Norwegian University of Life Sciences

Master's Thesis 2022 60 ECTS
Faculty of Veterinary Medicine

Parasite induced changes in neuronal activity: is the parasitic eye-fluke (*Diplostomum* spp.) manipulating its Arctic charr (*Salvelinus alpinus*) host?

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by

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September 2022

Acknowledgments

First of all, I would like to thank my main supervisor, Marco Vindas, for his excellent help and guidance throughout this process of project planning, lab work and thesis writing. I am truly grateful that he has challenged me to think and work independently, and at the same time ensured close supervision, which has resulted in great learning outcomes. By being encouraging and optimistic, he has contributed to making this whole process a great experience. A big thanks should also go to Øyvind Øverli, my co-supervisor, for taking me on board his team and for introducing me to Marco and to this really interesting project. He has been of great help during planning of the project as well as the planning and execution of the field work.

Further, I would like to thank Eirik Henriksen, and others, from the freshwater ecology group at UiT for helping out during the fish sampling and parasite quantification. Additionally, I want to thank Hong Qu at the Institute of Basic Medical Sciences at UiO for great help with scanning of IHC slides and Paul Whatmore at Strombus Genomics for excellent work with the analysis of RNAseq data.

Finally, I would like to thank my family and friends, for always supporting and motivating me. Without your support, this process would have been much more challenging.

Abstract

Many parasites possess complex lifecycles and depend on several host species to complete their lifecycle. Some parasites even manipulate their hosts phenotype in a way that facilitates trophic transmission to their final host (*i.e.*, parasite-induced trophic transmission; PITT) in order to complete their life cycle. The parasitic eye-fluke (*Diplostomum* spp.) has the Arctic charr (*Salvelinus alpinus*) as its second intermediate host where it establishes within the eyes and develops into metacercaria. For the eye-fluke to complete its life cycle and successfully reproduce, it depends on trophic transmission to a bird final host. Which means, the fish must be eaten by a bird. The physiological effects *Diplostomum* spp. infection might have on the Arctic charr and the possible effects on the fish's phenotype (e.g., coloration and behavior) is largely unknown. The aim of this study was to investigate the effects of *Diplostomum* spp. infection on neuronal activity and gene expression in the arctic charr. By quantification of immunohistochemistry for the neuronal activity marker phosphorylated Extracellular signal Regulated Kinase (pERK) a significantly lower neuronal activity was observed in the tuberal nucleus of the hypothalamus in arctic charr with high eye-fluke infection. Further RNA sequencing revealed an upregulation of gonadotropin-releasing hormone-3 (GnRH-3) and luteinizing hormone sub-unit β (LHβ) in heavily infected fish. These genes are involved in the hypothalamic-pituitary-gonadal (HPG) axis and serves as major regulatory genes in reproduction and steroid hormone production. Increased investment in reproduction is associated with a more conspicuous phenotype (*i.e.,* bright coloration, courting behavior and risk-taking behavior) which is linked to increased mortality by predation. For the parasite awaiting trophic transmission to its final host, interfering with the HPG-axis might be an effective way for the eye-fluke to indirectly influence the phenotype of the fish in a way that facilitates PITT. Further investigations are required to get a better understanding of the underlying physiological mechanisms involved in the potential phenotypic changes caused by *Diplostomum* spp. infection in the Arctic charr.

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List of Abbreviations

11kT	11-keto Testosterone
BSA	Bovine serum albumin
DE	Differential expression
DEGs	Differentially expressed genes
EGR-1	Early growth response protein 1
ERK	Extracellular signal Regulated Kinase
FA	Functional annotation
FDR	False Discovery Rate
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GSEA	Gene Set Enrichment Analysis
GTHs	Gonadotrophs
HPG	Hypothalamic-Pituitary-Gonadal
HPI	Hypothalamic-Pituitary-Interrenal
IEGs	Immediate early genes
IHC	Immunohistochemistry
IL-6Rβ	Interleukin-6 receptor subunit beta-like
KEGG	Kyoto Encyclopedia of Genes and Genomes
LH	Luteinizing hormone
LME	Linear mixed effects model
Log2FC	Log2 transformed fold change
NaH2PO4	Sodium dihydrogen phosphate
NAT	Nucleus anterior tuberis
NLT	Nucleus lateralis tuberis
NPT	Nucleus posterior tuberis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + 0,1%Tween
PCA plot	Principal Component Analysis plot
PCR	Polymerase Chain Reaction
pERK	phosphorylated Extracellular signal Regulated Kinase
PFA	Paraformaldehyde

PITT	Parasite-induced trophic transmission
qPCR	quantitative Polymerase Chain Reaction
Q-Q plot	Quantile-Quantile plot
ROI	Region of interest
SBN	Social behavior network
SDMN	Social decision-making network
SEM	Standard Error of Mean
SL	Somatolactin

1 Introduction

1.1 Study species

The arctic charr (*Salvelinus alpinus*) is the northernmost freshwater fish in the world and is referred to as the most variable vertebrate on earth. It is suggested that the charr's great variability in ecology, phenotype, size, life history and range has evolved due to adaptations to the highly unpredictable and variable environmental and ecological conditions of arctic lakes during several glacial periods (Klemetsen, 2013). *S. alpinus* has a circumpolar distribution and are found throughout the arctic, subarctic, boreal and temperate climate regions on the northern hemisphere, but it prefers cool or cold lakes (Klemetsen et al., 2003). In Norway the Charr migrated from the ocean in the west sometime after the last glacial period. The species has migrated to almost all parts of Norway, but it is more common the further north you and higher up in the mountains you go. Today the total number of charr populations in Norway is about 30 000 (Amundsen et al., 2015).

The charr is a generalist in relation to habitat choice. It lives in all habitats of oligotrophic lakes of all sizes and in rivers and small streams as well. Some of the charr populations also perform seasonal feeding migrations to the sea (anadromy) in the same way as Atlantic Salmon (*Salmo salar*) and Brown trout (*S. trutta*), but these migrations are most common among the northern populations. More commonly the charr migrates between lakes and rivers within a river system, and as they spawn in lakes many populations often only migrates between different areas within one lake (Klemetsen et al., 2003). This is also the case for the charr populations studied in this thesis, where the fish spends its entire life within the same lake (Amundsen et al., 2015). In the lake, the charr usually prefers the littoral zone, but under competition from larger individuals or other species they are flexible and able to exploit the profundal and pelagic zones (Klemetsen et al., 2003).

Throughout the charrs life it often undergoes an ontogenetic niche shift. That is, it changes both habitat and diet as it grows larger. The roe hatches in the littoral zone at shallow water and the fry quickly migrates to the profundal zone (deeper than 15m). They live there for about 2-3 years, protected by the dark and stay hidden from predators such as larger fish and diving birds. At this stage the diet mostly consists of crustacean plankton, insect larvae and small clams. As the Charr grows larger it adapts to a life in the pelagic zone and changes to a more silvery color

that makes the fish less visible and harder for predators to detect in the water. At this life stage the diet mostly consists of crustacean plankton and the juvenile charr stays here for about 2-3 years. When the fish reaches the age of 4-6, they migrate back to the littoral zone which is the most productive and nutrient rich part of the lake. At this point the charr has reached a size where the risk of predation from predatory fish and birds is strongly reduced (Amundsen et al., 2015). The mature charr in the littoral zone is a well-functioning predator with a morphology, senses, habitat tolerance and behavior that's well adapted to feeding on different prey-types varying from benthos to zoo-plankton, surface living insects and even other fish, including small charr (Klemetsen et al., 2003). It is also when the charr migrates to the littoral zone and matures it develops its characteristic spawning coloration with a brown/gray dorsal side and bright red abdomen with white fin margins(Amundsen et al., 2015).

Coloration is a phenotypic expression with large variations in the arctic charr, but the charr is possibly the most colorful of the northern fish species. The spawning coloration of both sexes varies a great deal, from almost colorless light pink to strong red with white fin margins (Figure *1*). The intensity of the coloration is probably closely linked to growth, fat contents and carotenoid-rich crustaceans in the diet(Klemetsen et al., 2003). This coloration is a carotenoid based sexual ornamentation, and the charr is a bisexually ornamented species where both sexes develops this spawning color(Skarstein & Folstad, 1996). During the spawning season the coloration gets more intense as the fish allocates carotenoid pigments from the muscles to the skin (Hatlen et al., 1996; Johansen et al., 2019; Skarstein & Folstad, 1996). This allocation of pigments important during the spawning season as the most colorful individuals are most likely to successfully reproduce (Johansen et al., 2019).

Additionally, there is some studies on teleost fishes showing positive associations between the intensity of the coloration and the parasite load (Folstad et al., 1994; Johansen et al., 2019).
In the arctic charr for example, the individuals with the most prominent breeding coloration are found to carry more eye flukes (trematode *Diplostomum* spp. metacercariae) than paler individuals (Skarstein & Folstad 1996).

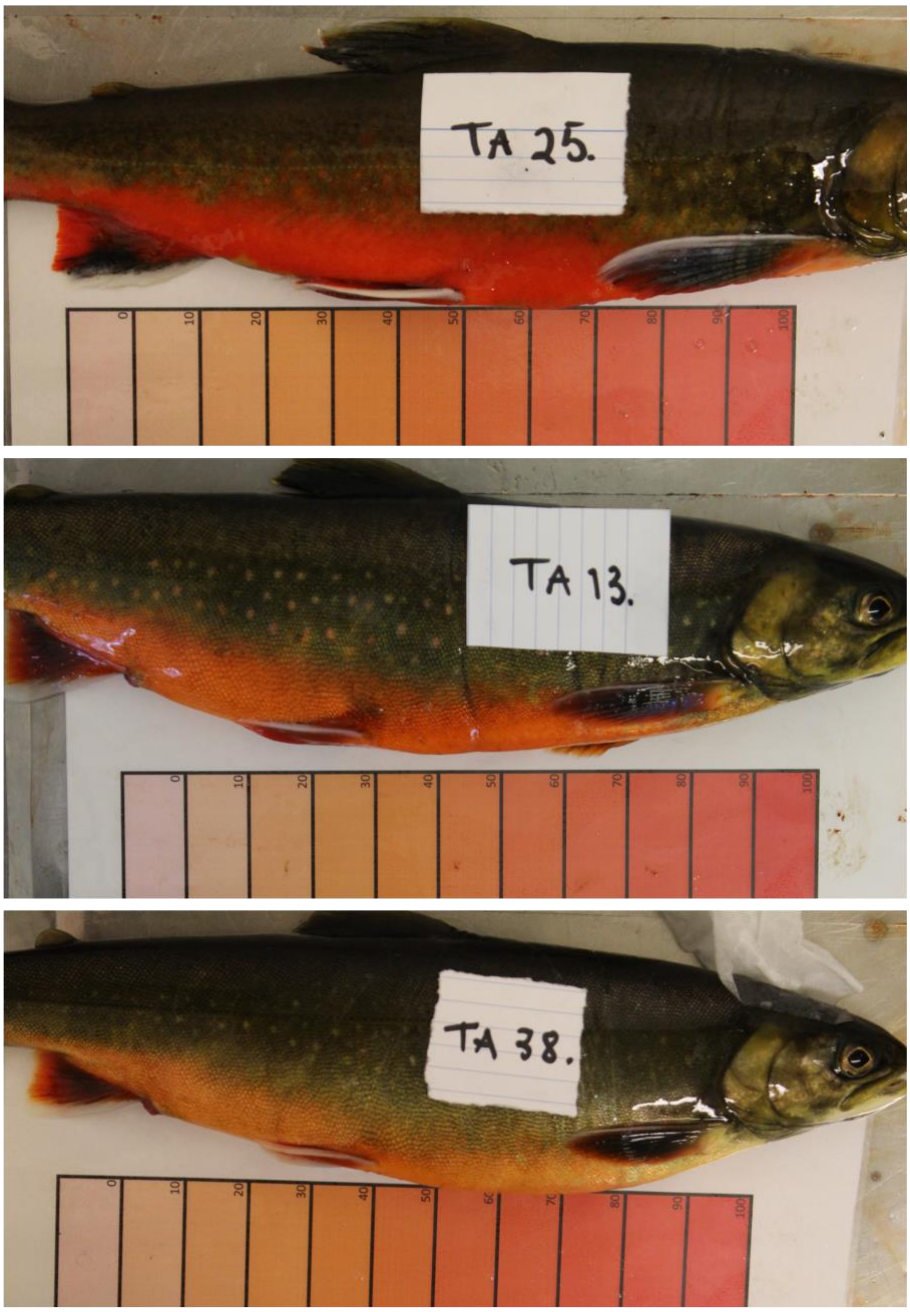


Figure 1: Picture of three individuals with different intensity of red coloration placed against a red scale from 0-100. Bright red (top), intermediate (middle) and light red/pink (bottom). Images taken during sampling in September 2021.

1.2 Parasites

There are many documentations of parasites being able to change their hosts phenotype in ways that boosts parasite fitness(Barber et al., 2000; Combes, 1991; Lefevre et al., 2009; Moore, 2013; Poulin, 1994). Berdoy et al. (2000) and Thomas et al. (2002) have sown some extraordinary examples where parasites are altering the host behavior in a way that facilitates parasite reproduction. These examples include rats being attracted to olfactory cues of cat-predators, and terrestrial crickets expressing water-seeking behavior, respectively.

An example of a teleost fish species being manipulated by a parasite is the California killifish (*Fundulus parvipinnis*). The parasite *Euhaplorchis californiensis* uses the killifish as its 2nd intermediate host and depends on the killifish being eaten by an avian predator (final host) to complete its lifecycle and reproduce. The parasite is modulating the serotonin signaling in the brain which is associated with conspicuous behavior resulting in increased predation by fish eating birds (Helland-Riise et al., 2020). Other studies using wild-caught, naturally infected Arctic charr has found a positive association between the abundance of parasites (*Diplostomum* sp.*)* awaiting trophic transmission to bird final hosts and skin redness (Johansen et al., 2019; Skarstein & Folstad, 1996), as well as for the ratio of skin versus muscle redness (*Diphyllobothrium* spp. and *Diplostomum* sp.) For these parasite species awaiting trophic transmission to a predatory bird to complete their lifecycle it is beneficial if the charr host expresses a more conspicuous phenotype (coloration, behavior etc.) making the fish more exposed to predation (Johansen et al., 2019). Additionally Johansen et al. (2019) found a negative association between a parasite using arctic charr as its final host (*Eubothrium salvelini*) and both skin redness and the ratio of redness in the skin versus muscle. *E. salvelini* completes its lifecycle in the charr where it can live and reproduce for a prolonged time (Kennedy, 1978). This species would benefit from a charr host with a less conspicuous phenotype regarding coloration and behavior leading to reduced predation risk. Even though there are many examples of parasites altering the hosts phenotype in a way that's beneficial for parasite fitness, the physiological mechanism behind these phenotypical changes remains poorly understood.

Arctic charr living in lakes in northern Norway possesses a diverse composition of parasites. The charr populations in this study are known to carry at least 11 species of parasites and the

fish usually carry several if not all parasite species at once. Nine of these parasite species are transferred to the charr via infected prey organisms (Amundsen et al., 2015). Some of the most common of these species are *Crepidostomum sp*., *Cyathocephalus truncatus, Cystidicola farionis, Diphyllobothrium* spp., *Eubothrium salvelini* and *Proteocephalus* sp. (Johansen et al., 2019). Only the crustacean gill lice (*Salmincola edwardsii*) (Amundsen et al., 2015) and the eye fluke (*Diplostomum* spp.) infect the fish directly through the water (Karvonen, 2012). As the *Diplostomum* infects the charr directly through the water and the infection isn't a result of the charrs diet it makes it easier to control for dietary effects on the char's phenotype. Therefore, I will focus on *Diplostomum* spp. throughout my thesis and the physiological effects *Diplostomum* spp. infection can potentially have on the char's phenotype.

It is common for parasites to have complex lifecycles. They commonly depend on several different host species to complete their life cycle. The *Diplostomum* spp. is not an exception. *Diplostomum* spp. is a trematode and the lifecycle of *Diplostomum* spp. like most other trematodes consists of three host species(Karvonen, 2012). The parasite's definite host is a fish-eating bird, a gull for instance. In the intestines of the bird, adult trematodes reproduce sexually and start producing eggs. It's common for a single bird to be infected by several hundred worms, each producing several hundreds of eggs per day (Karvonen et al., 2006).

Eggs are transferred to the water with the bird's feces and hatch to miracidia larvae (Figure *2*). The miracidia are short lived and actively swims through the water, seeking for its first intermediate host, a pond snail (*Lymnea stagnalis*). The larvae penetrate the snail and reproduces asexually, producing sporocysts within the snail (Karvonen, 2012). High numbers of cercariae emerge from the sporocysts and are transferred to the water. For several weeks, tens of thousands of cercariae can be released to the water every day from one single pond snail (Karvonen et al., 2004; Lyholt & Buchmann, 1996). The free living cercariae are now seeking out the fish as its second intermediate host. When the cercaria approaches the fish, it attaches and crawl around to find a suitable penetration site. As the cercariae reach the fish by chance the sites for attachment and penetration are usually random. As a result, cercariae are found to penetrate the fish at several sites including the head region, the gills, the skin and the fins. The gills are the area with the highest accumulation of penetrants. This could be a result of the inhalant respiratory water flow through the gills, passively pulling the cercariae into the gill chambers (Höglund, 1991).

