Baltic Sea. We investigated the aerobic performance, nutritional condition, organ masses, and plasma and proximate body composition of wild naturally infected *G. morhua* in relation to infection density with *C. osculatum*. Fish with high infection densities of *C. osculatum* had (i) decreased nutritional condition, (ii) depressed energy turnover as evidenced by reduced standard metabolic rate, (iii) reduction in the digestive organ masses, and alongside (iv) changes in the plasma, body and liver composition, and fish energy source. The significantly reduced albumin to globulin ratio in highly infected *G. morhua* suggests that the fish suffer from a chronic liver disease. Furthermore, fish with high infection loads had the lowest Fulton's condition factor. Yet, it remains unknown whether our results stem from a direct effect of *C. osculatum*, or because *G. morhua* in an already compromised nutritional state are more susceptible towards the parasite. Nevertheless, impairment of the physiological condition can lead to reduced swimming performance, compromising foraging success while augmenting the risk of predation, potentially leading to an increase in the natural mortality of the host. We hence argue that fish–parasite interactions must not be neglected when implementing and refining strategies to rebuild deteriorating populations.

Key words: Compromised liver function, liver worm, parasites, energetic cost, nutritional condition, Eastern Baltic cod

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Introduction

Parasitism is one of the most common animal lifestyles and can impact ecosystem functioning by affecting food-web stability, interaction strength and energy flow in both terrestrial and aquatic ecosystems (Marcogliese, 2004; Kuris *et al.*, 2008; Lafferty *et al.*, 2008; Hatcher *et al.*, 2014). At the level of the individual, parasites can cause adverse effects on the performance capacity of the host (McElroy and de Buron, 2014), e.g. by changing plasma protein and hormone levels (Akinyi *et al.*, 2019; O'Dwyer *et al.*, 2019), reducing aerobic and locomotor performances (Umberger *et al.*, 2013; Hahn *et al.*, 2018) and depleting energy reserves (Ferrer-Maza *et al.*, 2016). Together, this shapes the physiological condition of an infected individual, and impairment may lead to reduced growth and increased mortality (Marcogliese, 2004; Khan, 2005; Behrens *et al.*, 2014). For trophically transmitted parasites, such effects on transport hosts (transport hosts having only sexually immature stages of the parasite, and with no development of the parasite occurring while hosting it; Rohde, 2002) can either be a result of accidental side-effects associated with the infections (Hafer and Milinski, 2016) or host manipulations (McElroy and de Buron, 2014) or maybe even a combination of both (Heil, 2016). Yet, irrespectively of the reason, it makes the host more vulnerable to predators, increasing the probability of the parasite to reach its final host (Heil, 2016).

Marine fish are hosts to a high diversity of parasitic organisms (Marcogliese, 2002; Rohde, 2002), and parasite-induced impairment of the physiological condition has been suggested to reduce fish stock productivity, leading to declining catches of both freshwater and marine fish populations (Lloret *et al.*, 2012). However, establishing causality between parasite infection and physiological condition of the host can be difficult, and the mechanisms underlying parasite-altered host fitness remain largely unknown (Lloret *et al.*, 2012; McElroy and de Buron, 2014).

Here, we use the host–parasite system between the third stage larvae liver worm *Contracaecum osculatatum* (Zuo *et al.*, 2018) and Eastern Baltic cod *Gadus morhua* as a case study to investigate the physiological performance of wild fish with high parasite load. The Eastern Baltic cod stock has exhibited a decline in the nutritional condition during the past 20 years, an event that has occurred alongside deteriorating oxygen conditions and reduced prey abundance, now leaving the fish historically malnourished and growth impaired (Eero *et al.*, 2015; Casini *et al.*, 2016b; Hüssy *et al.*, 2018; Neuenfeldt *et al.*, 2020). This has challenged the management of the stock which at present shows no signs of recovery, and with high natural mortality (ICES, 2019) and a fishing ban introduced in 2019. The grey seal *Halichoerus grypus* population has been severely reduced due to hunting and breeding problems between the 1960s and the 1990s where after recovery slowly began (Harding *et al.*, 2007). Concurrent with the recovery

of *H. grypus*, an increase in infections with the trophically transmitted *C. osculatatum* that parasitizes the liver of cod has also been observed in *G. morhua* in the central and eastern Baltic Sea since the early 2010s (Haarder *et al.*, 2014; Nadolna and Podolska, 2014). This has coincided with even further deterioration of the health status and stock productivity of the fish (Eero *et al.*, 2012, 2015). *H. grypus* is the main final host of this parasite while cod act as the last transport host in the life cycle (Koie and Fagerholm, 1995; Nadolna-Altyrn *et al.*, 2018; Zuo *et al.*, 2018).

Field investigations have shown that infection intensity with *C. osculatatum* in *G. morhua* coincides with poor nutritional status, and that more Westerly and Northwesterly cod stocks with little or no *C. osculatatum* are in better nutritional condition (Horbowy *et al.*, 2016; Sokolova *et al.*, 2018). This parasite migrates to the liver of the cod following ingestion via smaller infected prey, e.g. sprat *Sprattus sprattus* (Zuo *et al.*, 2016; Nadolna-Altyrn *et al.*, 2018), where it accumulates over time, resulting in a larger parasite burden in older fish (Horbowy *et al.*, 2016; Zuo *et al.*, 2016). The liver is responsible for nutrient assimilation, bile production, maintenance of metabolic homeostasis and protein synthesis and also serves as an energy reserve and breeding capital for the fish (Hinton *et al.*, 2017). It is thus intuitive to think that a high liver parasite burden leads to reduced function of the organ with negative effects on the nutritional condition of the infected individual. Yet, disentangling potential effects of parasites on their hosts from effects arising in the wake of unfavourable abiotic and food conditions demands an interdisciplinary approach combining field and laboratory studies, and expert parasitologists, physiologists and biologists (McElroy and de Buron, 2014).

To elucidate how high parasite load may relate to the physiological condition of wild fish, we here investigated aerobic performance, nutritional condition, mass of selected organs and plasma and proximate body composition of wild, naturally infected *G. morhua* in relation to infection density with *C. osculatatum*.

Materials and methods

Pilot study

The number of *C. osculatatum* in cod livers increases with the length of the fish (Nadolna and Podolska, 2014; Horbowy *et al.*, 2016). In 2017, we therefore conducted a pilot study to identify the length interval of *G. morhua* needed to obtain fish samples with sufficient variability in infection intensity to make a solid study design. More specifically, wild and naturally infected *G. morhua* ($n = 86$) were captured by trawl East of Bornholm and used to assess the correlations between body mass (BM), total length (TL), liver mass (LM), gender and number of nematodes in the liver. A length range between 30 and 53 cm provided sufficient high variability in infection intensity of nematodes in the livers of the fish. Fish for

Table 1: Overview of fish within all six assessments. *n* = number of fish, TL = total length, BW = body weight, LM = liver mass, prevalence: percentage of infected fish in the sample, and intensity of infection: mean number of parasites per liver, including only infected individuals. The numbers in brackets represent ranges of variables. All numbers are mean ± SE

Assessment	<i>n</i>	TL (cm)	BW (g)	LM (g)	Prevalence (%)	Intensity
Nutritional condition	152	39 ± 0.4 (29–53)	510 ± 14.9 (209–1098)	21 ± 1.0 (4–80)	89	32 ± 2.7 (0–203)
Aerobic performance and plasma composition	60	42 ± 0.5 (34–53)	572 ± 22.2 (260–1077)	19 ± 1.4 (4–57)	93	46 ± 5.3 (0–203)
Proximate composition of fish and liver	33	40 ± 1.0 (29–53)	532 ± 41.7 (212–1098)	25 ± 3.3 (6–80)	85	23 ± 4.3 (0–104)
Organ size	59	36 ± 0.5 (28–45)	434 ± 16.7 (209–780)	20 ± 9.2 (5–41)	86	21 ± 2.5 (0–72)

experiments (see below) were consequently selected to cover this length range if possible.

Experimental animals

Wild naturally infected Eastern Baltic cod (*n* = 152) were captured by trawl East of Bornholm between 2017 and 2019 (Table 1). Cod were either used live for respirometry or sampling was made directly on dead fish for investigations of body composition and organ sizes. For the latter investigations, fish were killed and frozen at -20°C immediately after trawling, and transported to DTU Aqua, Lyngby, for later analysis (Table 1). For fish used live in experiments (Table 1), trawling time never exceeded 20 min in order to minimize stress and damage to the fish. Live cod were acclimated for 2 weeks in captivity at Bornholm's Salmon Hatchery before being transferred to the fish-holding facilities at DTU Aqua, Lyngby. Here, they were held in two circular tanks (2000 L each) with air-saturated recirculated water (10°C , 10‰ salinity, photoperiod of 8:16 light: dark, with a half-hour period of sunrise and sunset) and allowed 3 weeks of acclimation before initiation of experiments. Fish were fed three times weekly with cooked blue mussels corresponding to ~2% of their BM. All experiments were carried out according to the animal welfare regulations of the Technical University of Denmark and EU directive 2010/63/EU for animal experiments. Ethical permit 2017-15-0201-01282 from the Danish Animal Ethics Committee covered all experiments reported here.

Recovery of nematodes from cod livers

All livers from fish used in the present study were dissected out and frozen separately (-20°C) before they were analysed for the presence of nematodes. Individual livers, except those used for lipid and energy analysis, were placed in a plastic bag ($200 \times 400 \times 0.07$ mm) and compressed between two glass plates ($15 \times 15 \times 1$ cm) to a thickness of 1 mm by the addition of gentle pressure to the plates (Buchmann, 2007). Livers were subsequently examined under a Leica stereo microscope (6.3–40× magnification) (Leica Microsystems Germany), and detected nematodes were categorized as either small (<1-cm body length) or large (>1-cm body length). To minimize

oxidation processes and tissue breakdown in livers dedicated to lipid analysis, individual defrosted livers (*n* = 33) were placed on glass petri dishes kept on ice. Single nematodes were manually removed, using a tweezer, and the total number for each liver recorded and subsequently preserved in 70% ethanol. For all examined livers, nematode species identification was based on morphometric characteristics of the caudal and cephalic ends according to Fagerholm (Koie and Fagerholm, 1995). To compensate for differences in the number of nematodes related to liver size, infection density was calculated as the number of nematodes per gram of liver tissue (i.e. liver tissue = wet weight of the liver minus total weight of nematodes), rather than the total number of nematodes per liver.

Nutritional condition

The association between infection density with nematodes and the nutritional condition of all cod included in this study (*n* = 152) was analysed by calculating the Fulton condition factor:

$$\text{Fulton condition factor} = \left(\frac{\text{BM}}{\text{TL}^3} \right) * 100$$

Aerobic performance

To investigate potential associations between infections and the aerobic performance of the fish, we determined standard metabolic rate, maximum metabolic rate and aerobic metabolic scope (i.e. maximum metabolic rate minus standard metabolic rate) of cod (*n* = 60) with varying infection densities of nematodes. The standard metabolic rate represents the energy requirements of the individual at a resting, non-digesting state; maximum metabolic rate represents the maximum aerobic performance (Chabot *et al.*, 2016), while aerobic metabolic scope relates to the ability to perform aerobic work. Four static respirometers (6.6 L or 8.2 L, to accommodate for differences in TL of fish) were placed in a 250-L black tank supplied with a flow-through of aerated water from the same supply as the holding tanks (10°C , 10‰ salinity). To minimize disturbance from the neighbouring fish,

non-transparent polyethylene plates were placed vertically between the respirometers, and to minimize disturbance from the outside, a curtain shielded the setup.

For standard metabolic rate determinations, oxygen consumption rates ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$) were measured over a period of > 40 h, using intermittent-flow respirometry (Steffensen, 1989). Oxygen consumption rate was measured in 12-min loops consisting of a flush (420 s), a wait (60 s) and a measurement (240 s) period. To obtain maximum metabolic rate, individual fish were exposed to a chase protocol (intense continuous chasing for 2 min in a circular 300-L tank) and immediately thereafter (within maximally 2 min) placed in the respirometer, where the first oxygen consumption values were obtained rapidly, using measurement periods of 90 s, with 1 s of wait and without flushing. Measurements for maximum metabolic rate were terminated if oxygen saturation fell below 75% within the 90-s measurement period. Hereafter, oxygen consumption measurements continued in 12-min loops (as described above) for a minimum of 40 h (i.e. for standard metabolic rate determination).

The highest oxygen consumption measurement (i.e. maximum metabolic rate) occurred instantly after the chase protocol for 51 of the fish, whereas the maximum metabolic rate occurred later (following spontaneous activity inside the respirometer) for the remaining nine fish. For each fish, the standard metabolic rate was determined as the average of the 10% the lowest oxygen consumption (Chabot *et al.*, 2016) values, and aerobic metabolic scope was calculated as the difference between the maximum and standard metabolic rate. Background respiration was found by linear regression of the start and the final background measurements and subsequently subtracted from the corresponding oxygen consumption value. To eliminate potential effects of digestion on oxygen consumption, all cod were fasted 3–4 days prior to the experiment, the specific number of days being based on the cod gastric evacuation model made by Andersen, 2012. All cod were weighed upon initiation of the experiment. To assess for potential contributions from nematode oxygen consumption to the measured oxygen consumption of the cod, oxygen consumption ($\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$) was determined on 85 live nematodes (0.001–0.012 g) over a 24-h period, using a 24-well glass microplate containing 5-mL wells (Loligo Systems) with 10°C and 10‰ salinity water.

Organ size

To elucidate associations between infection density and size of selected organs, whole cod ($n=59$) were thawed, and BM, TL, LM, gender and weight to the nearest gram of the stomach (empty), intestine (empty), pyloric caeca and heart were recorded.

Plasma composition

To reveal potential associations between the nematodes and the function of the liver and the disease status of the fish, the

haematological analysis was performed. Following respirometry, fish ($n=60$) were stunned by a sharp blow to the head; blood was immediately sampled by caudal puncture with a lithium-heparinized 21-gauge hypodermic needle, and fish were euthanized by spinal transection. Blood samples were centrifuged at 1610G for 5 min, and the plasma fraction was stored at -18°C (Houston, 2002). Total blood protein content (g L^{-1}) was determined using an ADVIA 1800 Clinical Chemistry System (Siemens), while the separation of plasma protein fractions into pre-albumin, albumin and the globulins (alpha-1, alpha-2, beta-1, beta-2 and gamma) was done using capillary electrophoresis (MINICAP PROTEIN 6, Sebia, Lisses). A/G ratios were calculated by dividing individual plasma albumin and globulins values.

Proximate composition and energy content

In order to examine changes in body composition and energy content of cod in relation to different infection densities, 33 whole cod (livers removed and with empty stomachs) were individually autoclaved and homogenized (Table 1). Crude protein ($N \times 6.25$) content of the fish, and crude lipid content of fish and livers, were determined using the Kjeldahl (Foss Kjeltec 2200, Hillerød, Denmark) and the Bligh and Dyer methods (Bligh and Dyer, 1959), respectively. To obtain dry matter and water content of the fish and liver, samples were dried for 24 h at 105°C , and weight loss was determined (Memmert UN110, Büchenbach, Germany). Ash content determinations were based on weight loss after incineration of samples for 6 h at 550°C in a muffle furnace (Heraeus Instruments K1252, Hanau, Germany) (Obirikorang *et al.*, 2016), and glycogen content of the fish was calculated as the difference between the initial dry weight and the sum of the crude protein, fat and ash weights (Saint-Paul, 1984). The energy density of dry cod tissue was determined from dried tissue samples combusted in a Parr 6300 bomb calorimeter and subsequently converted to energy density per gram of wet BM (kJ g^{-1}) (Schloesser and Fabrizio, 2017).

Data handling and analysis

Prior to infection density calculations, the total weight of nematodes retrieved from individual livers was subtracted from the measured LM, small nematodes being assigned a weight of 0.004 g and large nematodes 0.009 g, based on the weight range of nematodes used in respirometry. Oxygen consumption measurements with $R^2 < 0.95$ (in total <0.8%) were excluded from the analysis. Two of the 60 fish used in respirometry never entered into a resting state (judged by observations of the raw oxygen consumption data) and were hence excluded from the dataset.

Log-linear Gaussian models were used to describe the associations between infection density, TL and gender in all assessed variables except in the analysis of changes in organ size where power function models were used instead. TL was included to account for the accumulation of nematodes in the liver over time (i.e. with the increasing length of the

fish) (Nadolna and Podolska, 2014; Horbowy *et al.*, 2016), and gender was included to test for any potential differences between males and females. TL was not included in the analysis of standard metabolic rate, maximum metabolic rate and aerobic metabolic scope as these were modelled as the mass-specific oxygen consumption because the scaling exponent for the relationship between oxygen consumption and body mass of the fish was 1. Likewise, TL was not included in the analysis of the Fulton condition factor as TL is included in this parameter. For the analysis of fish body composition, the effect of infection density on all the performed analyses was carried out together with analysis of changes in the whole-body energy content, as well as the redistribution between protein, lipid and glycogen and water within the fish. To test the robustness of the results (due to high variation in infection density), we divided fish into three groups of infection densities, non-, medium- and high-infected, to test for significant difference between these three groups for selected parameters.

All statistical tests were conducted in R (R Core Team, 2016). Before model fitting, collinearity between explanatory variables was assessed by using variance inflation factors (VIF) (Zuur *et al.*, 2009). No variables were excluded from the analysis due to collinearity (Tables S1 and S2). Model selection was performed using a stepwise backward selection routine based on the likelihood ratio test for each of the variables included and excluded in the models. The model assumptions of normality and independence were subsequently validated by visual inspection of model residuals (Figs S1–S12). ANOVA and post hoc (Tukey HSD) were used for the test of robustness.

Results

A total of 4309 nematodes were recovered from the 152 livers examined, all belonging to the species *C. osculatum*. The mean and range of infection density were 2 (\pm SE 0.3) and 0–22 nematodes per gram liver, respectively. Upon retrieval of the livers from the fish for recovery of nematodes, it was noticed that for the 12 most heavily infected livers (all with infection density above six nematodes per gram liver; Fig. 1), the organ seemingly was losing its structure/integrity, and ‘melted’ upon removal from the body cavity of the fish.

Nutritional condition

The Fulton condition factor of cod decreased significantly (GLM: $n = 152$, SE = 0.003, $t = -8.1$, $P < 0.001$) with increasing infection density (Fig. 2, Tables 2 and 3).

Aerobic performance

The standard metabolic rate decreased significantly (GLM: $n = 58$, SE = 0.003, $t = -3.2$, $P = 0.002$) with increasing infection density (Fig. 3, Tables 2 and 3). In contrast, the maximum metabolic rate was not associated with changes in



Figure 1: An example of a *G. morhua* with a liver having a high density of *C. osculatum* (19 nematodes per gram liver tissue), some visible on the surface of the organ, others hidden in the liver parenchyma

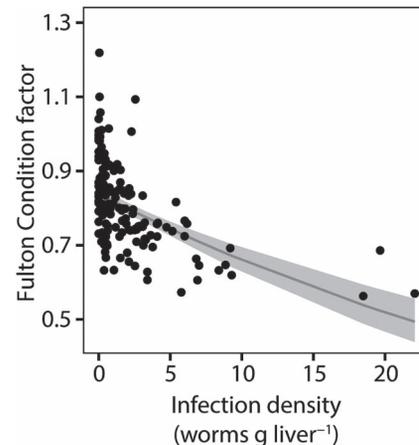


Figure 2: Fulton condition factor of cod ($n = 152$) with varying degrees of infection densities with *C. osculatum*. The thin grey line describes the model fit, a significant negative association between infection density and the Fulton condition factor. The 95% confidence interval is represented by the grey area

infection density and aerobic metabolic scope, and gender was not significant in any of the three cases. The oxygen consumption of *C. osculatum* was inconsiderable (mean \pm SE; $0.0002 \pm 2.2E^{-05}$, mg O₂ kg⁻¹ h⁻¹) and thus negligible for the measured oxygen consumption of the cod.

Organ size

The mass of intestine and pyloric caeca increased significantly (GLM: intestine, $n = 58$, SE = 0.350, $t = 6.3$, $P < 0.001$ and pyloric caeca, $n = 58$, SE = 0.357, $t = 7.3$, $P < 0.001$) with the length of the fish but decreased significantly with increasing infection density (GLM: intestine, $n = 58$, SE = 0.012, $t = -2.1$, $P = 0.03$ & pyloric caeca, $n = 58$, SE = 0.011, $t = -2.2$, $P = 0.03$). Females had larger intestines compared to males,

Table 2: Symbols reflect the estimates of the final models (SE in brackets): α = infection density (INF), β = intercept, γ = gender (estimate for female), μ = length and λ = total energy. - = variable not significant in the model, and empty columns = variable was not included in the full model. Units of parameters: 1 = mg O₂ kg⁻¹ h⁻¹, 2 = g, 3 = g L⁻¹, 4 = %, 5 = kJ g⁻¹ and 6 = g g liver⁻¹. SMR = standard metabolic rate. Asterisks indicate the significance level of the estimated parameters (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). R^2 indicates how much of the variation of data each model explains. All reported estimated model parameters are on log scale except for organ size where estimates are on a log-10 scale

Assessment	Parameter	α (INF)	β (intercept)	γ (gender)	μ (length)	λ (total energy)	R^2
Nutritional condition	Fulton condition	-0.02 (0.003)***	-0.17 (0.01)				0.30
Aerobic performance	SMR ¹	-0.01 (0.003)**	3.98 (0.02)	-			0.14
Organ size	Pyloric caeca ²	-0.02 (0.01)*	-3.44 (0.55)	-	2.60 (0.36)***		0.47
	Intestine ²	-0.02 (0.01)*	-2.94 (0.54)	0.08 (0.03)*	2.22 (0.35)***		0.43
Plasma composition	Total protein ³	-0.01 (0.005)**	3.49 (0.03)	-	-		0.13
	Globulins ⁴	0.01 (0.003)***	4.35 (0.02)	-	-		0.19
	A/G	-0.16 (0.03)***	-1.5 (0.17)	-	-		0.33
	Pre-albumin ⁴	-0.08 (0.02)***	2.35 (1.42)	0.64 (0.01)**	0.06 (0.03)*		0.21
	Gamma ⁴	0.02 (0.008)*	2.16 (0.04)	-	-		0.07
	Albumin ⁴	-0.15 (0.03)***	2.84 (0.16)	-	-		0.33
Proximate composition of fish	Total energy ⁵	-0.03 (0.01)**	1.28 (0.01)	-	-		0.18
	Water ⁴	0.003 (0.001)*	4.39 (0.002)	-	-		0.14
	Protein ⁵	-0.04 (0.01)**	1.03 (0.02)	-	-		0.25
	Glycogen ⁵	0.05 (0.01)**	0.36 (0.02)	-	-		0.20
	Ash ⁴	0.04 (0.01)*	1.25 (0.02)	-	-		0.14
	Dry matter ⁴	-0.01 (0.006)*	2.95 (0.01)	-	-		0.14
	Protein ⁵		-0.43 (0.09)	-	-	0.40 (0.02)***	0.89
Glycogen ⁵		1.71 (0.20)	-	-	-0.38 (0.06)***	0.57	
Proximate composition of liver	Lipid ⁶	-0.11 (0.03)***	3.59 (0.24)	0.15 (0.07)*	-		0.41
	Water ⁴	0.10 (0.03)***	3.76 (0.06)	-0.15 (0.07)*	-		0.37
	Dry mat ⁴	-0.10 (0.03)***	4.00 (0.06)	0.15 (0.07)*	-		0.41
	Ash ⁴	0.10 (0.03)**	-0.49 (0.06)	-	-		0.18

but gender could not explain any variation found in the weight of pyloric caeca (Table 2).

Plasma composition

Total protein in the plasma decreased significantly (GLM: $n = 60$, $SE = 0.005$, $t = -3.1$, $P = 0.01$; Fig. 4A) with increasing infection density, as did the albumin to globulin ratio (GLM: $n = 60$, $SE = 0.030$, $t = -5.4$, $P < 0.001$; Fig. 4B) (Tables 2 and 3). Albumin decreased significantly while the sum of globulins and gamma-globulins alone increased significantly with increasing infection density (Table 2). These changes in protein fractions of the plasma were reflected by the highly

significant decrease in albumin to globulin ratio. Pre-albumin, which is not a part of the albumin to globulin ratio, decreased significantly with increasing infection density (Table 2). On the contrary, there was no change in alpha-1, alpha-2 and beta-1-2 with increasing infection density. Gender and TL were only significant in the analysis of pre-albumin (Table 2).

Proximate composition and energy content

Overall, the body composition of the fish changed with increasing infection density. More specifically, total energy (GLM: $n = 33$, $SE = 0.009$, $t = -2.8$, $P = 0.006$; Fig. 5A) and protein content decreased significantly with increasing

Table 3: Results of post hoc analysis Tukey HSD test for eight variables where infection density is divided into three groups. For the first four variables, infection density (number of nematodes per gram liver tissue) in each group is non = 0, medium = 4 and high > 2 as a result of different range in infection densities between the two batches of fish. Units of parameters: 1 = mg O₂ kg⁻¹ h⁻¹, 2 = g L⁻¹, 3 = kJ g⁻¹, 4 = % and 5 = g g liver⁻¹. *P* value = overall significance level between the groups, letters = groups that do not differ statistically from each other. * visualizes how group b or c differs significantly from a group where **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. *n* represents the number of fish within each infection group (non, medium, high). SMR = standard metabolic rate. All numbers are mean ± SE

Parameter	Non	Medium	High	<i>P</i> value	<i>n</i>
Fulton condition	0.88 ± 0.2(a)	0.81 ± 0.1(b**)	0.68 ± 0.2(c***)	<0.001	(17, 115, 20)
SMR ¹	56.3 ± 0.5(a)	54.6 ± 0.2(a)	44.7 ± 0.3(b**)	0.002	(4, 39, 15)
Total protein ²	32.1 ± 0.6(ab)	32.4 ± 0.2(a)	27.8 ± 0.3(b*)	0.02	(4, 39, 15)
A/G	0.33 ± 0.9(a)	0.18 ± 0.4(a)	0.04 ± 1.3(b**)	<0.001	(4, 39, 15)
Total energy ³	3.67 ± 0.5(a)	3.49 ± 0.2(ab)	3.32 ± 0.4(b*)	0.05	(5, 21, 7)
Water fish ⁴	80.2 ± 0.4(a)	81.2 ± 0.2(ab)	81.4 ± 0.4(b*)	0.03	(5, 21, 7)
Lipid liver ⁵	0.58 ± 0.5(a)	0.45 ± 0.3(a)	0.30 ± 0.6(b**)	0.03	(5, 21, 7)
Water liver ⁴	36.6 ± 0.5(a)	44.2 ± 0.3(ab)	54.0 ± 0.5(b**)	0.02	(5, 21, 7)

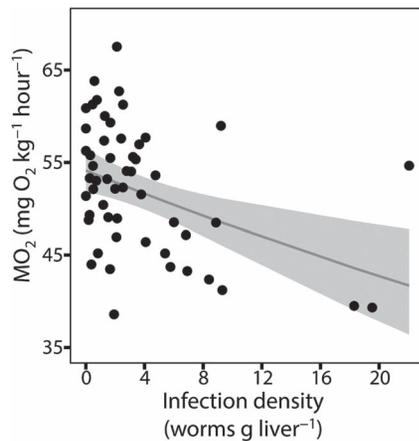


Figure 3: The standard metabolic rate (MO₂, in mg O₂ kg⁻¹ h⁻¹) of *G. morhua* (*n* = 58) with varying degrees of infection densities with *C. osculatum*. The thin grey line describes the association between infection density and standard metabolic rate, revealing a significant negative correlation. The grey boxes are 95% confidence intervals

infection density (Table 2), while water (GLM: *n* = 33, SE = 0.001, *t* = 2.5, *P* = 0.01; Fig. 5B) and glycogen content increased significantly (Table 2).

The decrease in total energy content of the fish was explained by a significant change in the source of energy: a significant increase in protein (GLM: *n* = 33, SE = 0.025, *t* = 16.1, *P* < 0.001; Fig. 6A) and a slight increase in lipid energy, and a significant decrease in glycogen energy (GLM: *n* = 33, SE = 0.057, *t* = -6.6, *P* < 0.001; Fig. 6C) with an increase in the total energy of the fish (Fig 6, Table 2).

The total lipid energy of the fish did not change with changing infection density, and gender could not be associated

with the observed changes in the body composition of the fish. Liver lipid content (GLM: *n* = 33, SE = 0.116, *t* = 2.1, *P* < 0.001; Fig. 5C) and dry matter decreased significantly with increasing infection density, while ash and water content (GLM: *n* = 33, SE = 0.074, *t* = -2.0, *P* = 0.001; Fig. 5D) significantly increased with increasing infection density. Lipid, water and dry matter contents of the liver differed significantly between males and females, as livers from females contained more lipid and less water and less dry matter content compared to livers from males (Table 2).

Test of robustness

Fulton condition factor decreased significantly between the non-, medium- and high-infected groups with the lowest value found in the high infected group (Table 3). The standard metabolic rate, A/G ratio and lipid content of the liver decreased significantly in the high-infected groups compared to the non- and medium-infected groups (Table 3). Total energy and water content of the fish and the liver did not differ significantly between the high and medium groups but only between the non- and high-infected groups (Table 3). Total protein in the plasma did not differ significantly between non- and high-infected groups, only between the high- and the medium-infected groups (Table 3).