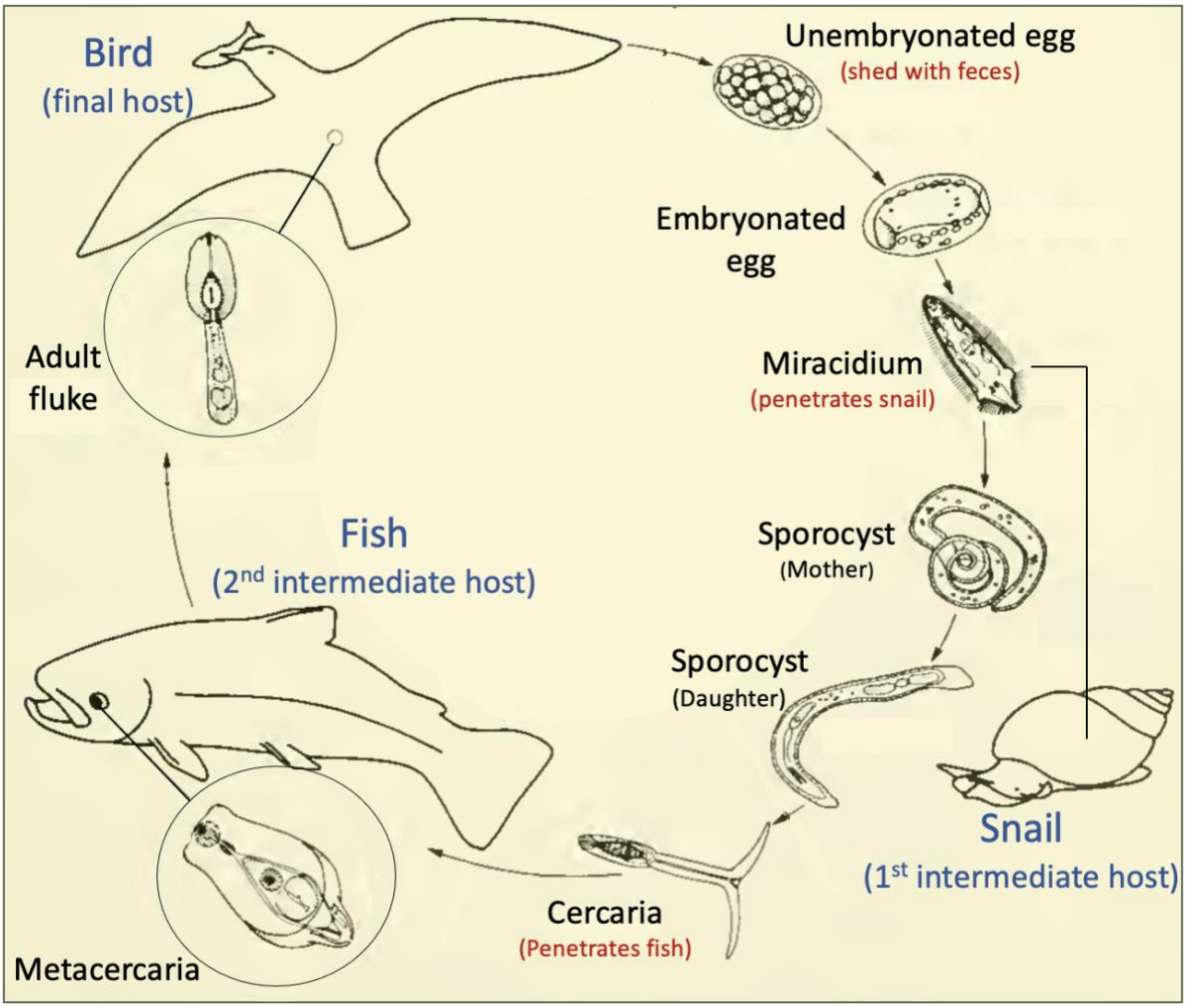


Figure 2: Life cycle of *Diplostomum* spp. Remodeled from Palmieri et al. (1976)

Once inside the fish, most of the cercariae reaches the eyes within 24 hours. The cercaria migrates from the penetration site to the eyes through both blood vessels and connective tissue. They move by muscle action and spines, contracting and elongating the body to create a forward movement (Höglund, 1991). When the cercaria has reached the eye, it develops into a metacercaria causing a disease called doplostomiasis. Subsequently, metacercaria grows in size and takes an oval or cylindrical shape. The internal structure of the eyes is a sterile environment separated from the immune system by the blood-retinal barrier making the eyes an immune privileged structure (Caspi, 2013). This gives the parasites a chance to escape immune responses mobilized by the host to reduce pathological effects induced by parasite infection (Padrós et al., 2018).When the metacercaria have entered the eye they are generally believed to live within the fish for several years, resulting in an accumulated infection over time. The

Diplostomum's lifecycle is completed when the infected fish is eaten by a predatory bird (Karvonen, 2012).

1.3 Neuroanatomy and function

The teleost brain consists of three main regions. It is the forebrain, midbrain and the hindbrain (Yamamoto, 2009). The forebrain consists of the telencephalon and diencephalon (Figure *3*). The midbrain is divided in the optic tectum dorsally and the tegmentum ventrally. And the hind brain is further divided into cerebellum and the brain stem (Wullimann, 1997). Further, the telencephalon is divided into the pallium dorsally and the subpallium ventrally, and the diencephalon is divided into the thalamus dorsally and the hypothalamus ventrally (Calvo & Schluessel, 2021).

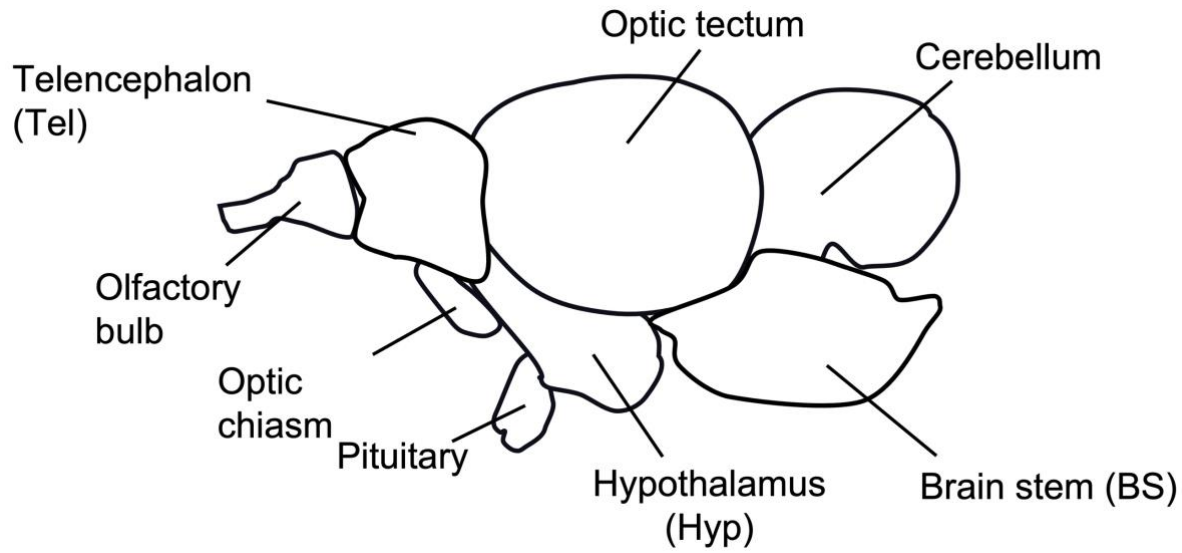


Figure 3:Simplified schematic illustration of the sagittal plane of the teleost brain. Modified from Vindas et al. (2017)

Telencephalon

The telencephalon is an important brain region for cognitive functions. Spatial cognition is one of the important cognitive functions of the telencephalon and is linked to orientation, spatial learning, and memory. Parts of the telencephalon is found to play a role in the motivation and reward pathways which is linked to enhanced learning. Decision making and choice behavior is also important features of the telencephalon. That is, making choices and expressing behaviors as response to these decisions. Parts of the telencephalon is associated with

processing external sensory stimuli (e.g. olfactory and visual stimuli). Avoidance learning and emotional memory which is linked to stress regulation and anxiety, conditioning and habituation is also some of the functions associated with the telencephalon. The telencephalon is also important for assessing numerical information and comparing quantities. This is beneficial for many behaviors such as foraging, socializing and reproduction. Further, the telencephalon is a part of the social behavior network (SBN) consisting of structures in the telencephalon, hypothalamus, midbrain as well as the cerebellum and brain stem. The SBN is important for social cognition. That is, individual recognition, social habituation to neighboring conspecifics, social behavior, social hierarchy, aggression, mate choice and reproductive behavior. Additionally, the telencephalon is a part of the social decision-making network (SDMN) which is linked to cooperative behavior e.g. cooperative territorial defense (Calvo & Schluessel, 2021 and references therein).

Hypothalamus

Another brain area that is associated with reproductive and other social behaviors is the hypothalamus. The hypothalamus receives information about internal (body homeostasis) and environmental signals as well as sensory inputs from other brain regions, including the telencephalon. This information is then integrated into hormonal and behavioral responses. This makes the hypothalamus the main region responsible for integrating and controlling the synthesis and release of hormones to the pituitary. The hypothalamus together with the pituitary is responsible for control and regulation of the endocrine system (Chandroo et al., 2004; Kime, 1998). There are several endocrine axes controlled by the hypothalamus. Some of these are the hypothalamic-pituitary-gonadal (HPG) axis and the hypothalamic-pituitary-interrenal (HPI) axis which is responsible for reproductive and stress regulation, respectively (Vissio et al., 2021; Winberg et al., 1997; Zohar et al., 2010).

Optic tectum

As with the telencephalon and hypothalamus mentioned above, the optic tectum has several functions required for rapid decision making which is necessary for behavioral reactions important for survival and reproduction. The optic tectum is the main region for processing visual stimuli as well as other sensory stimuli. The tectum is important for several behaviors such as navigating the environment, food and prey seeking, predator avoidance. The information processed by the optic tectum is directed to other structures in the midbrain and the

brain stem which have synapses with cell groups that activates motoneurons allowing eye movements and body movements as response to the sensory stimuli (Northmore, 2011).

Cerebellum

The cerebellum is associated with several important cognitive functions such as learning, memory and emotional conditioning. The cerebellum is also associated with classical conditioning of discrete motor control e.g. the eye-blink response (Reviewed by Rodriguez et al., 2005). Further, the cerebellum is associated with spatial cognition. Studies on cerebellum-ablated goldfish shows that fish with ablated cerebellum has a reduced spatial accuracy and shows abnormal exploratory behaviors and problems with navigating the environment (Durán et al., 2014). Additionally, reproductive behaviors such as mate choice and spawning is associated with increased neural activity in the cerebellum which may indicate that the cerebellum is involved in reproduction in fish (Okuyama et al., 2011).

Brain stem

The brainstem connecting the brain to the spinal cord is the main region responsible for somatosensation with the exceptions of olfactory and visual stimuli and is connected to the sensory organs through several cranial nerves (Kotrschal & Kotrschal, 2020). The brainstem is also associated with some life sustaining functions like regulating the respiratory rhythm and hart rate (Taylor et al., 2006). In addition, the main center for synthesis of serotonin (raphe nuclei) is located in the brain stem. This makes the brain stem involved in serotonin related functions like stress regulation, aggression, food intake and spontaneous locomotor activity (Winberg & Nilsson, 1993).

1.4 Neuronal activity

Neuronal activity in the brain is important for regulation of neuronal plasticity and the regulation of gene transcription which is important for development of the neuronal circuitry (Fiore et al., 2009). This contributes to the brain being able to respond and adapt to the environment the organism lives in. The neuronal activity is important for adjusting the development of new synapses to internal and external stimuli. These effects of neuronal activity are involved in regulating activity-dependent transcription factors that coordinate the expression of genes necessary for formation and maturation of dendrites and synapses. The

interaction between the environment and the genes contributes to sensory impressions influencing how the brain is organized and works. This way sensory information is used to organize the development of the brain and facilitates and adapts behaviors to the environment (West & Greenberg, 2011).

Immediate early genes

A group of markers for neuronal activity is called immediate early genes (IEGs). IEGs are genes which is activated in response to neuronal activity (Aggleton et al., 2012). These genes are rapidly activated (within minutes) after external stimulation as they don't require previous synthesis of proteins (Herrera & Robertson, 1996). Some IEGs are functioning as networks of proteins that are coordinately regulated and expressed as a result of neuronal activity. While others are only activated as a response to external stimuli. Some examples of immediate early genes are the c-Fos and EGR-1 (also called zif-268). These IEGs are working as a transcription factors. Together with other transcription factors, the c-Fos is involved in regulation of the gene expression of delayed response genes associated with neuronal plasticity (Zukin et al., 2004). Using IEGs in combination with techniques such as immunohistochemistry (IHC) provides great spatial resolution for detecting neuronal activity (Barbosa & Silva, 2018).

Extracellular signal-regulated kinase

Extracellular signal regulated kinases (ERKs) associated with activity-dependent regulation of neuronal functions, and can also be used as a marker for neuronal activity (Grewal et al., 1999). ERK is included in the mitogen-activated protein kinase (MAPK) group. And phosphorylation of upstream kinases leads to the activation of ERK (Widmann et al., 1999). When activated, phosphorylated ERK (pERK) can be transferred to the nucleus and activate several transcription factors like cAMP-response element binding protein (CREB; Gao & Ji, 2009). This transcription factor is associated with several neuronal genes as well as long-term synaptic plasticity(Ji & Rupp, 1997).

IEGs and pERK as neuronal activity markers

As neuronal activity markers both IEGs and pERK share some properties. Common for both IEGs and pERK is that they have relatively low baseline activity that rapidly increases after stimulation before decreasing to normal levels soon after activation (Herrera & Robertson, 1996; Ji et al., 1999). They are expression is specific and robust and it is relatively simple to perform techniques such as immunohistochemistry (IHC) for visualization and quantification. Even though, both IEGs and ERK has a rapid increase in activity after stimulation, pERK expression is much faster and more dynamic compared to the expression of c-Fos. The latency for activation after an acute noxious stimulus for the c-Fos is up to 30 min while the pERK is activated within 1-3 min. The activation then peaks within 1-2 hours for c-Fos and 10 minutes for pERK. The c-Fos expression then uses between 8 and 24 hours to return to baseline while pERK returns to baseline within two hours (Reviewed by Gao & Ji, 2009). This indicates that pERK can provide improved temporal resolution compared to IEGs (Randlett et al., 2015). A study on rats shows sustained expression of pERK in the spinal cord following nerve injury. After the injury, pERK is expressed in different cell types at different times, starting in the neurons and switching to microglia cells and astrocytes later during the different phases of the neuropathic pain development (Zhuang et al., 2005). While pERK can be expressed in the whole cell as well as other cell types, c-Fos is only expressed in the nuclei. This makes it possible to perform immunohistochemistry for c-Fos in combination with tract-tracing techniques to trace nerve pathways and identify the characteristics of the labeled neurons (Gao & Ji, 2009).

1.5 Aim

The main aim of the study is to investigate if the parasite *Diplostomum* sp. is able to affect the phenotype of the infected arctic charr in ways that facilitates transmission of the parasite to a final host, increasing the parasites fitness.

Hypotheses:

1. There is a difference in neuronal activity between arctic charr with high and low infection of the eye fluke *Diplostomum* sp. metacercaria.
2. The expression of genes with the potential to alter the fish's phenotype in a way that facilitates trophic transmission of the parasite to its final host is different between arctic charr with high and low *Diplostomum* infection.

2 Materials and Method

2.1 Field work 2016/2017

In addition to the fish sampled in September 2021, material sampled by Johansen et al. (2019) in 2016 was also used. Mature arctic charr was sampled from three lakes in Troms County, Fjellfrösvatn (69°05′N, 19°20′E), Takvatn (69°07′N, 19°05′E) and Sagelvvatn (69°11′N, 19°06′E). The fish were collected between the 7-9th of September 2016 using gillnets submerged for a maximum of two hours. The fish was placed in trays containing 0.25 mg/L MS-222 (Sigma-Aldrich, St. Louis, Missouri, USA) and transported back to land. A total of 62 sexually mature males were collected on from the spawning grounds across the three lakes. Back at land, fork length and skin coloration were registered. Following a blood sample was collected from the caudal vein for later analyses of steroid hormones (testosterone and 11-keto-testosterone; 11kT) before the fish were humanely killed by decapitation. Additionally, both eyes were dissected, and the rest of the body was kept on ice for parasite quantification. The lower jaw, and the very anterior part of the head was cut off and the rest of the head was covered in aluminum foil and stored at -80C until further analysis. For more details about the sampling and parasitological analysis, see Johansen et al. (2019).

2.2 Fish sampling 2021

The fieldwork was carried out at Lake Takvatn (69°06′N, 19°09′E) in Troms County in Northern Norway in the period 19th to 22nd September 2021. In total 43 mature arctic charr were sampled from the littoral zone along the shoreline on the south-eastern part of the lake close to the spawning grounds (Figure *4*). The gillnets used to catch the fish were 40 m long and 1.5 m deep with a multi-mesh consisting of eight randomly distributed 5 m panels (mesh size 10, 12.5, 15, 18.5, 22, 26, 35 and 45 mm.). Three gillnets were placed three different locations (Figure *4*) and retrieved after one to three hours. The nets didn't stay for more than three hours in order to control for differences in sampling-latency between the first and last fish caught at every fish bout. In addition, each gillnet was retrieved one at a time and all the fish in the net were placed in a plastic container with a lethal dose of anesthesia (Benzoak vet. 200 mg/ml) and immediately transported back to the field laboratory within 5 min. When the required samples from each batch were obtained, the gillnet was placed back in the water and the next gillnet was retrieved.

Figure 4: Map over lake Takvatn. The areas the gillnets were placed is illustrated with the red fields on the right side of the lake. The field station is marked with the red star on the bottom right of the lake (ref: kartverket.no)

At the field lab the fish were processed after confirmation that there were no signs of life. The fish were first measured and weighted, their sex was determined and their intensity of the red coloration on the abdomen (*i.e.* red-scale) was estimated using a color scale (adapted from methodology by Johansen et al., 2019). A picture was taken from each side of the fish using an SLR camera (canon EOS4000D) before blood samples were taken by using 1ml syringes. The blood was transferred to an Eppendorf tube and centrifuged for 2 min at 6000 rpm and outdoor temperature (varying between 8 and 15ºC). Blood plasma was transferred to a new Eppendorf and placed at -20ºC for later analysis of steroid hormones. The fish were then decapitated, and the brain was dissected out by using surgical scissors. The top of the skull was split, and the bone and cartilage were bent to the side exposing the brain. The optic nerve and connective tissue around the brain was cut and the brain was removed and placed in small glass jars (Numbered 1-43) with 4% PFA (paraformaldehyde) in Sorensen's Buffer (28 mmol/L NaH_2PO_4 and 71 mmol/L Na_2HPO_4, pH 7.2) to fixate the brain tissue. The jars containing the brains were stored at 4ºC over night (approx. 16 h). The brains were then washed 3 times for 20 min in Sorensen´s buffer followed by cryopreservation in a 25% sucrose solution in Sorensen´s buffer for approximately 48-72hours. Following decapitation, the rest of the body was processed by cutting a 1 cm^3 cube from right side of the body behind the pelvic fin (where

the color was most intense) to obtain a skin and muscle sample for carotenoid analysis. Once removed, the skin and muscle were placed in separate Eppendorf tubes and stored at -20ºC until further analysis. The color of the muscle was also registered by using the red scale mentioned above, in order to calculate the ratio between the color of the skin and muscle.

In order to quantify the number of parasites the eyes and intestines were dissected, placed in physiological salt water (0,9% NaCl) and frozen at -20ºC, allowing parasitological analysis in the laboratory at a later time. Additionally, the gills were examined for gill lice and the number of *Diphyllobothrium* sp. cysts on the stomach wall was counted before the stomachs were cut open to visually determine the total degree of fullness on a scale from empty (0%) to full (100%). This work as not part of my thesis, but it is relevant for our project partners at the freshwater ecology group at the University of Tromsø (UiT) and their long-term studies of the ecology of the fish populations in Takvatnet).

2.3 Parasite quantification

The intestines were thawed and held on ice to prevent the tissue and parasites from degrading. The intestines were placed in petri dishes with physiological salt water and cut open. The content of the intestines as well as the pyloric caeca was scraped out using tweezers and sieved using a 180-micron mesh size nylon net under running water to remove mucus and microparticles. The cleaned intestinal content was transferred over in a clean petri dish with physiological salt water for identification and quantification of the parasites using a stereo microscope.

The eyes were thawed and placed in separate petri dishes. Using small eye scissors, the eyes were cut open from the entrance of the optic nerve, and the tissue was scraped clean and rinsed using physiological salt water. The small eye flukes (*Diplostomum sp.*) were then counted under a stereo microscope. After parasite quantification the charr was sorted by parasite load and divided into high and low contrast groups (see Table 1 in the results for further details).

2.4 Lab work

Mounting and cryosectioning

The fresh brains collected during the 2021 sampling were taken out of the sucrose solution and were mounted in Tissue-Tek® O.C.T. compound (Sakura Finetek, USA) before freezing and storing at -80 C. A layer of Tissue-Tek was put in a silicone mold placed in a Styrofoam box with dry ice until the Tissue-Tek started to crystalize. The brain was then immediately placed horizontally on top before being filled completely with Tissue-Tek before the mold was placed back on the dry ice until frozen (Figure 5). Once frozen the sample was removed from the mold and placed in a small zip-lock bag labeled with sample number and stored at -80ºC for further cryosectioning. The frozen heads collected during the 2016 sampling also mounted in Tissue-Tek as described above.

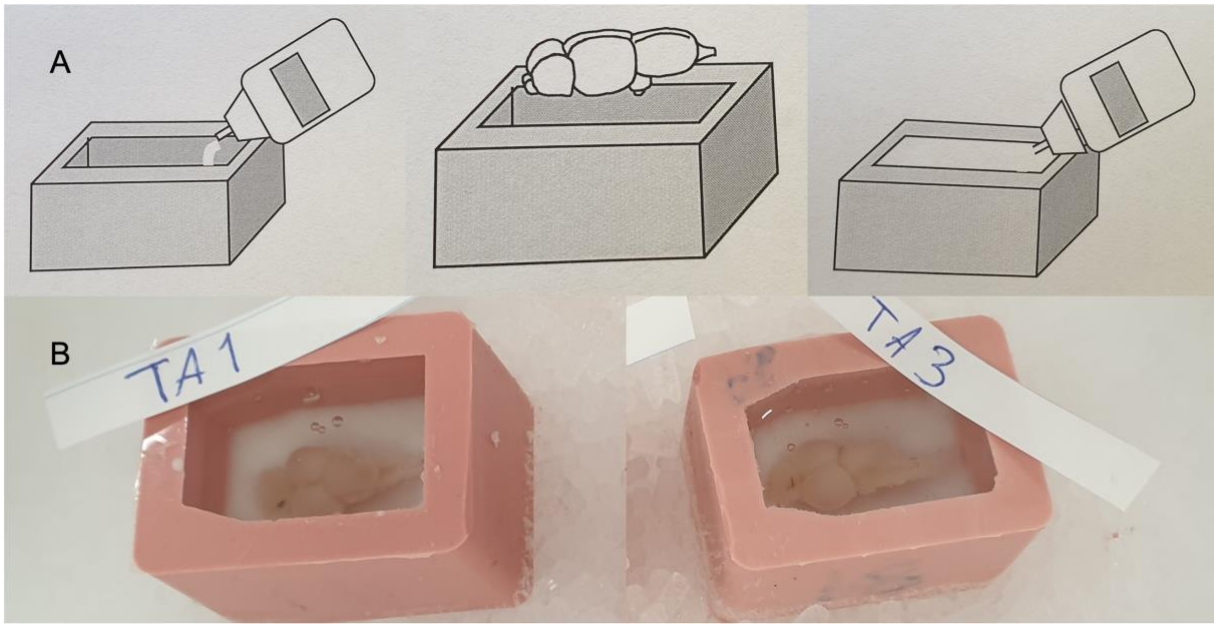


Figure 5: Brain mounting procedure. A: Schematic drawing of the mounting procedure. B: Silicone molds with arctic char brains covered in Tissue-Tek placed on dry ice.

Cryosectioning was done using a Leica CM3050 cryostat (Leica, Wezlar, Germany) set at -24ºC. The thickness of the slices was adjusted to 14 µm for the histology (*i.e.* frozen fixed brains) samples and 300 µm thickness for the gene expression (*i.e*. frozen heads) samples. All the equipment was cleaned with RNase-Zap before being placed in the machine to cool down. Tissue-Tek was poured onto a stub in order to mount the brain and head samples for slicing. For the histology samples, previously marked glass slides (Fisherbrand™ Tissue Path Superfrost™ Plus Gold Slides) were kept outside the machine in batches of six. Sliced tissue

was then thaw mounted on the glass slides by gently pressing the slide on the tissue section. The first slice on the first glass slide, the second slice on the second glass slide and so on until all six slides were full. Then six new slides were marked, and the procedure repeated. When the whole brain was sliced, all the glass slides were placed in an oven at 65ºC for 10 minutes and stored at -80ºC for further analysis by immunohistochemistry (IHC). Each brain resulted in approximately 100 glass slides of 8-12 slices (approximately 1000 slices per brain). As the brains was so large and the sectioning was time-consuming, only six brains from each contrast group were cryosectioned. That is, the six with the highest and lowest parasite loads (12 in total).

For the frozen head samples one or two of the previously marked glass slides were kept cold inside the cryostat. The sectioned tissue was placed onto the cold slides one by one until the slide was full. Consequently, the slide was taken out in order to quickly thaw mount the slices before freezing the slide again. Full slides were placed on dry ice until the whole head was sectioned and then stored at –80ºC until further processing (*i.e.*, microdissections).

Immunohistochemistry (IHC)

As there are no studies on immunostaining of the neural activity marker Phosphorylated Extracellular Signal-Regulated kinase (pERK) on the arctic charr a study by Randlett et al., (2015) on brain activity mapping for zebrafish was used to test and a adapt a protocol for pERK immunohistochemistry (Appendix A). The testing of the protocol was first carried out on 14μm sections of zebrafish brains to control that the protocol was working. As the initial test of the protocol was working for zebrafish tissue, further testing was carried on with the 14μm brains sections from the arctic charr with the same protocol in addition to one slide with zebrafish tissue for positive control. The staining on the charr tissue was very superficial in the first test, resulting in some modifications on the retrieval step of the protocol in order for the antibodies to penetrate deeper into the tissue. Additionally, a test including negative controls both for zebrafish and artic charr samples was also included. This was done by excluding the primary antibody and only applying the secondary antibody. As each charr brain resulted in a high number of slides, an initial qualitative analysis was conducted on 5 samples for the whole brain in order to determine regions of interest (ROI). This resulted on a final selection of 10 slides (80 in total) ranging from the middle/end of the telencephalon (starting right after the start of

the optic chiasm) to the middle/end of the hypothalamus (finishing at the end of the hypothalamus) from each fish was chosen for the final immunostaining (Figure *6*).

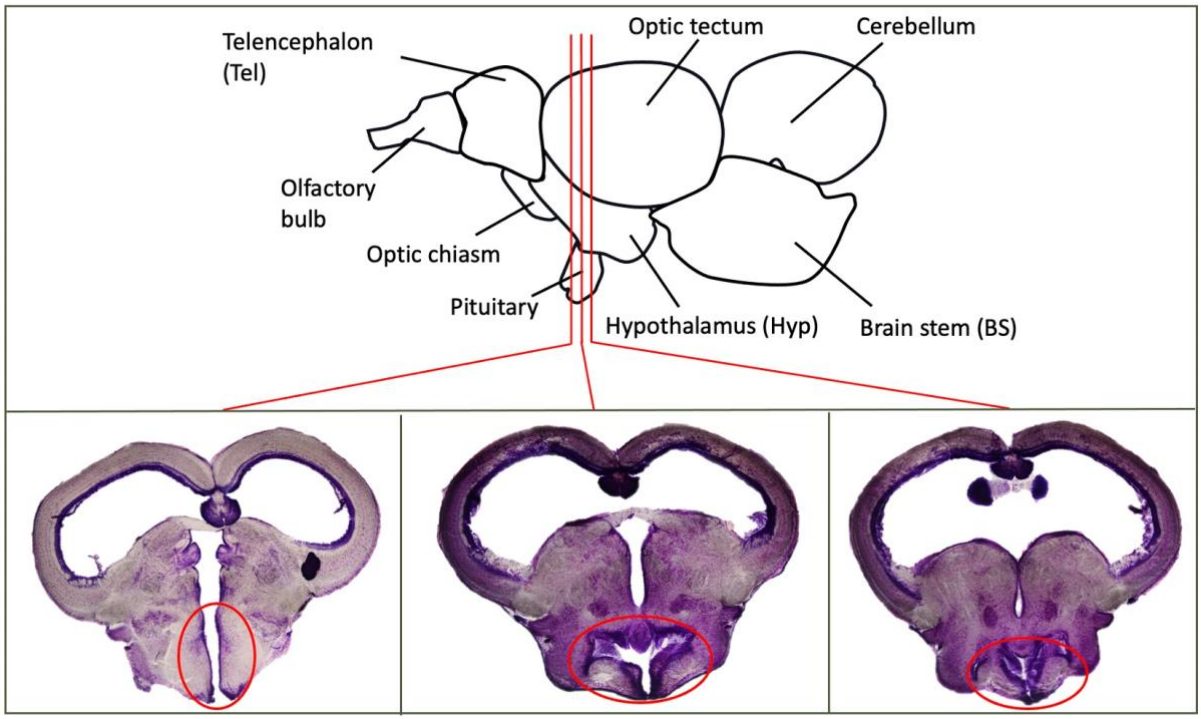


Figure 6: Schematic illustration of the whole brain in the sagittal plane with cross sections showing the region of interest (ROI) with red circles (the tuberal nucleus of the hypothalamus). The illustration is modified from Vindas et al. (2017) and an Atlantic salmon atlas by Vindas (unpublished).

The slides were evenly distributed (2-3 from each fish) through 4 batches of 20 slides. The slides were left at room temperature for one hour before antigen retrieval by incubation in 150mM Tris-HCL (pH 9) for 10 minutes at room temperature in a plastic container with a lid, followed by heating at 70ºC for 25 minutes and 5 min cool down at room temperature. Next, the slides were washed 3x5 min in PBST (Phosphate buffered saline + 0,1%Tween) in two Couplin staining jars (10 slides in each; Figure *7*). After washing, the slides were taken out one at a time and a PAP-pen (Abcam, Cambridge, United Kingdom, Cat. ab2601) was used to mark the boarder of the slides to create a hydrophobic barrier. Subsequently, the tissue was blocked in a blocking-solution consisting of PBST + 1% Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) + 2 % Normal Goat Serum (NGS; provided by the immunology unit, PREPAT, Faculty of Veterinary Medicine, NMBU) + 1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in the staining jars for one hour. Following this, the slides were placed in a humid-chamber and the pERK primary antibody (Cell Signaling, # 4370) was added by pipetting. The primary antibody was diluted 1:500 in 200µl x slide of PBST + 1% BSA + 1% DMSO. The

primary antibody was incubated at 4ºC over night. The following day, the primary antibody was rinsed off by washing three times with 1000µl PBST. The slides where then immediately soaked in PBST for 15 minutes, followed by soaking twice in in Phosphate buffered saline (PBS) for 15 min. The slides were then placed in the humid chamber and the secondary antibody (Alexa fluor 568 Donkey Anti-rabbit IgG red; catalog #A10042; ThermoFischer scientific, Waltham, MA, USA) was added by pipetting, followed by incubation for 45 min at room temperature in the dark. The secondary antibody was diluted 1:500 in 200µl per slide in PBS + 1% BSA. Following incubation, the slides were placed in the staining jar and washed three times with PBS for five min. Vectashield antifade reagent (Vector laboratories, Inc, Newark, USA) was added before mounting the cover glass. Finally, the cover glass was sealed to the slides by applying transparent nail polish around the edges. The tissue from one of the fish with low parasite infection was too damaged after slicing or immunostaining to be used for quantifications. Due to this, five more samples (two from the high parasite group and three from the low parasite group) were sliced and immunostained as described above, at a later time.