Discussion

Using an integrative approach, we show that wild naturally infected *G. morhua* with heavy infections with the parasitic *C. osculatum* have impaired nutritional condition, reduced functionality of the digestive system, as indicated by the observed reduction in digestive organ masses (intestine and pyloric caeca), reduced baseline metabolism and changes in the body and plasma composition and fish energy source. Notably, fish with high infection densities had decreased plasma albumin

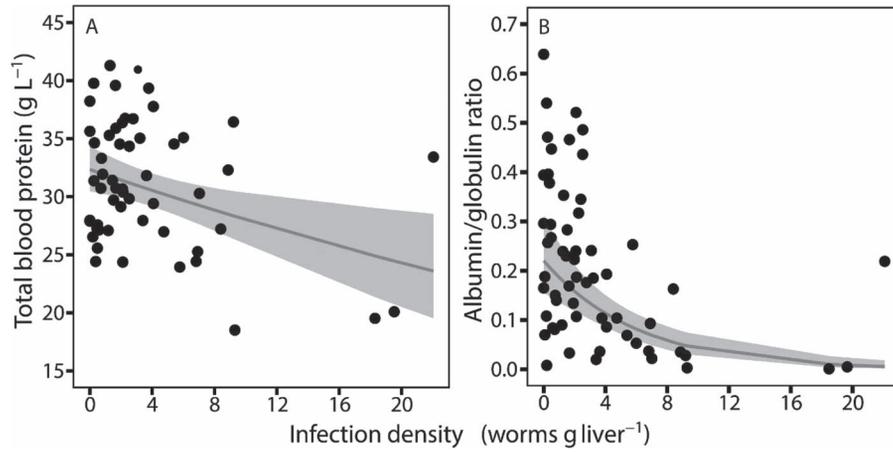


Figure 4: (A) Total blood protein (g L^{-1}) and (B) albumin/globulin ratio in *G. morhua* ($n = 60$) in relation to varying degrees of infection densities with *C. osculatum*. Both parameters decreased significantly with increasing infection density, as described by the thin grey lines. The grey boxes are 95% confidence intervals

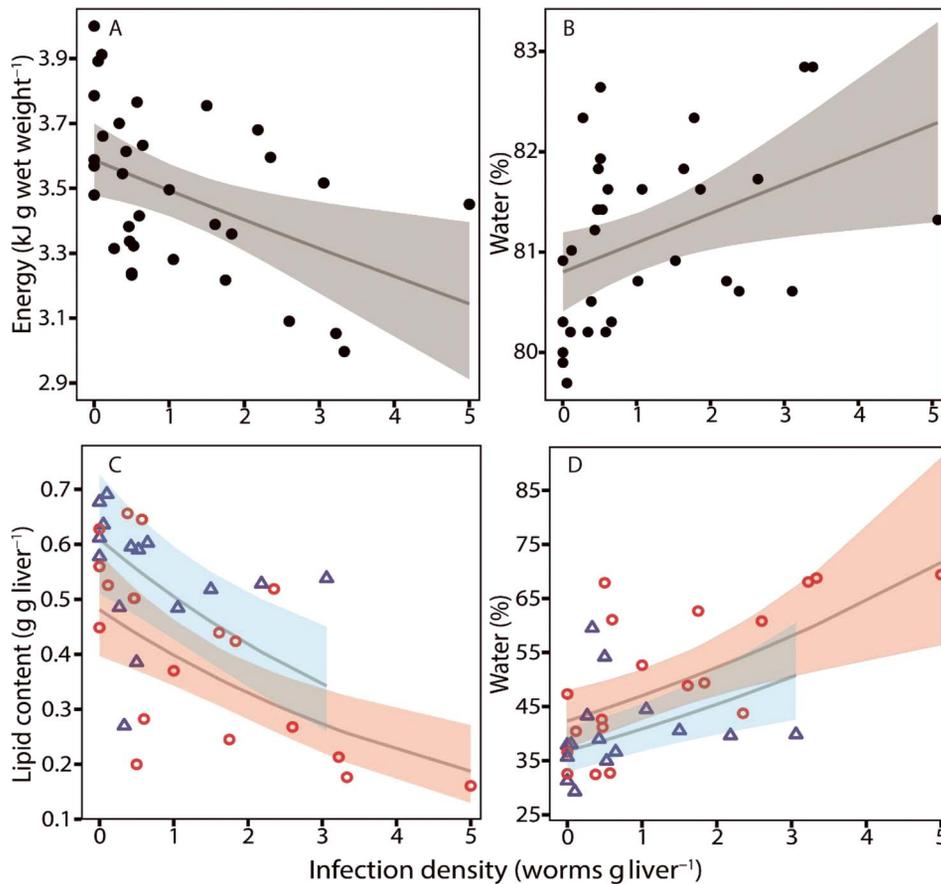


Figure 5: (A) Total energy content ($\text{kJ g wet-weight}^{-1}$) of the whole fish excluding the liver, (B) water content (%) of the whole fish, (C) lipid content of the liver (g g liver^{-1}) and (D) water content of the liver (%), all in relation to changes in infection density as described by the thin grey lines. Both the total energy of the fish and lipid content of the liver decreased significantly with increasing infection density while water content increased significantly in both the fish and the liver with increasing infection density. In C and D, colours and symbols represent: blue and Δ = female, red and \circ = male. Grey, blue and red areas represent 95% confidence intervals.

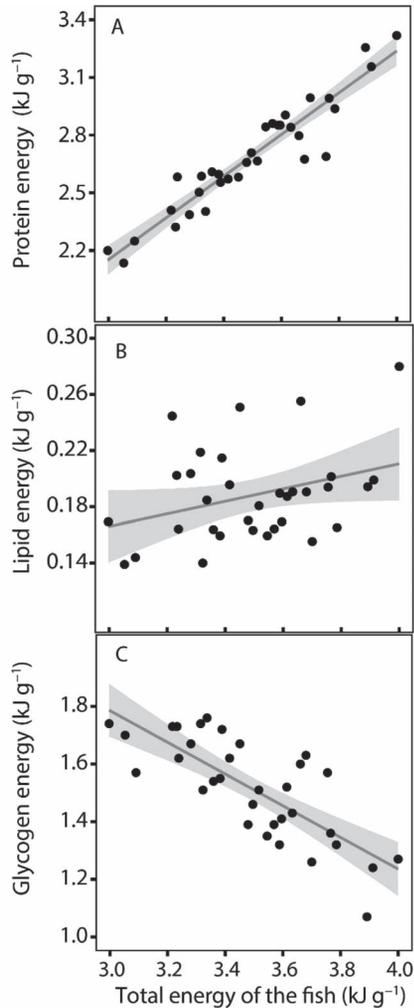


Figure 6: Proportions of the fish energy source coming from (A) protein energy (kJ g^{-1}), (B) lipid energy (kJ g^{-1}) and (C) glycogen energy (kJ g^{-1}), all in relation to the total energy of the fish ($n = 33$). As described by the thin grey lines, the proportion of energy in the fish coming from protein increased significantly with increasing energy of the fish, and likewise for lipid energy (though not significantly), whereas the amount of energy coming from glycogen sources decreased with the energy of the fish. The grey areas represent a 95% confidence interval

and increased globulin levels, resulting in reduced albumin to globulin ratio.

Synthesized in the liver, albumin is the main protein of the blood plasma. It has several functions, e.g. maintaining osmotic pressure, capillary permeability and transport and metabolism of an extraordinarily diverse range of molecules (McDonald and Milligan, 1992; Garcia-Martinez *et al.*, 2013). In humans, reduced albumin to globulin ratios (driven by reduced albumin and increased globulins) are seen in individuals with chronic liver diseases associated with parenchymal damage, such as cirrhosis and liver cancer. Here,

the increased globulin levels are caused by alternations in the gamma fraction (synthesized in lymphatic tissues), with alpha and beta globulins remaining stable (Teloh, 1978; Suh *et al.*, 2014). Changes in plasma protein composition also occur in diseased fish, where the albumin to globulin ratio has been used to reveal the physiological effects of specific pathogens (Aydin *et al.*, 2001; Osmani *et al.*, 2009). Notably, for some fish species, gamma globulins are considered to represent the complex nature of parasitic nematode antigenicity (Meade and Harvey, 1969), which is in agreement with the present response of *G. morhua* to a parasitic nematode, where the gamma fraction of the globulins drives the observed increase in the plasma globulins. In support, in *G. morhua*, genes related to immune response are overall affected by infections with *C. osculatum* (Marnis *et al.*, 2019). More specifically, expression of immune-related genes in *G. morhua* tends to be downregulated in the liver but upregulated in the spleen, suggestively due to local immune suppression in the liver caused by *C. osculatum* (Marnis *et al.*, 2020). Taken together, we argue that the significant decrease in the albumin to globulin ratio (caused by concomitant changes in plasma albumin and gamma globulin) that occurs with increasing *C. osculatum* infection reveals that highly infected *G. morhua* suffer from a chronic pathological liver condition. This in turn probably impairs the organ functionality, likely due to extensive tissue damage by *C. osculatum* (Fig. 1) (Buchmann and Mehrdana, 2016).

Many parasites impose an energetic cost to their host (Lester, 1971; Östlund-Nilsson *et al.*, 2005; Binning *et al.*, 2013); yet, we saw reduced standard metabolic rate in heavily infected fish. Because standard metabolic rate represents the summated energy expenditure *in vivo*, pinpointing the specific cause for this with certainty is not possible. However, as the site of infection of this particular parasite is the liver, and because the specific site of infection often determines how the parasite affect its hosts (Lafferty and Shaw, 2013), we suggest that a main cause of the reduced standard metabolic rate is an impaired functionality of this accessory digestive organ, leading to a compromised digestive system. This would result in reduced efficiency in protein turnover, reflected in a loss of body protein, low plasma total protein and albumin, and decrease in body energy content and a shift in the composition of the body energy in fish as seen in the present study for *G. morhua* with high infection densities. Furthermore, as a large proportion of the maintenance costs are directed towards internal organs (Hulbert and Else, 2000). The observed decreased mass of intestinal tissue and pyloric caeca in highly infected fish may also partly explain the reduced standard metabolic rate. It is nevertheless important to note that the present results are derived from wild, naturally infected fish, with unknown feeding histories, potentially including periods of starvation. Starvation elicits a cascade of physiological responses, and many ectotherms (including fishes) and endotherms have been reported to reduce the mass of their gastrointestinal tissues to reduce energetic demands during starvation (McCue, 2010; Day *et al.*, 2014).

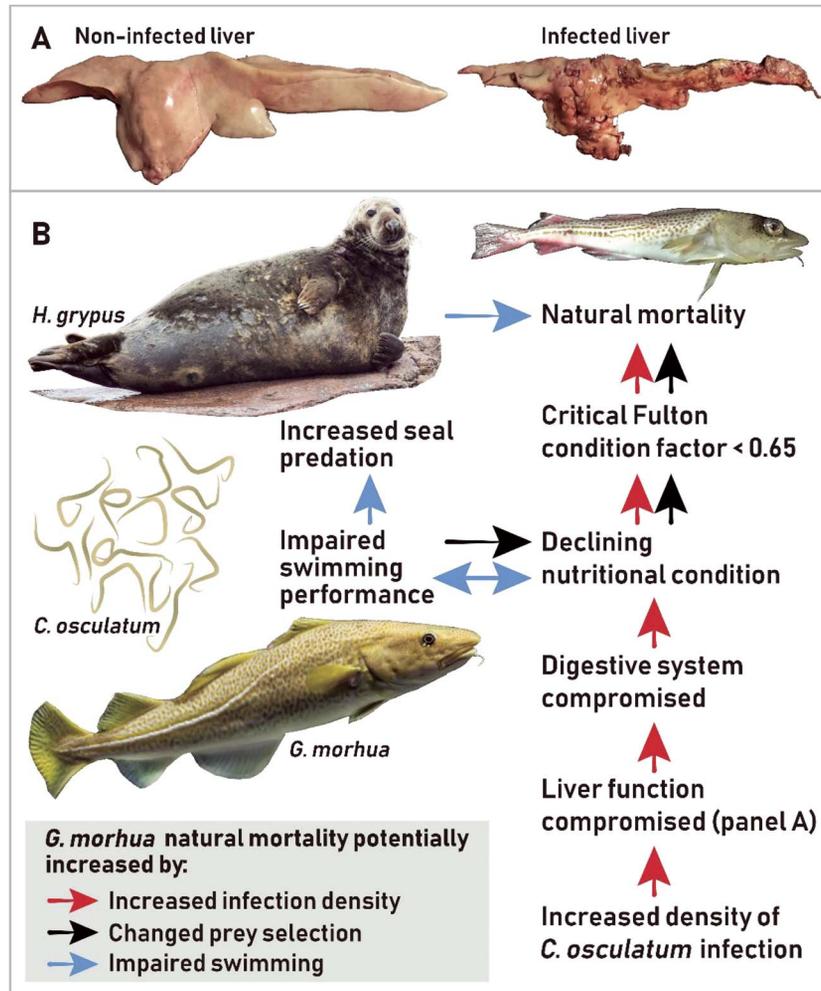


Figure 7: Schematic overview that summarizes possible mechanisms that suggestively can lead to increased natural mortality of *G. morhua* with high infection densities of *C. osculatum*, a parasitic nematode that infects the liver of the fish. Infections are associated with compromised function of the liver and digestive system that can lead to declining nutritional condition (red arrows) and potentially impaired swimming performance resulting in augmented susceptibility towards predation by seal (blue arrows) and changes in prey selection (black arrows). All mechanisms may lead to an increase in natural mortality. The critical Fulton condition factor where cod are considered dying is defined in Casini *et al.*, 2016a based on findings of Dutil and Lambert, 2000

Furthermore, during starvation, *G. morhua* initially exhausts its hepatic lipid and glycogen storage, and muscle glycogen, followed by mobilization of muscle protein (substituted by water) (Black and Love, 1986; Navarro and Gutiérrez, 1995). Thus, the reduced protein and lipid contents observed in the present study on *G. morhua* with high infection densities in some aspects resemble a starvation response. Yet, one aspect differs markedly; fish that have starved long enough to deplete their hepatic lipid and muscle glycogen resources rely on muscle protein as the main source of energy (Navarro and Gutiérrez, 1995), whereas in the present study, glycogen energy was found to constitute the main proportion of the fish energy source for the fish with the highest infection intensities. Based on this, it seems plausible that high infection densities, and

not starvation, are the main driver of the observed changes in body composition and preferred substrate utilization by the fish in the present study.

Although the present results reveal major associations between infections with *C. osculatum* and the physiological condition of *G. morhua*, the causality is unclear, and we are still, to some extent, dealing with ‘the chicken or the egg’ dilemma—especially in relation to the strong negative association between the Fulton condition factor and the increasing infection density. In various taxa including fish, the nutritional state of an individual is recognized to impair immune function and thereby increasing the risk of being infected by a pathogen (Gulland, 1992; Chandra, 1997; Johansen *et al.*, 1997;

Oliva-Teles, 2012). A gradual decrease in the nutritional condition of Eastern Baltic *G. morhua* has occurred since the 1990s, in all likelihood caused by reduced quantity and quality of prey in combination with deteriorating oxygen conditions (Plambech *et al.*, 2013; Eero *et al.*, 2015; Casini *et al.*, 2016b; Neuenfeldt *et al.*, 2020). In an already compromised nutritional state, *G. morhua* may be more susceptible to infection with *C. osculatum*. Notably, and irrespectively of potential causalities, history seems to repeat itself; in the late 1940s, the number of *H. grypus* (i.e. the main final host of *C. osculatum*) in the Baltic Sea was comparable to present days (Harding *et al.*, 2007), and at that time, *G. morhua* as now suffered high *C. osculatum* infection rates, with liver lipid content being highly comparable to our study when comparing parasite-free livers with livers infected with *C. osculatum* (Petrushevsky and Shulman, 1955). Furthermore, as in the present study, *G. morhua* infected with *C. osculatum* also had a lower condition as compared with uninfected conspecifics.

Although admittedly speculative, we suggest infections to lead to augmented mortality for the most heavily infected fish (Fig. 7), as also suggested by Horbowy *et al.*, 2016. Natural mortality increases for cod in critical condition (Dutil and Lambert, 2000), and the observed very low nutritional condition (Fulton condition factor) combined with low lipid levels in the liver in heavily infected *G. morhua* may thus have fatal consequences for the individual. One could speculate that heavily infected fish exhibit impaired swimming performance where predation on *S. sprattus* may become increasingly challenging, contributing further to the negative association between high infection load and low nutritional condition (Fig. 7). Likewise, cod in poor condition exhibit reduced swimming endurance and cannot achieve as high swimming speeds as more well-conditioned conspecifics (Martínez *et al.*, 2003). Ultimately, this may lead to increased susceptibility towards predation, for example, by the end host of the parasite (Fig. 7).

To fulfil its life cycle, *C. osculatum* depends on its main final host, the oceanic-living mammal *H. grypus*. As such, *H. grypus* not only has the role as a top predator in the Baltic Sea ecosystem, but this marine mammal is also important in structuring part of the community and disease dynamics of *G. morhua* by introducing and maintaining the parasite burden of *C. osculatum*. Oceanic-living mammals are final hosts to all the major groups of parasites in the oceans, many possessing complex life cycles with several intermittent hosts, including invertebrates and fish (McClelland *et al.*, 1990). Although marine mammals during periods of time in history have suffered from intense hunting and humans have depleted their populations, in the 20th century, a shift from resource exploitation towards wildlife conservation resulted in the recoveries of many of these populations (Magera *et al.*, 2013). The worldwide occurrence of these marine mammals thus stresses the need of further investigations of potential

influence of their parasite fauna on community dynamics, not least in relation to the rebuilding of deteriorating fish populations.

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Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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Chapter III

Paper II: Linking physiological mechanisms and growth processes: using bioenergetics modelling to study growth rate of Eastern Baltic cod, *Gadus morhua*, infected with a parasitic nematode, *Contracaecum osculatum*

Title: Linking physiological mechanisms and growth processes: using bioenergetics modelling to study growth rate of Eastern Baltic cod, *Gadus morhua*, infected with a parasitic nematode, *Contracaecum osculatum*

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Abstract

Fish stocks are facing increasing pressures from stressors related to human activities and climate change, which can affect reproduction, growth and mortality, leading to changes in stock productivity. Yet, the magnitude and directionality of these effects can be difficult to predict. Physiology has been defined as the transfer function that links environmental conditions to changes in behaviour and fitness in fish stocks, and the effects of temperature, oxygen and salinity on the metabolism of fish have been the focus of numerous experimental studies, whereas effects of parasites have been largely ignored. This is despite that parasites can affect host physiology, behaviour, reproduction or morphology. Individual-level findings are however difficult to scale to the level of populations. One way to solve this is the use of modelling, in particular bioenergetics models. Building on an established bioenergetics model, we for the first time examine the potential effects of parasites on growth rate and body condition of a wild fish stock. As a case study, we use the host-parasite system of Eastern Baltic cod infected with the parasitic nematode *Contracaecum osculatum*. The Eastern Baltic cod is in distress suffering from poor individual growth and nutritional conditions, high natural mortality and low productivity. Fish in this stock have during the latest decade experienced increasing prevalence and intensity of infection with *C. osculatum*, and all larger cod are now heavily infected. We found that growth rate and body condition decrease with increased infection density and that highly infected cod reach a point of no return where their energy intake gets too low to maintain a surplus energy balance that may eventually lead to starvation-induced mortality. In conclusion, we argue that parasite infections should not be ignored when assessing drivers of fish stock productivity, and we here provide a tool – a mechanistically based bioenergetics model – that across host-parasite systems can be used to estimate population-level impacts of parasites.

1. Introduction

Marine ecosystems and fish stocks are facing increasing pressures from stressors related to human activities and climate change (Dahlke et al., 2020; Halpern et al., 2008; Hing et al., 2016; Metcalfe et al., 2012). Effects of these stressors on vital rates such as reproduction, growth and mortality can lead to changes in stock productivity, but this can be very difficult to assess and predict (Halpern *et al.*, 2008; Watson *et al.*, 2020). Correlations in field survey data can identify important relationships of the dynamics, but they do not identify the mechanisms behind (Krohn *et al.*, 1997; Sokolova *et al.*, 2018).

Physiology has been defined as the transfer function that links environmental conditions to behaviour and fitness in fish stock because physiological processes (e.g. metabolism, enzyme activity, digestion and cardiovascular activity) respond to changes in the environment (Ricklefs and Wikelski, 2002; Young *et al.*, 2006; Horodysky *et al.*, 2015). Consequently, a mechanistic understanding of physiological processes is essential to our understanding of the diversification of life histories in fish stocks (Ricklefs & Wikelski, 2002). There is therefore a need to address questions of physiological responses at the level of the individual and scale this to the level of the population in order to determine fish stock responses to exploitation and changes in the environment (Horodysky et al., 2015; Metcalfe et al., 2012; Young et al., 2006).

The effects of abiotic factors like temperature, oxygen and salinity on the metabolism of fish have been the focus of numerous experimental studies (e.g. Clark et al., 2013; Metcalfe et al., 2016; Rogers et al., 2016). Such individual-level findings are, however, difficult to scale to the level of populations (Metcalfe et al., 2012). One way to solve the scaling issue is to use modelling, in particular bioenergetics models, which function as a theoretical tool for quantifying energy allocation in fishes under different environmental conditions (Holt and Jørgensen, 2015; Deslauriers *et al.*, 2017). These type of models can both be used to obtain a mechanistic understanding of physiological and energy requiring life-history processes (e.g. growth, reproduction and survival; Cooke et al., 2016; Deslauriers et al., 2017; Jørgensen et al., 2016; Rice, 1990) and reveal non-intuitive outcomes e.g. from combinations of stressors (Behrens *et al.*, 2018). Moreover, extension beyond pure bioenergetics has resulted in more holistic bioenergetics models that complement bioenergetic measurements with trade-offs often related to survival or reproduction of fish (Jørgensen et al., 2016; Jørgensen & Fiksen, 2006).

Parasites are the most common animal lifestyle and universal components of biological systems (Marcogliese, 2004; Kuris *et al.*, 2008; Lafferty *et al.*, 2008; Hatcher *et al.*, 2014). Per definition, parasites exploit their hosts for development and reproduction (Combes, 2001).

Across taxa, they may affect host physiology, behaviour, reproduction or morphology (Sánchez *et al.*, 2018; Timi and Poulin, 2020), with subsequent effects on food-web stability, interaction strength and energy flow in both terrestrial and aquatic ecosystems (Marcogliese, 2004; Lafferty *et al.*, 2008; Kuris *et al.*, 2008; Hatcher *et al.*, 2014). Yet, infection with parasites is often neglected when describing stressors in wild fish populations (Lloret *et al.*, 2012; Timi and Poulin, 2020) and have so far has been ignored in mechanistically founded bioenergetics models for fish.

Building on a previous bioenergetics modelling approach used to study growth and maturation of Northeast Arctic cod (Jørgensen & Fiksen, 2006), we here for the first time assess the effects of parasite load on the energy budget and subsequent growth potential of an infected wild host population. We use the host-parasite system between the Eastern Baltic cod stock and the parasitic nematode, *Contracaecum osculatum*, as a case study. The Eastern Baltic cod stock has suffered from poor individual growth and condition since the early 1990s and the stock is in distress, with historically low productivity (Casini *et al.*, 2016a; Eero *et al.*, 2015; Hüseyin *et al.*, 2018; Mion *et al.*, 2020). Furthermore, natural mortality is now estimated to be considerably higher than fishing mortality (ICES, 2020). Suggested drivers behind this trend are the historical fisheries in the 1980s that resulted in removal of larger fish (ICES, 2019a) and hypoxia-induced habitat compression where fish have to live and forage under suboptimal conditions, and, in addition, low feeding levels (Casini *et al.*, 2016; Neuenfeldt *et al.*, 2009; Neuenfeldt *et al.*, 2020; Plambech *et al.*, 2013). Notably, in the last decade Eastern Baltic cod has also suffered from increase in infections with *C. osculatum* (Haarder *et al.*, 2014; Nadolna and Podolska, 2014), a parasite to which cod is one of several transport hosts (Koie and Fagerholm, 1995). Cod gets infected following ingestion of smaller infected prey items such as e.g. sprat *Sprattus sprattus* (Zuo *et al.*, 2016; Nadolna-Ałtyn *et al.*, 2018) and the parasites accumulate in the cod liver with time, where older/larger cod typically have higher parasite load (Horbowy *et al.*, 2016; Zuo *et al.*, 2016). The liver is a vital organ being responsible for nutrient assimilation, bile production, maintenance of metabolic homeostasis and protein synthesis, and in gadoid species, such as cod, the liver is furthermore the main storage site of lipid, thus acting like a 'lunchbox' for the fish (Hinton *et al.*, 2017; Lambert & Dutil, 1997). Recent studies have revealed a negative association between parasite load and poor nutritional condition and impaired physiological condition of Eastern Baltic cod infected with *C. osculatum* (Horbowy *et al.*, 2016; Sokolova *et al.*, 2018; Ryberg *et al.*, 2020).

In the present study, we hypothesize that infection with parasites can be a stressor that can affect growth processes of fish. We tested this hypothesis by parameterizing our model with data from recent experimental work that reveal changes in physiological mechanisms (i.e. reduced standard metabolic rate, impaired liver function, disease status) of Eastern Baltic cod infected with *C. osculatum* (Ryberg *et al.*, 2020), and we validated the output against field observations. More specifically, we examined the quantitative loss in 1) growth rate and 2) body condition of cod in relation to different infection densities.

2. Method and material

2.1. Processes

In this model, the free energy is allocated to growth and/or energy storage the latter serving as energy for reproduction or metabolism for times where energy intake is low (Figure 1). The amount of energy stored over a growth season can be invested in irreversible somatic growth during the season or gonads at the end of the season. Changes in growth and/or the amount of energy stored can be reflected in changes of the state of the fish, such as length, weight and condition (Figure 1).

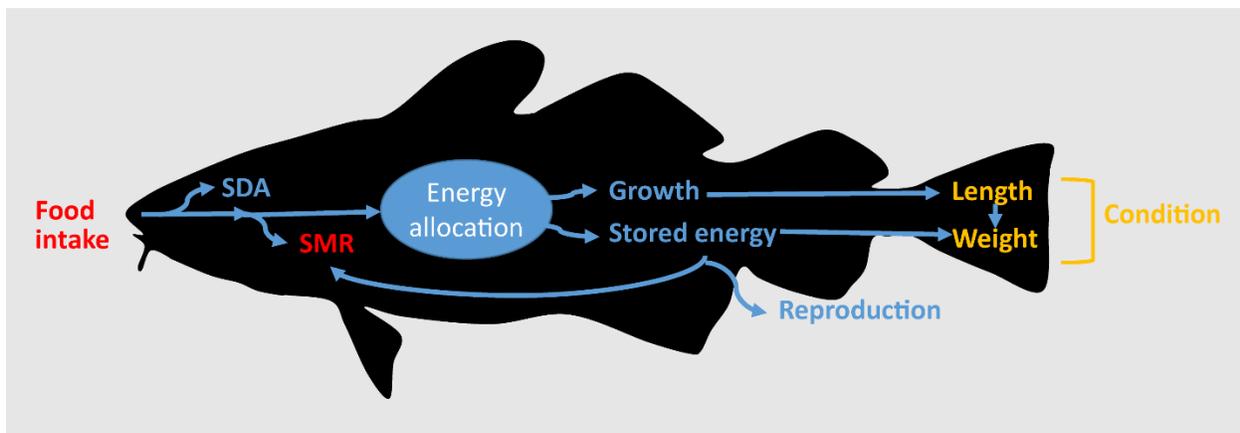


Figure 1. Schematic overview of the bioenergetic model examining growth and condition of fish infected with parasites. In the model the parasite causes a reduction of maintenance cost (standard metabolic rate: SMR) as well as in food intake (red colour) and hereby a reduction in energy intake. The standard dynamic action (SDA) is covering all energy related to processing of food for use and storage. When both SMR and SDA are covered energetically, the surplus of energy needs to be allocated toward different processes. In the present model the fish store its energy and use it on growth and/or stored energy where the latter can be allocated to reproduction and SMR depending on the energetic status of the fish (blue colour). Length, weight and condition are states of the outcome of the energy allocation (yellow colour).

2.2. Initialization

Four state variables (i.e. initial state of the fish to time 0 in the model) were defined; age, body length, Fulton condition factor and number of nematodes. Fish were introduced in the model at age 4 and with a body length of 30 cm based on age-length relationships estimated by Hüseyin *et al.* (2018) resulting in only adult fish in the models based on the reaction norm for Eastern Baltic cod (estimated by linear regression on maturity ogives at length and age; ICES, 2019b). This body length was chosen based on the previous studies revealing that the proportion of Eastern Baltic cod infected with *C. osculatum* increases rapidly above 30-35 cm (Zuo *et al.*, 2016). Start Fulton condition factor was set to 0.875 for a 30 cm cod, based on a model fit of Fulton condition factor in relation to length (Ryberg *et al.*, 2020) and initial number of nematodes was set to zero. Age-specific rates of mortality was not included in the model.

2.3 Model description and key parameters describing infection dynamics

The physiological energy budget of fish can be used to study changes in for example growth, condition and survival:

$$(1) \quad \text{Free energy} = \gamma * \text{energy}_{\text{intake}} - \text{metabolic}_{\text{cost}}$$

The free energy (kJ/time unit) available to somatic growth or energy storage depends on the energy intake and the food assimilation efficiency (γ) minus the metabolic cost associated with life processes (excluding the standard dynamic action $(1-\gamma)*\text{energy}_{\text{intake}}$, which has been deducted from the intake). In the present study, the basic structure of the model was adopted from Jørgensen and Fiksen (2006) who developed a state-dependent energy allocation model to study growth and maturation in the Northeast Arctic cod stock. The model was then adapted to account for specific biological knowledge including infections with parasites of the Eastern Baltic cod stock.

Key points of the present model were as follows; 1) medium complexity in relation to process representation, 2) Fulton condition factor was an emergent prediction from the model as the model was capable of simulating changes in condition, 3) the model only covered mature cod and followed the cod in yearly time steps for 8 years and 4) allocation of energy to growth and storage (u) was kept constant. The model was implemented in Python (Harris *et al.*, 2020; Python Software Foundation, 2020) and plots were done in R (R Core Team, 2016). Both trajectories patterns and trend lines were developed in the model.

Four new parameters were added to the model to represent infections with the parasitic nematode; α (nematodes/kJ), $metabolicINF_{tolerance}$, $metabolicINF_{reduc}$ and $intakeINF_{tolerance}$, where INF is the infection density of the cod. A basic assumption in this study is that the liver (and subsequent physiological) response to infection does not depend on the number of nematodes in the liver per se, but rather the number of nematodes per gram liver. Therefore, the auxiliary variable INF provided number of nematodes per gram liver tissue (E.q. 2):

$$(2) \quad INF = \frac{N}{L_w}$$

The actual liver weight (L_w) used for normalization was based on estimation of the minimal liver weight in relation to length ($H_0(L)$):

$$(3) \quad L_w = H_0(L) + \left(\frac{E}{\rho_E}\right)$$

Where E is the stored energy and ρ_E (kJ/g) is the average energy density for all energy stored. Individual cod with high INF are known to have reduced standard metabolic rate (i.e. maintenance costs) (Ryberg *et al.*, 2020). To implement this, a $metabolic_{factor}$ was defined and implemented in the function for the total $metabolic_{cost}$ (E.q. 4):

$$(4) \quad metabolic_{cost} = metabolic_{factor} * SMR_{rate}$$

Where SMR_{rate} reflects the standard metabolic rate of a healthy cod and the metabolic factor is defined as:

$$(5) \quad metabolic_{factor} = metabolicINF_{reduc} + (Act_{health} - metabolicINF_{reduc}) * \exp\left(-\frac{INF}{metabolicINF_{tolerance}}\right)$$

Act_{health} set the standard activity level (i.e. the energy related to activity) of a healthy cod and $metabolicINF_{reduc}$ represents the reductions in metabolic rate of severely infected individuals.

The metabolic $INF_{tolerance}$ reveals at what level of INF the standard metabolic rate of cod starts to be reduced. From a mechanistic point, the reduction in standard metabolic rate leaves more free energy (E.q. 1) available for the infected compared to the non-infected cod. Thus, many factors point in the opposite direction, that infected cod has less free energy available for e.g. growth and energy storage reflected by impaired physiological and nutritional condition and reduced liver function of Eastern Baltic cod infected with *C. osculatum* (Ryberg *et al.*, 2020) potentially causing lower energy intake and reduced assimilation efficiency. To reflect this in the model, the energy intake is reduced with increase in INF reflected by the $intake_{reduc}$ variable in equation 6:

$$(6) \quad energy_{intake} = satiation_{level} * eat_{lim} * intake_{reduc}$$

Satiation level reflects the feeding level in relation to ad libitum intake, eat_{lim} the maximum intake (kJ/year) of a healthy cod and $intake_{reduc}$ depends on INF:

$$(7) \quad Intake_{reduc} = \exp\left(-\frac{INF}{intake_{INF_{tolerance}}}\right)$$

The $intake_{INF_{tolerance}}$ reveals at what level of INF the energy intake of cod starts to be reduced. The changes in number of nematodes (N) over time is depending on nematodes per kJ (α) and the $energy_{intake}$:

$$(8) \quad \frac{dN}{dt} = \alpha * energy_{intake}$$

Since $energy_{intake} \geq 0$, the number of nematodes in the cod liver cannot decrease, whereas the liver weight (E.q. 3) can fluctuate above the minimum organ size.