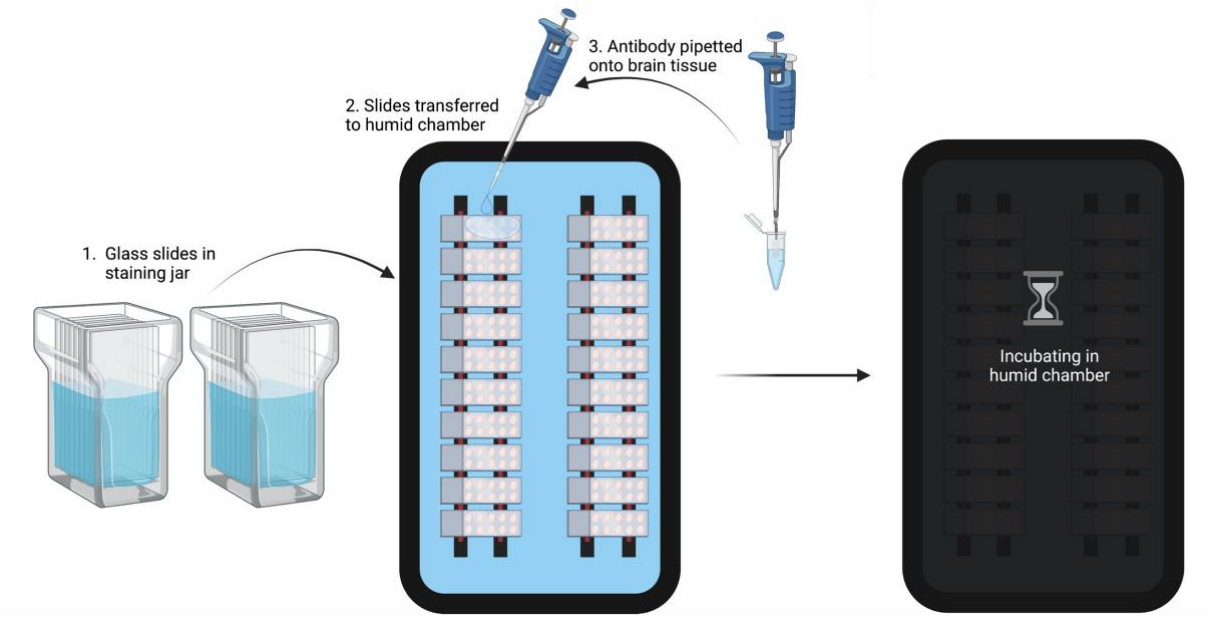


Figure 7: IHC procedure. 1. Glass slides soaked in phosphate buffer in staining jar. 2. Glass slides transferred to humid chamber. 3. Antibody pipetted onto tissue sections before left for incubation.

The initial qualitative analysis of the immunoreactive cells, was conducted by using an immunofluorescence microscope (Leica DM6 B) with the LAS X software (Leica, Wezlar, Germany). The suitable excitation filter for Alexa fluor 568 was used for imaging at 5x magnification and atlases over both the salmon and trout brain were used to identify the

different brain regions (Carruth et al., 2000; Folgueira et al., 2004; Pérez et al., 2000; Vindas, unpublished).

IHC quantification

All the slides from the pERK immunostaining were sent to the Norbrain Slidescanning Facility (Institute of Basic Medical Sciences, University of Oslo) and high-resolution images of the histological sections were acquired using an automated slide scanning system (Axio Scan Z1, Carl Zeiss Microscopy, Munich, Germany). The images were inspected using the Zeiss ZEN Lite Blue software (Carl Zeiss Microscopy) and the images were saved in a CZI format. Following this, the Zen Lite Blue software were used to adjust brightness and contrast as well as split each tissue section into individual image files. The files were further converted to TIFF format for further analysis.

The Fiji platform (Schindelin et al., 2012; **RRID**:SCR_002285) in ImageJ2 (Rueden et al., 2017; **RRID**:SCR003070) were used to quantify the pERK labeled cells. Brain ROIs were identified using the aforementioned salmonid stereotaxic atlases and immunoreactive cells were counted in the whole tuberal nucleus (NAT, nucleus anterior tuberis; NPT, nucleus posterior tuberis; NLT, nucleus lateralis tuberis) of the hypothalamus (Figure *6*). The IHC toolbox (Shu et al., 2014) plugin for ImageJ was used to quantify the immune stained cells. To use the IHC quantification toolbox a training process had to be done for the software to recognize the specific color of interest. The rectangular tool in imageJ was used to select a region of interest (a pERK immunoreactive cell) and the training button was pressed. A slide bar was presented to adjust the color threshold so that only the immunoreactive cells were visible. This procedure was repeated for five images from different individuals and sections throughout the ROI in order to establish a statistical model for the software to recognize the color of interest.

For each image the left and right hemisphere were quantified separately to control for differences in neuronal activity between the two hemispheres. The area of interest was selected manually, and the image were cropped. In sections with tissue damage or bright background staining these areas were removed. The “color” was then used, and a new image popped up containing only the stained tissue of interest (Appendix B). This image was made binary (black and White) and the “analyze particles” function was used. A new pop-up window appaired with a summary of the analyzed image containing “count”, “total area”, “avg. size” and “%Area”.

The data from the summary was then transferred to an excel sheet for later analysis. The number of immunoreactive cells were counted within the ROI for both lobes in each section. Due to differences in brain size and tissue damage in some sections there was a difference in the number of counted sections per fish and the number of counted sections from each sample varied between 12 and 30. To control for the difference in number of brain sections per fish, the average number of labeled cells were calculated for each fish. These average number of labeled cells per fish were further used in the statistical analysis.

Microdissections

The differences in expression of pERK immunoreactive cells between the two contrast groups, were used to establish ROIs for microdissections. A total of 24 fish were microdissected. That is, the four fish with the highest and lowest parasite load from each of the three lakes (Takvatn, Sagelvvatn and Fjellfrösvatn) sampled in 2016 (Appendix D). Microdissections were performed in order to extract tissue from the anterior, posterior and lateral tuberal nucleus of the hypothalamus, which were pooled together (*i.e.,* the collected samples are for the tuberal nucleus as a whole). A hypodermic needle (needle size 21G) was flattened and sanded down on all sides into an approximately 45º angle using a Dremel tool (Figure *8*A; DREMEL Europe, Bosch Power Tools B.V., Breda, The Netherlands). The needle was connected to a 5ml syringe via a rubber tube. Before beginning the procedure, all the equipment and the workstation was cleaned with RNA-Zap and a cooling plate was set at constant -14ºC to keep the glass slides cold during the procedure. The cooling plate was placed under a stereo microscope and the area of interest was identified using the aforementioned stereotaxic atlases (Carruth et al., 2000; Folgueira et al., 2004; Pérez et al., 2000; Vindas, unpublished). The plunger was pulled out and the desired area was obtained by pressing and twisting the needle against the tissue in a 90º angle (needle punching). The tissue was then transferred to an Eppendorf tube with 350µl RLT buffer (QIAGEN RNeasy Mikro kit) by pressing in the plunger (Figure 8B). The number of microdissected punches for each fish was noted, and on average 12 punches were collected from each fish. The Eppendorf with the RLT buffer and tissue were immediately vortexed to break down the tissue and frozen on dry ice and placed at –80ºC until further RNA extraction.

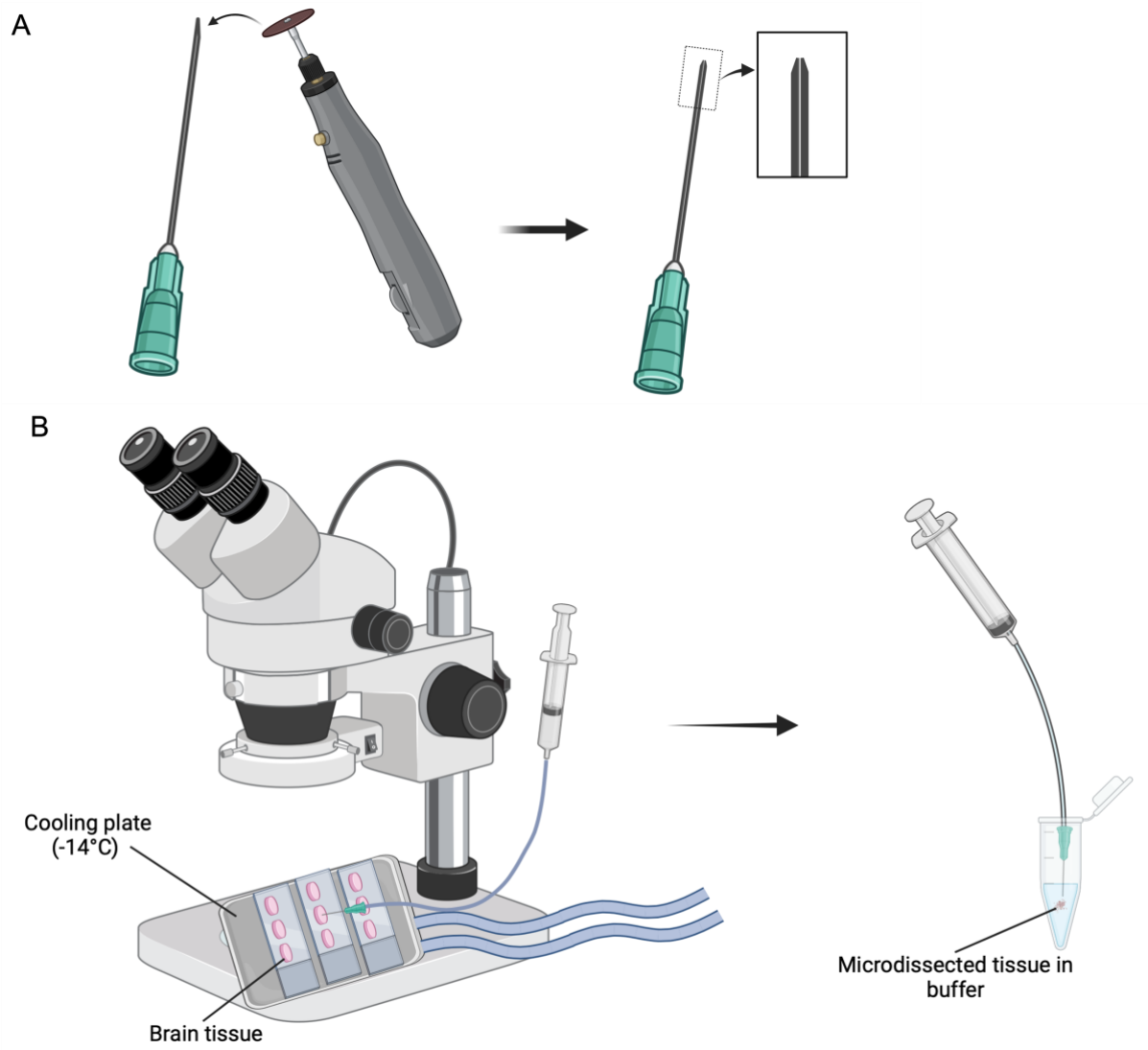


Figure 8: Illustration of the microdissection procedure. A: A Dremel tool is used to change the angle of the hypodermic needle to approximately 45º. B: Glass slides with brain slices placed on a cooling plate under the stereo microscope. Tissue from the region of interest is collected with the syringe and transferred to the buffer by pressing the plunger.

RNA extraction

The samples were thawed and vortexed until all the tissue was dissolved. Next, the sample was transferred to a 2ml tube with a screw top containing 4-5 beads for disruption and the samples was homogenized at 5500 RPM for 20sec. The following steps of total RNA extraction were performed in accordance with the instructions for RNeasy®

Plus Mini kit from the manufacturer (QIAGEN, Crawley, West Sussex, UK). The extracted RNA was placed on ice and 4µl from each sample was transferred to separate tubes for measuring concentration and quality. The rest of the samples (10 µl) were stored at -80ºC.

The RNA concentrations were measured using an Epoch microplate spectrometer (Biotek Instruments, Winooski, VT, USA) and the values was obtained using the Gen 5 3.00 software (BioTek® Instruments, Inc). All the samples had acceptable concentrations (47.5 ± 22.2 ng/µl; mean ± SD;) and were chosen for further assessment. Following, the RNA quality was analyzed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The quality of the RNA is assessed with a RIN scale with a number from 1 to 10 where values above 5 are acceptable and values above 8 represent the best quality. All the samples had RIN values above 9,1 except for one, for which it was not possible to obtain a value (see Appendix E for further details on RNA concentrations and quality). As the RNA quality was assessed with excellent RIN values all the samples (including the one with no RIN value) were accepted for further processing and shipped to NovoGene (NovoGene, Cambridge, UK) for further RNA sequencing.

Transcriptome sequencing

The transcriptome sequencing was carried out in the same was as in the study by Midttun et al. (2020). At NovoGene additional quality testing of the sample RNA were performed before sequencing of total RNA. Oligo(dT) magnetic beads were used to enrich the total RNA samples for extraction of mRNA. The mRNA was further randomly fragmented in a fragmentation buffer, combining random hexamers and assembling with M-MuLV reverse transcriptase to synthesize first-strand cDNA. Nick translations were used to synthesize complementary strands by using a custom (Illumina) synthesis buffer containing RNase H, dNTP's and *Escherichia coli* polymerase I. Following this, the cDNA went through adapter ligation, terminal repair, poly A-tailing, size selection and PCR enrichment, followed by a final quality assessment. The final quality assessment was performed by concentration with Qubit 2.0 fluorometer (life Technologies), insert size by Agilent 2,100 Bioanalyzer and quantification by qPCR. An Illumina Hiseq 2,500 instrument were used to sequence the cDNA libraries as 150 base pair, paired-end reads. For sequencing analysis, the samples from each of the three lakes were compared to each other to check for common regulated genes between the populations.

2.5 Statistical analysis

The statistical analysis for the neural activity count were performed using the Rstudio software, R version 4.2.1 (RStudio Team, 2020). The Rstudio packages "nlme", "MuMIn" and "lme4"

were used to generate a linear mixed effects model (LME) with “Activity” (neuronal activity count) as the response variable and “Treatment” (High/Low parasite load) as the explanatory variable, with “Fish” (Fish ID) as a random effect. To check the assumptions for the LME the fitted values were plotted against the standardized residuals. Additionally, a quantile-quantile plot (Q-Q plot) were used to check if the data met the assumption of normality (see Appendix F for R-script). Examination of the plots assured that the data met the required assumptions for the LME. A Welch Two Sample t-test were used to assess the difference in neuronal activity between the two groups (High/low parasite load). P-value <0.05 was considered statistically significant.

The data from the RNAseq were statistically analyzed by dr. Paul Whatmore at Strombus Genomics (Strombus Genomics, Maroochydore, Australia) as described by Løvmo et al. (2022), Løvmo et al. (2021) and Mochol et al. (2021). Paired-end sequence reads were aligned to the Arctic Charr reference genome (ASM291031v2; 12/10/2019) using the latest version of HISAT2 (v2.2.1). The gene expression was quantified, and the data was imported to R (v4.2.0) where the subsequent downstream analysis was completed. Principal Component Analysis (PCA) plots, density plots, pairwise distance dendogram and heat map were used to analyze outliers and batch effects. The analysis of differentially expressed genes (DEGs) was completed using the R package “DESeq2” (v1.36.0) and the data was presented as Log2 transformed fold change (Log2FC). The group with low parasite infection were set as the baseline group in all comparisons, hence a positive Log2FC represent upregulation and negative Log2FC represent downregulation. Further, a Wald test was used to test the significance of expression strength between groups generating p-values for each gene, and the p-values were adjusted by Benjamini-Hochberg procedure to control for the false discovery rate (FDR). Through over-representation testing and Gene Set Enrichment Analysis (GSEA) the list of DEGs were used for functional annotation (FA) of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways which resulted in a list of pathways comparing the differences between the groups (High/Low). DEGs and KEGG pathways with $p < 0.05$ was considered statistically significant.

3 Results

3.1 Parasite quantification and red-scale

As mentioned in the methods section the number of Diplostomum sp. were quantified and the fish were divided into contrast groups (High/Low parasite load). The number of *Diplostomum* sp. in the fish sampled in September 2021 varied between 3 at the lowest to 123 at most (Table 1). For the contrasts groups the mean number of *Diplostomum* sp. counted were 73 ± 10,4 (SEM) for the High parasite load group and 15 ± 1,6 (SEM) for the Low parasite group. No difference in abdominal red coloration was observed between the two contrast groups. Mean red-scale for the high parasite group was 45 ± 3,4 (SEM), while the mean red-scale for the low parasite group was 44,2 ± 5,8 (SEM).

Table 1: Data from fish sampling September 2021 sorted by parasite load (Total *Diplostomum* sp.) from high to low. Redscale is the intensity of coloration estimated using the color scale adopted form Johansen et al. (2019). The samples are divided into contrast groups marked with red (High parasite load) and green (Low parasite load). These are the samples used for quantification of neuronal activity.

Fish ID	Total *Diplostomum* sp.	Redscale	Fish ID	Total *Diplostomum* sp.	Redscale
TA28	123	35	TA42	28	35
TA30	72	45	TA29	27	30
TA2	69	50	TA33	27	80
TA37	59	35	TA38	26	25
TA13	57	50	TA41	24	55
TA18	57	55	TA1	23	50
TA5	55	70	TA34	23	65
TA12	55	70	TA15	21	75
TA25	51	80	TA16	21	30
TA3	47	40	TA39	21	75
TA31	45	50	TA8	20	60
TA20	44	60	TA24	20	50
TA21	38	65	TA4	16	60
TA26	38	60	TA14	16	30
TA22	37	65	TA9	15	35
TA32	34	35	TA11	15	60
TA7	32	70	TA35	8	30
TA17	32	75	TA6[a]	3	60
TA19	32	45	TA43[b]	N/A	55
TA40	32	45	TA23[c]	N/A	90
TA36	31	40	TA44[c]	N/A	55
TA10	30	55	TA45[c]	N/A	60
TA27	29	40			

[a] Sample was used as a test for cryosectioning and IHC. The quality of the sections were not accepted for neuronal activity quantification.