2.4. Parametrization of key parameters describing infection dynamics

Due to the diverse diet of cod (Neuenfeldt *et al.*, 2020), it can be difficult to estimate α in the present model. Calculations of α based on data from literature on energy content of sprat (i.e. 5.5 kJ/g; Pedersen and Hislop, 2001), intensity of infection (i.e. number of nematodes per fish, only including infected individuals) of sprat from the Eastern Baltic Sea (i.e. 1.6 nematodes per fish; Zuo *et al.*, 2016) and average mean weight of a 18cm sprat (i.e. 10 gram; Casini *et al.*, 2006) revealed that one sprat on average contains 0.03 nematodes/kJ. However, validation of this α with previous data on number of nematodes with length of cod (Figure 2) revealed that this α value was too high and cod contained unrealistic number of nematodes in the model in relation to length. Therefore, parametrization of α was based on a sensitivity analysis resulting in $\alpha=8^{-4}$ nematodes/kJ, possibly reflecting a limited attack success of parasites present in cod prey (Appendix B, Figure S1, Table 1).

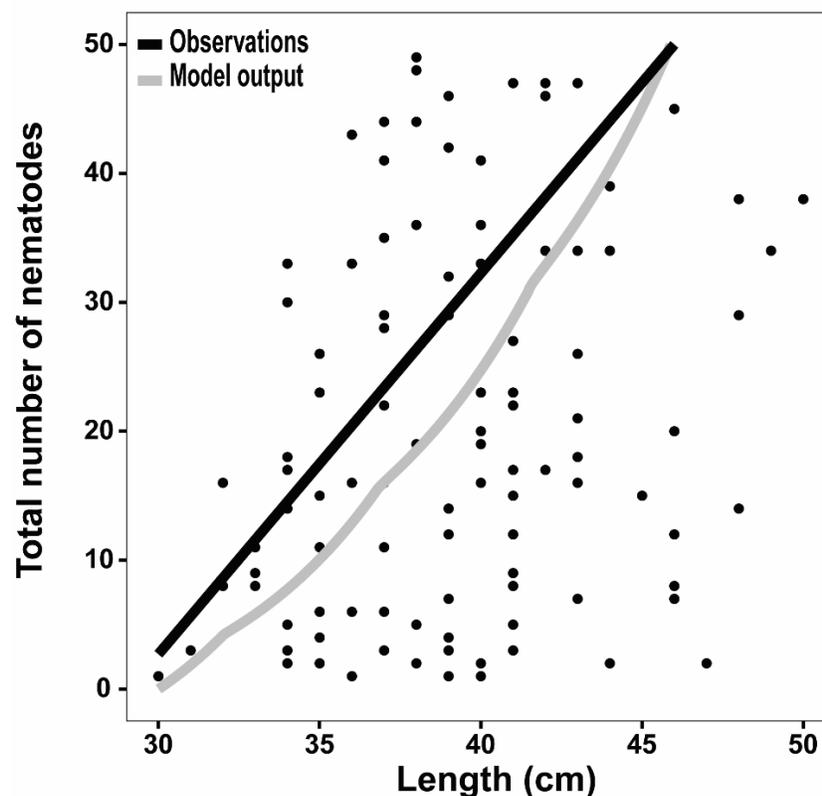


Figure 2. The parametrization of α (nematodes/kJ) was done by comparing the observed (black line, linear regression; $\alpha=2.95$, $SE=0.58$, $p<0.001$) and the modelled (grey line) total number of nematodes where the model was tested with different α values. The observed data is derived from a previous study (Ryberg *et al.*, 2020). The resulting α value was defined to 8^{-4} nematodes/kJ based on this comparison.

The metabolic $INF_{tolerance}$ is parametrized to 4 based on previous experimental work (Figure 3, Table 1, Ryberg *et al.*, 2020). At this level of INF , the variation in the standard metabolic rate of cod tend to be reduced and most individuals are found with a metabolic rate below the model fit (Figure 3).

Definition of the metabolic INF_{reduc} was based on the 23% reduction in standard metabolic rate when comparing highly infected with non-infected individuals (Figure 3, Table 1). It was assumed that the energy related to Act_{health} and standard metabolic rate was reduced with 23%. In a healthy cod at an ambient temperature of 5 °C the ACT_{health} is set to 1.25 (Hansson *et al.*, 1996). Thus, the reduction in energy related to activity was calculated by reducing 0.25 with 23%, where 0.25 reflects the increase in energy related to activity:

$$(9) \quad metabolicINF_{reduc} = 1.19 * 0.77 = 0.918$$

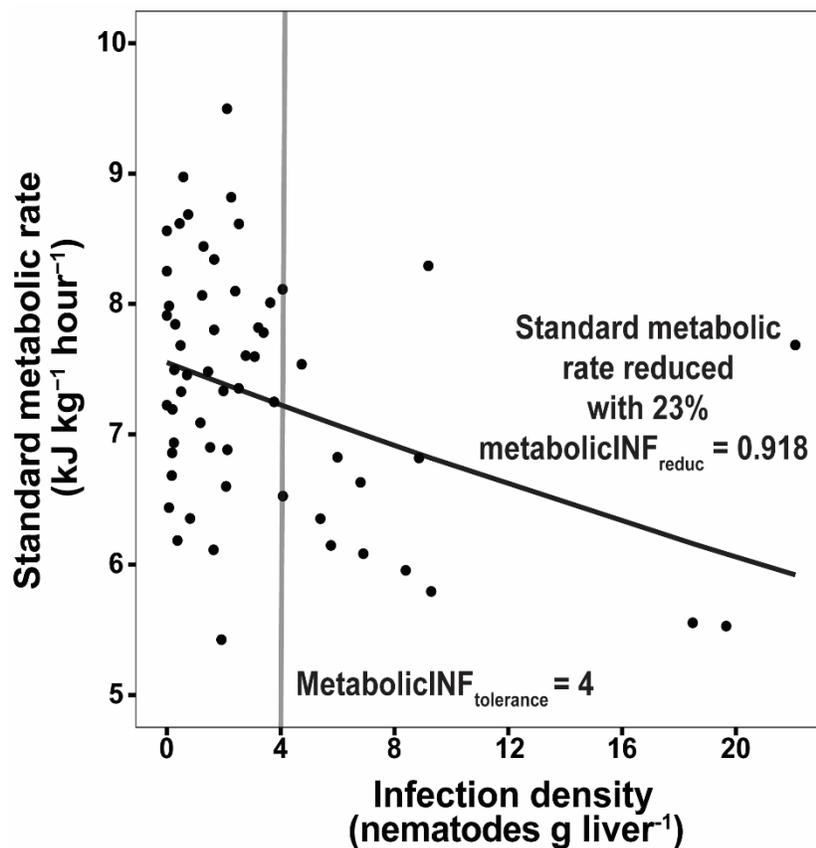


Figure 3. The standard metabolic rate in relation to different levels of infection densities. Data points represent individual cod and the black solid line illustrates the model fit of a previous analysis on this data (Ryberg *et al.*, 2020). In the present model, this data has been used to parametrize the metabolic $INF_{tolerance}$ (grey vertical line) and the metabolic INF_{reduc} (see E.q. 9)). The metabolic $INF_{tolerance}$ is defined to 4 nematodes/gram liver, indicating effects from the infections on the maintenance cost above this infection density level. Redrawn after Ryberg *et al.*, 2020.

The intake $INF_{tolerance}$ was parametrized based on data revealing a strong negative relationship between the nutritional condition and INF of Eastern Baltic cod (Ryberg *et al.*, submitted, paper III). Suggestively, the reduced nutritional condition must somehow reflect a decrease in food intake and intake $INF_{tolerance}$ was therefore defined to be 5 nematodes per gram liver (Figure 4, Table 1).

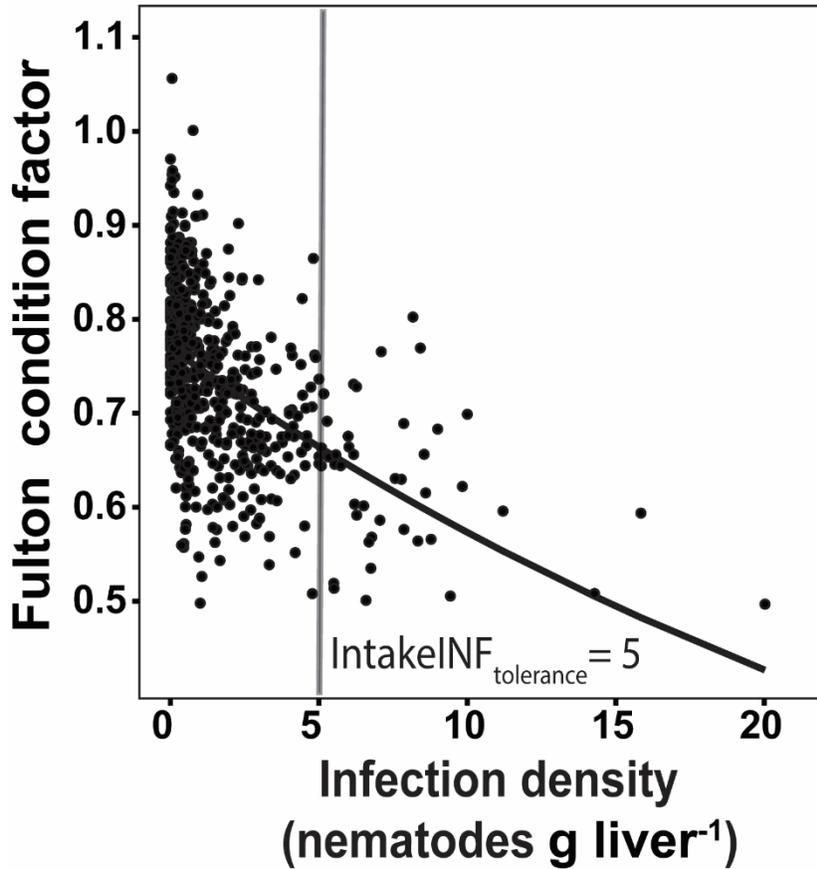


Figure 4. Fulton condition factor in relation to different levels of infection densities. Data points represent individual cod and the black solid line illustrates the model fit of a previous analysis on this association (Ryberg *et al.*, submitted, paper III). In the present model, this data has been used to parametrize the intakeINF_{tolerance} (grey vertical line) defined to 5 nematodes/gram liver, indicating reduction in the energy intake above this infection density level. Redrawn after Ryberg *et al.*, submitted, paper III.

2.5. Parametrization of parameters for Eastern Baltic cod stock

The allometric scaling between body mass and length (ϵ), the maturation reaction norm (mat_{reac}), fisheries mortalities ($F_{\text{feeder \& spawner}}$), natural mortality (M_{natural}), mortality related to spawning (M_{spawn}) and temperature (Temp) were all parametrized based on data derived from scientific surveys (Margit Eero, pers. communication, 2018; Table 1). The minimum condition factor (K_{min}) reflects the minimum body mass required for structures (i.e. below this level the fish dies) and is based on definitions made by Casini *et al.* (2016) and experimental findings by Dutil and Lambert (2000). On the other hand the maximum condition factor (K_{max}) reflects the anatomic limitation and the need to maintain other body functions (Jørgensen and Fiksen, 2006). K_{max} was reduced from 1.25 to 1.22 in the present study to meet the current condition of Eastern Baltic cod. Furthermore, the migration distance (D_m) for Eastern Baltic cod is expected to be shorter compared to Northeastern Arctic cod (Table 1). Historic (H_{sat}) and recent (R_{sat}) satiation levels were parametrized based on two feeding regimes by Neuenfeldt *et al.* (2020). Finally, data from 828 post-spawned non-infected Western Baltic cod caught between 1996-2018 was used to estimate the minimal liver weight in relation to length ($H_0(L)$) by using a power law function (ICES, 2019b). The length estimate for the minimal liver weight was 3.71 for the Western Baltic cod stock. However, using this estimate in the bioenergetics model resulted in unrealistic high liver weights of Eastern Baltic cod (compared to observations). The length estimate for the minimal liver weight was therefore reduced to 3.4 based on comparison between observed liver weights and modelled liver weights (Table 1). Field data from the Eastern Baltic cod stock from 2010-2020 was used to compare observations on weight-at-length with weight-at-length derived from the bioenergetics model (ICES, 2019b).

Table 1. Parameters adapted and implemented in the present bioenergetic model to meet the biological information of Eastern Baltic cod including parasites.

Parameter	Value and unit	Biological interpretation	Reference
ε	0.03879	Excess value of allometric scaling between body mass	ICES, 2019b
K_{\min}	0.65 g/cm ³	Minimum condition factor at standard length L_{std}	Casini <i>et al.</i> , 2016; Dutil and Lambert, 2000
K_{\max}	1.22 g/cm ³	Maximum condition factor at standard length L_{std}	Own estimate
D_m	40*10 ⁵ cm	Distance for spawning migration	Own estimate
U_{current}	0.0 cm/year	Current during spawning migration	Own estimate
Mat_{reac}	1.3754*age + 0.1194*length - 8.146 = 0	Reaction norm	ICES, 2019b
$Spawn_{\min}$	50 %	Percentage of energy store for spawning	Own estimate
F_{feeder}	0.4	Fisheries mortality	M. Eero, pers. comm. 2018
F_{spawner}	0.4	Fisheries mortality	M. Eero, pers. comm. 2018
M_{natural}	0.5*(length/20) ^{-0.75}	Natural mortality	M. Eero, pers. comm. 2018
M_{spawn}	0.1*(length/20) ^{-0.75}	Increased mortality during spawning and migration	M. Eero, pers. comm. 2018
Temp	7.0 (C°)	Baltic reference temperature	M. Eero, pers. comm. 2018

Parameter	Value and unit	Biological interpretation	Reference
H_{sat}	$0.40 - 0.1 * (1.0 - \exp(\frac{\text{length} - 20.0}{30}))$	Historic satiation level	Neuenfeldt <i>et al.</i> , 2020
R_{sat}	$0.25 - 0.15 * (1.0 - \exp(\frac{\text{length} - 20.0}{30}))$	Recent satiation level	Neuenfeldt <i>et al.</i> , 2020
$H_0(L)$	$10^{-4.64 * \text{length}^{3.4}}$	Minimal liver weight in relation to length	Own estimate
α (nematodes/kJ)	8^{-4} nematodes/kJ	Nematodes per kJ	Own estimate
$\text{metabolicINF}_{\text{tolerance}}$	4 nematodes/gram liver	INF level where the metabolic cost starts to decrease	Own estimate
$\text{MetabolicINF}_{\text{reduc}}$	0.918	Reduction in metabolic cost of infected individuals	Own estimate
$\text{intakeINF}_{\text{tolerance}}$	5 nematodes/gram liver	INF level where energy intake starts to decrease	Own estimate

3. Results

3.1. The modelled growth rate and condition

Irrespectively of size/age of the cod, the modelled growth rate was lower in infected individuals compared to non-infected individuals. This difference in growth rate between non-infected and infected cod was most pronounced for the largest/oldest fish, the latter being driven by a more pronounced decline in growth rate of the larger, most heavily infected cod (Figure 5A). This was as a result of decreasing energy intake with increasing infection density as the fish become larger/older (Figure 5B). Furthermore, for fish below 45cm, the modelled energy intake was lower for fish under a historic satiation level as compared with fish under a more recent satiation level (Figure 5B). For the modelled growth rate and energy intake of infected fish, the four minimum spikes reflect the short periods directly after spawning, where infection density becomes very high due to the low liver size that results from energy allocated to spawning.

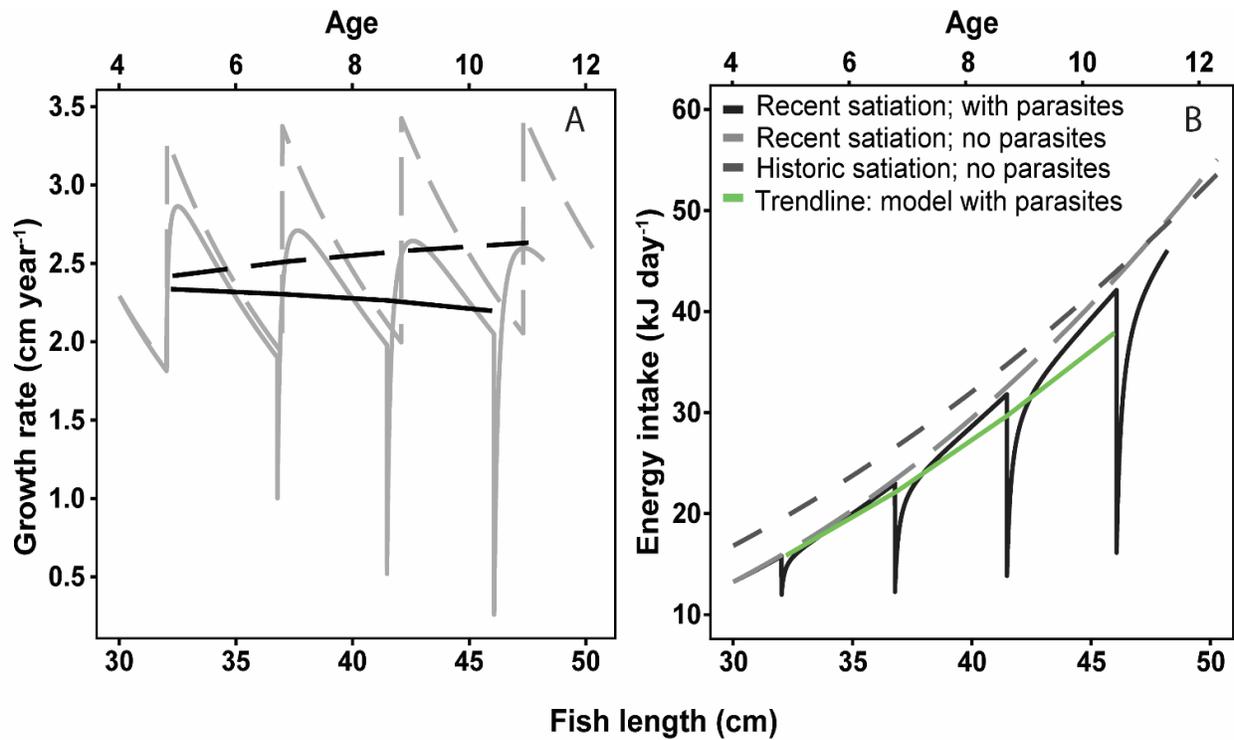


Figure 5. A) Trajectories patterns (grey lines) and overall trends (black lines) of yearly growth rate in individual non-infected cod (dashed lines) with α (nematodes/kJ)=0 and infected cod (solid lines) with $\alpha>0$, and overall trends of B) daily energy intake (kJ/day) in relation to length of infected Eastern Baltic cod. The four different lines reflect non-infected individuals with either recent satiation level (light-grey dashed line) or historic satiation level (dark-grey dashed line), and infected individuals with recent satiation level (black solid line) and overall trend (green solid line). The four minimum spikes in both panel A and B reflect the time after spawning where infection density (nematodes per gram liver) is very high due to the use of energy from the liver. The model output for food intake has been validated by comparison with intake levels with previous data from Neuenfeldt *et al.* (2020).

Fulton condition factor decreased with length in infected individuals, but remained constant with length in non-infected individuals (Figure 6). The maximum peaks of Fulton condition factor reflect that cod close to maturation build up their liver lipid reserves in preparation for spawning (Figure 6). As describe for figure 5, the four minimum spikes reflect the short periods directly after spawning, where infection density per definition is very high due to the low liver weight that results from energy allocated to spawning. Notably, if spawning occurs every year, a minimum spike (i.e. where liver resources are depleted and infections density become very high) is expected on a yearly basis for each of the individual trajectory patterns for growth rate, weight-at-length and condition as well as energy intake (only for infected individuals). However, irrespectively of the cod being infected or not, these minimum spikes only occurred on a biannual basis, suggesting that the modelled cod performed skipped spawning, thus only depleting liver reserves every second year (Figures 5 and 6).

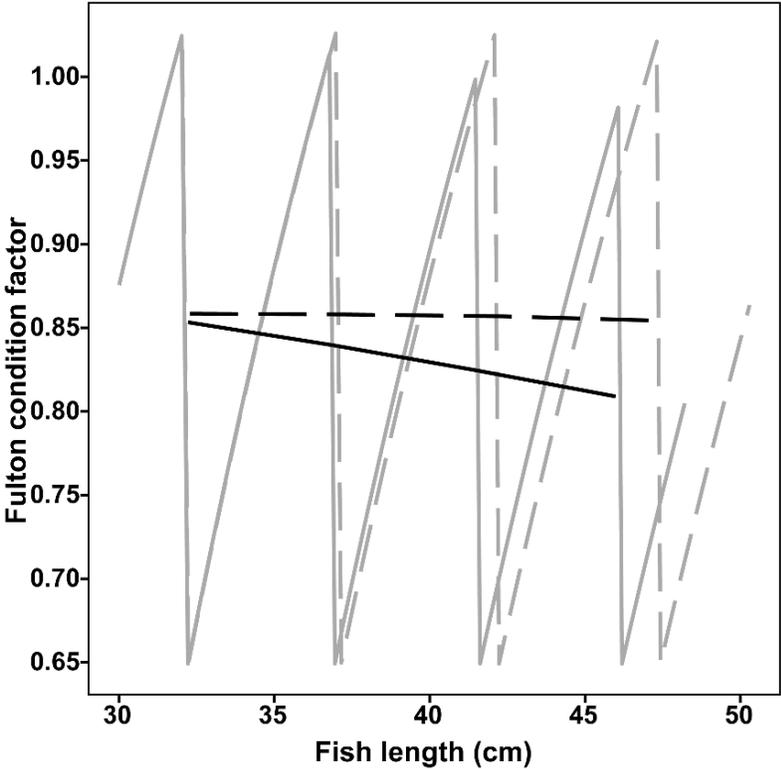


Figure 6. Trajectory patterns (grey lines) and overall trend (black lines) of Fulton condition factor in individual non-infected cod (dashed lines) with α (nematodes/kJ)=0 and infected cod (solid lines) with $\alpha > 0$ in relation to length. Start Fulton condition factor in the model was set to 0.875 (predicted condition for a 30 cm cod from a previous experimental study, Ryberg *et al.*, 2020).

3.2 The point of no return

In the present model, the standard metabolic rate (i.e. metabolic cost) and the energy intake were reduced with increasing infection density. This means that beyond a certain infection density (defined as the maximum infection level) at a given weight at length, the surplus energy balance (Eq. 1) becomes negative and the cod enters point of no return where the condition factor steadily declines, with no option of reversing this negative spiral (Figure 7). Because metabolism is weight-dependent, thin individuals have a higher tolerance level of maximum infection at a given length and weight. Notably however, it does prevent a downward spiral of a cod that passes the infection density limit in Figure 7. The minimum infection density level of the contour scale reflects a resilience level of infection density (<2 nematodes per gram liver weight) where infection load does not result in a negative energy output.

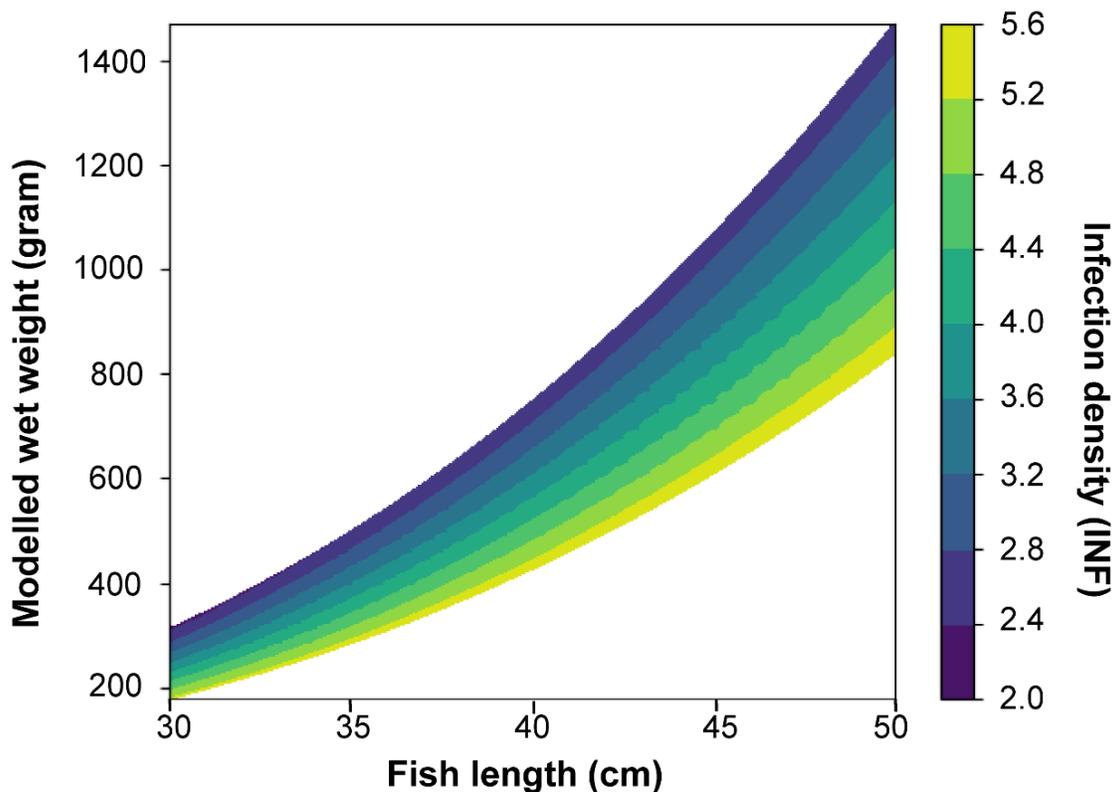


Figure 7. Infection level at given length and weight (contour scale) where the energy output becomes negative in the model and the cod enters a 'point of no return' with respect to nutritional condition, unable to grow in length because its surplus energy balance is negative.

3.3. Comparisons with field data

Modelled weight-at-length of Eastern Baltic cod compares well with field observations, both regarding the general trends (black lines) and the variations (grey lines and dots) (Figure 8A and B). Starting from approximately 40cm of length, infected cod reveal a higher weight at length, where an infected cod after 8 years of simulation only weigh ~790 gram as compared to 910 gram of a non-infected conspecific (Figure 8A).

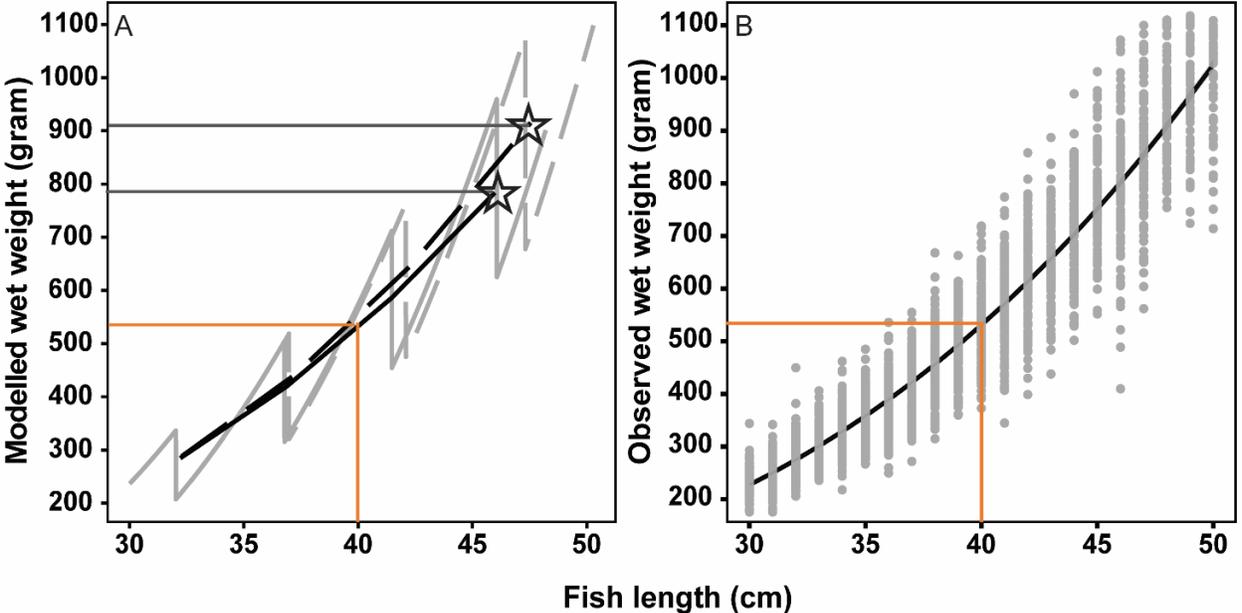


Figure 8. A) Trajectory patterns (grey lines), overall trends (black lines) and final modelled weights (grey solid horizontal lines and stars) of modelled wet-weight of individual non-infected cod ($\alpha=0$; dashed lines) and infected cod ($\alpha>0$; solid lines) in relation to fish length and B) predictions from model fit (power law relation) of observed weight-at-length (black solid line) based on the observations (grey dots) of weight-at-length for Eastern Baltic cod between 2010-2020. Comparison between modelled and observed wet weight for a 40cm cod is illustrated by the orange lines in A and B.

4. Discussion

With increasing pressures from anthropogenic disturbances and a changing climate, the disturbances to the aquatic environment are pronounced. Yet, the magnitude and directionality of the various stressors on wild populations, including fish, are difficult to assess. Such information is however imperative to achieve best possible management of our natural resources. Using Eastern Baltic cod infected with the parasitic nematode *C. osculatum* as a case study, we here present the first mechanistic bioenergetics model that includes the physiological consequences of parasite infection on growth and condition in a fish stock. When we implement a reduced standard metabolic rate and energy intake of infected cod in the model, we find that growth rate and body condition decrease with increased infection density and that the modelled weight and length are lower in infected compared to non-infected individuals, resulting in smaller infected fish after 8 years of simulation compared to their non-infected conspecifics. Notably also, in the present model cod with a certain infection density reach a point of no return where energy intake of infected individuals becomes so low, that maintaining a surplus energy balance is impossible and starvation-induced mortality is subsequently unavoidable. We believe that the model predictions of growth and condition patterns are reliable as 1) the weight-at-length generated by the model is consistent with monitoring data of weight-at-length from 2010 to 2020, 2) the energy intake of the model cod fits well with previous data on feeding levels (Neuenfeldt *et al.*, 2020) and 3) the parametrization of α (i.e. nematodes/kJ) in the model is consistent between the modelled and observed total number of nematodes in relation to cod length (Figure 2).

The findings of the present bioenergetics model are in agreement with recent findings showing that nutritional condition decreases with increase in infection load of infected Eastern Baltic cod (Horbowy *et al.*, 2016; Sokolova *et al.*, 2018; Ryberg *et al.*, 2020). Moreover, the reduction in growth rate and the smaller final size in the model of infected individuals found in the present model are supported by recent findings showing exceptionally poor growth of Eastern Baltic cod (Svedäng and Hornborg, 2014; Hüsey *et al.*, 2018; McQueen *et al.*, 2020). It has been suggested that the currently observed low feeding levels of young Eastern Baltic cod, together with mild hypoxia, induce severe growth limitations of the stock (Brander, 2020; Neuenfeldt *et al.*, 2020). Interestingly, there are clear inter-stock and inter-regional differences in growth between the Eastern and Western Baltic cod stocks, with individuals from the Western stock exhibiting a higher growth rate than individuals from the Eastern stock (McQueen *et al.*, 2020). This finding likely reflects that the current ecosystem conditions with pronounced hypoxic areas, poor prey quantity and quality and increase in prevalence and intensity of infections with *C. osculatum* are worse for Eastern Baltic cod than for the Western Baltic cod stock (Haarder *et al.*, 2014; Casini *et al.*, 2016a; Sokolova *et al.*, 2018; Neuenfeldt *et al.*, 2020).

Parameterization of models is not trivial and needs careful consideration. We assumed that energy intake was reduced with increased infection density. This assumption is based on previous findings on the physiological condition of Eastern Baltic cod infected with *C. osculatum* (Ryberg *et al.*, 2020). As for all higher taxa, the liver of the fish is a vital organ that carries out multiple functions, including maintaining whole body homeostasis (Rui, 2014), and especially two factors point towards impaired liver function of Eastern Baltic cod infected with *C. osculatum*: Firstly, encapsulation and migration of the nematodes in the liver destroys hepatocytes, most likely causing functional damage to the organ (Zuo *et al.*, 2017; Chondromatidou, 2020; Ryberg *et al.*, 2020) and, secondly Eastern Baltic cod with high infection loads show reduced body protein and liver lipid and increased body glycogen, revealing a loss of energy reserves (Ryberg *et al.*, 2020). Ultimately, increasing infection load is associated with decreased body condition (Horbowy *et al.*, 2016; Sokolova *et al.*, 2018; Ryberg *et al.*, 2020) and highly infected Eastern Baltic cod suffer from a severe liver disease (Ryberg *et al.*, 2020). In the present model, the reduction in energy intake in relation to infection density in the model was effectuated when infection density reached five nematodes per gram liver (i.e. $\text{intakeINF}_{\text{tolerance}}$), while the reduction in the standard metabolic rate was effectuated at four nematodes per gram liver ($\text{metabolicINF}_{\text{tolerance}}$). Both values were based on recent experimental data in Ryberg *et al.* (2020), where a series of investigations were performed to evaluate effects of *C. osculatum* on the health status of Eastern Baltic cod. The threshold tolerance level of the $\text{MetabolicINF}_{\text{tolerance}}$ was set lower than the $\text{intakeINF}_{\text{tolerance}}$ because infections with parasites first change physiological mechanisms or morphological features, which then subsequently change the performance of the infected individual (Binning *et al.*, 2017). Thus, infections with *C. osculatum* lead to changes in physiological mechanisms first (i.e. reduced standard metabolic rate, impaired liver function, disease status; Ryberg *et al.*, 2020) and secondly to changes in performance for example reduced swimming performance (which again may lead to reduced foraging success and hence decreased food intake) and impaired metabolism (likely causing malnutrition due to malabsorption of nutrients; Henkel and Buchman, 2006).

Growth, recruitment and survival constitutes the three main vital rates defining stock productivity (Hilborn and Walters, 1992). Seasonal changes in population growth, recruitment and survival rates of Eastern Baltic cod reveal a seasonal productivity pattern where critical condition of individuals after spawning is suggested to increase natural mortality (Mildenberger *et al.*, 2020). Low body condition was also found after spawning in the present study, associated with a sudden high infection density due to depletion of liver energy reserves. Rather interestingly, and somewhat initially to our surprise, the modelled individual cod exhibited skipped spawning every second year, irrespectively of being infected or not. This may reflect that cod in this stock at present have insufficient energy intake to meet the energy requirements of spawning (Rideout and Tomkiewicz,

2011). In support, compiling information from five decades of stomach data, Neuenfeldt *et al.* (2020) recently documented that present feeding levels are low as compared to previous decades, likely a result of decrease in benthic prey abundance due to increased hypoxic areas, with no possibility of compensatory shift in diet towards more pelagic species. Skipped spawning has likewise been shown in another bioenergetics model for North East Atlantic cod (Jørgensen *et al.*, 2006).

The choice of using infection density (nematodes per gram liver tissue) instead in infection intensity (total number of nematodes per liver) to parametrize the present model, is based on the believe that the liver (and the subsequent physiological) response to infection likely depends on the number of nematodes per unit of tissue mass, and not on the number of nematodes in the liver per se. The argument is that increase in infection density leads to increase in functional damage of the liver (as shown in Ryberg *et al.*, 2000), leaving the infected fish more vulnerable post-spawning where infection density is very high, due to energy-allocation from the liver to gonadal build-up (Marshall *et al.*, 1999). Mortality wise this period may thus be especially critical for individuals with high infection densities. Moreover, poor body condition is known to reduce fecundity, and induce skipped spawning and increase in natural mortality (Dutil and Lambert, 2000; Lambert and Dutil, 2000; Rideout and Tomkiewicz, 2011; Mion *et al.*, 2018). Thus, scaling the present results on individual level to population level suggest that both growth, recruitment and mortality rates in the Eastern Baltic cod stock are affected by high infection densities with *C. osculatum*.

In conclusion, building on established bioenergetics concepts, we show that a broadly applicable modelling approach can be used as a complement to laboratory and field investigations to investigate effects of parasites on vital parameters driving fish stock productivity. It hence contribute to understanding of an often overlook driver of wild fish stocks (and wild animals in general), namely parasites. Furthermore, the present modelling framework offers a platform to incorporate candidate drivers of growth and condition simultaneously, for example temperature, hypoxia, prey availability and energy composition of the prey, in order to understand their partial and synergistic effects. With such information at hand, the most likely scenarios of stock development can be predicted, which for the Eastern Baltic cod stock in particular, will enable fisheries scientists to evaluate how the poor situation of the cod stock could possibly be improved by ecosystem based management measures.

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Chapter IV

Paper III: Parasite load of Eastern Baltic cod, *Gadus morhua*, assessed by the liver category method, and the association between infection density and critical condition

Title: Parasite load of Eastern Baltic cod, *Gadus morhua*, assessed by the liver category method and the association between infection density and critical condition

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Abstract

Parasites may affect fish physiology and behaviour, influencing stock dynamics and productivity. Yet parasite occurrence is rarely part of routine samplings on monitoring surveys of wild fish stocks, likely because collection of such data requires expert knowledge, is cumbersome and difficult to conduct board. One way to overcome this is to collect less ambitious but robust proxies of parasite loads. Livers of Eastern Baltic cod *Gadus morhua* presently have high infection loads with the parasitic nematode *Contracaecum osculatum*, with negative effects on the health status of infected fish. From 2021, it is mandatory on monitoring surveys to assign a 'liver category' to individual livers, based on the number of nematodes visible on the surface. Although very useful as a proxy to follow the spatiotemporal development of infections, the liver category overlooks nematodes hidden inside the liver and calculated infection densities (number of nematodes per gram liver) will be underestimated. Because infection density associates with reduced condition and health status of the infected cod, it is important to get a more precise number of total parasites in individual livers. We here present a tool for estimation of the total number of nematodes in livers, and test the are-specific robustness of the liver category method based on liver categorical data and counted total number of nematodes from 642 cod livers sampled from ICES subdivisions 22, 24 and 25. Furthermore, to reveal the importance of estimating infection density, we examined the probability of cod having a critical Fulton condition factor below 0.65 depending on infection density. We found that the liver category is a good predictor of total number of nematodes, and that the probability of cod having a critical condition increased with infection density. The present results may inspire peers working with disease in other fish stocks to include monitoring of parasites.

Keywords:

Contracaecum osculatum, Baltic Sea, disease monitoring, Fulton condition factor, liver worm, natural mortality

1. Introduction

Assessment of fish stocks is based on a series of input data such as numbers and/or biomass of fish, natural and fishing mortality, growth and recruitment. These data are classically derived from monitoring surveys and used for evaluation of the present status and prediction of the future development of exploited fish stock (Jennings *et al.*, 2001). Despite that parasites can affect their hosts ranging from the level of the individual to the level of population (Timi and Poulin, 2020), fish health indicators such as parasite occurrence are usually not part of the routine sampling on monitoring surveys (Lloret *et al.*, 2012). This exclusion may be due to that collection of such data requires expert knowledge, is often time-consuming, cumbersome, difficult to conduct on board, and expensive. One way to overcome this discrepancy is to collect less ambitious but robust proxies of parasite loads on board of standard surveys.

The Eastern Baltic cod, *Gadus morhua*, stock a key species in the Baltic Sea ecosystem and fisheries, represents an emerging case where information on infections with parasitic nematodes in the liver may assist in improving stock assessment, scientific advice and management. Fish in this stock are in a historically low nutritional condition, have reduced individual growth and low productivity, and natural mortality are in later years estimated more than three times higher than fishing mortality (Eero *et al.*, 2015; Sokolova *et al.*, 2018; Mion *et al.*, 2020). Several ecosystem changes such as deteriorating oxygen conditions and reduced quality and quantity of prey has been suggested to contribute to the poor state of the Eastern Baltic cod (Plambech *et al.*, 2013; Eero *et al.*, 2015; Casini *et al.*, 2016b; Neuenfeldt *et al.*, 2020). During the latest decade fish in this stock have also experienced a marked increase in infections with a parasitic nematode, *Contracaecum osculatum*, which parasitizes the liver of cod, and almost all larger cod in the central and eastern Baltic are now infected (Haarder *et al.*, 2014; Nadolna and Podolska, 2014; Horbowy *et al.*, 2016). Yet, so far infection levels remain low in the more Westerly stocks (Sokolova *et al.*, 2018). The parasite may negatively affect the condition of the fish as field observations reveal that cod with many parasitic nematodes in the liver have lower body condition than conspecifics with no or few of these parasites (Horbowy *et al.*, 2016; Sokolova *et al.*, 2018; Ryberg *et al.*, 2020). Low body condition can lead to increased natural mortality, reduced fecundity and skipped spawning hereby affecting stock productivity (Dutil and Lambert, 2000; Gislason *et al.*, 2010; Casini *et al.*, 2016a; Mion *et al.*, 2018).

Cod become infected with *C. osculatum* when ingesting smaller infected fish, such as sprat, *Sprattus sprattus*, herring, *Clupea harengus*, or great sandeel, *Hyperoplus lanceolatus* (Valtonen *et al.*, 1988; Rodjuk, 2014; Zuo *et al.*, 2016; Nadolna *et al.*, 2017, 2018). Grey seal, *Halichoerus grypus*, is the main final host of *C. osculatum* in the Baltic Sea, whereas cod is one of several transport hosts (Koie and Fagerholm, 1995).

Abundance of grey seal has increased markedly in recent years, now counting approximately 38,000 animals (status 2019) compared to a few thousands in the 1980s (Harding *et al.*, 2007; HELCOM, 2018; ICES, 2020). This trend has coincided with a marked increase in infections with *C. osculatum* in cod in the central and Eastern Baltic, with 90-100% of the individuals above 35 centimetre in ICES subdivision (SD) 25 and 26 being infected during recent years. Yet, so far infection levels remain low in the more Westerly cod stocks (Sokolova *et al.*, 2018).

Poland made the first detailed determinations of number of *C. osculatum* in individual cod livers (National Marine Fisheries Research Institute) in 2010 (Nadolna and Podolska, 2014), followed by Denmark in 2012 (Haarder *et al.*, 2014). However, such detailed investigations where all individual nematodes are identified and counted are complex, expensive and time-consuming. As a supplement, a few Baltic countries have during recent years on a voluntary basis on monitoring surveys (e.g. the German and Danish Baltic International Trawl Survey) implemented a more easily accessible way to follow the spatiotemporal development in infection levels in cod, the so-called 'liver category' method (Figure 1).

Using a scale from 0 to 4, the liver category method assigns a category to each inspected liver, depending on the number of visible nematodes on the surface of the organ. This approach requires only basic training of staff and is inexpensive, easy and fast to implement. From 2021, the Baltic International Fish Survey Working Group (WGBIFS) has made it a mandatory part of the standard Baltic International Trawl Survey protocol for participating countries to assign a liver category to all individual livers. Integration of the spatiotemporal development of *C. osculatum* infection loads in Baltic cod livers in assessment may thus improve the biological realism of explaining the causes of the deterioration of this stock, and hence improve management advice. Yet, the robustness of the liver category method has never been evaluated. This is however imperative to assure that managers and scientist obtain a reliable proxy of the spatio and temporal development in prevalence and abundance of the infections. Although some nematodes are visible on the surface of the liver, several specimens usually reside inside the liver parenchyma and are not visible (Nadolna and Podolska, 2014). Therefore, one obvious limitation of the liver category method is that it likely underestimates the true number of nematodes in the liver, the actual discrepancy being determined by the size and shape of the organ. A more precise estimate of the true number of nematodes in livers will enable us to evaluate potential health effect on the infected individual, as high infection density (i.e. number of nematodes per gram liver tissue) associates with impaired physiological condition of the fish (Ryberg *et al.*, 2020). It is therefore imperative to develop a tool that predicts the total number of nematodes based on the assigned liver category, and evaluate the area-specific strength of this tool.



Figure 1. Photo of the five liver categories (0-4) of nematode infection levels of cod livers used in the Baltic Sea. A liver category scale is assigned according the number of nematodes counted on the surface of the liver and the categorical boundaries given by the scale. Photo by Bastian Huwer.

Using data from the main contemporary distribution areas of Baltic cod (ICES SD22, 24 and 25) (Figure 2), our main objectives were to 1) develop an estimation tool that converts liver categorical data obtained from inspections of cod liver surfaces into estimates of total number of nematodes, 2) assess the area-specific robustness of the liver category method, and 3) examine the probability of cod having a critical Fulton condition factor below 0.65 depending on infection density.

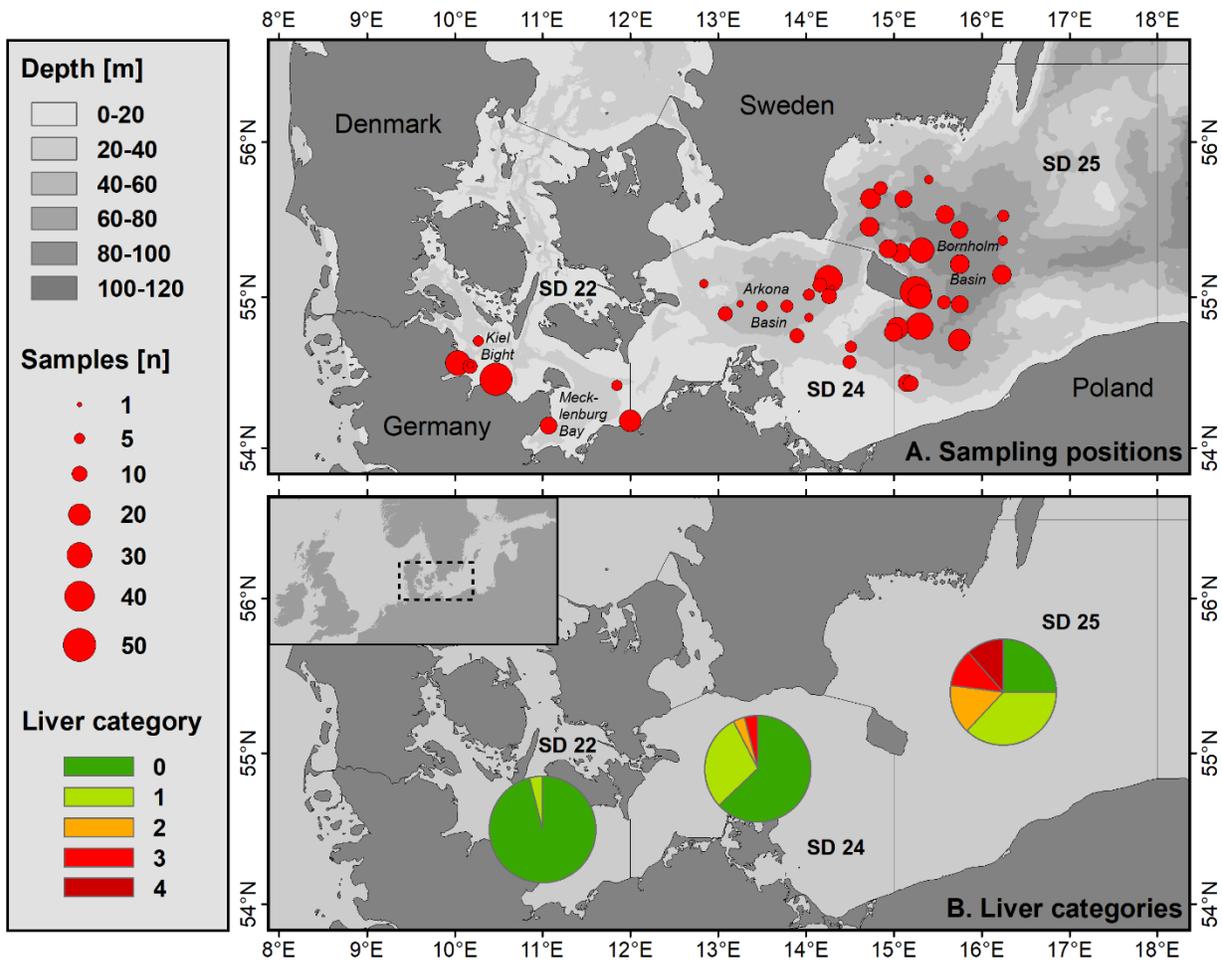


Figure 2. A) Study area and sampling positions within the three ICES subdivisions where cod *Gadus morhua* were collected for analysis of liver nematodes. Grey scale provides information of water depth and size of the bubbles represents number of samples examined from each position. B) Percentage of the total number of livers assigned to each liver category by subdivision.

2. Material and method

2.1 Study areas and fish collection

Altogether 642 cod, *Gadus morhua*, from SD 22 (Kiel Bight and Mecklenburg Bay – western Baltic), SD24 (Arkona Basin) and SD25 (Bornholm Basin) were used in the analysis (Figure 2A, Table 1). Cod were caught by trawling in SD24 and SD25 and by trawling, gillnets and trammel nets in SD22 (Table 1). All fish were processed fresh (without freezing or other conservation) either (i) directly on board, or (ii) within a few hours after capture and transported on ice to the laboratory, and total length, wet weight of whole fish and of livers, gutted weight and gender recorded for each fish. To cover the length distribution commonly caught in monitoring cruises and to assure variability in infection load of fish, individuals ranging from 20-58 cm were included in the study (Table 1).

Table 1. Overview of 642 cod sampled between 2017-2020 from ICES subdivisions (SD) 22, 24 and 25 for the analysis of infections with liver nematodes (see also Figure 2 for sampling positions and numbers). Values are mean \pm SE and brackets represent range of data. TL= total length; W= total wet weight; GW=gutted wet weight; LW= wet-weight of liver; HSI = hepatosomatic index; Prev. = prevalence: percentage of infected fish in the sample; Intensity: mean number of counted parasites per fish, only including infected individuals.

SD	# livers	TL (cm)	W (g)	GW (g)	LW (g)	HSI	Prev. (%)	Intensity
22 [†]	132	44 \pm 0.5 (28-71)	910 \pm 31 (190-2972)	827 \pm 30 (178-2751)	23 \pm 1.5 (2-87)	2.7 \pm 0.1 (0.4-6.8)	13	2 \pm 0.5 (0-10)
24 [‡]	115	39 \pm 0.4 (35-50)	562 \pm 18 (300-1450)	481 \pm 15 (270-1212)	23 \pm 1.3 (3-72)	4.6 \pm 0.2 (0.6-10.6)	74	17 \pm 2.0 (0-113)
25 [‡]	395	39 \pm 0.3 (20-58)	607 \pm 16 (54-2280)	483 \pm 11 (47-1648)	26 \pm 0.9 (1-115)	5.4 \pm 0.1 (1.0-14.9)	89	28 \pm 1.6 (0-180)

Fishing gears: [†]gillnet set, otter trawl bottom and trammel net (gillnet consisting of three layers of net); [‡] scientific otter trawl, operated at the sea floor, similar to otter trawl bottom.

2.2. Analysis of livers for nematodes and parasite identification

To develop an estimation tool for prediction of the total number of nematodes based on liver categories, we established a database containing both liver category and counted total number of nematodes for each individual liver. All individual livers of the 642 sampled cod were assigned a liver category by using the so-called “liver category method” on fresh livers introduced by Thünen Institute in western Baltic areas. Today the liver category covers liver categories 0, 1, 2, 3 and 4 corresponding to counts of 0, 1-10, 11-20, 21-30 and >30 nematodes on the liver surface, respectively (Figure 1, in SD22 and SD24: scale 0-3, in SD25: scale 0-4). After assignment of liver categories, individual livers were kept at -20 °C until subsequent analysis of total number of nematodes using the so-called “compression method”. Nematode species identification was based on morphometric characteristics of the caudal and cephalic ends according to Fagerholm (1982) and carried out at the Laboratory of Aquatic Pathobiology, University of Copenhagen (Frederiksberg, Denmark). For further details on the methodologies, see Buchmann (2007) and Sokolova *et al.* (2018).

2.3. Statistical analysis

A full generalized linear model (GLM) including data from all three areas was defined to test for associations between liver categories and the counted total number of nematodes in livers and to provide estimates for further predictions of the total number of nematodes. In addition, to account for the area effect (i.e. SD22, SD24 and SD25), one model was defined for each area, resulting in another three separate models. In all four models, the counted number of nematodes was defined as the response variable (Eq. 1), which followed a negative binomial distribution where variance increases quadratically with the mean (Eq. 2). A log link function was defined by default in the model (Hardin *et al.*, 2007). The mean Y_i was independent with μ and over dispersion parameter $\theta > 0$. This implied that the variance of the i 'th observation becomes $\mu(1+\mu/\theta)$. A poisson distribution was tested, but this could not account for the variance in the data sets.

$$Y_i = \text{nematodes} \quad (1)$$

$$Y_i \sim \text{NBin}(\mu_i, \theta) \quad (2)$$

Total fish length (TL) was included as an explanatory variable to account for the accumulation of nematodes in the liver over time (Horbowy *et al.*, 2016). To account for seasonal changes in the size of the livers, a hepatosomatic index (HSI) was calculated (Eq. 3) and also included as an explanatory variable in the model (Lambert and Dutil, 1997).

$$HSI = \frac{LW}{GW} * 100 \quad (3)$$

Where LW represents liver wet weight and GW represents gutted weight of the fish. GW of the fish was used in the calculation of HSI to eliminate potential bias related to gonad size and stomach fullness. Two-order interactions between TL and liver category as well as HSI and liver category were included in the full models to examine any differences of the effect of TL and HSI within each liver category (Eq. 4).

$$\text{Log}(Y_i) = \alpha(\text{liver category}_i) * \text{HSI}_i + \beta(\text{liver category}_i) * \text{length}_i \quad (4)$$

However, due to data limitations for SD22 (dominance of category 0 livers), it was not possible to test any two-order interactions in the full model for this area. In this respect it is noteworthy that the differences in the number of livers assigned to the different liver categories throughout the Baltic Sea do not reflect bias in sampling of livers, but simply reflect the spatial differences in infection load with nematodes in the livers of Baltic cod (Sokolova *et al.*, 2018).

The statistical tests were carried out using the statistical program R with Rstudio (version 3.4.1.) (R Core Team, 2016). The four GLM models were fitted with glmmTMB using the package “glmmTMB” (Brooks *et al.*, 2017). Before model fitting, collinearity between explanatory variables was assessed by using variance inflation factors (VIF) (Zuur *et al.*, 2009). No variables were excluded from the analysis due to collinearity (Table S1). Model selection was performed using a stepwise backward selection routine based on a likelihood ratio test for each of the variable included and excluded in the models. Extraction of residuals for model validation of each final model was done by using one-step predictions which is an implemented function in the R package “Template Model Builder” used to extract quantile residuals of models (Thygesen *et al.*, 2017). The model assumptions of normality and independence were hereafter validated by visual inspection of model residuals (Figures. S1-S4).

2.3.1. Fulton condition factor

To examine associations between Fulton condition factor and infections with *C. osculatum* in cod, two different analyses were performed on data from SD25. Calculation of Fulton condition factor was based on gutted weight (Eq. 5):

$$\text{Fulton condition factor} = \frac{GW}{(TL)^3} * 100 \quad (5)$$

In the first case, we tested for significant difference of Fulton condition factor within the five levels of the liver category for each month represented in data. This was done by using Anova and a post hoc analysis (Tukey HSD). In the second case we included more own data (N=594) on total number of nematodes in livers from cod in SD25 (Table S2) in order to examine the association between Fulton condition factor and infection density and to estimate the probability of cod with critical condition factor (i.e. below 0.65) in relation to infection density. The critical Fulton condition factor reflects the level where cod are considered dying (Dutil and Lambert, 2000; Casini *et al.*, 2016a). The association between Fulton condition factor and infection density was examined by an exponential model (Eq. 6):

$$\log(\text{Fulton condition factor}_i) \sim N(\alpha * \text{infection density}_i, \sigma^2) \quad (6)$$

The probability can be calculated as a tail probability directly from the model in equation 6 from each infection density. A parametric bootstrap was used to propagate the uncertainty from the estimates of α and σ to the probability estimates.

3. Results

3.1. Number and species of nematodes in analysed livers

A total of 11352 nematodes were recovered from the 642 livers examined, 32, 1487 and 9833 nematodes from SD22, SD24 and SD25, respectively. In SD22, 9 out of the 32 parasitic nematodes belonged to two other species of nematodes: herring or whale worm, *Anisakis simplex*, (n=8) and seal worm or cod worm, *Pseudoterranova decipiens* (n=1). In SD24, 4 out of 789 parasitic nematodes were identified as *A. simplex* and the remaining as *C. osculatum*. In SD25, only *C. osculatum* was identified. Given the low contribution of species other than *C. osculatum*, the counted total number of nematodes used in the statistical analysis included all counted nematodes irrespectively of the species of nematodes.

3.2. Observed liver categories and counted number of nematodes

Overall, there were pronounced differences between SD22, SD24 and SD25, both in relation to the number of livers assigned to each liver category, and in the counted number of nematodes within each liver category (Figures 2B and 3, Table 2). Irrespectively of area, variance in counted number of nematodes within a liver category was highest in the highest liver category (Figure 3). When combining data from the three areas, the variance of counted number of nematodes within each liver category increased (Figure 3D). In area SD22 most livers were assigned liver category 0 and no livers were assigned to liver category 2 and 3 (Figures 2B, 3A and Table 2), while in area SD24 most livers were assigned to both liver category 0 and 1 and a few to liver category 2 and 3 (Figures 2B, 3B and Table 2). In area SD25, all five liver categories were present (Figures 2B, 3C and Table 2).

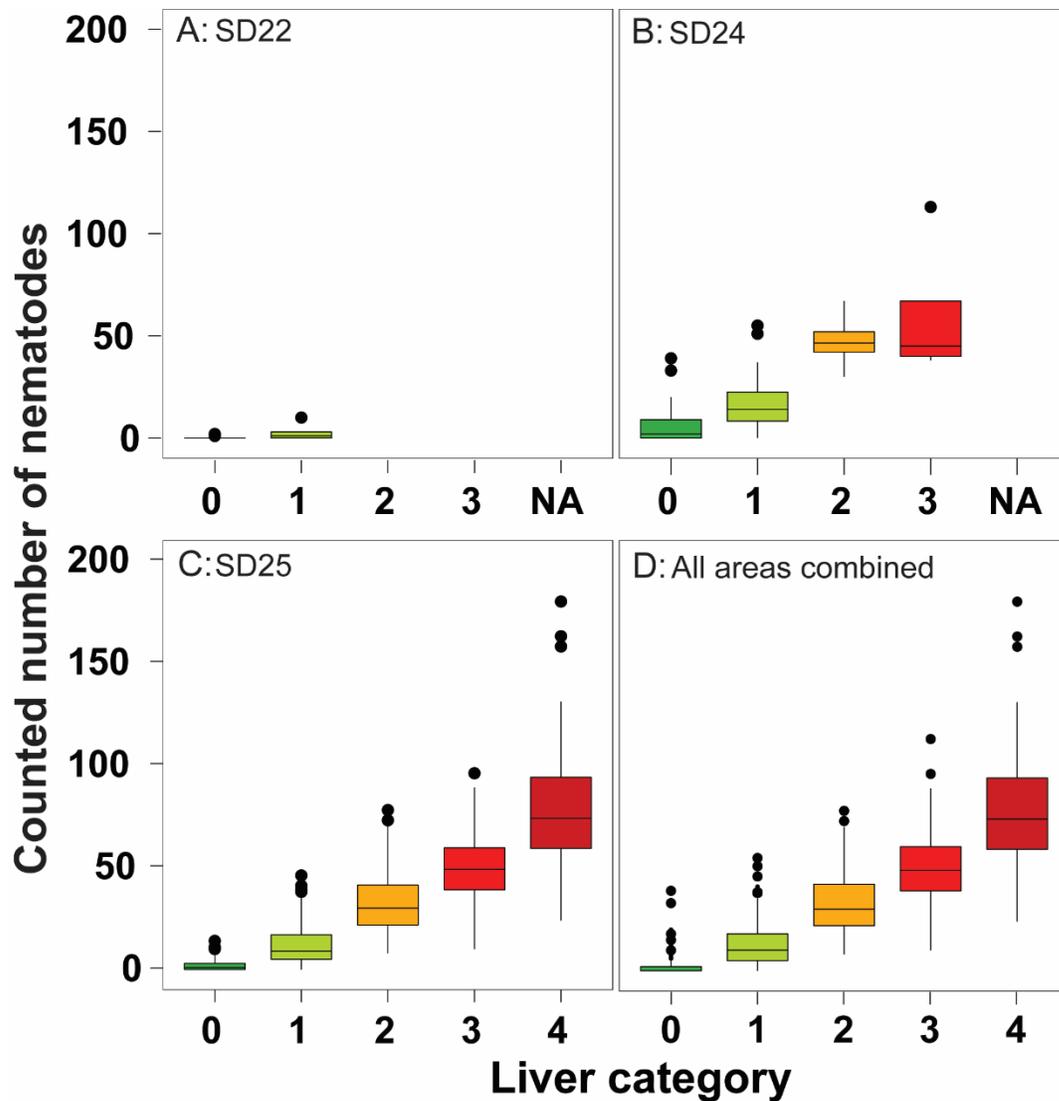


Figure 3. Relationship between the assigned liver categories and the counted total number of nematodes inside the livers of Baltic cod sampled in ICES SD22 (A), SD24 (B), SD25 (C) and all areas combined (D). See figure 1 for visual appearance and a detailed description of the five liver categories. NA in panel A and B illustrates that to date this category has not been used in SD22 and SD24. For the box plots, the solid line is the median, the box is the interquartile area (bottom and top are 25th and 75th percentiles, respectively). Whiskers show either the max/min observation if within 1.5 of the interquartile range or 1.5 times the interquartile range. See table 2 for details on the total number of livers assigned to each category.

Table 2. Total number of livers assigned to each liver category for each area (SD22, SD24 and SD25) and month. For further details on liver categories, see Figure 1.