[b] One eye raptured during sampling. Total number of *Diplostomum* is missing.

[c] Number of *Diplostomum wasn't quantified.*

3.2 Neuronal activity

Qualitative analysis

Qualitative comparison of IHC images from the whole brain of Arctic Charr with high and low parasite infection revealed some clear differences in the expression of pERK immunoreactive cells between the two contrast groups. Looking through the images section for section, the immunohistological expression pattern looked more or less similar throughout the brain, but there was one area in particular, the tuberal nucleus of the hypothalamus, where one of the groups seemed to have clearly more expression of pERK immunoreactive cells (Figure 9).

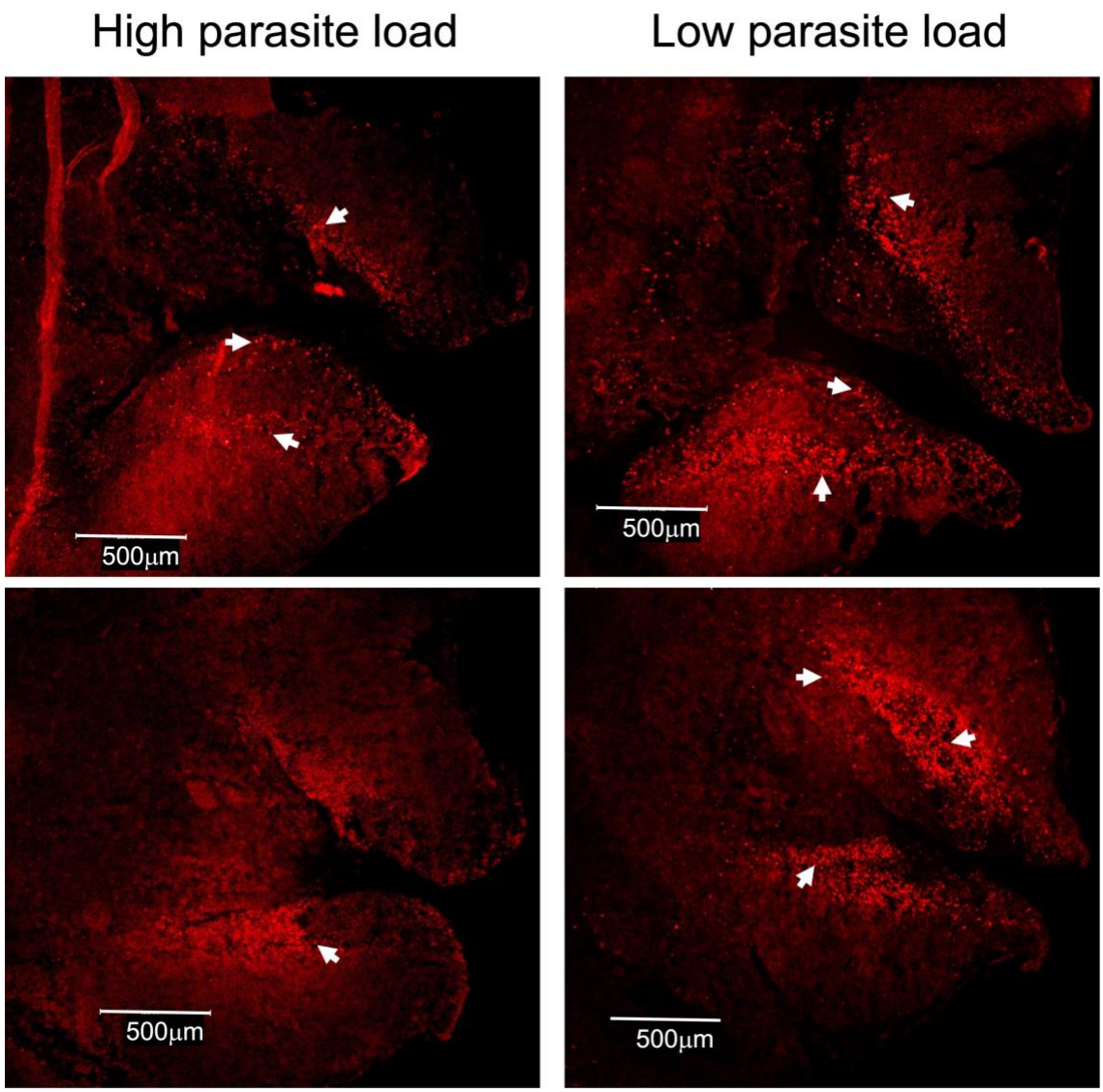


Figure 9: Example images of IHC for pERK in the tuberal nucleus of the hypothalamus in Arctic Charr used for qualitative analysis of neuronal activity between fish with high and low parasite load. Arrows indicate stained cells. The scale bars represent 500µm.

Quantitative analysis

The number of pERK labeled cells was significantly lower in the tuberal nucleus of fish with high parasite infection of *Diplostomum* sp. (Welch two sample t-test, $t_{(8.2)} = -3.91$, P = 0.004; Figure 10; see Appendix C and F for supplementary data and R-script)

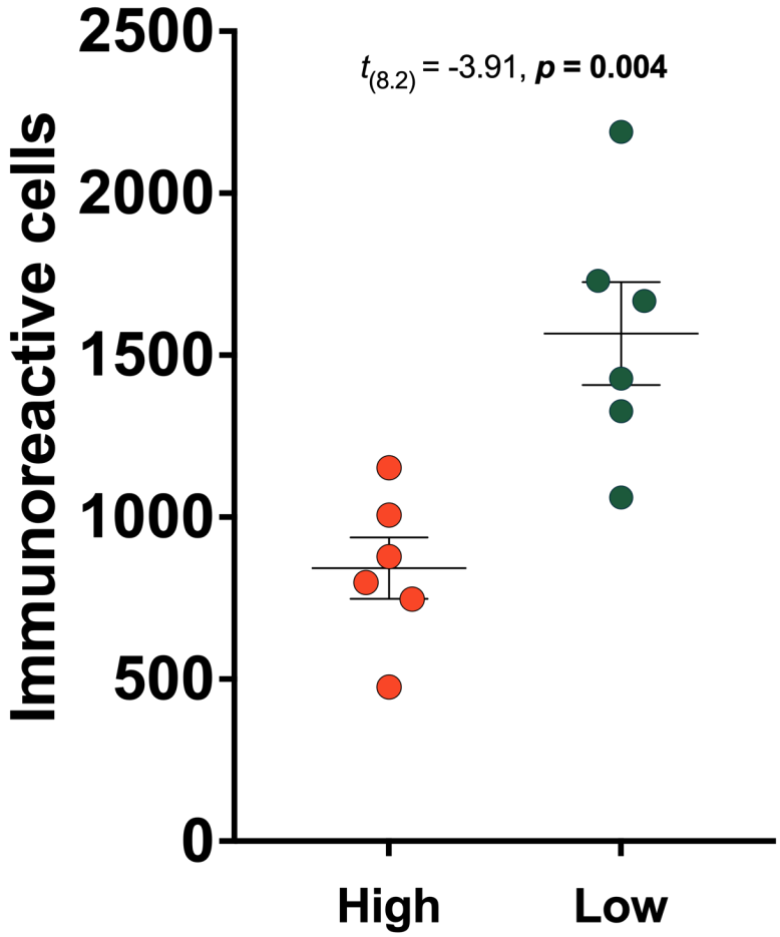


Figure 10: Immunohistochemistry labeled cells of the neuronal activity marker pERK in the tuberal nucleus of the hypothalamus in the Arctic Charr with High (red) and Low (green) infection of *Diplostomum* sp. Data is presented as mean ± SEM.

3.3 RNAseq results:

Differential expressed genes analysis

Analysis of differential expression revealed that between charr with low and high parasite infection, a total of 1803 genes were significantly differentially expressed ($p < 0.05$) for the samples from Takvatn. Of these 1803 genes, 653 were upregulated (36.22% of total significantly DEGs), while 1150 were downregulated (63.78% of total significantly DEGs; Figure 11-12). For the samples from Sagelvvatn and Fjellfrösvatn, the total number of significantly DEGs between high and low infected fish were 6 (5 up, 1 down) and 2 (1 up, 1 down) respectively. From the Takvatn samples somatolactin (SL; Log2FC = 4.72) is one of the most upregulated genes, while interleukin-6 receptor subunit beta-like (IL-6Rβ; Log2FC = -5.48) in one of the most downregulated genes (see Appendix G for more details).

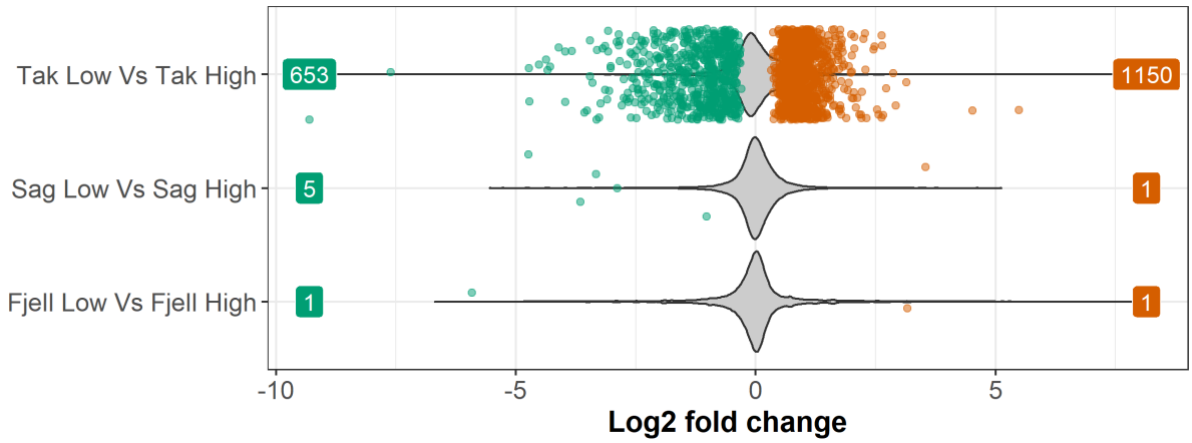


Figure 11: Proportion of upregulated and downregulated genes. Log2FC scores of significantly DEGs are represented by jittered dots (green = significantly upregulated, orange = significantly downregulated). Violin plots represent non-significant Log2FC scores.

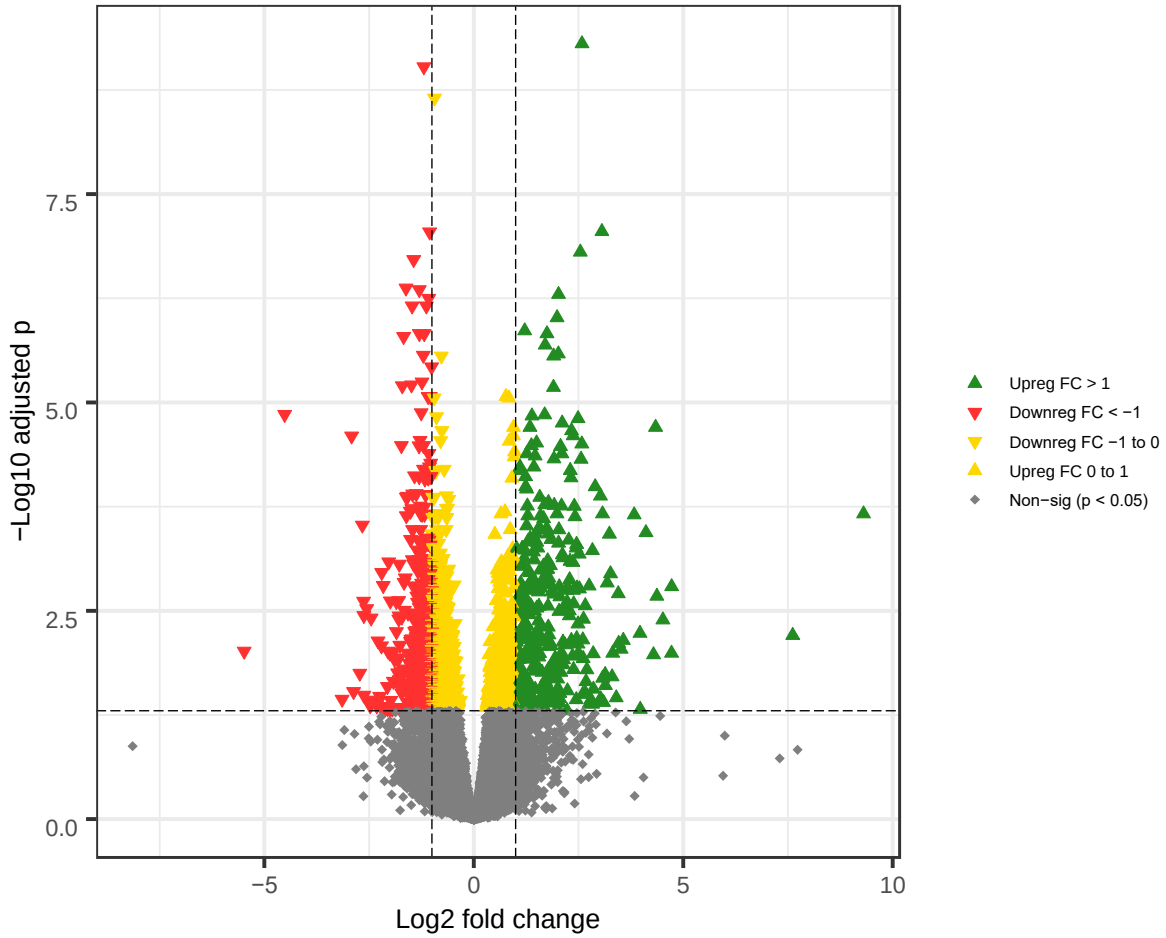


Figure 12:Volcanoplot of up and downregulated genes in the tuberal nucleus between the contrast groups from Takvatn.

KEGG pathways

Six significantly enriched KEGG pathways were identified during over-representation analysis (Table 2). These pathways contain an over-represented number of significantly DEGs ($p < 0.05$) compared to the number of background genes in each pathway in the analyzed samples. The pathways are extracellular matrix (ECM) -receptor interaction, gonadotropin-releasing hormone (GnRH) signaling pathway, Focal adhesion, forkhead box O (FoxO) signaling pathway, Apelin signaling pathway and Vascular smooth muscle contraction. The number of significantly DEGs in each pathway varies between 23 and 34 (see Figure 13 for number of up- and downregulated DEGs in each pathway).

Table 2: List enriched KEGG pathways in the tuberal nucleus of Arctic charr with high *Diplostomum* infection compared to charr with low infection of *Diplostomum* from Takvatn. Pathways were uncovered based on significantly DEGs ($p < 0.05$). Adjusted p-values are Benjamini-Hochberg adjusted and gene count represent number of significantly DEGs found in that pathway.

KEGG Pathway ID	Pathway name	Adjusted p-value	Gene count
salp04512	ECM-receptor interaction	5.8e-06	25
salp04912	GnRH signaling pathway	2.5e-03	23
salp04510	Focal adhesion	2.0e-02	34
salp04068	FoxO signaling pathway	3.0e-02	27
salp04371	Apelin signaling pathway	3.7e-02	25
salp04270	Vascular smooth muscle contraction	4.2e-02	23

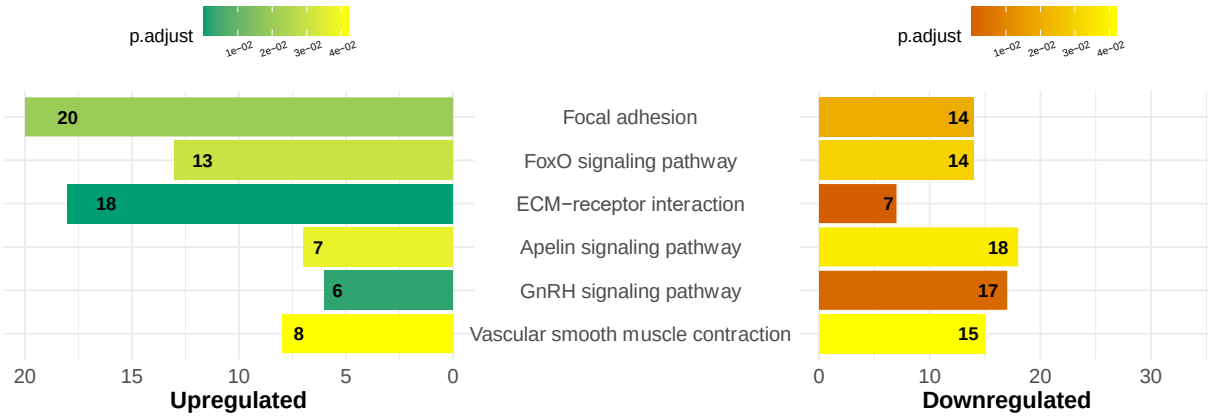


Figure 13: Mirrored bar plot of the number of up- and downregulated DEGs in each significantly (adjusted $p < 0.05$) enriched KEGG pathway in the tuberal nucleus of Low vs. High infected Arctic Charr from Takvatn. X-axis is number of upregulated and downregulated DEGs. Bars are shaded by adjusted p-values per pathway.