Liver category	SD22			SD24		SD25			Sum	
	Jun	Aug	Nov	Apr	Nov	Mar	Jun	Sep		Nov
	2018	2018	2017	2018	2017	2020	2018	2017		2019
0	14	65	48	35	37	29	21	12	36	297
1	0	2	3	30	4	41	39	30	35	184
2	0	0	0	2	2	11	26	16	7	64
3	0	0	0	1	4	6	30	6	4	51
4	NA	NA	NA	NA	NA	7	36	3	0	46
Sum	14	67	51	68	47	94	152	67	82	642
Sum per area	132			115		395				

3.3. The estimation tool

3.3.1. Associations between liver categories and estimated number of nematodes, HSI and fish length

The model output of the final model for all data combined revealed that there was a significant increase in the estimated number of nematodes with increasing liver category ($p=0.009$, Table 3 and S3). Furthermore, the estimated number of nematodes increased significantly with TL for all liver categories, except for liver category 0 where an increase in TL resulted in a decrease of the estimated number of nematodes ($p<0.001$, Table 3). There was no effect of HSI on the estimated number of nematodes (Table S3).

Table 3. Estimates of significant parameters and standard errors (SE) of the final model, including all data combined, describing how the total number of nematodes changes within each category and with length. Wald Z provides the statistical result of each variable and factor level and p values below 0.05 are considered significant. Numbers are on log scale.

Parameter	Estimate	SE	Wald Z	p-value
Intercept	$\alpha(\text{category})$			0.009
0	3.137	0.546	5.750	
1	0.326	0.588	0.555	
2	1.745	1.172	1.488	
3	3.135	1.307	2.399	
4	2.920	1.535	1.903	
Slope w.r.t length	$\beta(\text{category})$			<0.001
0	-0.060	0.014	-4.365	
1	0.056	0.015	3.740	
2	0.042	0.028	1.498	
3	0.018	0.030	0.602	
4	0.033	0.034	0.947	

3.3.2. Area-specific patterns in estimated number of nematodes

In all three area models, the estimated number of nematodes increased significantly with increasing liver category ($p < 0.001$, Table 4). In SD22, the estimated number of nematodes increased significantly with TL ($\beta = 0.094$, $p = 0.04$) and HSI ($\gamma = 0.331$, $p = 0.04$) (Table S3 and 4), whereas there was no effect of HSI and TL on estimation of nematodes in SD24 (Table S3). In SD25, estimation of the number of nematodes increased with TL ($\beta = 0.049$, $p < 0.001$) but decreased with increased HSI ($\gamma = -0.043$, $p = 0.004$) (Table S3 and 4, Figure 4). For a 40 cm cod with a category 4 liver (i.e. highly infected) from SD25, the predicted number of nematodes was e.g. 39% lower in fish with highest observed HSI (HSI=14.9; 42 nematodes) compared to cod with a medium HSI (HSI=5.4; 61 nematodes) (Figure 4). Model reduction of the three area models revealed that no interactions between liver category and HSI and liver category and TL were significant for the estimation of nematodes (Table S3).

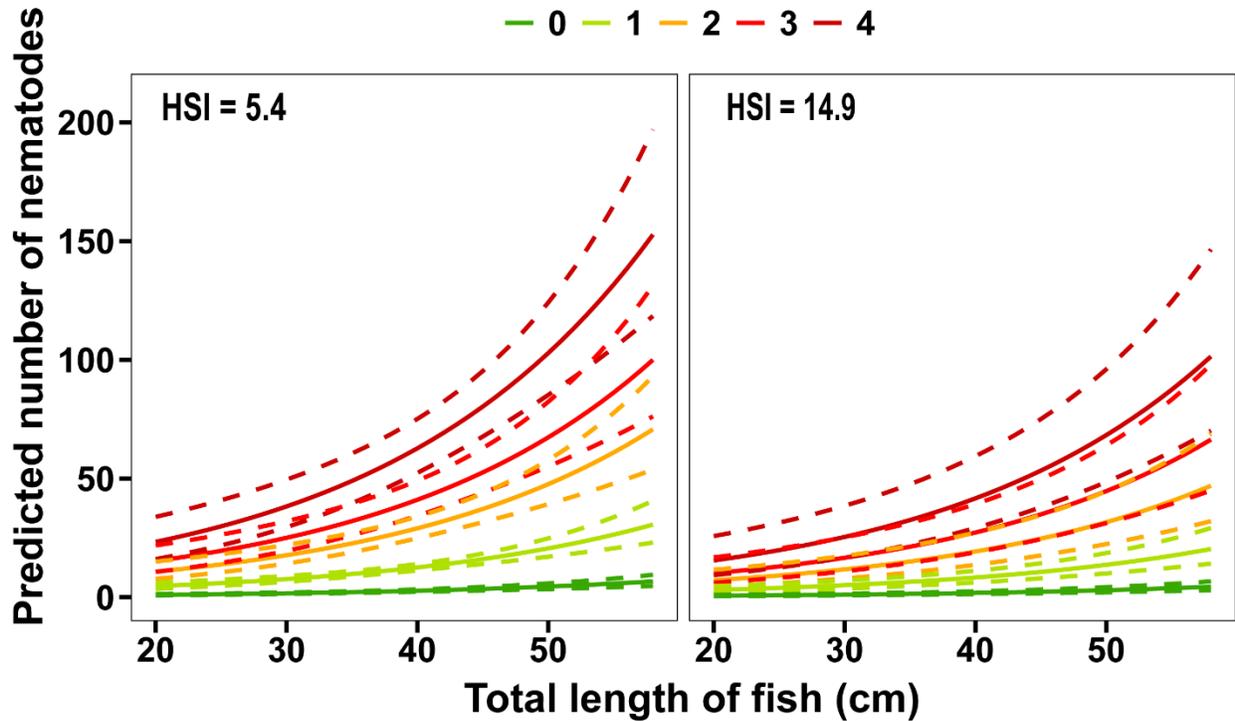


Figure 4. Predictions of the total number of nematodes in livers of Baltic cod from area SD25 derived from the final GLM model for different cod sizes with mean HSI index = 5.4 (A) and the highest observed HSI index = 14.9 (B). Colours represent the five liver categories 0-4. See Figure 1 for visual appearance and a detailed description of the five liver categories. Solid lines: mean predictions of the total number of nematodes, dashed lines: confidence intervals (0.95) of the model predictions.

Table 4. Estimates of significant parameters and standard errors (SE) of the final models describing how the estimated total number of nematodes changes for each area within each liver category (intercept). Wald Z provides the statistical result of each variable and factor level and p values below 0.05 are considered significant. Numbers are on log scale.

Area	Parameter	Estimate	SE	Wald Z	p-value
SD22	Intercept	$\alpha(\text{category})$			<0.001
	0	-7.347	2.574	-2.854	
	1	-3.897	2.458	-1.585	
	Slope w.r.t. length	β			0.04
		0.094	0.054	1.726	
	Slope w.r.t. HSI	γ			0.04
	0.331	0.162	2.076		
SD24	Intercept	$\alpha(\text{category})$			<0.001
	0	1.756	0.152	11.559	
	1	2.831	0.213	13.267	
	2	3.861	0.615	6.282	
	3	4.104	0.549	7.478	
SD25	Intercept	$\alpha(\text{category})$			<0.001
	0	-0.731	0.264	-2.768	
	1	0.787	0.271	2.908	
	2	1.625	0.289	5.629	
	3	1.971	0.299	6.602	
	4	2.395	0.310	7.734	
	Slope w.r.t. length	β			<0.001
		0.049	0.007	7.235	
Slope w.r.t. HSI	γ			0.004	
	-0.043	0.015	-2.880		

3.3.3. Robustness of the liver category and estimation tool: liver categories and predicted number of nematodes

To evaluate the robustness of the three areas models, and to assess the strength of the estimation tool, predicted number of nematodes and confidence intervals (0.95) of the three area models were extracted (Table 5). Due to significance of TL and HSI in for SD22 and SD25, predictions were based on a cod of 40 cm length with the mean HSI of 2.7 for SD22 and 5.5 for SD25 (Table 5). Predicted number of nematodes for area SD24 were calculated for all sizes and values of HSI, due to non-significance of the variables in this area (Table S2 and 4). Comparing predicted number of nematodes between the three areas revealed that predicted numbers within each liver category were highest in SD25 in all categories except for category 0, where the predicted number was highest in SD24 (Table 5). Accuracy of the three models decreased with increasing liver category as the confidence intervals became broader with each liver category level (Table 5).

Table 5. Mean predicted numbers of nematodes based derived from the estimation tool for the three different areas. In SD22 and SD25 predictions and confidence intervals (CI = 95%) of the total number of nematodes within each liver category are represented for a 40 cm cod and the average HSI in area SD 22 (HSI = 2.7) and area SD25 (HSI=5.4). In SD24 predictions and confidence intervals (CI = 95%) of total number of nematodes are only based on the four liver category levels as length and HSI turned out to be non-significant in the model for this area.

	Liver category				
	0	1	2	3	4
SD22					
Mean	0.1	2.1	-	-	NA
CI (lower-upper)	0.0 – 0.2	0.5 – 9.6	-	-	NA
SD24					
Mean	5.8	17.0	47.5	60.6	NA
CI (lower-upper)	4.3 - 7.8	11.1 – 26.0	13.9 – 162.4	20.2 – 181.6	NA
SD25					
Mean	2.7	12.6	29.1	41.1	62.7
CI (lower-upper)	2.3 – 3.3	11.3 – 14.0	24.9 – 34.0	34.4 – 49.1	52.4-75.1

3.3.4. Fulton condition factor

For all months, there was a tendency for Fulton condition factor to decrease (the decrease was only significant for June, $p=0.008$) with increasing liver category, except for liver category 4 (Figure 5).

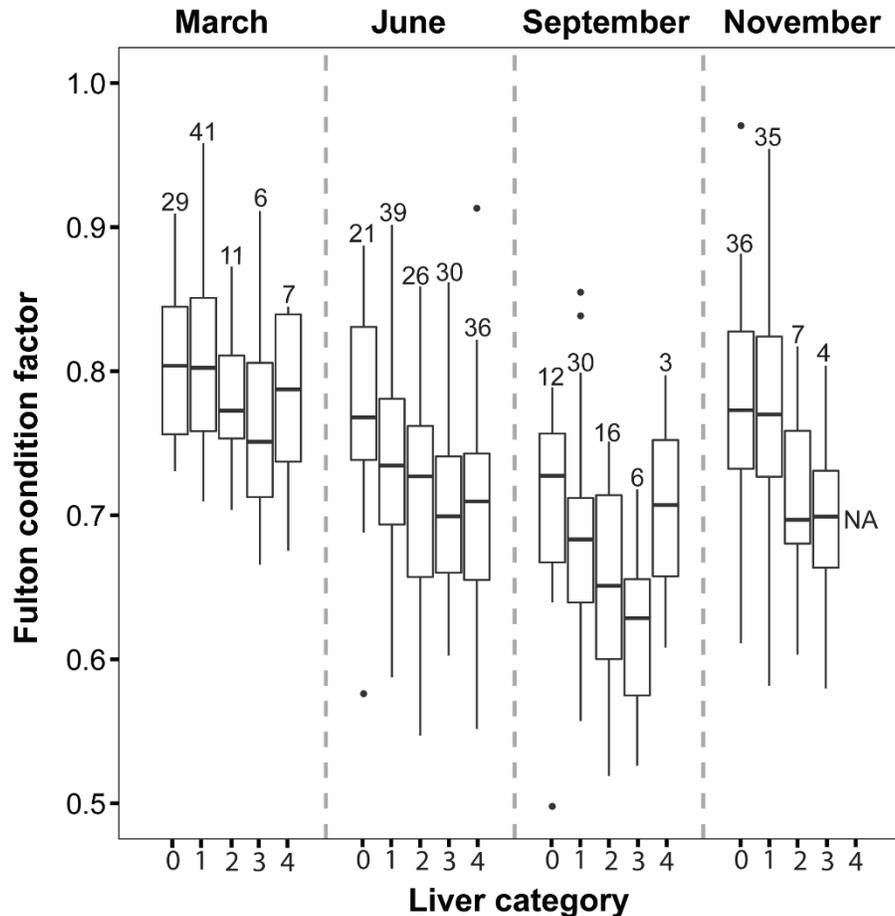


Figure 5. Fulton condition factor in relation to liver category of cod from SD25 (N=395) illustrated for four different months. Numbers above each box present number of cod within the given category. For the box plots, the solid line is the median, the box is the interquartile area (bottom and top are 25th and 75th percentiles, respectively). Whiskers show either the max/min observation if within 1.5 of the interquartile range or 1.5 times the interquartile range. See table 2 for details on the total number of livers assigned to each category.

However, Fulton condition factor decreased significantly with increase in infection density ($p<0.001$, $\alpha=\exp(-0.029)$, $sd=0.002$, $\text{intercept}=\exp(-0.262)$). The subsequent probability model revealed a sigmoid pattern between infection density and the probability of the fish having a critical Fulton condition factor, e.g. fish with an infection density of 6 had a probability of having a critical condition factor of 50%, whereas fish with infection densities of 10 almost had 100% probability of having a critical condition factor (Figure 6).

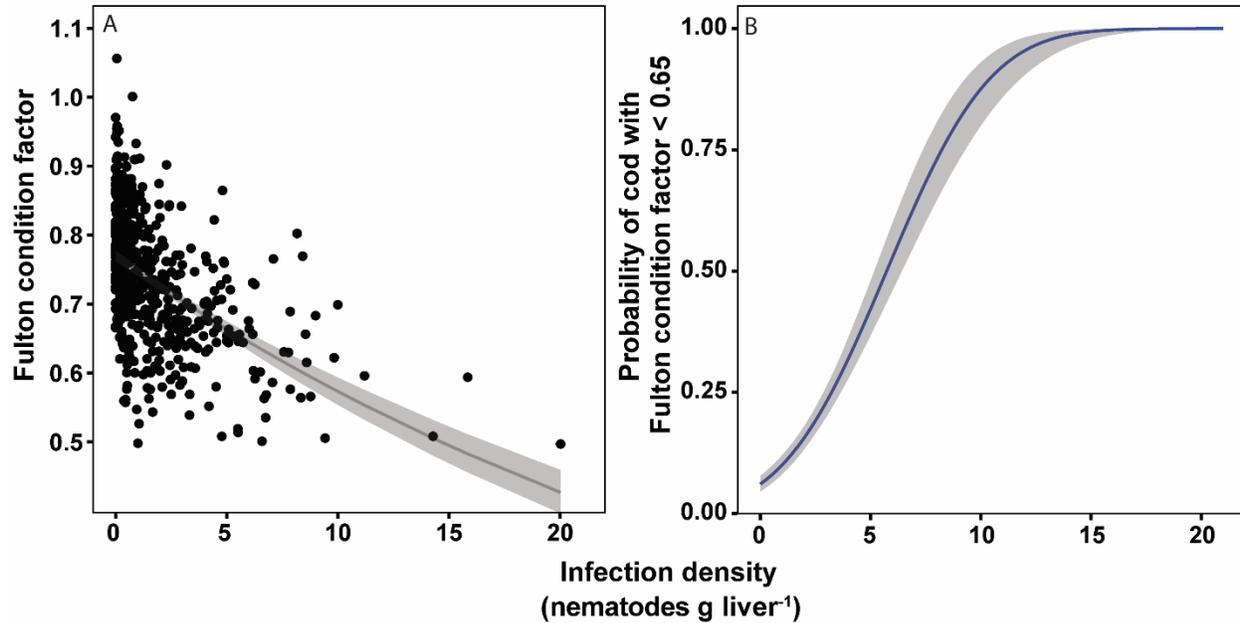


Figure 6. A) Model fit (grey solid line) and uncertainty (grey area) of Fulton condition factor in relation to infection density for the 594 Eastern Baltic cod sampled between 2016-2020 from SD25 included in the present study. B) Mean (blue solid line) and uncertainty (grey area) of the probability for cod having Fulton condition factor below 0.65 (i.e. critical Fulton condition factor) for different levels of infection density. The probability model fit and the uncertainty are calculated based on the predictive output of the model shown in panel A. The level of the critical Fulton condition factor where cod are considered dying is defined in Casini *et al.* (2016a) based on findings of Dutil and Lambert (2000).

4. Discussion

We here provide an estimation tool for prediction of number of nematodes in cod livers based on the assigned liver category and detailed analysis of number of nematodes in the same liver. From this, infection densities can be obtained, describing the severity of infections, which is known to relate to the health status of the infected individual. Moreover, we verify the robustness of the liver category method by showing that estimates of total number of nematodes increase significantly with increasing liver category. Thus, collection of liver categorical data during monitoring surveys provides an inexpensive and easy way to obtain pan-Baltic information on spatiotemporal changes in infection load with *C. osculatum*. We show that the explanatory variables (i.e. TL and HSI) for estimation of nematodes differed between areas, implying the need to include a spatial component in the analysis. Finally, we illustrate how infection densities are related to the probability of cod having a critical Fulton condition factor, believed to lead to induced mortality. Fisheries scientists should thus not ignore potential effects of parasites on fish stocks, as this may lead to flawed interpretations e.g. of factors driving natural mortality or stock productivity (Timi and Poulin, 2020).

4.1. The estimation tool: One size does not fit all

To simplify things, we initially set out to make one model for the estimation tool, covering SD22, 24 and 25. However, the effects of length and HSI on the estimations of the total number of nematodes differed substantially between the areas. Furthermore, the number of livers assigned to the different categories varied markedly from West to East, as also seen in a recent detailed investigation where a West-East gradient was found in prevalence and abundance of infection with *C. osculatum* in cod livers (Sokolova *et al.*, 2018). Therefore, the spatial component (i.e. a model for each of the three areas) had to be included to improve the strength of our estimations and predictions. Notably, the area-based models will enable identification of present and emerging areas with high infection densities, thus providing a spatial component to the description of future disease dynamics in cod.

4.2. How biological parameters affect estimations of total number of nematodes

Previous detailed investigations of cod in SD25 have shown that total number of nematodes in individual livers increase with TL, likely because the nematodes accumulate in the liver as cod consume infected prey (Horbowy *et al.*, 2016; Zuo *et al.*, 2016). The same pattern was found in the present study for fish in SD22 and SD25.

The lack of significance of TL on estimated number of nematodes in SD24 may be a result of the narrow length range (35-50 cm) of the cod sampled for the analysis within this area, disabling the model to capture a potential length effect, and/or be a result of stock mixing in this area (Hüssy *et al.*, 2016). Inter-individual differences in infection patterns in SD24 is linked to the population of origin (Sokolova *et al.*, 2018), and, although speculative, a potential length effect may be balanced out by the mixture of smaller infected EBC cod and larger, less infected Western Baltic cod.

We chose to use HSI in the models to account for the seasonal difference in liver weight in relation to fish weight. In SD25, fish with low HSI had significantly higher predicted total number of nematodes for a given liver category than fish with high HSI. The opposite was the case in SD22, where increase in HSI resulted in higher predictions of total number of nematodes. The reason(s) for this difference between areas remains speculative, but maybe high numbers of nematodes (as seen in SD25) may cause destruction of liver structure and subsequent decrease in organ size. Previous detailed investigations of cod in SD25 have revealed that high liver parasite burdens cause reduced lipid content of the organ, resulting in reduced HSI (Petrushevsky and Shulman, 1955; Ryberg *et al.*, 2020). On the contrary, livers in SD22 are only assigned to category 0 or 1 (i.e. no or low infection load) and a high HSI might leave more nematodes hidden inside the organ.

4.3. *Contracaecum osculatum*, Fulton condition factor and natural mortality

Fish body condition is a key parameter in the dynamics of fish stocks. When cod reach a critically low Fulton condition factor of 0.65 it is expected to die, and natural mortality for use in stock assessment of Eastern Baltic cod has recently been adjusted for the observed low condition (Dutil and Lambert, 2000; Casini *et al.*, 2016a). Before a lethal low level is reached, low condition associates with reduced reproductive potential (Lambert *et al.*, 2000; Rätz and Lloret, 2003; Mion *et al.*, 2018) and slow growth rates (Dutil *et al.*, 1999; Hüssy *et al.*, 2018). The combined effects of low condition has been suggested as one of the causes of the lack of recovery of the Gulf of St. Lawrence cod stock despite the fishery moratorium in the 1990s (Lambert and Dutil, 2000), thus stressing the importance of fish being in a good nutritional health for stock productivity. In the present study, there was a tendency for reduced Fulton condition factor with increase in liver category, except for cod with a liver category 4. The higher Fulton condition factor for fish with liver category 4 may be a result of skipped spawning due to impaired health of fish with high infection loads, and/or reflect increased mortality of highly infected cod with very poor condition, as suggested by Horbowy *et al.* (2016). Notably also, based on data from 594 cod, we found a significant decrease in Fulton condition factor and increase in the probability of cod with Fulton condition factor below 0.65 with increased infection density.

Considering that the relative importance of the different ecosystem drivers on the poor state of the Eastern Baltic cod still remains uncertain, our results reveals the importance of including the link between infection density and Fulton condition factor when promoting the understanding for the main factors driving the cod stock status.

4.4. Limitations of the estimation tool

Although the categorization of cod livers now applied on board Baltic International Trawl Surveys, in combination with the estimation tool provided here, undoubtedly will enable an essential step forward towards future assessments of cod liver parasite load, it also has a major shortcoming. While a liver contains a definite number of worms in its three-dimensional structure (unknown at the moment of liver categorization), the thoroughness and experience of the individual technicians looking at the liver surface to assign a category may well differ between persons. Furthermore, there may be an increasing uncertainty in assignment of liver category with increase in the number of nematodes on the surface of the liver. This call for careful intercalibration and training of technicians to minimize this error source.

4.5. Perspectives

Marine ecosystems are like dynamic landscapes where conditions and biological interactions change constantly over time (Barange *et al.*, 2011), and one way to ensure sustainable ecosystem-based management is to integrate solid time-series data from biological and ecological monitoring into stock assessment and management of exploited resources such as fish stocks. Fish disease monitoring is in general sparse, and mainly provides snapshot data in space and time. One previous effort is the establishment of the Fish Disease Index (FDI) (Lang and Wosniok, 2008), that e.g. has been used to monitor the health status of North Sea dab in relation to impacts of hazardous substances (Lang *et al.*, 2018). *C. osculatum* has a complex life cycle, relying on several hosts (Valtonen *et al.*, 1988; Koie and Fagerholm, 1995; Nadolna-Ałtyn *et al.*, 2018; Zuo *et al.*, 2018), and their distribution and abundance – and hence interactions – will change through time. With this, *C. osculatum* burdens may increase also in the more Westerly cod stocks, although it remains unclear if the parasite will thrive in these high salinity waters (Sokolova *et al.*, 2018). Though, the assignment of liver categories, combined with the present estimation tool, will enable fishery scientists to follow the spatiotemporal development in both prevalence, abundance and infection density of this parasite. In addition, while there is still some way to go to actually estimate mortality rates based on infection density, we consider the probabilistic approach presented here has an important step ahead that hopefully also will inspire colleagues working with disease in other fish stocks.

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Chapter V

Discussion and summary

Summary of findings for all three papers

This thesis presented studies based on experimental work, collection of field data and bioenergetics and statistical modelling. The results shed new light on the potential causes for the poor nutritional status of individual Eastern Baltic cod and on the consequences this has for the population (Figure 9).

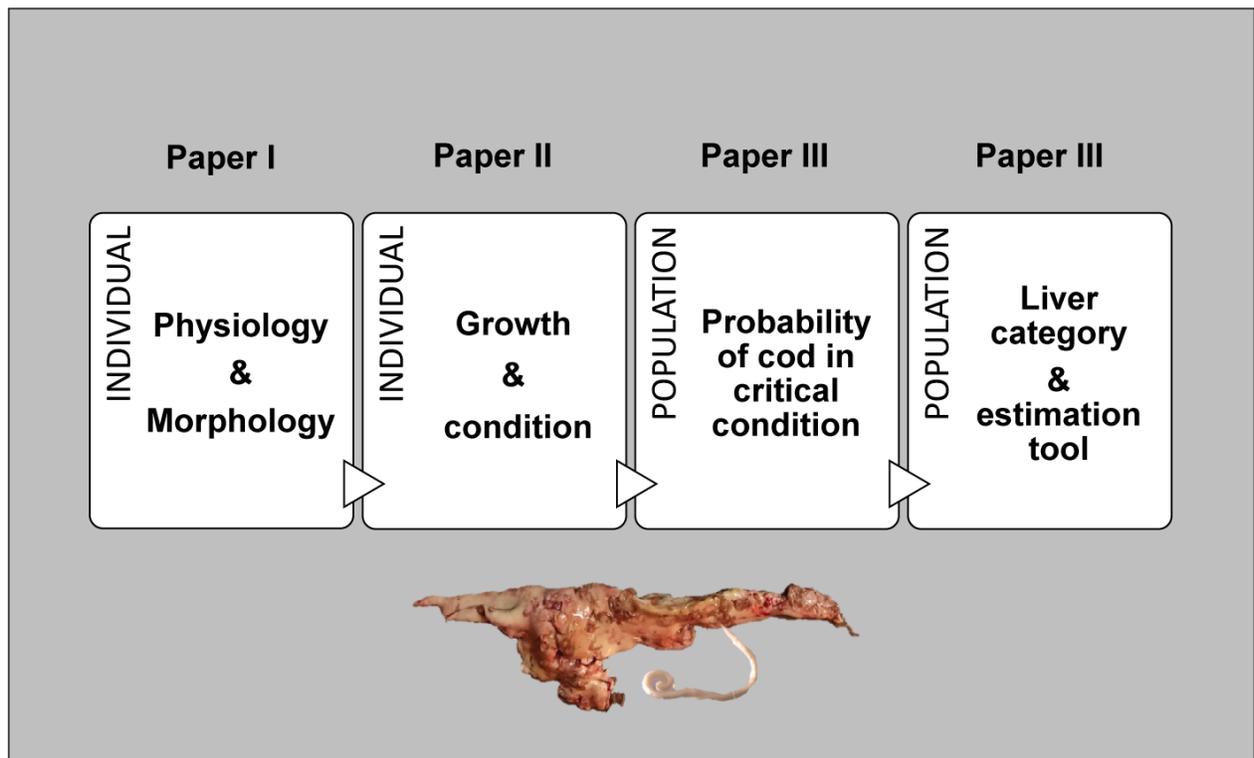


Figure 9. Schematic summary of all findings in this PhD project, divided into individual and population-level processes. **Paper I** covers changes in morphological features and physiological mechanisms; **paper II** covers the energy requiring process growth and hence condition; and **paper III** includes a monitoring perspective and places nutritional condition versus infection density into probabilities of cod being in critical condition when infected with *C. osculatum*. See the main text for more detailed information on the findings.

At the individual level, the experimental and field data revealed how several important physiological mechanisms and morphological features change with infections of *C. osculatum* (**paper I**, Figure 10). Specifically, the standard (maintenance) metabolic rate decreased with an increase in infection density. This result was in contrast to what was originally hypothesised, that infection with *C. osculatum* would pose an energetic cost to the host that would be reflected in an augmented metabolic rate of the fish (hypothesis I).

However, maintenance of internal organs constitutes a major part of the daily energy budget (i.e. is energetically expensive; Hulbert and Else, 2000), and the reduced size of digestive organs (intestine and pyloric caeca) in fish with high parasite loads may well, at least partly, explain the lower metabolic rates of these fish. Moreover, the lipid content of the liver and protein content of the fish were both significantly reduced in infected fish, while glycogen content was increased relative to non-infected individuals, possibly as a result of impaired liver function (**paper I**, hypothesis II). Examination of the plasma composition revealed changes in the protein profile with parasite load, much like the clinical signs that characterise severe liver disease in humans (e.g. cirrhosis of the liver or liver cancer), including a pronounced immune response with increased gamma globulins. Thus, as hypothesised (hypothesis III), Eastern Baltic cod with high infection densities of *C. osculatum* appear to suffer from severe liver disease (**paper I**, Figure 10).

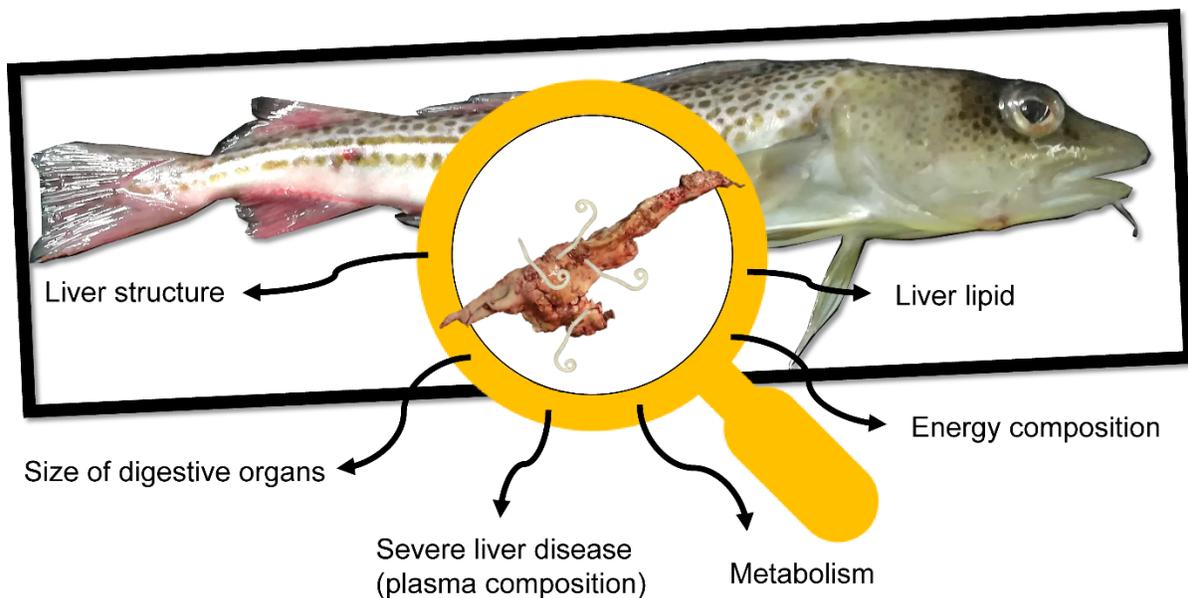


Figure 10. Summary of the physiological mechanisms that revealed negative impacts of infection with *C. osculatum* in Eastern Baltic cod (**paper I**). See main text and **paper I** for details.

The results obtained from experimental work on individuals and the field data (**paper I**) were integrated into a bioenergetics model. The model revealed that growth was reduced with increasing infection density, and condition factors of the individual fish were lower in infected compared with non-infected individuals (**paper II**). Interestingly, the model output revealed that infected and non-infected cod followed the same temporal and seasonal trajectories throughout their life history, which was reflected by skipped spawning.

Notably also, cod reach a point of no return where energy intake of infected individuals becomes so low, that maintaining a surplus energy balance is impossible. At this point starvation-induced mortality is subsequently unavoidable. In summary, the findings of **paper II** improve our understanding of how growth processes can be affected by changes in physiological mechanisms and reveal that the present feeding levels of Eastern Baltic cod are simply too low to avoid skipped spawning and to avoid starvation-induced mortality of infected individuals.