The KEGG pathway map for the GnRH signaling pathway (Figure 14) indicates that luteinizing hormone subunit beta (LHβ; Log2FC = 3.1) and gonadotropin-releasing hormone 3(GnRH-3; Log2FC = 2.68) are the most upregulated genes in the Arctic Charr with high parasite infection compared to charr with low parasite infection (see Appendix H for more details on DEGs in the GnRH signaling pathway).

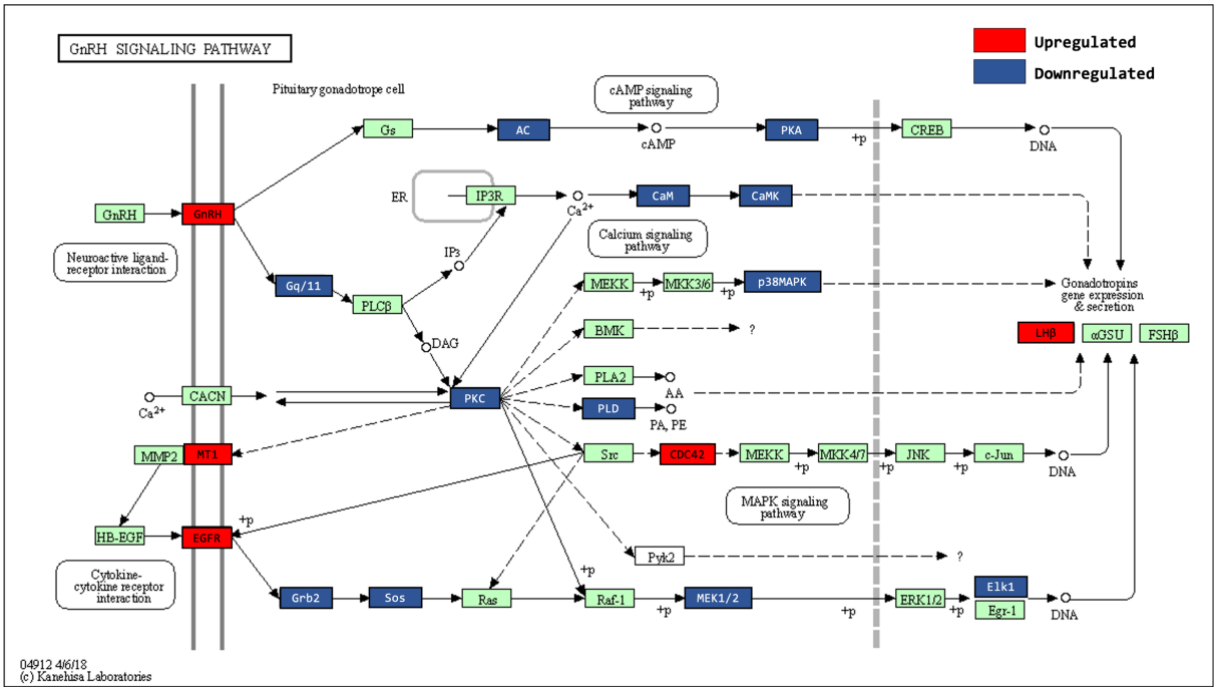


Figure 14: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map for the GnRH signaling pathway containing significantly DEGs in the tuberal nucleus of arctic charr from Takvatn with Low vs. High parasite infection. Colors represent upregulation (red) and downregulation (blue).

4 Discussion

4.1 Methodological considerations

Cryosectioning

The brains sampled from Takvatn in September 2021 used for quantification of neuronal activity were sliced in 14μm sections using a cryostat. As the brains were sampled form sexually mature arctic charr of relatively large size, the brains were almost too large to fit in the cryostat when mounted in Tissue-Tek. Before the sectioning could be done a razor blade was used to remove excess Tissue-Tek in order to reduce the size of the mounted brain. For the largest samples the most rostral part of the brain (the olfactory bulbs) had to be removed as well for the brain to fit in the cryostat.

The large brain size also resulted in a large area that were in contact with the blade during sectioning. This made it difficult to prevent the thin tissue sections from curling up before they were thaw mounted on the glass slides. Additionally, the large size increases the risk of damaging the tissue during sectioning. Another challenging aspect of slicing such large brains in 14μm sections is that each brain takes a long time to section, and it results in a very large number (approximately 1000) of sections per brain. This makes the temperature inside the cryostat increase, which in turn increases the risk of the tissue being damaged or curling up. As the sections were thaw mounted on glass slides in bathes of six, that is, the first section was mounted on the first slide, the second section on the second slide and so on until all six glass slides were full, there were six slides containing the same region of the brain. This made some tissue damage or curling acceptable as there were several slides to choose from for the IHC.

Immunohistochemistry

IHC staining for pERK is an effective method to investigate differences in neuronal activity between the charr with high and low parasite infection with great spatial and temporal resolution. When the fish is caught using gillnets it is difficult to know how long the fish has been in the net, and how this affects neuronal activity. An issue with pERK and other neuronal activity marker like IEGs is the rapid expression after neuronal activation. As pERK is expressed almost instantaneously it is likely that the time the fish have spent in the gillnet have

an effect on neuronal activity. When immunostaining for pERK, it is difficult to control for the potential effects the gillnet has on neuronal activity.

As there were such a high number of glass slides from each brain, the IHC was performed in several batches. To control for batch-differences, slides from each sample were evenly distributed throughout all batches as described in the methods section. Regardless of this, some differences may have occurred. But when the slides are organized in this manner, the slides within in a batch contains the same brain area from all samples and batch-differences will not have a great effect on the results when comparing samples from low- and high infected fish.

To our knowledge there are no studies on how *Diplostomum* sp. infection may affect neuronal activity in the arctic charr, or other species. Therefore, the main purpose of the IHC staining for the neuronal activity marker pERK was to investigate if there were differences in brain neuronal activity in charr with high vs. low *Diplostomum* infection. And where these differences in neuronal activity were located for further transcriptome analysis of these areas.

The quantification of the IHC were performed semi-automatic as described in the methods section. Even though the quantification was done with the help of computer software, there is a possibility for biased results. Both the brain-sectioning, IHC and quantification was performed by the same person. This may have unknowingly challenged the objectivity and experimenter bias is a possibility. This potential experimenter bias could have been avoided by having another experimenter analyzing the images without knowing which samples that belong to each of the contrast groups.

Another challenge with the quantification of the IHC images is that the brain was of varying size, as well as some tissue damage and excessive background staining in some section which were excluded from the analysis. This resulted in varying number of sections analyzed from each fish. To control for this the mean number of immunoreactive cells per section were calculated for the tuberal nucleus as a whole. Ideally the brains should have been of the same size and the same amount of tissue sections should have been analyzed for each fish. This could potentially have resulted in improved spatial resolution of the differences in neuronal activity between the two contrast groups.

Microdissections and RNAseq

The samples from 2016 used for microdissections were sliced at 300μm. This thickness is effective for microdissecting large brain areas. But for smaller areas it becomes difficult to maintain high precision and the risk of accidentally contaminate the samples with tissue from other neuronal subpopulations increases. Due to the thickness of the slices the tissue from the tuberal nucleus as a whole were pooled together instead of microdissecting the anterior-, lateral- and posterior tuberal nucleus separately. This could have provided more details on differentially expressed genes in the different neuronal sub populations of the tuberal nucleus between the two contrast groups.

Based on the findings by Johansen et al. (2019) that there is a positive association between *Diplostomum* infection and intensity of the red breeding coloration in arctic charr which is closely linked to reproduction, an early hypothesis was that the *Diplostomum* have an effect on the brain pituitary gonadal-axis (BPG-axis). This was prior to investigating the differences in neuronal activity between the two contrast groups. As we didn't know if there were any differences in neuronal activity or where in the brain these differences were located at the time, the preoptic area (POA) and Dorsomedial pallium (Dm) in the telencephalon were microdissected from fish from both contrast groups. Further, RNA extraction and cDNA synthesis were performed, and qPCR were used to quantify gene expression of gonadotropin releasing hormone (GnRH). None of the primers for GnRH found in the literature worked as expected, and the experiment didn't produce any results. This is something that could have been avoided if we had performed the IHC for neuronal activity and investigated which brain areas that were interesting to look at first.

For the RNAseq there were some problems working with the species (arctic charr) itself, as the genomic databases for the species were under development. Additionally, the sample size for each of the contrast groups from each of the three lakes were small (n=4). Ideally the sample sizes would have been larger, but some of the samples from 2016 were missing. The samples were most likely lost during relocation of the veterinary school from Oslo to Ås. In order to make the difference regarding parasite load between the groups with low and high parasite infection large enough to see some differences between the groups it resulted in such small sample sizes.

The reason for the low number of DEGs found in the samples from Sagelvvatn and Fjellfrösvatn (Figure 11) is most likely due to too many confounding variables that overwhelmed the gene expression due to parasite load. As of this, it is uncertain if these results are credible or not, and the results from these locations will not be discussed further. An issue with a small sample size, is that the smaller the sample size, the more likely the signal will disappear in the noise of confounding variables. In the samples from Takvatn on the other hand, there is a good number of DEGs. This fits well, as the samples from 2021 used to compare neuronal activity between high and low infected fish is from Takvatn as well. Additionally, the arctic charr used in the study are wild caught, naturally infected. That results in a high number of variables, such as dietary effects, age differences, infection from other parasites and pathogens, and so on, which you cannot control for. This is one of the problems with studying wild caught fish.

4.2 Biological implications

Neuronal activity

The quantification of pERK immunoreactive cells show that arctic charr with high infection of *Diplostomum* sp. have lower neuronal activity in the tuberal nucleus of the hypothalamus compared to charr with low *Diplostomum* infection. This indicates that the parasite has an inhibitory effect on the neuronal activity in this brain area. As the tuberal nucleus is located in the hypothalamus close to the pituitary it is likely that this area is involved in the hypothalamo-pituitary complex which plays an important role in several endocrine systems related to reproduction, stress, and metabolism (Bernier et al., 2009). There might be a possibility that this inhibition of neuronal activity can have an effect on how the fish receives and reacts to information about body homeostasis and external sensory information.

Differentially expressed genes

The results from the RNAseq demonstrates large differences in DEGs in the tuberal nucleus of arctic charr from Takvatn with high parasite load compared to arctic charr with low parasite load. One of the genes which is most upregulated is somatolactin. Somatolactin is involved in regulation of skin pigmentation and a loss of somatolactin in medaka and rainbow trout is associated with lighter skin coloration. Increased somatolactin makes the skin darker and more colorful by reducing the density of light colored chromatophores (leucophores in medaka) while the number of carotenoid pigmented xanthophores or dark melanophores increases (Bertolesi

& McFarlane, 2021). Additionally, somatolactin plays a role in several other physiological processes such as gonadal maturation and reproduction, stress, osmoregulation and energy metabolism (Kawauchi et al., 2009). Even though we did not see a difference in red coloration between high and low infected fish from the 2021 samples, the upregulation of somatolactin in char with high parasite load supports the positive association between parasite load and coloration found by Johansen et al. (2019). This can potentially make the fish more colorful and suspicious, and easier for predatory birds to see. Bright conspicuous coloration is associated with increased predation risk from visually hunting predators (Endler, 1984; GöTmark & Olsson, 1997). This makes arctic charr with brighter coloration potentially more prone to predation by birds, which can facilitate trophic transmission of the parasite from the fish to its definite host.

Interleukin-6 receptor subunit beta-like (IL-6Rβ) is one of the genes which is most downregulated in the arctic charr with high *Diplostomum* infection. The information about the dynamics of interleukin-related gene expression and their function during parasite infections are limited. Interleukins is a group of cytokines important for regulating the immune system. Cytokines are normally produced at the entry site of a pathogen and produce inflammatory signals that regulates the capacity of phagocytes to fight of the intruding pathogen, in this case, the parasite (Chabalgoity et al., 2007). The IL-6 is considered pro-inflammatory (Secombes et al., 2011), that is, it is promoting inflammation in response to infection. The IL-6Rβ is downregulated in arctic charr with high *Diplostomum* infection, meaning the receptors responsiveness to IL-6 is potentially reduced. This might indicate that the accumulating parasite infection have some anti-inflammatory effects which may increase survival during the migration to the eyes. As mentioned in the introduction, the *Diplostomum* sp. metacercaria is located in the eyes of the fish, which is considered an immune privileged structure, separated from the immune system by the blood-retinal barrier (Caspi, 2013). Therefore, the parasite gets the chance to escape immune responses mobilized by the host as a response to infection (Padrós et al., 2018). As of this, another possible reason for the downregulation of the IL-6Rβ is to reduce tissue damage caused by the inflammatory response to the parasite infection.

Gonadotropin releasing hormone signaling pathway

From functional annotation of KEGG pathways which resulted in a list of pathways that were different between the high and low infected fish, especially one pathway (GnRH signaling

pathway) stood out as a potential way for the parasite to affect the phenotype of its host. GnRH-3 also called salmon gonadotropin-releasing hormone (sGnRH) is the major form of GnRH found in salmonids (Bailhache et al., 1994) and is the main hormone responsible for regulating sexual maturation and reproduction through the hypothalamic-pituitary gonadal (HPG) axis. The HPG-axis the main neuroendocrine system controlling reproduction (Weltzien et al., 2004). In teleosts, the GnRH plays a central role in the synthesis and regulation of gonadotrophs (GTHs), that is, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by direct innervation of GTH-producing cells in the anterior pituitary (Amano et al., 1997). Hence, an upregulation of GnRH-3 as seen in this study will potentially increase the production if LH as reflected by the significant upregulation of the LHβ observed in the GnRH signaling pathway. The GTHs are released into the circulatory system and binds to specific receptors in the gonads. The GTHs function at the gonads to stimulate the production of gametes and the gonadal synthesis of steroid hormones (e.g., 11kT; as opposed to testosterone in mammals). The amont of GnRH is fluctuating between the seasons, with an increase in the spawning season, which leads to reproductive maturation. The production of LH follows this reproductive maturation, and the amount of LHβ accumulates in the pituitary prior to spawning, resulting in a major releaase during the spawning period (Ando & Urano, 2005). As the arctic charr in from this study with the heaviest *Diplostomum* sp. infection awaiting trophic transmission is found to have an upregulation of GnRH-3 and LHβ, these observations might indicate that the most heavily infected fish also demonstrates the strongest investment in reproduction.

As mentioned above, one of the main functions of LH is the process of producing steroid hormones (steroidogenesis). An upregulation of the GnRH and LH in the most heavily infected arctic charr will potentially result in an increased production of 11kT and other steroid hormones in the gonads. The changes in brain and endocrine function (*i.e.*, increased steroid levels and associated neurobiological control) the arctic charr undergoes during reproductive maturation and spawning preparations leads to several coordinated changes in the fish phenotype. These changes involve increased ornamental coloration (Bass, 1992; Borg, 1994), reproductive behavior (Borg, 1994; Stacey, 1987) and reduced immune function(Segner et al., 2017). All of which can serve as potential ways the trophically transmitted *Diplostomum* can influence for increased chance of transmission to its final host.

11-keto testosterone is produced at the end of the HPG-axis in male teleosts and is known to induce secondary sexual characteristics such as bright coloration in teleosts (Bass, 1992; Borg, 1994). This is supported by the findings from Bjerkeng et al. (1999), that carotenoid metabolism and distribution from muscle to skin is mediated by 11kT in arctic charr as well as the positive association between 11kT and redness ratio (ratio of red coloration between skin and muscle) in the arctic charr observed by Johansen et al. (2019). Even though visual sexual signals are important for mate choice in numerous species, included the arctic charr, the bright coloration may attract the attention of potential predators. Consequently, this coloration may be costly in terms of predation risk and thus increased mortality (Godin & McDonough, 2003).

In many animal species included the arctic charr, there is normally a decline in feeding behavior during spawning-migrations and other reproductive behaviors such as territoriality, courting, spawning, and mate guarding. Sex steroids might have a regulatory effect on feeding, and it is likely that these effects are more pronounced during the spawning season (Miquelle, 1990; Tveiten et al., 1996; Volkoff et al., 2009). Increasing levels of steroid hormones as a response to increased GnRH and LH signaling might lead further decline in feeding. This might lead to anorexic fish with impaired physical condition and reduces ability to avoid predators.