Monitoring of parasite loads in wild fish stocks is rarely done (Lloret *et al.*, 2012). Thus, based on the finding that infection with *C. osculatum* is associated with impaired physiological condition of Eastern Baltic cod (**paper I**), and abundance and prevalence of infections are increasing, it has been decided by WGBIFS that, from 2021, it will be mandatory to conduct visual inspections of cod livers on all BITS using the ‘liver category’ method. The assigned liver category is based on the number of nematodes visible on the surface of individual livers. However, it has never been tested whether livers assigned to different categories differ in terms of the number of nematodes. In **paper III**, the robustness of the liver category method was therefore tested based on liver categorical data and detailed analysis of nematodes in livers from 642 Eastern Baltic cod. The liver category was found to be a good predictor of the total number of nematodes, and the estimation tool developed in the study was found to be suitable for providing estimates of the total number of nematodes in livers, which is needed for calculations of infection density (**paper III**). The latter turned out to be an important result, as a strong negative association between Fulton condition factor and infection density of cod from the Bornholm Basin (area SD25) was found together with an increase in probabilities of Eastern Baltic cod having a critical condition with an increase in infection density. This could imply that infection with *C. osculatum* indirectly relates to the observed increase in natural mortality of Eastern Baltic cod in recent years.

Based on the findings mentioned above, it is clear that infections with *C. osculatum* in Eastern Baltic cod cannot, and should not, be ignored when working towards an understanding of the drivers that are currently resulting in the distress and low productivity of the stock. However, one issue remains unresolved: Does high parasite load reduce nutritional condition or does low nutritional condition increase susceptibility to parasites “the chicken or the egg” conundrum?

Severity of infections

Whether growth and condition are negatively or positively associated with a given parasitic infection depends on whether the parasite is a so-called low- or high-cost parasite to its host (Henriksen *et al.*, 2019). High-cost parasites are defined as causing mechanical damage to their hosts or evoking energetically costly immune responses, where the negative effects of the parasite exceed the benefits from the energy gain obtained from eating the infected prey (Henriksen *et al.*, 2019). In contrast, infections with low-cost parasites can lead to an energetic gain to the fish host when eating the infected prey. In addition, I would suggest that there are three main factors, that alone or together, determine the severity of parasitic infections to the host; these are as follows: 1) the site of infection of the parasite; 2) the immune response of the host; and 3) the condition of the fish when it becomes infected.

The site of a parasitic infection may influence how the parasite affects its host, resulting in different outcomes of the physiological condition of the host (Lafferty and Shaw, 2013). Some parasites infect sites that lead to physiological and/or behavioural changes in the host, while others choose sites as a result of nutritional preference (Lafferty and Shaw, 2013; Strømnes, 2014; Mattiucci *et al.*, 2017). For example, a parasite may infect the central nervous system of the host, likely as an attempt to induce behavioural manipulation (Prandovszky *et al.*, 2011), while other parasites infect the eye of their host, causing disrupted visual abilities (Flink *et al.*, 2017). In both cases, the physiological and behavioural changes make the host more susceptible to predation (Lafferty and Shaw, 2013). The fact that *C. osculatum* specifically infects the liver of Eastern Baltic cod, resulting in tissue damage (Chondromatidou, 2020) and reduced function of the organ (**paper I**), implies that the site of infection in this host-parasite system may have vital impacts on the physiological condition and hence performance of the infected individual. This may make the infected cod more prone to predation, for example, by the final host of the parasite, the grey seal. This may at first glance appear as host manipulation, but it may also merely be a side effect of the parasite. Although purely speculative, the site of infections by *C. osculatum* may instead be a result of nutritional preference by this parasite species. An *in vitro* study revealed that L3 larvae of *Anisakis simplex* showed signs of nutritional preferences, preferring tissues with a high-fat content (Strømnes, 2014), which may also be the case for *C. osculatum*.

The specific immune response of fish towards parasitic infections may play a crucial role in how severe infections become in the individual. Like cod, Atlantic salmon, *Salmo salar*, is an apex predator in the Baltic and feeds on some of the same forage fish (e.g. sprat). Not surprisingly, a recent study showed that salmon also become infected with *C. osculatum* in the same area as infected cod are seen. However, salmon have a substantially lower prevalence (46.8%) and intensity (between one and four nematodes per liver; Setyawan *et al.*, 2019) of infections compared with cod infected in the central and eastern part of the Baltic (90–100% infection prevalence and on average 27–40 nematodes per liver; Sokolova *et al.* 2018). Why do we see this difference between two species occupying the same trophic level and feeding on the same infected transport hosts? One explanation may be that salmon have a more effective immune response towards infection with *C. osculatum* in the liver than cod do. In salmon, nematodes are found in small granulomas, and they appear to reach a smaller size (6–10 mm, compared to 3–27 mm in cod livers), indicating that something inhibits their growth in the salmon liver (Mohamed *et al.*, 2020; Setyawan *et al.*, 2020).

Notably, the immune system of Atlantic cod is unique compared with other teleost species. It relies more on the innate immune system than the adaptive immune system, which is uncommon in teleosts (e.g. salmon) and vertebrates in general (Øverland *et al.*, 2010). The innate immune system, also referred to as the non-specific immune system, is the first to be activated when a teleost fish is attacked by a pathogen. In contrast, the adaptive immune system, also referred to as the specific immune system, has a longer response time but is more specialised in attacking specific pathogens by antibodies discovering antigens on the pathogen (Murphy *et al.*, 2012). In mammals, the response to parasitic nematodes involves components of the adaptive immune system (Buchmann, 2012; Murphy *et al.*, 2012).

Cod have lost the genes related to the major histocompatibility complex (MHC) II, cluster of differentiation 4 (CD4, a glycoprotein on the surface of immune cells) and invariant chain (Ii), which are crucial components of the adaptive immune system (Star *et al.*, 2011). Instead, cod tend to have upregulated pattern-recognition receptors and an increased variability of expressed MHC I sequences (Solbakken *et al.*, 2016). It has been shown that the immune system of cod recognises infections with *C. osculatum* in the liver, subsequently activating various granulocytes (Marnis *et al.*, 2020). Thus, the larger size range of *C. osculatum* found in cod compared with salmon livers indicates different immune responses between the two hosts. It is suggested that the excretory–secretory compounds released by *C. osculatum* may induce local immune depression in the liver of cod, thereby protecting the nematodes and ensuring their survival (Mehrdana *et al.*, 2017; Marnis *et al.*, 2020).

Across taxa, including teleosts, it has been shown that reduced nutritional condition can lead to increased susceptibility to pathogens, most likely because the immune response is less effective when the individual is in a poor nutritional state (Gulland, 1992; Chandra, 1997; Johansen *et al.*, 1997). Malnutrition in fish species other than cod is known to reduce antibody production and the immune response (Salomoni *et al.*, 1987; Mackinnon, 1998; Oliva-Teles, 2012). Likewise, Atlantic cod in poor condition have been found to be more susceptible to lethal disease outbreaks than individuals in good condition (Dutil *et al.*, 2006). For fish acting as hosts in the lifecycle of trophically transmitted parasites, the energy gain of eating infected prey comes with an increased probability of being infected with parasites (Henriksen *et al.*, 2019). Thus, the relationship between condition and parasite load is highly complex (Sánchez *et al.*, 2018). In some cases, the association between the prevalence of infections and the nutritional condition of the host indicates more or less favourable feeding conditions for the given host, as an increase in prevalence simply can reflect a higher food intake of the host or vice versa (Podolska and Horbowy, 2003; Levsen *et al.*, 2016). However, many studies also show the opposite, that an increase in infection load with parasites is associated with a reduced body condition factor of the infected fish (Neff and Cargnelli, 2004; Oliva-Teles, 2012; Horbowy *et al.*, 2016; Sánchez *et al.*, 2018; Sokolova *et al.*, 2018). Therefore, it is not straightforward to disentangle the causes and effects of parasites on the physiological and nutritional condition of the host.

‘The chicken or the egg’

For a long time, measures of body condition have been used to infer the impacts of parasites on the nutritional state or general health status of the infected animal in numerous taxonomic groups (Lloret *et al.*, 2012; Sánchez *et al.*, 2018). Notably, the nutritional condition factor of Eastern Baltic cod has been decreasing since the early 1990s, with the strongest decrease found for intermediate-sized and larger individuals (Eero *et al.*, 2015; Casini *et al.*, 2016). Thus, the decline in condition started to occur almost two decades before the prevalence and intensity of infection with *C. osculatum* in cod started to emerge and increase (Haarder *et al.*, 2014; Nadolna and Podolska, 2014; Eero *et al.*, 2015; Casini *et al.*, 2016). The observed decline in nutritional condition was likely caused by deteriorating oxygen conditions with increasing (and more severe) hypoxic areas of the Baltic Sea (Casini *et al.*, 2016), in combination with changes in feeding levels (i.e. reduced feeding intake and changes in prey composition; Neuenfeldt *et al.*, 2020). Therefore, it is likely that the poor nutritional state of Eastern Baltic cod increased the susceptibility towards infections with *C. osculatum*. However, the extent of this outcome is unknown.

In this PhD thesis, the word ‘effects’ has been used with care. Nevertheless, I would argue that all three factors described above, which contribute to the severity of parasitic infections of the host (i.e. site of infection, different immune response and poor nutritional condition at infection time) are present in the host-parasite system between *C. osculatum* and Eastern Baltic cod. First, *C. osculatum* infects and, at high infection loads, destroys one of the most vital organs in cod, the liver. Second, cod have a unique immune system compared to other teleosts, as cod rely mostly on the innate immune system and lack crucial elements of the adaptive immune system. Third, Eastern Baltic cod were already in poor nutritional condition when *C. osculatum* was likely emerging in the ecosystem along with an increasing number of the parasite’s final host, the grey seal. Thus, the individual cod may have been more susceptible to infections compared with more well-fed fish in better condition. I believe that the changes found in energy and plasma composition with increasing infection load in this PhD are indeed direct effects of the parasite, and not a result of poor nutritional condition per se (**paper I**). The changes in energy composition, observed as decreased protein and increased glycogen content with increasing infection density (**paper I**), are not characteristic of a starved cod (a starved cod exhausts its hepatic lipid and glycogen stores first, before its muscle glycogen; Black and Love, 1986; Navarro and Gutiérrez, 1995). Moreover, the observed plasma protein profile, with its reduced albumin-to-globulin ratio and increased gamma globulins, most likely relates to the disease status of the cod (**paper I**). It is not possible to disentangle whether the reduced standard metabolic rate and organ sizes are effects of the parasite or simply due to the reduced feeding level that cod are experiencing now (Neuenfeldt *et al.*, 2020) or a combination of both.

However, although it is still not possible to fully answer “the chicken or egg” question for the present host–parasite system, scaling the results derived at the level of the individual in this PhD project to the population level may reveal potential effects of *C. osculatum* on the productivity of the Eastern Baltic cod stock.

Scaling from individual physiological responses to population-level effects

The most common definition of stock productivity involves three vital rates, which are as follows: 1) somatic growth, 2) recruitment (number of offspring produced) and 3) survival rate (Hilborn and Walters, 1992). Today, the spawning stock biomass of Eastern Baltic cod consists of small individuals in poor condition (ICES, 2019), which is hampering the productivity of the population. Based on findings in this PhD project, it may be suggested that *C. osculatum* is a high-cost parasite for Eastern Baltic cod because infections are associated with changes in cod physiology, growth, condition and lipid content of the liver (**papers I, II and III**). These changes can affect productivity. For example, recruitment is positively associated with the liver weight of spawners in Barents Sea cod (Marshall *et al.*, 1999), and the potential fecundity of Eastern Baltic cod is reduced in fish in poor condition (Mion *et al.*, 2018). Moreover, swimming performance is known to be impaired in Atlantic cod with reduced body condition (Martínez *et al.*, 2003; Lapointe *et al.*, 2006). Impaired swimming ability of Eastern Baltic cod resulting from low body condition may lead to reduced foraging success and increased predation by grey seals, thereby increasing natural cod mortality (Figure 11, **paper I**). Reduced food intake due to poor abilities to catch prey may subsequently lead to depletion of energy reserves in the liver of cod, and with a shrinking liver, the infection density will increase further. Thus, infected cod enter a vicious circle, which inevitably results in starvation-induced mortality at some point (Dutil and Lambert, 2000; **paper II**, Figure 11).

Eastern Baltic cod show a seasonal productivity pattern, with seasonal changes in population growth, recruitment and survival rates (Mildenberger *et al.*, 2020), and the natural mortality tends to be high for old individuals (ICES, 2019). The weight loss after peak spawning (in May to August) may explain this trend, at least partly, as individual cod may suffer from critical conditions immediately after spawning, which could lead to increases in natural mortality (Mildenberger *et al.*, 2020) if the cod decide to spawn. Thus, the trajectory patterns from the bioenergetics modelling outputs in **paper II** revealed that individual Eastern Baltic cod, irrespective of whether they were infected with *C. osculatum*, exhibited skipped spawning and only spawned every second year in the model. This result probably reflects that fish in this stock are currently experiencing very low feeding levels (Neuenfeldt *et al.*, 2020) and are in poor nutritional condition.

Scaling linear processes into probabilities, such as the probability of Eastern Baltic cod being in critical condition when infected with *C. osculatum* at different densities (**paper III**), increases the application of the results from **papers I and II** in this PhD project. Since mortality, fecundity and skipped spawning are all related to body condition, a parasite-induced reduction in condition may negatively impact the productivity of fish populations (Lambert and Dutil, 2000; Rätz and Lloret, 2003; Neff and Cargnelli, 2004; Birkeland and Dayton, 2005; Mion *et al.*, 2018)

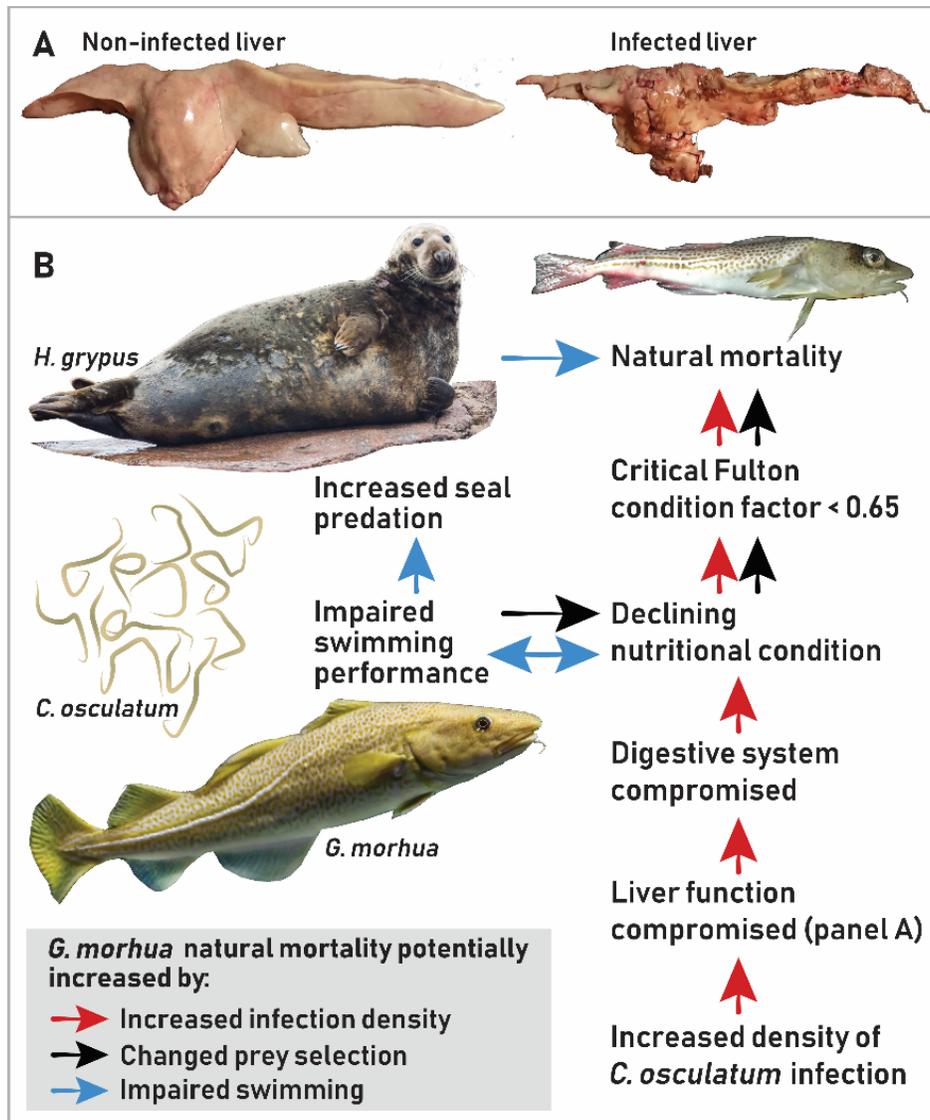


Figure 11. A) Non-infected versus infected liver of Eastern Baltic cod. B) Summary of possible mechanisms and performances that may lead to increased natural mortality of Eastern Baltic cod infected with *C. osculatum*. The three colours of arrows represent different mechanisms leading to a potential increase in natural mortality. Increased infection density (red) is suggested to have a direct effect on the mortality of cod and an indirect effect on swimming performance (blue), leading to changes in prey selection and increased seal predation due to impaired swimming performance (black). (Figure from Ryberg *et al.* 2020).

Perspectives

Little evidence exists on the causes and effects of the infections. A promising place to start examining this is by considering food assimilation efficiency and the specific growth rate of Eastern Baltic cod in relation to different infection densities. Measurement of the specific dynamic action (SDA), which is the total energetic cost related to ingestion, digestion and assimilation of a meal (Secor, 2009), could be one way to elucidate whether infected individuals differ from non-infected individuals in terms of their ability to use the energy in a meal for growth. Alongside SDA, examination of changes in the activity of enzymes related to growth, such as citrate synthase, lactate dehydrogenase and trypsin, could likewise be relevant for improving our knowledge of the causes and effects of *C. osculatum* infections (Pelletier *et al.*, 1995; Lemieux *et al.*, 1999; Bélanger *et al.*, 2002). If infected cod were found to perform more poorly than non-infected conspecifics do, this would support the reduced growth of infected individuals identified in **paper II** and imply that infections have a direct effect on the poor nutritional condition of Eastern Baltic cod.

Notably, the number of grey seals in the Baltic Sea has been high in the past at comparable levels to present-day numbers during the 1930s to 1950s, and even higher than now at the beginning of the 19th century (Harding *et al.*, 2007; HELCOM, 2018). This suggests that *C. osculatum* also could have been abundant in the ecosystem in previous periods. Scrutinising and combining information from the available older literature describing the condition of cod, fishery pressure, food availability and oxygen conditions, together with information on grey seal abundance and infection load with *C. osculatum* in cod, would certainly make us wiser about the present situation. For example, literature from around 1940–1950 exists on Baltic cod infected with *C. osculatum* together with information on their health status, although this literature is in Russian and needs to be translated to make it accessible to the wider academic community.

Another avenue for future studies could be to investigate whether infected cod exhibit behavioural fever. In response to infections, ectotherms can move to warmer places, thereby increasing their body temperature, which is likely to accelerate the immune response (Rakus *et al.*, 2017). This phenomenon has been observed in bluegill sunfish (*Lepomis macrochirus*) infected with bacteria (Reynolds *et al.*, 1976), in brown trout (*Salmo trutta*) infested with glochidia larvae (Horký *et al.*, 2019) and in rainbow trout (*Oncorhynchus mykiss*) following injections with bacteria (Gräns *et al.*, 2012). For behavioural fever to take place, it is clearly necessary for the fish to access warmer waters. Eastern Baltic cod have this opportunity, for example, in some areas of the Bornholm Basin (SD25), where the water column is stratified, causing different temperatures (Andersen *et al.*, 2017).

If Eastern Baltic cod seek warmer waters, their metabolism and energetic demand increase (Chabot and Gu nette, 2013). Because of the low feeding levels at present (Neuenfeldt *et al.*, 2020), it is likely that Eastern Baltic cod would be unable to meet such increased energetic demands induced by behavioural fever, resulting in an even worse condition of infected individuals.

The mentioned experiments and historic review could all provide important knowledge on the mechanisms and relationships behind *C. osculatum* infection in Eastern Baltic cod. Moreover, studies on the performance and productivity of the Eastern Baltic cod population would be of high relevance. For instance, it would be interesting to study whether the speculated impaired swimming performance of cod infected with *C. osculatum* would result in increased predation by seals and reduced food intake by the cod. More specifically, it would be interesting to examine whether it is simply the cod's poor condition (regardless of infection) that impairs their swimming performance or if *C. osculatum* infection has a direct effect on the swimming performance of cod. In this respect, information on both burst swimming performance and sustainable swimming speed at different infection densities would provide valuable information on the ability of cod to do the following: 1) escape predation by seals, 2) catch forage fish, 3) avoid being caught by trawl and 4) migrate to spawning grounds. Finally, empirical studies on skipped spawning and potential fecundity in relation to different infection densities would enlighten fisheries scientists with a more direct measure of how the infections can contribute to reduced productivity of the population.

Conclusion

The Eastern Baltic cod stock is on the verge of collapse, and the future perspectives for the stock are currently unknown. The size and severity of hypoxic areas are not improving, there are no indications that the quantity and quality of food for cod will improve in the near future and the parasite infection pressure remains high alongside the number of grey seals. To understand how the poor situation of this cod stock may be improved by ecosystem-based management measures, we need to enhance our understanding of the impacts that these drivers have on the low productivity and general poor health status of individuals in the stock. Although much remains unresolved, the present thesis provides a significant step forward in our understanding of how infections with *C. osculatum* act as a driver of the Eastern Baltic cod stock dynamics. In summary, the four objectives of the thesis have been met and there is no doubt that Eastern Baltic cod with high infection densities of *C. osculatum* suffer from severe liver disease and have impaired physiology, reduced growth and increased probability of being in critical body condition (**papers I, II and III**). Thus, the findings of the thesis range from the level of the individual cod to the population level and they highlight that infection load with *C. osculatum* should not be ignored by fisheries scientists when providing scientific advice on the Eastern Baltic cod stock.

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Supplementary material A

**Physiological condition of Eastern Baltic cod,
Gadus morhua, infected with the parasitic
nematode *Contracaecum osculatum***

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Supplementary section A1: Results

Test of collinearity

Test of variation inflation factor: $VIF = \left(\frac{1}{1-R_i^2} \right)$ is used to assess the collinearity between the variables. The tests are only performed once for each batch of fish, and a VIF value is given for each variable. VIF is tested for the models including INF+length, length+total energy and in models including sum worm+ length+liver weight. The latter has been chosen to show that collinearity was low between liver weight and length of fish. Variables with VIF above 10 are defined as critical in relation to collinearity.

Table S1. Results from the test of collinearity of explanatory variables used in the different analyses.

Batch number of fish	Assessment	INF+length	Sum worm + Length + Liver weight	Length +Total energy
VIF				
Batch 1+2+3	Nutritional condition	1.05 & 1.05	1.3 & 1.8 & 1.5	-
Batch 1	Aerobic performance + plasma composition	1.0 & 1.0	1.2 & 1.6 & 1.4	-
Batch 2	Organ size	1.0 & 1.0	1.5 & 2.5 2.1	-
Batch 3	Prox com fish + liver	1.0 & 1.0	1.2 & 2.2 & 2.0	1.0 & 1.0

Effect of length on estimation of infection density (INF)

To show that the association between infection density and the different examined parameters is not a result of a length effect, we have tested all the models again, but this time length was kept in the models despite being non-significant in most cases. This analysis revealed that the effect of length did not influence estimates of infection density in any of the cases (Table S2).

Table S2. Additional analysis of the associations between infection density (INF) and the different parameters when length is included in the model, even though length is not significant in most models. In this analysis gender was included in the models where it was significant, however this estimate is not shown here.

Assessment	Parameter	α (INF)	μ (length)	λ (total energy)
Nutritional condition	Fulton condition	-0.02(0.003) ^{***}	-0.006(0.002) ^{***}	
Aerobic performance	SMR ¹	-0.01(0.003) ^{**}	-0.004(0.004)	
Organ size	Pyloric caeca ²	-0.02(0.01) [*]	2.60(0.36) ^{***}	
	Intestine ²	-0.02(0.01) [*]	2.22(0.35) ^{***}	
Plasma composition	Total protein ³	-0.02(0.005) ^{**}	0.009(0.006)	
	Globulins ⁴	0.01(0.003) ^{***}	0.002(0.004)	
	A/G	-0.16(0.03) ^{***}	0.003 (0.04)	
	Prealbumin ⁴	-0.07(0.02) ^{**}	0.06(0.03) [*]	
	Gamma ⁴	0.02(0.008) [*]	0.007(0.009)	
	Albumin ⁴	-0.15(0.03) ^{***}	0.005(0.034)	
Proximate composition of fish	Total energy ⁵	-0.03(0.01) ^{**}	-0.001(0.002)	
	Water ⁴	0.003(0.001) [*]	-3.1 ⁻⁵ (3.1 ⁴)	
	Protein ⁵	-0.04(0.01) ^{**}	-0.002(0.003)	
	Glycogen ⁵	0.05(0.01) ^{**}	0.000(0.003)	
	Ash ⁴	0.04(0.01) [*]	0.003(0.003)	
	Dry matter ⁴	-0.01(0.006) [*]	0.000(0.001)	
	Protein ⁵		0.000(0.001)	0.40(0.02) ^{***}
	Glycogen ⁵		-0.002(0.002)	-0.38(0.06) ^{***}
Proximate composition of liver	Lipid ⁶	-0.18(0.04) ^{***}	0.018(0.009)	
	Water ⁴	0.10(0.03) ^{***}	-0.009(0.006)	
	Dry mat ⁴	-0.10(0.03) ^{***}	0.011(0.006)	
	Ash ⁴	0.10(0.03) ^{**}	-0,009(0.007)	

Model validation plots

Fulton condition factor is calculated based on data compiled from all three batches of fish (i.e. all fish used in this study, n=152) (Figure S1). Examination of aerobic performance and plasma composition was performed on the same batch of fish (Figures. S2-S5). The study where changes in organ size was investigated involved fish from the second batch which was only used in in this context (Figure S6). Analysis of composition of the fish and the liver was based on a third batch of fish and figure S7-S12 are therefore showing the same fish ID's.

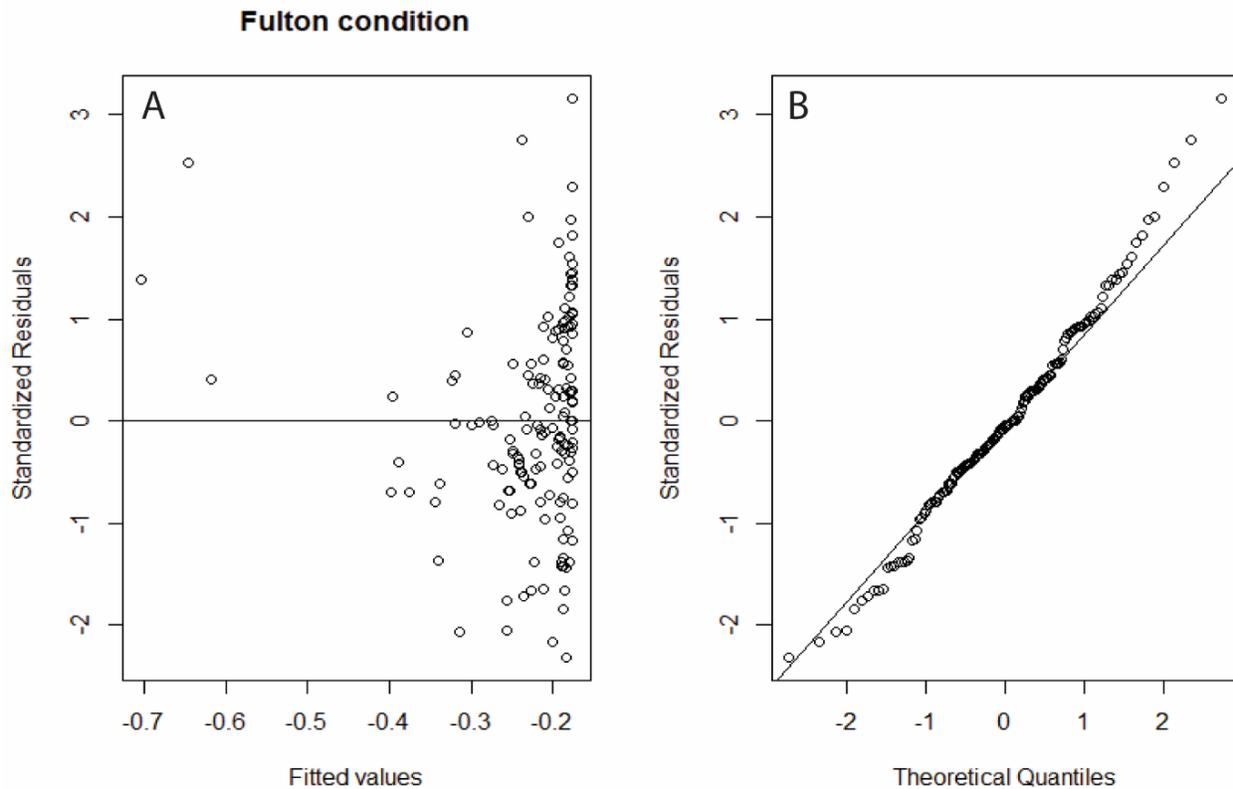


Figure S1. Model diagnostic plots for the final model of Fulton condition factor showing A) standardized residuals and fitted values and B) Q-Q plot for normal distribution of residuals.

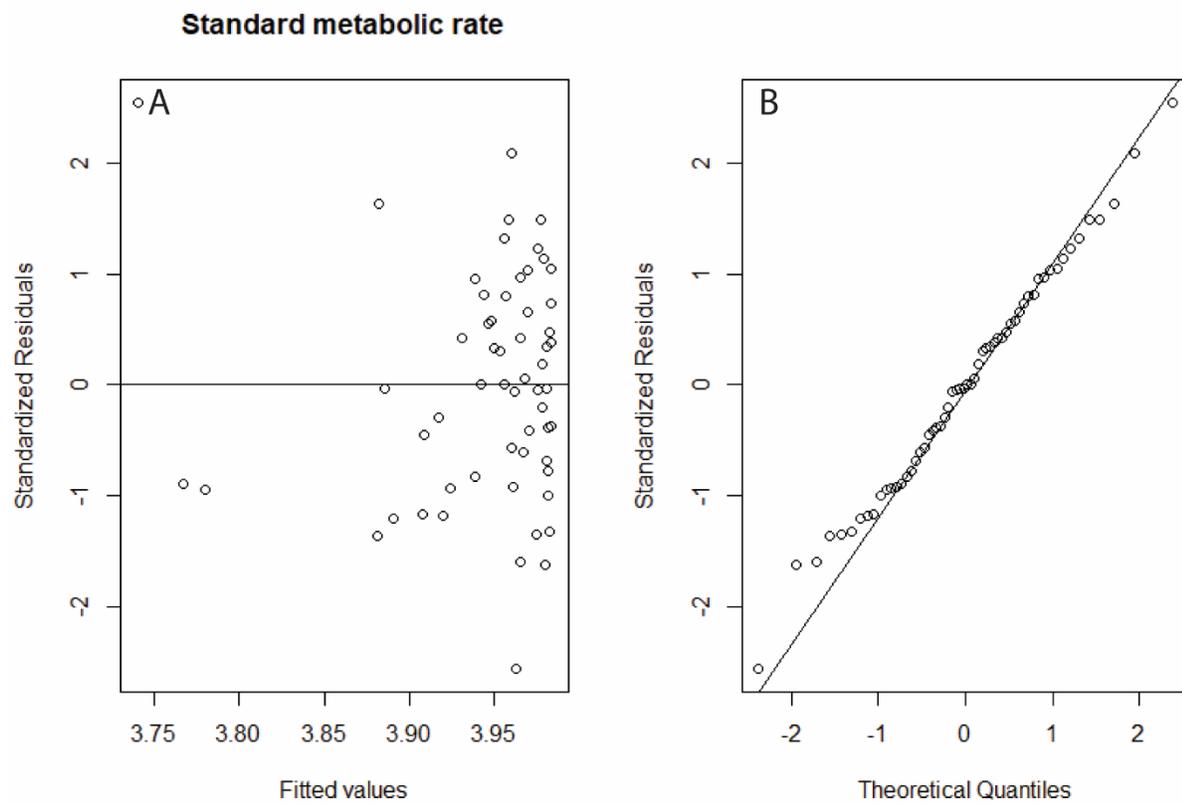


Figure S2. Model diagnostic plots for the final model of standard metabolic rate (SMR) showing A) standardized residuals and fitted values and B) Q-Q plot for normal distribution of residuals.

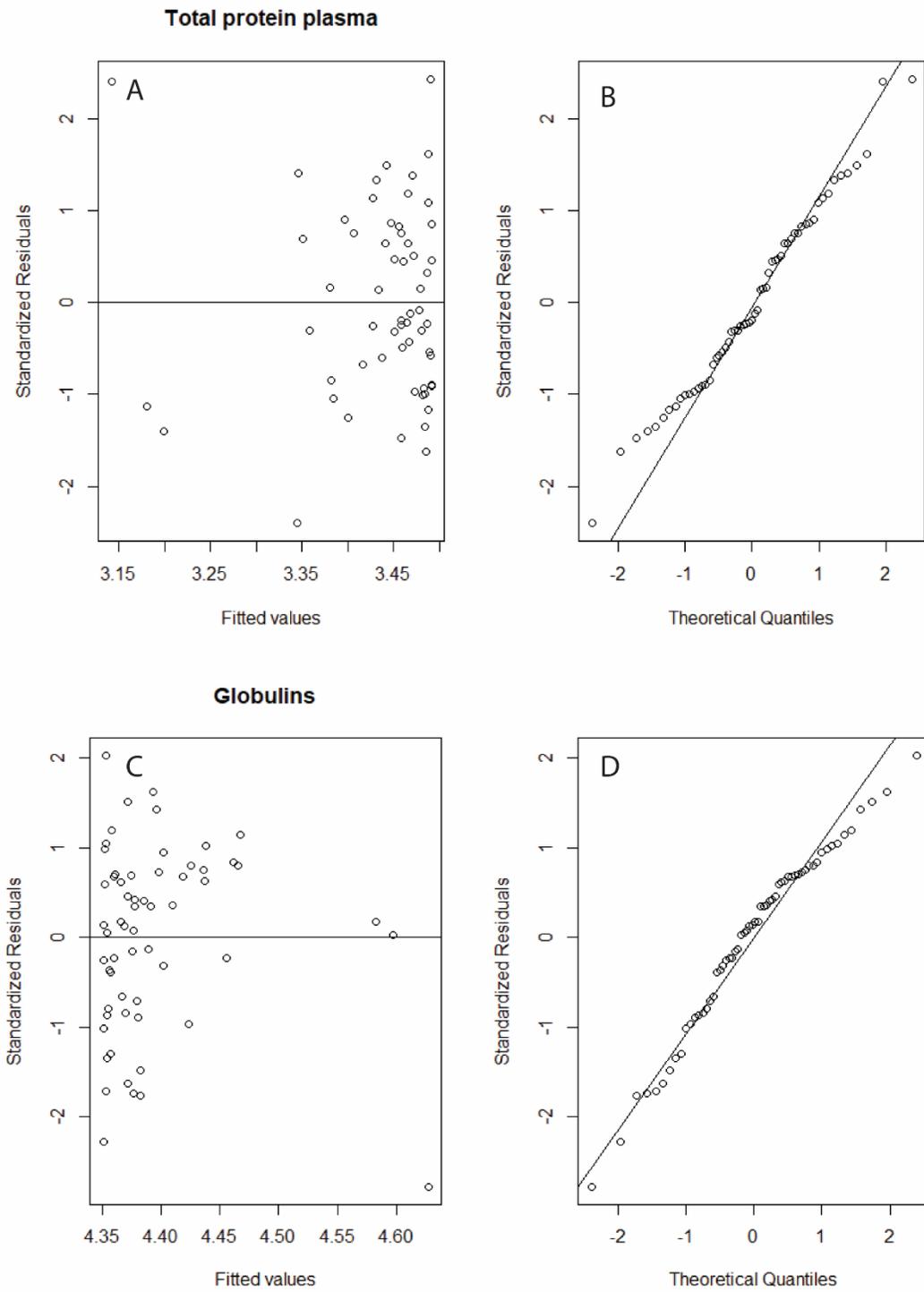


Figure S3. Model diagnostic plots for the final model of total protein (A+B) and globulins (C+D) in blood showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

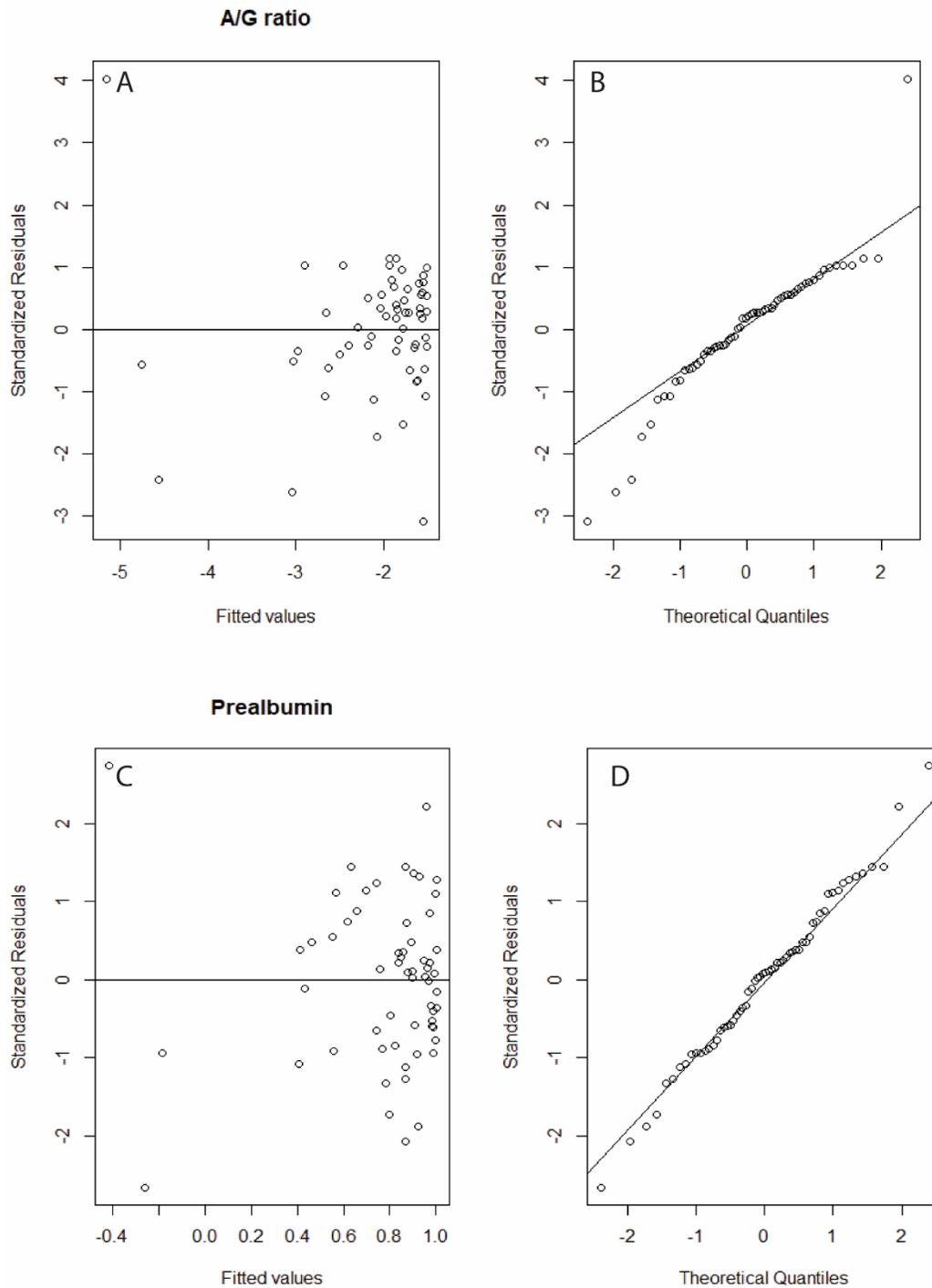


Figure S4. Model diagnostic plots for the final model of A/G ratio (A+B) and prealbumin (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

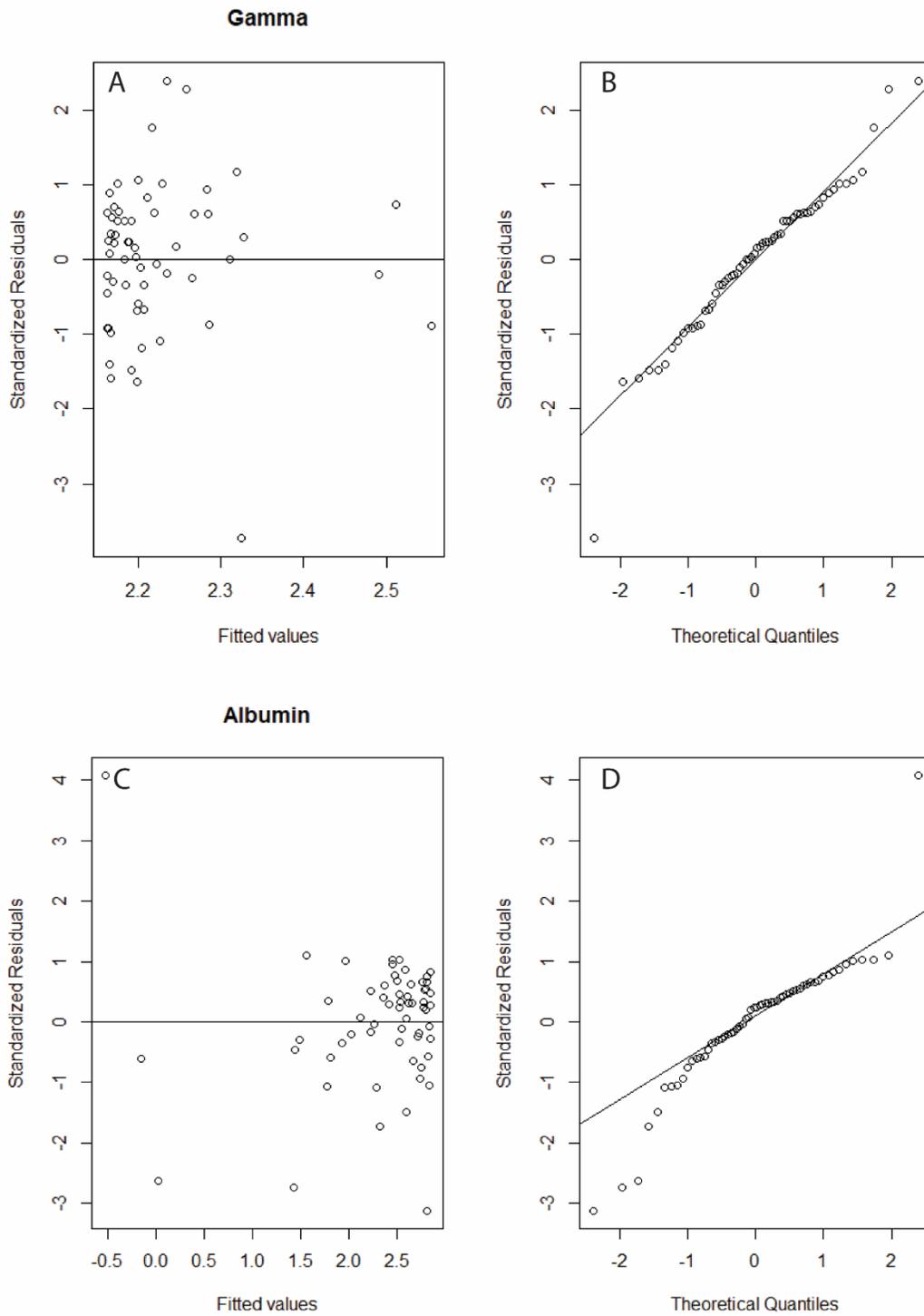


Figure S5. Model diagnostic plots for the final model of gamma (A+B) and albumin (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

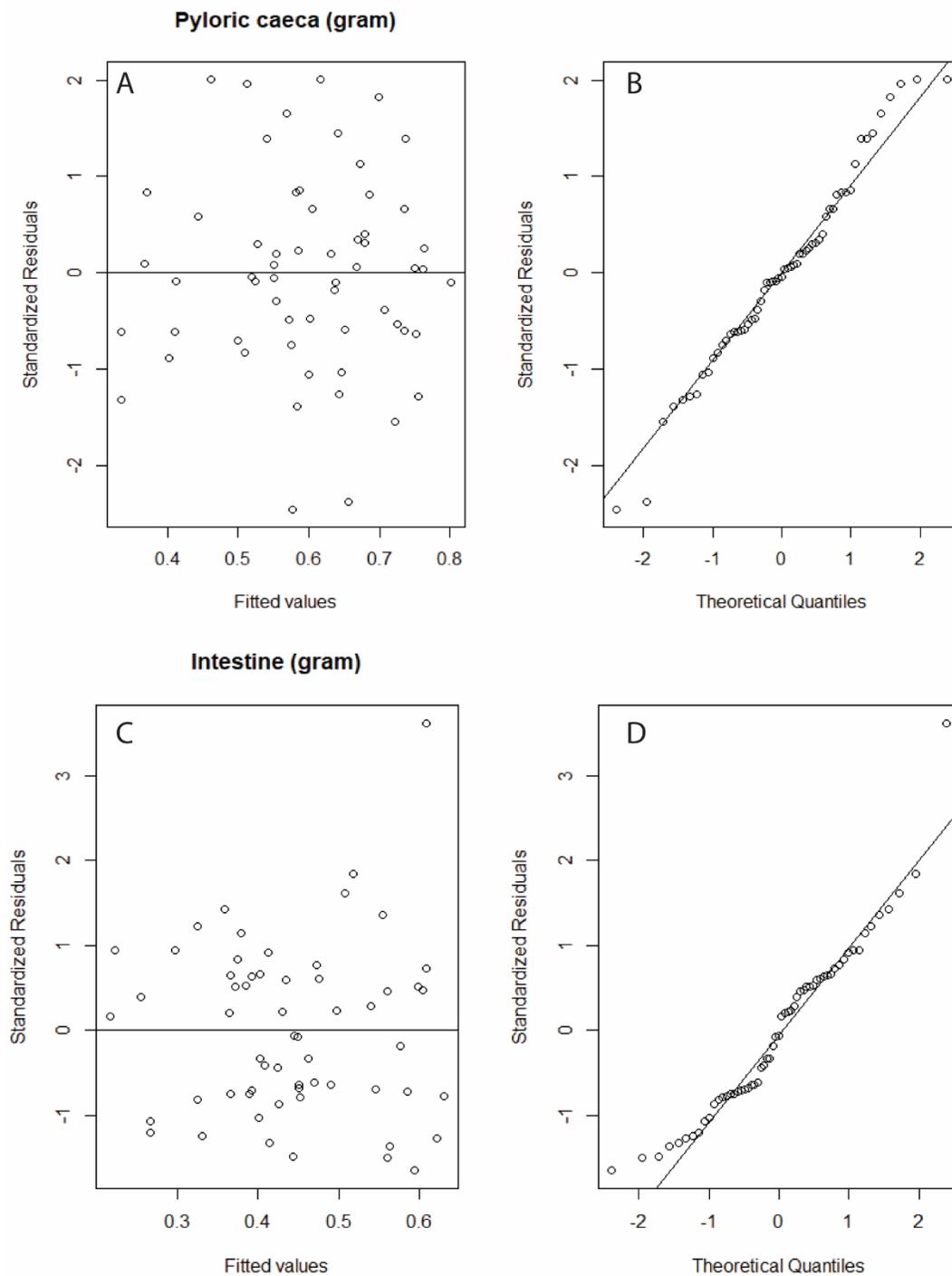


Figure S6. Model diagnostic plots for the final model of pyloric caeca (A+B) and intestine (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

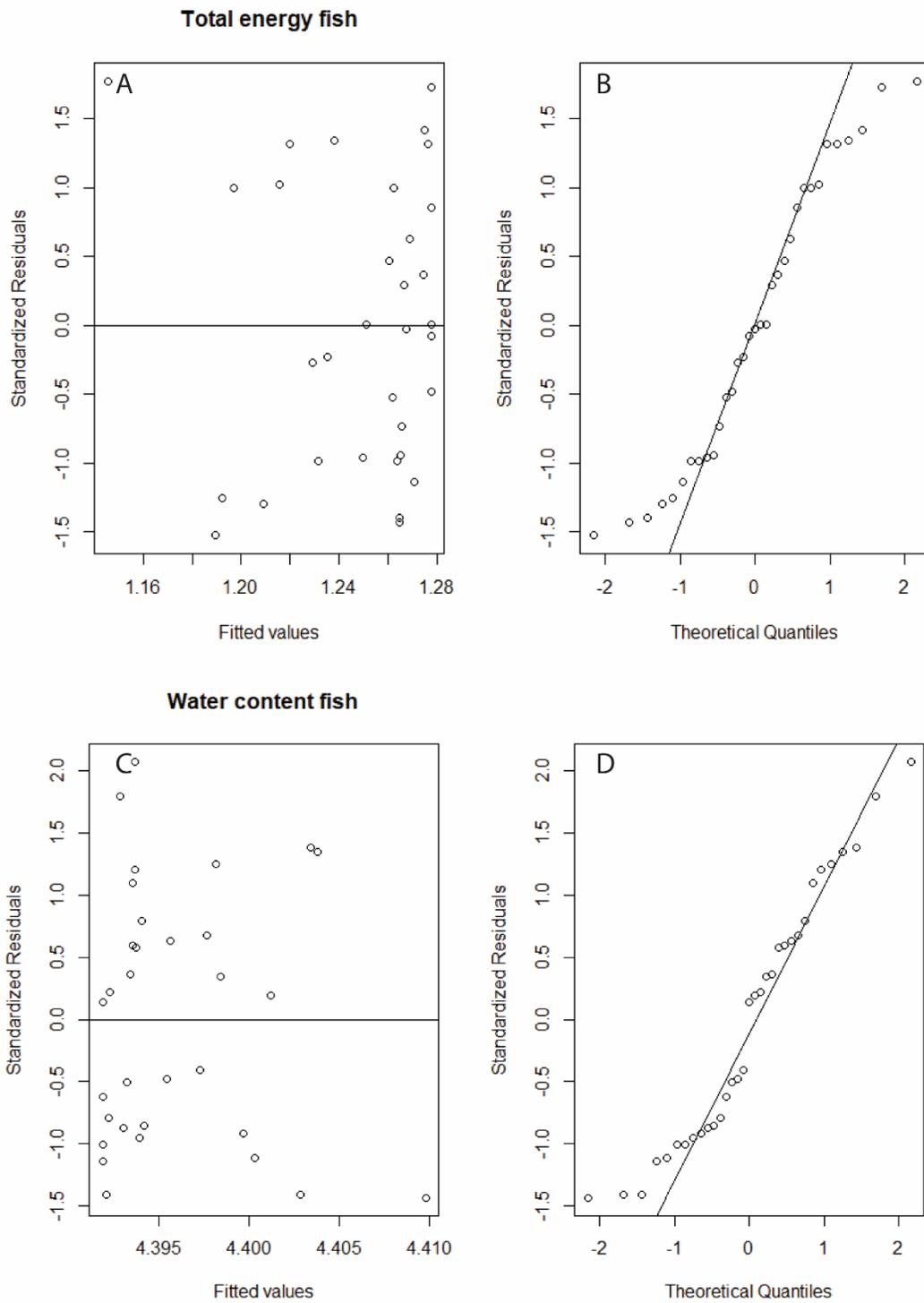


Figure S7. Model diagnostic plots for the final model of total energy (A+B) and water of fish (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

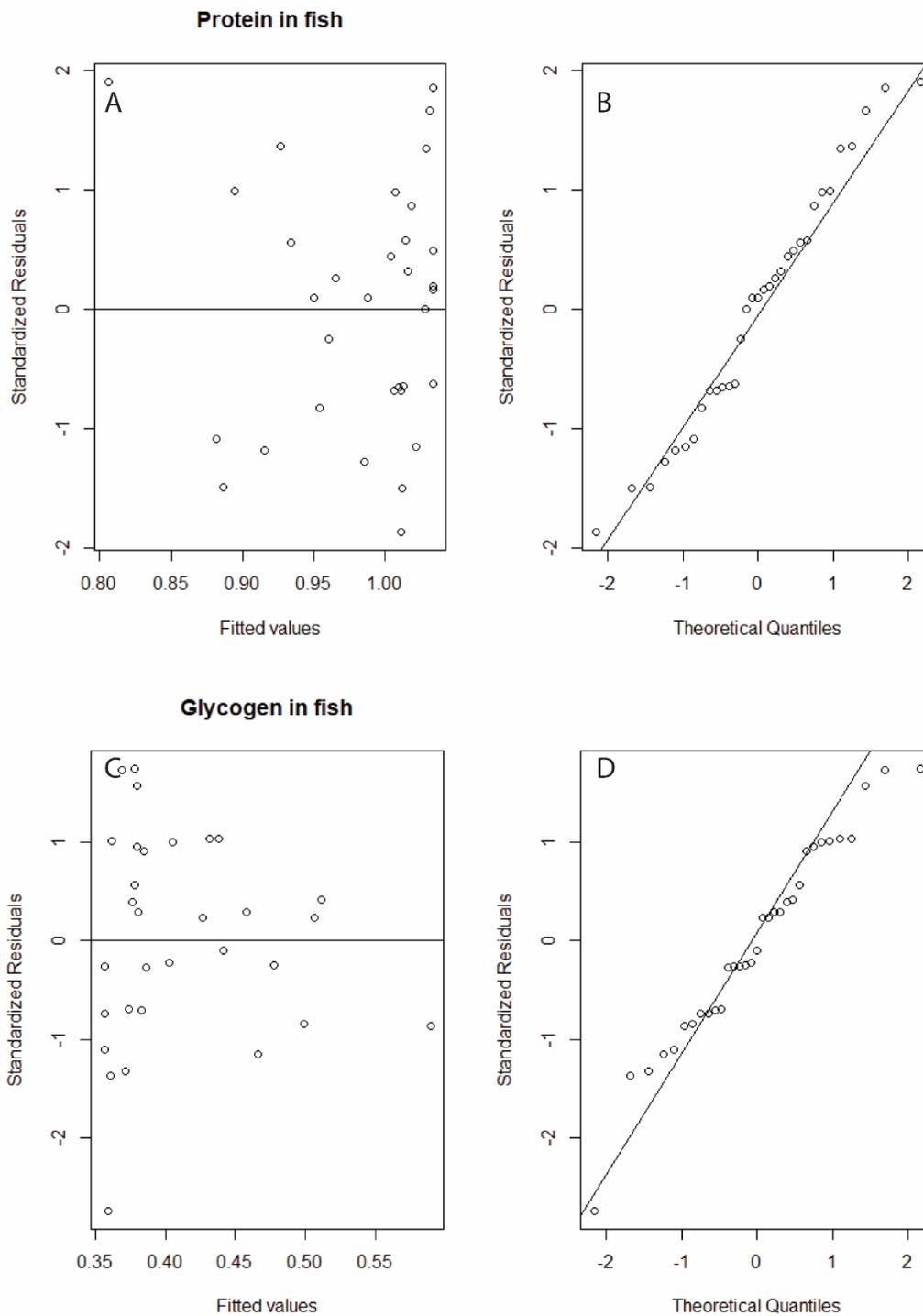


Figure S8. Model diagnostic plots for the final model of protein (A+B) and glycogen in fish (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

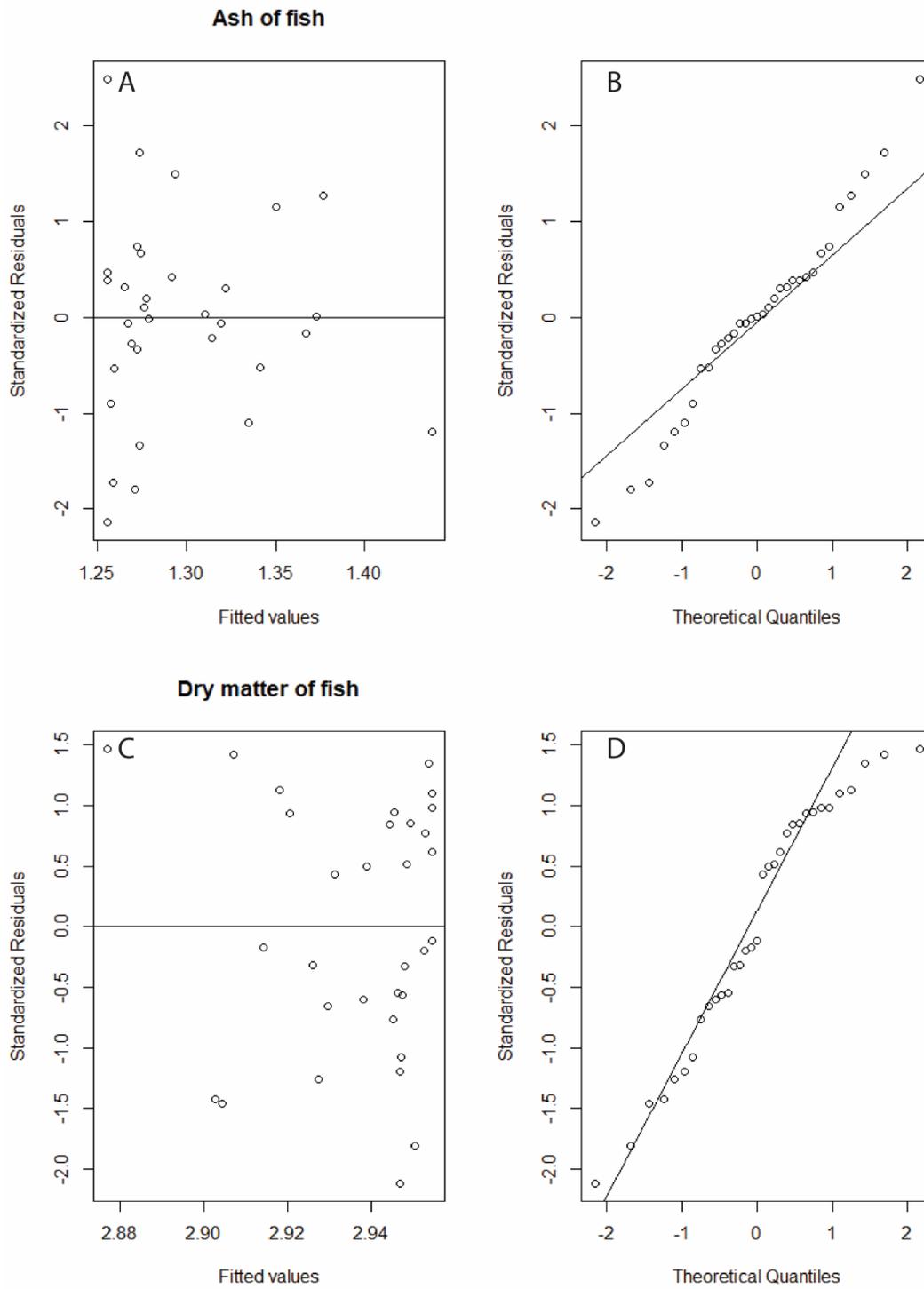


Figure S9. Model diagnostic plots for the final model of Ash (A+B) and dry matter of fish (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

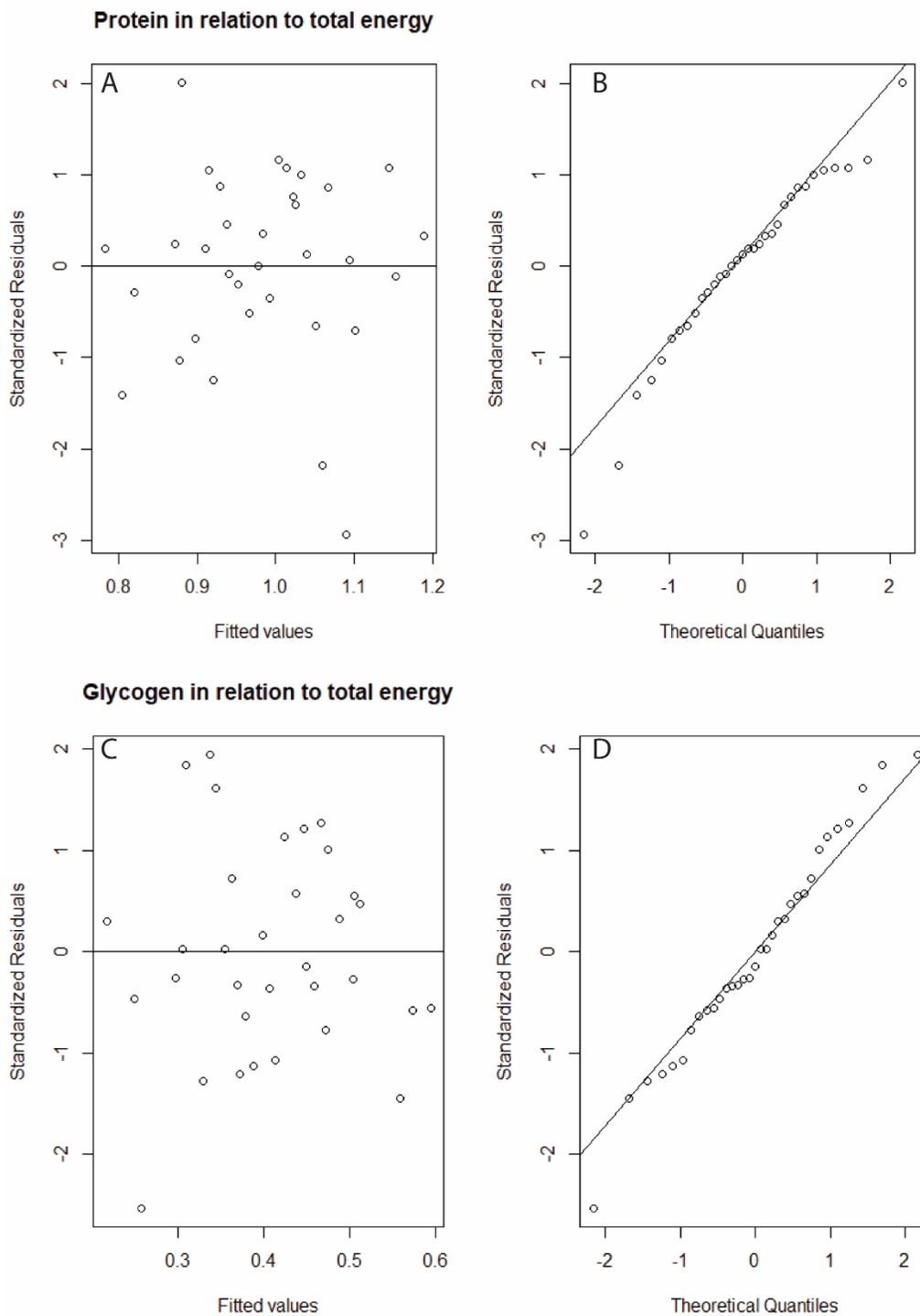


Figure S10. Model diagnostic plots for the final model of protein (A+B) and glycogen versus total energy (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