Increased risk-taking behavior is another highly pronounced trait in the arctic charr during the spawning period. In a study by Brattli et al. (2018) on spawning behavior in the arctic charr, video cameras were deployed within 0.3-1 meter from spawning females to record spawning behavior. This indicates that during spawning the arctic charr shows a clear indifference to the presence of humans. This also matches personal observations from the sampling in September 2021. We could sit in the boat at shallow waters (1-3meters depth) with the engine running and observe numerous arctic charr expressing courtship and guarding behaviors directly underneath the boat without showing signs of insecurity about our presence. This indicates increased expression of risk-taking behavior in the spawning arctic charr, making the fish easy prey for predatory birds.

All of the aforementioned phenotypic changes in arctic charr expressed during the spawning season is indirectly regulated by GnRH and LH through the HPG-axis. Thus, interfering with the GnRH signaling pathway by increasing the production of GnRH and LH in the HPG-axis, could be an efficient approach for the parasite to achieve substantial manipulation of the host

phenotype. This can potentially increase the chances of trophic transmission to its bird final host resulting in increased parasites fitness.

4.3 Future perspectives

Very little is known about the potential effects of *Diplostomum* infection on the phenotype of the arctic charr. The results of this study suggests that the *Diplostomum* sp. has an effect on the expression of GnRH and LH in the in the hypothalamus of the arctic charr, but potential effects of this increased expression is not known. As I decided to focus on the GnRH signaling pathway with special emphasis on GnRH and LH, the possible effects of the rest of the up- and downregulated genes in this pathway should be further investigated. Further, the other significantly enriched KEGG pathways from the RNAseq should be further analyzed to reveal potential effects of the differentially expressed genes on the arctic charr phenotype.

Some further immunohistochemistry analysis should also be considered. Dopamine is found to possess some modulatory effects on GnRH neuronal activity in teleosts by interfering with release processes in both the brain and pituitary. This results in an inhibition of the gonadotropin secretion from the pituitary(Yu et al., 1991). Double staining with IHC for both the neuronal activity marker pERK and dopaminergic markers could therefore be interesting. This way it is possible to investigate if pERK and dopamine is co-expressed in the same region which might give some insight in the differences in GnRH and LH expression between high and low infected charr. Future projects should also consider investigating the effects of *Diplostomum* infection on brain neurochemistry and analyze monoamine (e.g. serotonin, dopamine and nor-adrenaline) concentrations using high performance liquid chromatograph (HPLC).

A more comprehensive experiment which could potentially give a deeper understanding of the effects of *Diplostomum* infection on arctic charr phenotype could be to rear arctic char in a lab-facility and perform experimental infections with *Diplostomum* and compare effects with a non-infected control group. This way it is possible to avoid a lot of confounding variables such as, diet, infection of other parasites species, and age differences when analyzing the biological data.

5 Conclusions

The aim of this thesis was to investigate whether the parasite *Diplostomum* sp. awaiting trophic transmission are able to affect the phenotype of the arctic charr host in ways that facilitates transmission a bird final host, increasing the parasites fitness. The first hypothesis was that there is a difference in neuronal activity between arctic char with high and low infection of *Diplostomum.* The second hypothesis stated that the expression of genes with the potential to influence the fish's phenotype in a way that facilitates trophic transmission of the parasite to a final host is different between arctic charr with high and low *Diplostomum* infection. The analysis of IHC for the neuronal activity marker pERK reviled a significantly lower neuronal activity in the tuberal nucleus of the hypothalamus in arctic charr with high *Diplostomum* infection compared to charr with low infection. This might suggest that the parasite has an inhibitory effect on neuronal activity in this brain region in the arctic charr. These results provided useful spatial information for further transcriptome sequencing.

The analysis of the transcriptome sequencing resulted in a good number of genes differentially expressed between the two contrast groups as well as some significantly enriched KEGG pathways. As the arctic charr used in this study were caught during the spawning period, the GnRH signaling pathway is one of the significantly enriched pathways, which is believed to have a potential effect on the arctic charr phenotype. An upregulation of GnRH-3 and LHβ in arctic charr with high parasite infection might suggest that the *Diplostomum* metacercaria might interfere with the HPG-axis in a way that indirectly results in an arctic char host with a more conspicuous phenotype (*i.e.*, bright sexual ornamentation, conspicuous reproductive behavior or increased risk-taking behavior). Such conspicuous phenotypes are associated with increased predation risk, which is beneficial for the parasite awaiting trophic transmission to its final host. Very little emphasis has been put on the physiological effects of *Diplostomum* infection in the arctic charr and how this may facilitate parasite-induced trophic transmission of the *Diplostomum* to its final host. More experiments are needed in order to get a deeper understanding of the influence *Diplostomum* metacercaria on the phenotype of arctic charr.

6 References

Aggleton, J. P., Brown, M. W. & Albasser, M. M. (2012). Contrasting brain activity patterns for item recognition memory and associative recognition memory: Insights from immediate-early gene functional imaging. *Neuropsychologia*, 50 (13): 3141-3155. doi: https://doi.org/10.1016/j.neuropsychologia.2012.05.018.

Amano, M., Urano, A. & Aida, K. (1997). Distribution and function of gonadotropin-releasing hormone (GnRH) in the teleost brain. *Zoolog Sci*, 14 (1): 1-11. doi: 10.2108/zsj.14.1.

Amundsen, P.-A., Smålås, A., Kristoffersen, R., Knudsen, R., Siwertsson, A. & Klemetsen, A. (2015). Takvatnprosjektet-Forskning og kultivering av en overbefolka røyebestand. *Septentrio Reports* (5).

Ando, H. & Urano, A. (2005). Molecular Regulation of Gonadotropin Secretion by Gonadotropin Releasing Hormone in Salmonid Fishes. *Zoological Science*, 22 (4): 379-389, 11.

Bailhache, T., Arazam, A., Klungland, H., Aleström, P., Breton, B. & Jego, P. (1994). Localization of salmon gonadotropin-releasing hormone mRNA and peptide in the brain of Atlantic salmon and rainbow trout. *J Comp Neurol*, 347 (3): 444-54. doi: 10.1002/cne.903470310.

Barber, I., Hoare, D. & Krause, J. (2000). Effects of parasites on fish behaviour: a review and evolutionary perspective. *Reviews in Fish Biology and Fisheries*, 10 (2): 131-165.

Barbosa, F. F. & Silva, R. H. (2018). Chapter 18 - Immediate-Early Gene Expression in Neural Circuits Related to Object Recognition Memory. In Ennaceur, A. & de Souza Silva, M. A. (eds) vol. 27 *Handbook of Behavioral Neuroscience*, pp. 261-271: Elsevier.

Bass, A. (1992). Dimorphic male brains and alternative reproductive tactics in a vocalizing fish. *Trends in Neurosciences*, 15 (4): 139-145. doi: https://doi.org/10.1016/0166-2236(92)90356-D.

Berdoy, M., Webster, J. P. & Macdonald, D. W. (2000). Fatal attraction in rats infected with Toxoplasma gondii. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 267 (1452): 1591-1594.

Bernier, N. J., Van Der Kraak, G., Farrell, A. P. & Brauner, C. J. (2009). *Fish physiology: Fish neuroendocrinology*: Academic Press.

Bertolesi, G. E. & McFarlane, S. (2021). Melanin-concentrating hormone like and somatolactin. A teleost-specific hypothalamic-hypophyseal axis system linking physiological and morphological pigmentation. *Pigment Cell & Melanoma Research*, 34 (3): 564-574. doi: https://doi.org/10.1111/pcmr.12924.

Bjerkeng, B., Johnsen, K., Mayer, I., Storebakken, T. & Nilssen, K. J. (1999). Influence of 11-ketotestosterone, 17β-estradiol, and 3,5,3′-triiodo-L-thyronine on distribution and metabolism of carotenoids in Arctic charr, Salvelinus alpinus L.*. *Fish Physiology and Biochemistry*, 21 (4): 353-364. doi: 10.1023/A:1007802803008.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 109 (3): 219-245. doi: https://doi.org/10.1016/0742-8413(94)00063-G.

Brattli, M. B., Egeland, T. B., Nordeide, J. T. & Folstad, I. (2018). Spawning behavior of Arctic charr (Salvelinus alpinus): Spawning synchrony, vibrational communication, and mate guarding. *Ecology and evolution*, 8 (16): 8076-8087.

Calvo, R. & Schluessel, V. (2021). Neural substrates involved in the cognitive information processing in teleost fish. *Animal Cognition*, 24 (5): 923-946.

Carruth, L. L., Jones, R. E. & Norris, D. O. (2000). Cell density and intracellular translocation of glucocorticoid receptor-immunoreactive neurons in the kokanee salmon

(Oncorhynchus nerka kennerlyi) brain, with an emphasis on the olfactory system. *Gen Comp Endocrinol*, 117 (1): 66-76. doi: 10.1006/gcen.1999.7391.
Caspi, R. R. (2013). In this issue: Immunology of the eye—Inside and out. *International Reviews of Immunology*, 32 (1): 1-3.
Chabalgoity, J. A., Baz, A., Rial, A. & Grille, S. (2007). The relevance of cytokines for development of protective immunity and rational design of vaccines. *Cytokine & growth factor reviews*, 18 (1-2): 195-207.
Chandroo, K. P., Duncan, I. J. & Moccia, R. D. (2004). Can fish suffer?: perspectives on sentience, pain, fear and stress. *Applied Animal Behaviour Science*, 86 (3-4): 225-250.
Combes, C. (1991). Ethological Aspects of Parasite Transmission. *The American Naturalist*, 138: 866 - 880.
Durán, E., Ocana, F. M., Martín-Monzón, I., Rodríguez, F. & Salas, C. (2014). Cerebellum and spatial cognition in goldfish. *Behavioural brain research*, 259: 1-8.
Endler, J. A. (1984). Natural and sexual selection on color patterns in poeciliid fishes. In Zaret, T. M. (ed.) *Evolutionary ecology of neotropical freshwater fishes: Proceedings of the 1st international symposium on systematics and evolutionary ecology of neotropical freshwater fishes, held at DeKalb, Illinois, U.S.A., June 14–18, 1982*, pp. 95-111. Dordrecht: Springer Netherlands.
Fiore, R., Khudayberdiev, S., Christensen, M., Siegel, G., Flavell, S. W., Kim, T. K., Greenberg, M. E. & Schratt, G. (2009). Mef2-mediated transcription of the miR379–410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. *The EMBO journal*, 28 (6): 697-710.
Folgueira, M., Anadón, R. & Yáñez, J. (2004). An experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). I: Olfactory bulb and ventral area. *Journal of Comparative Neurology*, 480 (2): 180-203. doi: https://doi.org/10.1002/cne.20340.
Folstad, I., Hope, A. M., Karter, A. & Skorping, A. (1994). Sexually Selected Color in Male Sticklebacks: A Signal of Both Parasite Exposure and Parasite Resistance? *Oikos*, 69 (3): 511-515. doi: 10.2307/3545863.
Gao, Y. J. & Ji, R. R. (2009). c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J*, 2: 11-17. doi: 10.2174/1876386300902010011.
Godin, J.-G. J. & McDonough, H. E. (2003). Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. *Behavioral Ecology*, 14 (2): 194-200. doi: 10.1093/beheco/14.2.194.
Grewal, S. S., York, R. D. & Stork, P. J. S. (1999). Extracellular-signal-regulated kinase signalling in neurons. *Current Opinion in Neurobiology*, 9 (5): 544-553. doi: https://doi.org/10.1016/S0959-4388(99)00010-0.
GöTmark, F. & Olsson, J. A. N. (1997). Artificial colour mutation: do red-painted great tits experience increased or decreased predation? *Animal Behaviour*, 53 (1): 83-91. doi: https://doi.org/10.1006/anbe.1996.0280.
Hatlen, B., Arnesen, A. & Jobling, M. (1996). Muscle carotenoid concentrations in sexually maturing and immature Arctic charr, Salvelinus alpinus (L.). *Aquaculture Nutrition*, 2 (4): 207-212.
Helland-Riise, S. H., Vindas, M. A., Johansen, I. B., Nadler, L. E., Weinersmith, K. L., Hechinger, R. F. & Øverli, Ø. (2020). Brain-encysting trematodes (Euhaplorchis californiensis) decrease raphe serotonergic activity in California killifish (Fundulus parvipinnis). *Biology open*, 9 (7): bio049551.
Herrera, D. G. & Robertson, H. A. (1996). Activation of c-fos in the brain. *Progress in Neurobiology*, 50 (2): 83-107. doi: https://doi.org/10.1016/S0301-0082(96)00021-4.

Höglund, J. (1991). Ultrastructural observations and radiometric assay on cercarial penetration and migration of the digeneanDiplostomum spathaceum in the rainbow troutOncorhynchus mykiss. *Parasitology Research*, 77 (4): 283-289.
Ji, R. R. & Rupp, F. (1997). Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. *J Neurosci*, 17 (5): 1776-85. doi: 10.1523/jneurosci.17-05-01776.1997.
Ji, R. R., Baba, H., Brenner, G. J. & Woolf, C. J. (1999). Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci*, 2 (12): 1114-9. doi: 10.1038/16040.
Johansen, I. B., Henriksen, E. H., Shaw, J. C., Mayer, I., Amundsen, P.-A. & Øverli, Ø. (2019). Contrasting associations between breeding coloration and parasitism of male Arctic charr relate to parasite species and life cycle stage. *Scientific reports*, 9 (1): 1-9.
Karvonen, A., Kirsi, S., Hudson, P. & Valtonen, E. (2004). Patterns of cercarial production from Diplostomum spathaceum: terminal investment or bet hedging? *Parasitology*, 129 (1): 87-92.
Karvonen, A., Cheng, G.-H., Seppälä, O. & Valtonen, E. (2006). Intestinal distribution and fecundity of two species of Diplostomum parasites in definitive hosts. *Parasitology*, 132 (3): 357-362.
Karvonen, A. (2012). Diplostomum spathaceum and related species. In *Fish parasites: pathobiology and protection*, pp. 260-269: CABI Wallingford UK.
Kawauchi, H., Sower, S. A. & Moriyama, S. (2009). The neuroendocrine regulation of prolactin and somatolactin secretion in fish. *Fish Physiology*, 28: 197-234.
Kennedy, C. (1978). Studies on the biology of Eubothrium salvelini and E. crassum in resident and migratory Salvelinus alpinus and Salmo trutta and in S. salar in North Norway and the islands of Spitsbergen and Jan Mayen. *Journal of Fish Biology*, 12 (2): 147-162.
Kime, D. E. (1998). Disruption of hypothalamic and pituitary function. In *Endocrine Disruption in Fish*, pp. 109-120: Springer.
Klemetsen, A., Amundsen, P. A., Dempson, J., Jonsson, B., Jonsson, N., O'connell, M. & Mortensen, E. (2003). Atlantic salmon Salmo salar L., brown trout Salmo trutta L. and Arctic charr Salvelinus alpinus (L.): a review of aspects of their life histories. *Ecology of freshwater fish*, 12 (1): 1-59.
Klemetsen, A. (2013). The most variable vertebrate on Earth. *Journal of Ichthyology*, 53 (10): 781-791.
Kotrschal, A. & Kotrschal, K. (2020). Fish brains: anatomy, functionality, and evolutionary relationships. In *The Welfare of Fish*, pp. 129-148: Springer.
Lefevre, T., Adamo, S. A., Biron, D. G., Missé, D., Hughes, D. & Thomas, F. (2009). Invasion of the body snatchers: the diversity and evolution of manipulative strategies in host–parasite interactions. *Advances in parasitology*, 68: 45-83.
Lyholt, H. & Buchmann, K. (1996). Diplostomum spathaceum: effects of temperature and light on cercarial shedding and infection of rainbow trout. *Diseases of Aquatic Organisms*, 25 (3): 169-173.
Løvmo, S. D., Whatmore, P., Sundh, H., Sigholt, T., Madaro, A., Bardal, T. & Olsen, R. E. (2021). Effects of Atlantic salmon (Salmo salar) fed low- and high HUFA diets on growth and midgut intestinal health. *Aquaculture*, 539: 736653. doi: https://doi.org/10.1016/j.aquaculture.2021.736653.
Løvmo, S. D., Sundh, H., Whatmore, P., Nordvi, M. F., Sigholt, T., Madaro, A., Bardal, T. & Olsen, R. E. (2022). Intestinal health in Atlantic salmon post-smolt (Salmo salar) when fed low- and high HUFA diets. *Aquaculture*, 557: 738318. doi: https://doi.org/10.1016/j.aquaculture.2022.738318.

Midttun, H. L. E., Vindas, M. A., Whatmore, P. J., Øverli, Ø. & Johansen, I. B. (2020). Effects of Pseudoloma neurophilia infection on the brain transcriptome in zebrafish (Danio rerio). *Journal of Fish Diseases*, 43 (8): 863-875. doi: https://doi.org/10.1111/jfd.13198.

Miquelle, D. G. (1990). Why don't bull moose eat during the rut? *Behavioral Ecology and Sociobiology*, 27 (2): 145-151. doi: 10.1007/BF00168458.