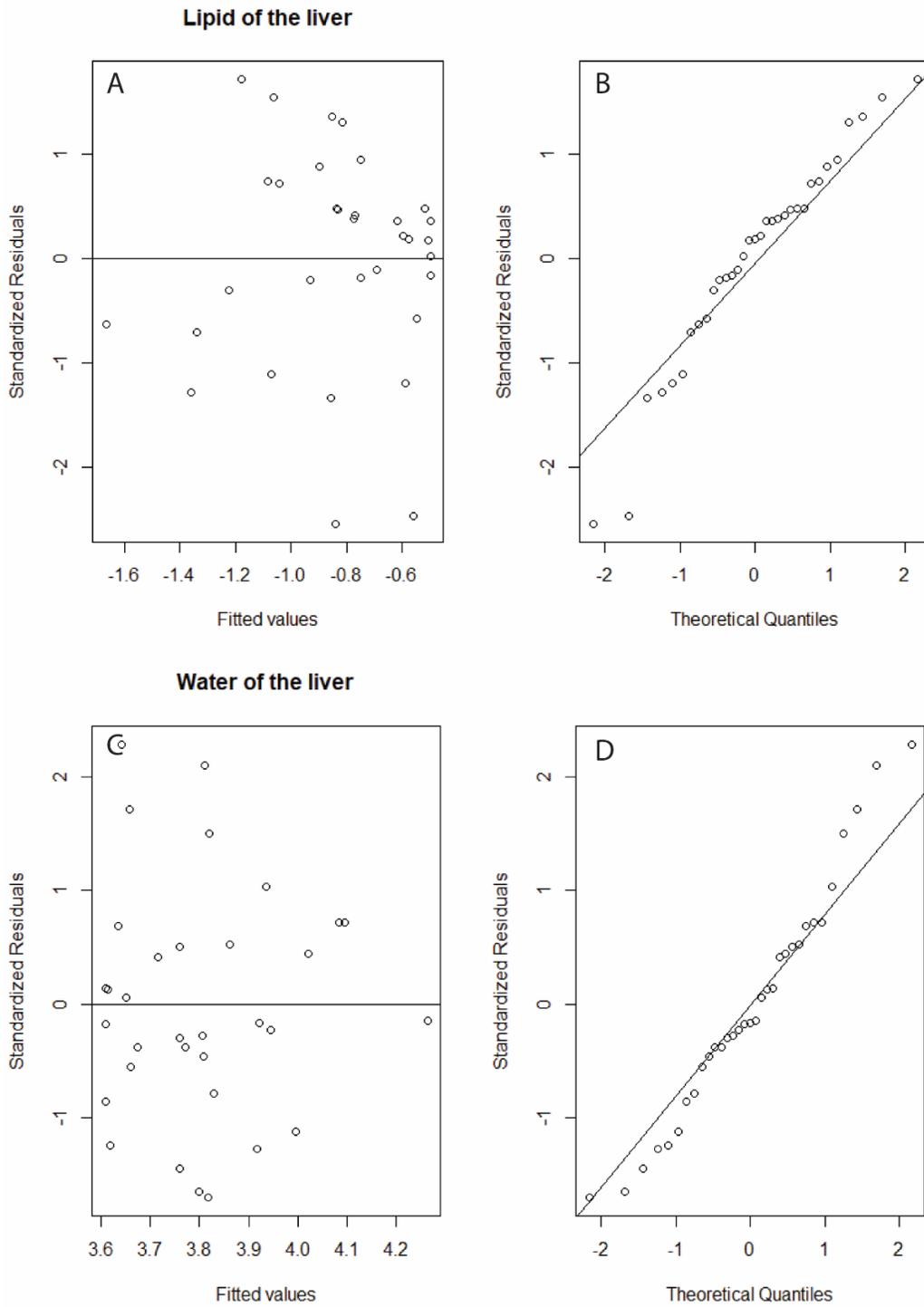


Figure S11. Model diagnostic plots for the final model of lipid (A+B) and water of liver (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

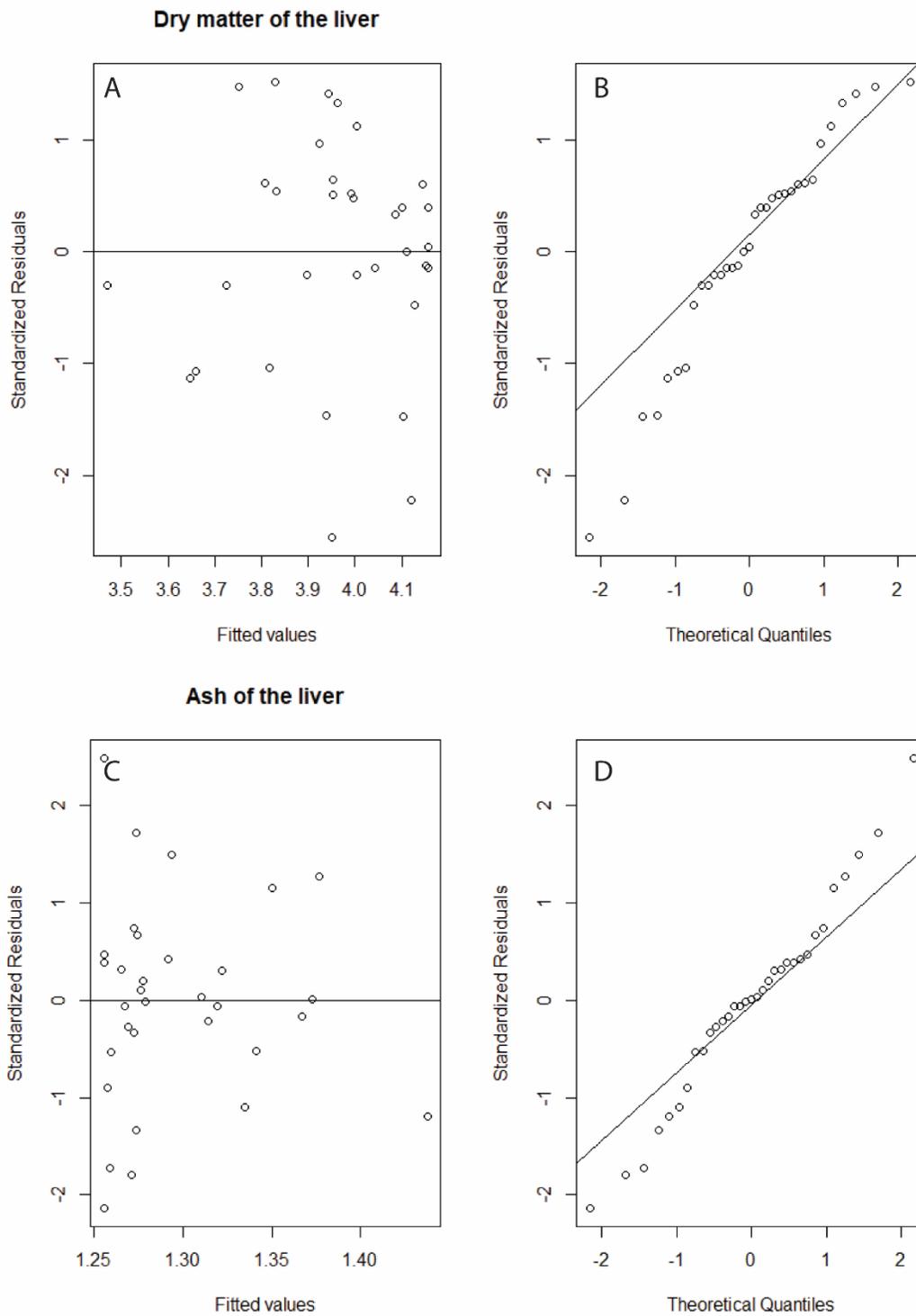


Figure S12. Model diagnostic plots for the final model of dry matter (A+B) and ash of liver (C+D) showing A+C) standardized residuals and fitted values and B+D) Q-Q plot for normal distribution of residuals.

Supplementary material B

Linking physiological mechanisms and growth processes: using bioenergetics modelling to study growth rate of Eastern Baltic cod, *Gadus morhua*, infected with a parasitic nematode, *Contracaecum osculatum*

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Stefan Neuenfeldt, Peter, V. Skov and Jane W. Behrens

Supplementary section B1: Results

Sensitivity analysis of α (nematodes/kJ)

The model is very sensitive to α (nematodes/kJ) in terms of number of nematodes for given lengths. The figure below (Figure S1) shows model outputs of total number of nematodes in relation to length with different α values (factor(type)). The α value with that best reflect the observed number of nematodes in the cod livers was found to be $8 \cdot 10^{-4}$ (light brown line in Figure S1).

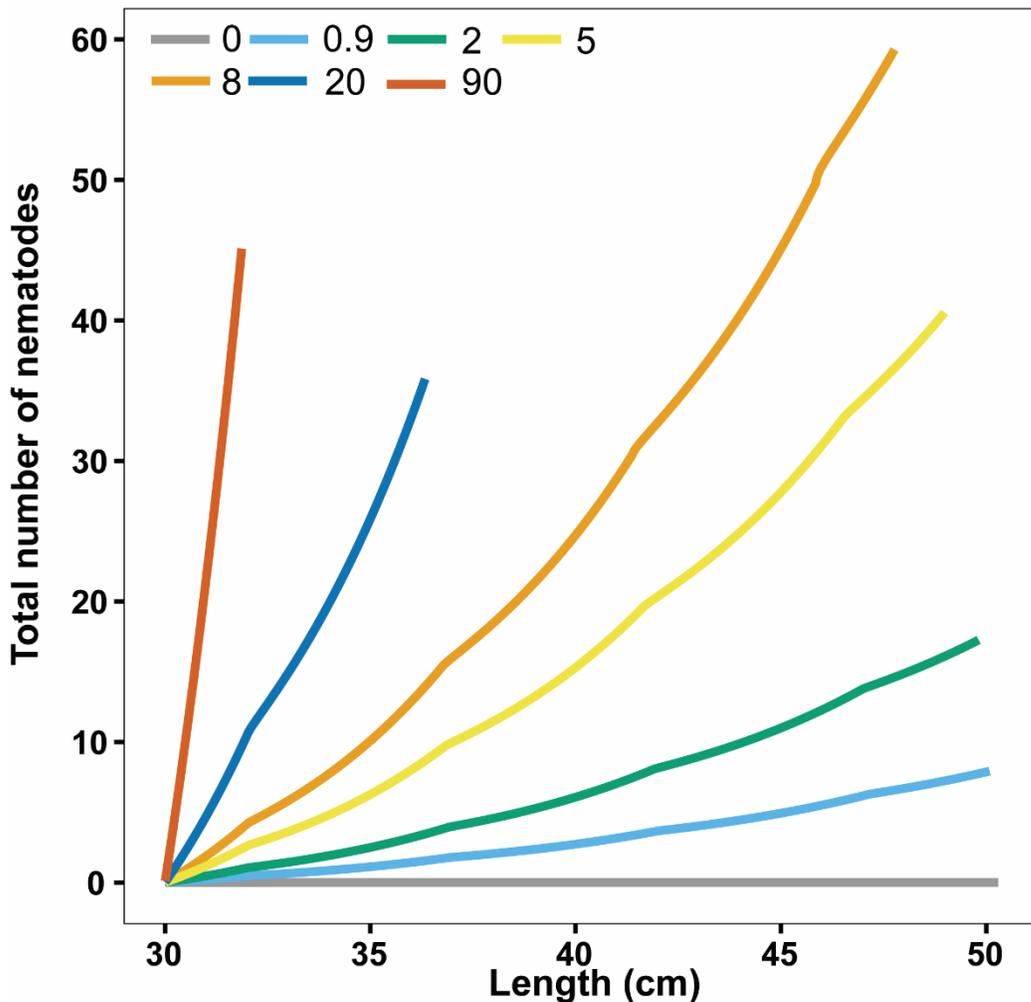


Figure S1. Relation between total number of nematodes and length for seven different values of α (nematodes/kJ) used in the model. The α values (10^{-4}) are shown in the figure and represented by seven different colours. The light brown line and α value 8 is used in the model.

Supplementary material C

Parasite load of Eastern Baltic cod, *Gadus morhua*, assessed by the liver category method and the association between infection density and critical condition

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Jane W. Behrens

Supplementary section C1: Methodology

Test of collinearity:

To test for collinearity between HSI and length a test of variation inflation factor was performed (Table S1).

Table S1. Results from the test of collinearity of explanatory variables used in the different analyses. Test of variation inflation factor: $VIF = \left(\frac{1}{1-R_i^2}\right)$ is used to assess the collinearity between the variables. Variables with VIF above 10 are defined as critical in relation to collinearity.

Variables	VIF
HSI+length	1.01

Information of data added to the probability analysis

To assure consistency in data used in the probability analysis all data available on infection density and Fulton condition factor was compiled (Table S2).

Table S2. Cod caught in SD25 between 2016-2020, used in the analysis of the association between Fulton condition factor (gutted) and infection density (nematodes per gram liver weight). Pre. = prevalence. All cod were caught by bottom trawl.

# livers	TL (cm)	W (g)	GW (g)	LW (g)	HSI	Pre. (%)	Intensity
594	39 ± 0.2 (20-58)	572 ± 11 (54-2280)	469 ± 8 (47- 1648)	24 ± 0.6 (1-115)	5.1 ± 0.1 (1.0- 14.9)	91	30 ± 1.3 (0-190)

Supplementary section C2: Complementary results

Model selection

Table S3. Comparisons of model fits for all data combined and each of the three areas (SD22, SD24, SD25). The first line (1) states the full model and the final model is represented in bold. Df = degrees of freedom, p-value is given for the difference in model reduction. Model selection was performed using a stepwise backward selection routine based on likelihood ratio tests.

Model	Variables included	df	P
All data			
1	$Log(\mu_i) = \alpha(liver\ category_i) * HSI_i + \beta(liver\ category_i) * length_i$	16	0.17
2	$Log(\mu_i) = \alpha * HSI_i + \beta(liver\ category_i) * length_i$	12	0.08
3	$Log(\mu_i) = \alpha(liver\ category_i) + \beta(liver\ category_i) * length_i$	11	
SD22			
1	$Log(\mu_i) = \alpha(liver\ category_i) + \beta * length_i + \gamma * HSI_i$	6	
SD24			
1	$Log(\mu_i) = \alpha(liver\ category_i) * HSI_i + \beta(liver\ category_i) * length_i$	13	0.7
2	$Log(\mu_i) = \alpha(liver\ category_i) * HSI_i + \beta * length_i$	10	0.9
3	$Log(\mu_i) = \alpha(liver\ category_i) + \beta * length_i + \gamma * HSI_i$	7	0.56
5	$Log(\mu_i) = \alpha(liver\ category_i) + \beta * length_i$	6	0.2
6	$Log(\mu_i) = \alpha(liver\ category_i)$	5	
SD25			
1	$Log(\mu_i) = \alpha(liver\ category_i) * HSI_i + \beta(liver\ category_i) * length_i$	16	0.2
2	$Log(\mu_i) = \alpha(liver\ category_i) * HSI_i + \beta * length_i$	12	0.8
3	$Log(\mu_i) = \alpha(liver\ category_i) + \beta * length_i + \gamma * HSI_i$	8	

Model validation plots

Model validation plots are provided for the final model combining all data (Figure S1) and the three models one for each of the three areas examined, SD 22, 24 and 25, respectively (Figure S2-4).

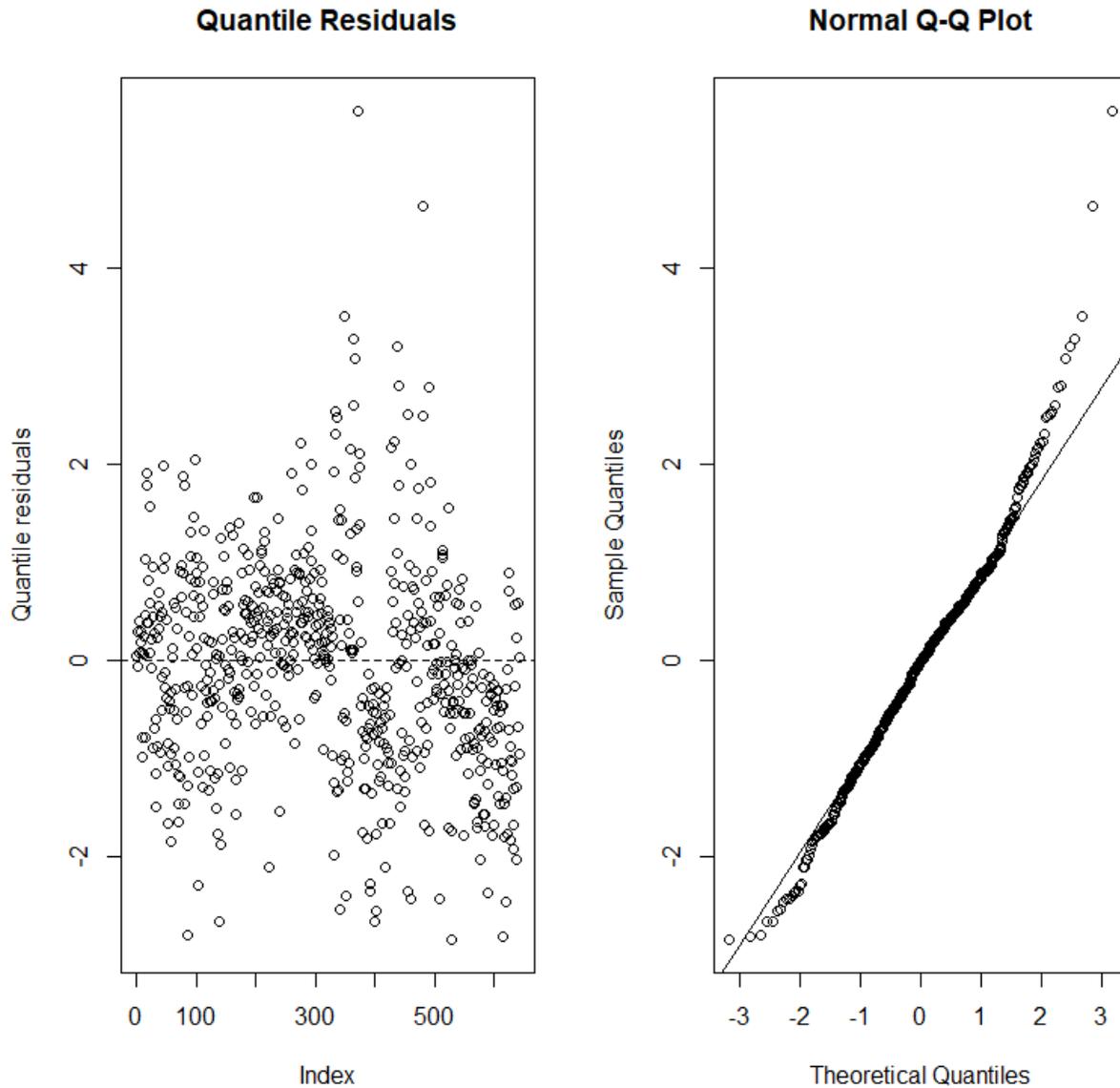


Figure S1. Model diagnostic plots for the final model including all data combined showing A) quantile residuals and B) Q-Q plot for normal distribution of residuals.

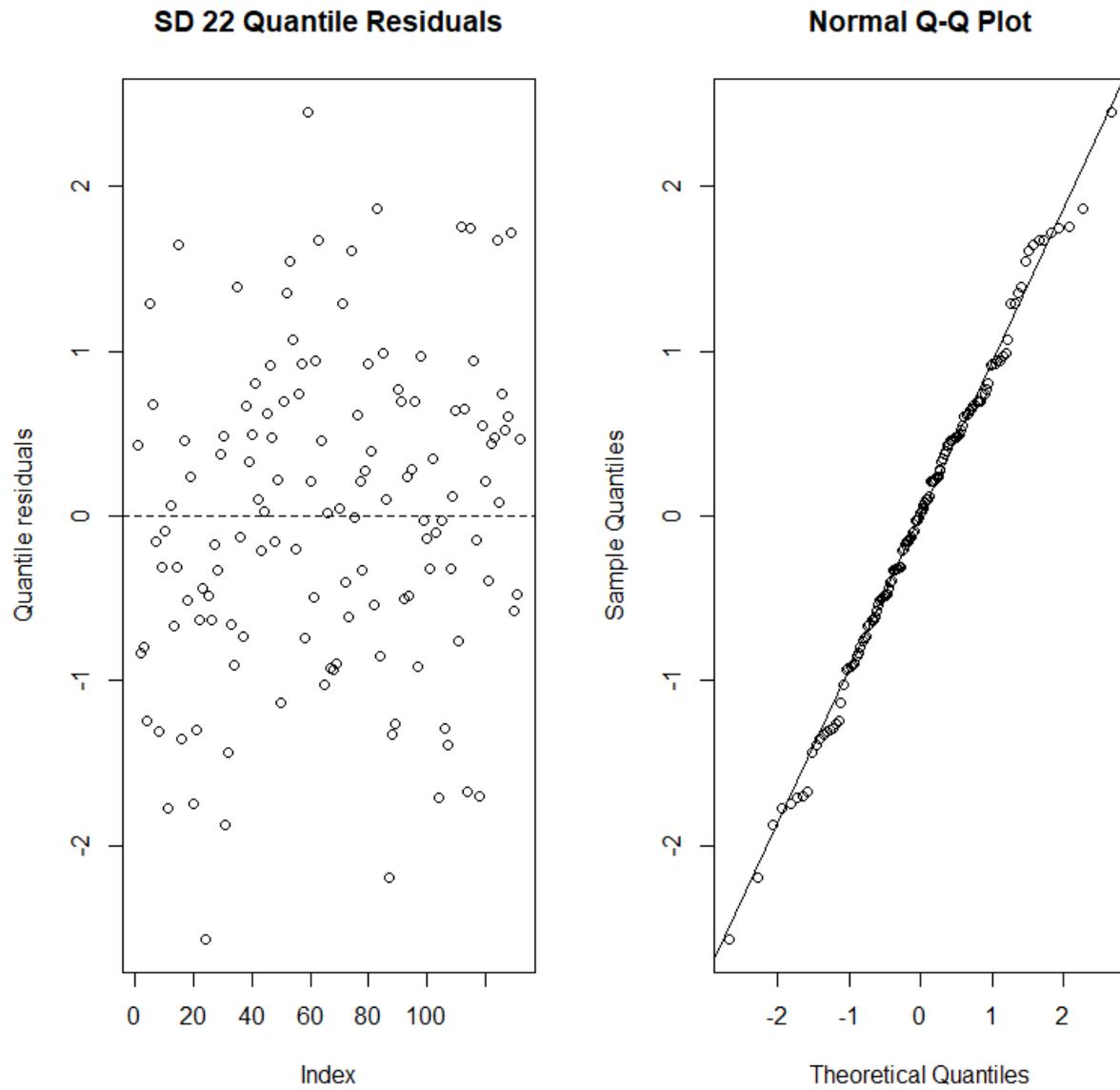


Figure S2. Model diagnostic plots for the area model for SD22 showing A) quantile residuals and B) Q-Q plot for normal distribution of residuals.

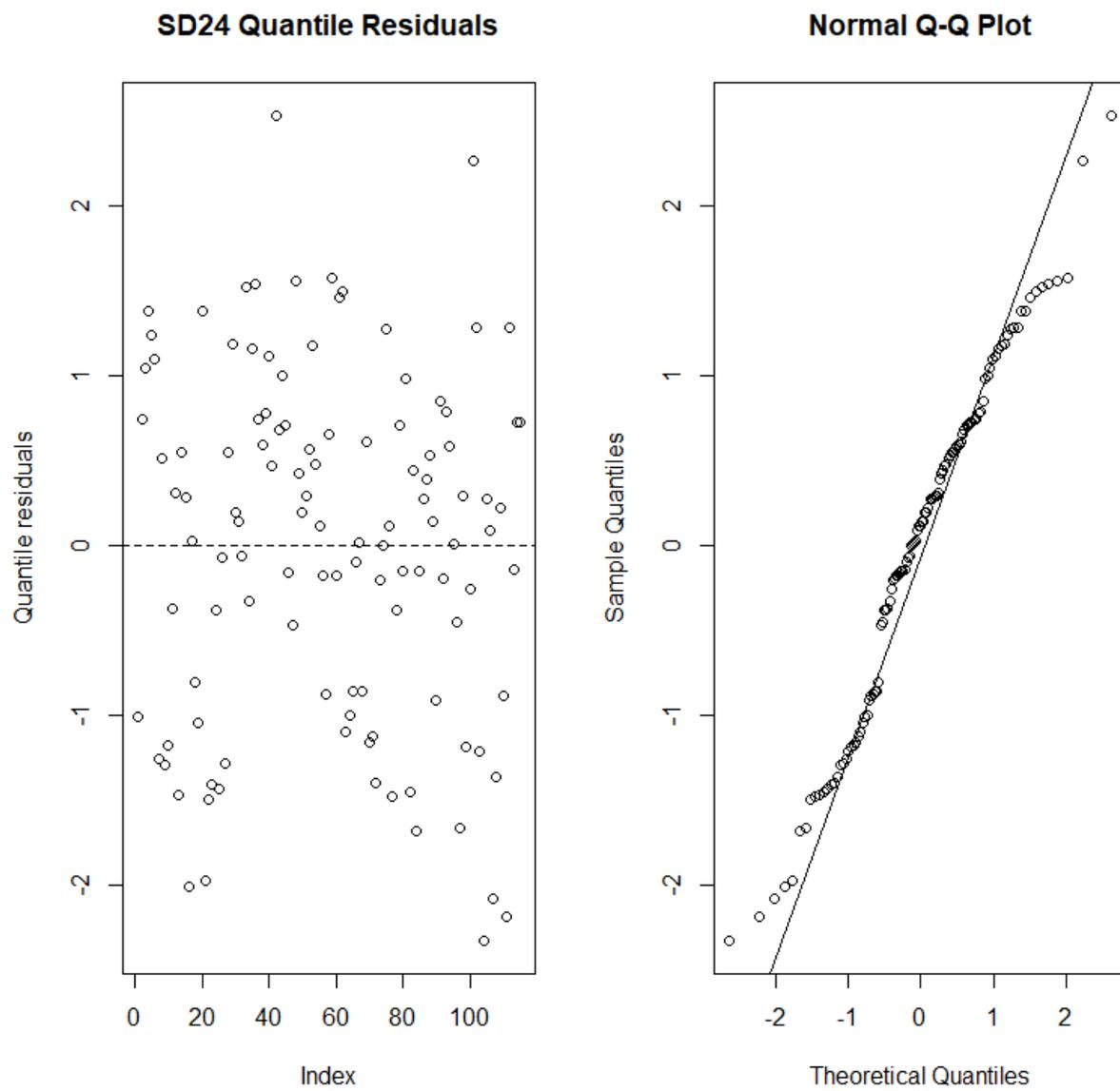


Figure S3. Model diagnostic plots for the area model for SD24 showing A) quantile residuals and B) Q-Q plot for normal distribution of residuals.

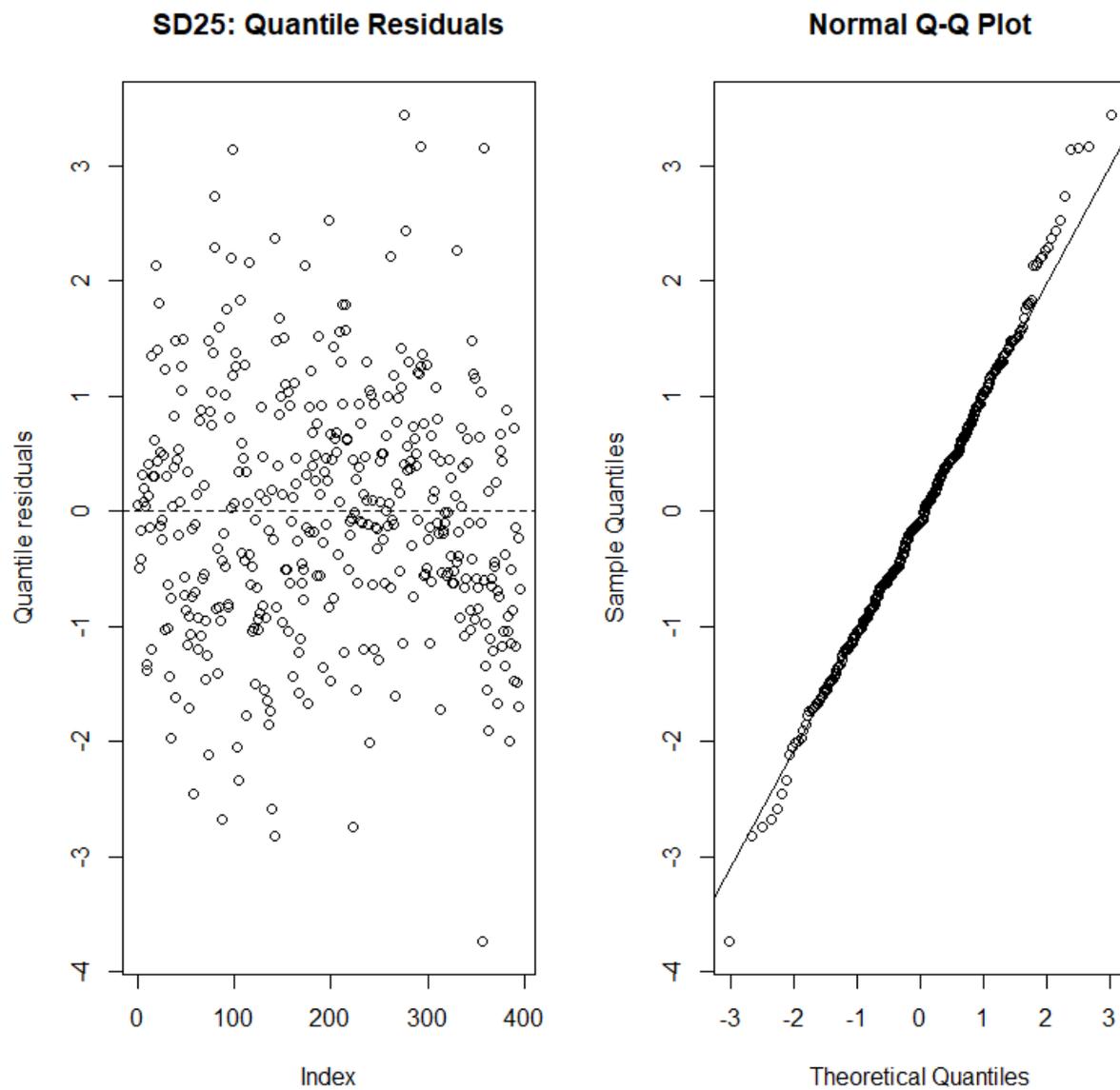


Figure S4. Model diagnostic plots for the area model for SD25 showing A) quantile residuals and B) Q-Q plot for normal distribution of residuals.