Mochol, M., Whatmore, P., Taubøll, E., Landmark, C. J., Ropstad, E., Svalheim, S. & Fraser, T. W. K. (2021). Lamotrigine effects on immune gene expression in larval zebrafish. *Epilepsy Research*, 178: 106823. doi: https://doi.org/10.1016/j.eplepsyres.2021.106823.

Moore, J. (2013). An overview of parasite-induced behavioral alterations–and some lessons from bats. *Journal of Experimental Biology*, 216 (1): 11-17.

Northmore, D. (2011). Encyclopedia of fish physiology: from genome to environment. 131-142.

Okuyama, T., Suehiro, Y., Imada, H., Shimada, A., Naruse, K., Takeda, H., Kubo, T. & Takeuchi, H. (2011). Induction of c-fos transcription in the medaka brain (Oryzias latipes) in response to mating stimuli. *Biochemical and biophysical research communications*, 404 (1): 453-457.

Padrós, F., Knudsen, R. & Blasco-Costa, I. (2018). Histopathological characterisation of retinal lesions associated to Diplostomum species (Platyhelminthes: Trematoda) infection in polymorphic Arctic charr Salvelinus alpinus. *International Journal for Parasitology: Parasites and Wildlife*, 7 (1): 68-74.

Palmieri, J. R., Heckmann, R. A. & Evans, R. S. (1976). Life cycle and incidence of Diplostomum spathaceum Rudolphi (1819) (Trematoda: Diplostomatidae) in Utah. *The Great Basin naturalist*, 36: 6.

Pérez, S. E., Yáñez, J., Marín, O., Anadón, R., González, A. & Rodríguez-Moldes, I. (2000). Distribution of choline acetyltransferase (ChAT) immunoreactivity in the brain of the adult trout and tract-tracing observations on the connections of the nuclei of the isthmus. *J Comp Neurol*, 428 (3): 450-74. doi: 10.1002/1096-9861(20001218)428:3<450::aid-cne5>3.0.co;2-t.

Poulin, R. (1994). The evolution of parasite manipulation of host behaviour: a theoretical analysis. *Parasitology*, 109 (S1): S109-S118.

Randlett, O., Wee, C. L., Naumann, E. A., Nnaemeka, O., Schoppik, D., Fitzgerald, J. E., Portugues, R., Lacoste, A., Riegler, C. & Engert, F. (2015). Whole-brain activity mapping onto a zebrafish brain atlas. *Nature methods*, 12 (11): 1039-1046.

Rodriguez, F., Durán, E., Gómez, A., Ocana, F., Alvarez, E., Jiménez-Moya, F., Broglio, C. & Salas, C. (2005). Cognitive and emotional functions of the teleost fish cerebellum. *Brain research bulletin*, 66 (4-6): 365-370.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T. & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC bioinformatics*, 18 (1): 1-26.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. & Schmid, B. (2012). Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9 (7): 676-682.

Secombes, C. J., Wang, T. & Bird, S. (2011). The interleukins of fish. *Developmental & Comparative Immunology*, 35 (12): 1336-1345. doi: https://doi.org/10.1016/j.dci.2011.05.001.

Segner, H., Verburg-van Kemenade, B. M. L. & Chadzinska, M. (2017). The immunomodulatory role of the hypothalamus-pituitary-gonad axis: Proximate mechanism for reproduction-immune trade offs? *Developmental & Comparative Immunology*, 66: 43-60. doi: https://doi.org/10.1016/j.dci.2016.07.004.

Shu, J., Qiu, G. & Ilyas, M. (2014). *Immunohistochemistry (IHC) Image Analysis Toolbox*. Available at: https://imagej.nih.gov/ij/plugins/ihc-toolbox/index.html.
Skarstein, F. & Folstad, I. (1996). Sexual dichromatism and the immunocompetence handicap: an observational approach using Arctic charr. *Oikos*: 359-367.
Stacey, N. E. (1987). Roles of hormones and pheromones in fish reproductive behavior. *Psychobiology of reproductive behavior: an evolutionary perspective*: 28-60.
Taylor, E., Campbell, H. A., Levings, J. J., Young, M. J., Butler, P. J. & Egginton, S. (2006). Coupling of the respiratory rhythm in fish with activity in hypobranchial nerves and with heartbeat. *Physiological and Biochemical Zoology*, 79 (6): 1000-1009.
Team, R. (2020). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, PBC. Available at: http://www.rstudio.com/.
Thomas, F., Schmidt-Rhaesa, A., Martin, G., Manu, C., Durand, P. & Renaud, F. (2002). Do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial hosts? *Journal of Evolutionary Biology*, 15 (3): 356-361.
Tveiten, H., Johnsen, H. K. & Jobling, M. (1996). Influence of maturity status on the annual cycles of feeding and growth in Arctic charr reared at constant temperature. *Journal of Fish Biology*, 48 (5): 910-924. doi: https://doi.org/10.1111/j.1095-8649.1996.tb01486.x.
Vindas, M. A., Gorissen, M., Höglund, E., Flik, G., Tronci, V., Damsgård, B., Thörnqvist, P. O., Nilsen, T. O., Winberg, S., Øverli, Ø., et al. (2017). How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. *J Exp Biol*, 220 (Pt 8): 1524-1532. doi: 10.1242/jeb.153213.
Vissio, P. G., Di Yorio, M. P., Pérez-Sirkin, D. I., Somoza, G. M., Tsutsui, K. & Sallemi, J. E. (2021). Developmental aspects of the hypothalamic-pituitary network related to reproduction in teleost fish. *Frontiers in Neuroendocrinology*, 63: 100948.
Volkoff, H., Unniappan, S. & Kelly, S. P. (2009). Chapter 9 The Endocrine Regulation of Food Intake. In vol. 28 *Fish Physiology*, pp. 421-465: Academic Press.
Weltzien, F.-A., Andersson, E., Andersen, Ø., Shalchian-Tabrizi, K. & Norberg, B. (2004). The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 137 (3): 447-477.
West, A. E. & Greenberg, M. E. (2011). Neuronal activity–regulated gene transcription in synapse development and cognitive function. *Cold Spring Harbor perspectives in biology*, 3 (6): a005744.
Widmann, C., Gibson, S., Jarpe, M. B. & Johnson, G. L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiological reviews*, 79 (1): 143-180.
Winberg, S. & Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 106 (3): 597-614.
Winberg, S., Nilsson, A., Hylland, P., Söderstöm, V. & Nilsson, G. E. (1997). Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish. *Neuroscience letters*, 230 (2): 113-116.
Wullimann, M. F. (1997). The central nervous system. 245-282.
Yamamoto, N. (2009). Studies on the teleost brain morphology in search of the origin of cognition. *Japanese Psychological Research*, 51 (3): 154-167.
Yu, K. L., Rosenblum, P. M. & Peter, R. E. (1991). In vitro release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female

goldfish. *General and Comparative Endocrinology*, 81 (2): 256-267. doi: https://doi.org/10.1016/0016-6480(91)90010-4.

Zhuang, Z.-Y., Gerner, P., Woolf, C. J. & Ji, R.-R. (2005). ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain*, 114 (1): 149-159. doi: https://doi.org/10.1016/j.pain.2004.12.022.

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A. & Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. *General and Comparative Endocrinology*, 165 (3): 438-455. doi: https://doi.org/10.1016/j.ygcen.2009.04.017.

Zukin, R. S., Jover, T., Yokota, H., Calderone, A., Simionescu, M. & Lau, C. G. (2004). Chapter 42 - Molecular and Cellular Mechanisms of Ischemia-Induced Neuronal Death. In Mohr, J. P., Choi, D. W., Grotta, J. C., Weir, B. & Wolf, P. A. (eds) *Stroke (Fourth Edition)*, pp. 829-854. Philadelphia: Churchill Livingstone.

7 Appendices

Appendix A. Immunohistochemistry protocol for pERK in zebrafish

1st day:

Prepare all the solutions needed for the 1st day before starting the protocol. Keep the Eppendorf tube with the antibody and PBST+BSA+DMSO on ice.

1. Take slides out of the -80° freezer and leave them in room temperature for one hour.
2. Place the slides in a container with a lid and incubate in 150 mM Tris-HCL pH9 for **5 min** in room temperature followed by heating at 70°C for **15 min**.
3. Wash in PBST **3x5min** in staining jar
4. Mark the boarder of the slides with a PAP-pen in order to create a hydrophobic barrier to contain the liquid.
5. Block tissue in PBST + 1% bovine serum albumin (BSA) + 2% normal goat serum (NGS) + 1% dimethyl sulfoxide (DMSO) for **one hour** in staining jar.
6. Take the slides out one at the time and dry of the boarder of the slide to activate the PAP-pen barrier.
7. Put the slides in the humid chamber and add the primary antibody (pERK (Cell Signaling, #4370) **or** tERK (Cell Signaling, #4696)). The primary antibody is diluted in 200 ul x slide of PBST + 1%BSA + 1% DMSO (Dilution 1:500).
8. Incubate over night at 4°C (**make sure the slides don't dry up**).

2nd day:

Prepare the antibody solution (PBS + 1%BSA + secondary antibody) before starting the protocol. Cover the tube with aluminum foil and keep on ice until use.

1. Use 1000ul PBST 3 times per slide in order to rinse off antibody holding the slide vertically.
2. Soak the slides in PBST for **15 min** in staining jar.
3. Soak **2x15 min** in **PBS** in staining jar.
4. Take slides out one at the time and dry the boarder to activate the PAP-pen barrier.
5. Put the slides in the humid chamber and add the secondary antibody (the antidbody is diluted in 200 ul x slide of PBS + 1% BSA, the dilution depends on the antibody you use) by pipetting.

6. Incubate for 45 min in the dark (Make sure the slides don't dry up).
7. Wash the slides 3 x 5 min in staining jar with PBS.
8. Take all the slides out of the staining jar and mount by adding one drop of Vectashield antifade reagent with DAPI and place coverslides. Use transparent nail polish and apply a thin layer just around the edges of the cover slides to mount the cover slides. Let slides dry for 10 min on lab bench, store finished slides at 4°C.

Buffers:

- **150mM Tris-HCl pH 9:**

for 1L:

150ml 1M Tris-HCl pH9,5 + **850 ml** MQ H2O

For 200 ml:

30ml 1M Tris-HCl pH9,5 + **170ml** MQ H2O

Measure the new pH to be to make sure the pH is right.

- **PBST + 1% bovine serum albumin (BSA) + 1% dimethyl sulfoxide (DMSO):**

For 100 ml:

100ml PBST + 1g BSA + 1ml DMSO

You need 70 ml for one staining jar.

PBST + 1%BSA + 1% DMSO + 2% Normal goat serum (NGS):

For 70 ml:

70 ml PBST+ 1%BSA + 1%DMSO + 1,4ml Normal goat serum (NGS

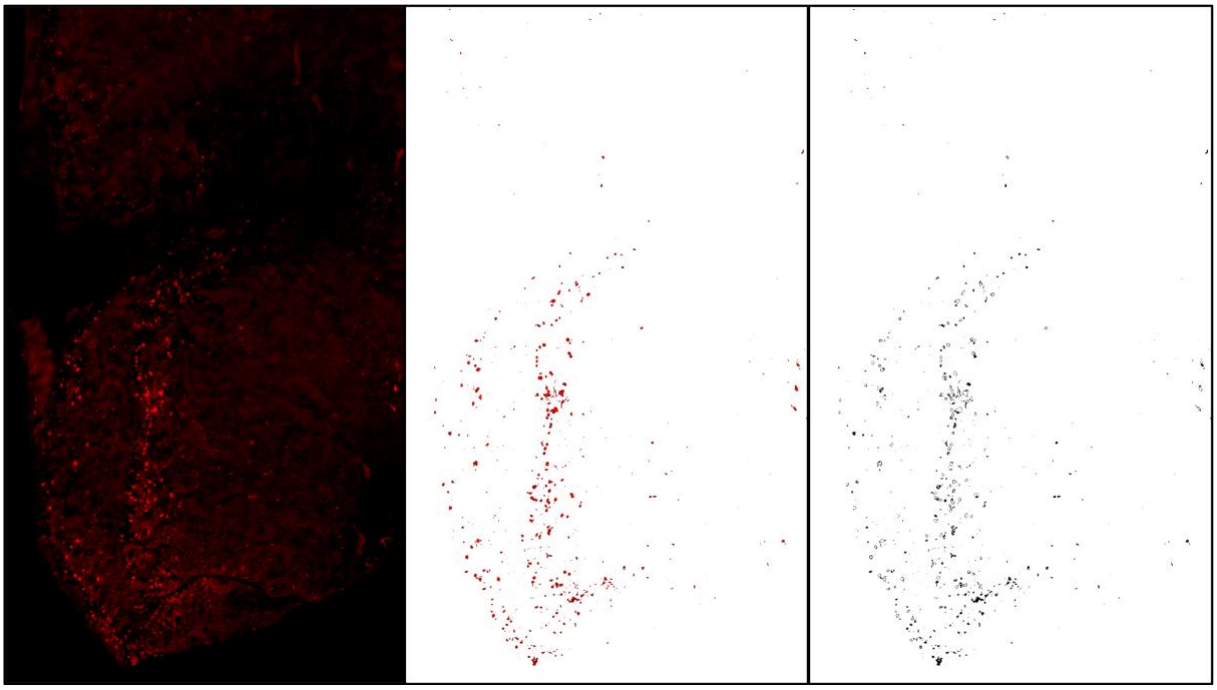
PBS + 1% BSA for secondary antibody:

For up to 20 slides:

5ml PBS + 0,05g Bovine serum albumin (BSA)

The pERK antibody (Cell Signaling, #4370), and tERK antibody (Cell Signaling, #4696), diluted 1:500. (https://www.cellsignal.com). The secondary antibody for pERK is **Anti Rabbit** secondary antibody (dilution 1:500. - 0,4µl per slide). The secondary antibody for tERK is **Anti Mouse** secondary antibody (dilution 1:500; 0,4µl per slide).

Appendix B. IHC quantification procedure



Supplementary figure 1: Example of the IHC quantification procedure of the right hemisphere. Left: The ROI cropped from the original image. Middle: After the "color" function is used showing only immunoreactive cells. Right: Binary (black and white) of the middle image, "analyze particles" function is used to count all the particles in this image.

Appendix C. IHC quantification data

Supplementary table 1: Summary of the IHC quantification data. “Fish” is the fish ID, “Treatment” is contrast group (High or Low parasite load), “Parasites” is number of *Diplostomum*, “Activity” is the mean number of counted pERK immunoreactive cells per section per fish and “LogActivity” is the log-transformed Activity.

Fish	Treatment	Parasites	Activity	LogActivity
28	High	128	747,10	2,87
30	High	72	798,90	2,90
2	High	69	475,88	2,68
37	High	59	1152,39	3,06
13	High	57	878,63	2,94
18	High	57	1006,63	3,00
24	Low	20	1667,76	3,22
4	Low	16	2189,43	3,34
14	Low	16	1327,00	3,12
9	Low	15	1427,84	3,15
11	Low	15	1061,54	3,03
35	Low	8	1730,11	3,24

Appendix D. Microdissected samples from 2016

Supplementary table 2: Information about parasite count, contrast group and "redscale" for the samples used in RNAseq.

Location	Sample ID	Total *Diplostomum* sp.	Contrast group High/Low parasite	Redscale
Fjellfrøsvatn	F10	216	High	70
	F26	202	High	80
	F11	197	High	55
	F21	170	High	40
	F16	104	Low	35
	F3	98	Low	65
	F22	57	Low	15
	F20	52	Low	40
Sagelvvatn	S1	114	High	50
	S4	111	High	55
	S28	87	High	40
	S20	80	High	35
	S2	38	Low	60
	S3	37	Low	45
	S6	34	Low	60
	S27	34	Low	30
Takvatn	T109	148	High	70
	T112	80	High	65
	T111	76	High	55
	T126	75	High	50
	T105	30	Low	20
	T106	28	Low	50
	T104	26	Low	50
	T102	11	Low	50

Appendix E. RNA concentration and quality

Supplementary table 3: Information about RNA concentration (ng/µl) and quality (RIN) for each of the samples used for RNAseq.

Sample ID	Concentration ng/µL	Quality RIN
F11	122.13	10
F20	70.56	9.3
F21	31.27	9.8
F16	76.62	9.1
T126	22.86	9.4
T106	25.82	10
T112	43.61	N/A
T109	35.03	9.5
S6	62.19	9.2
S27	11.27	10
S20	38.96	10
S3	51.28	10
F10	47.36	10
F26	37.97	10
F22	36.36	9.7
F3	40.70	9.4
T105	39.91	10
T111	64.41	10
T104	46.61	10
T102	40.44	10
S1	52.89	9.3
S4	35.18	9.7
S2	40.87	10
S28	66.29	9.2

Appendix F. Statistical analysis for neuronal activity– R script

```
> # import from clipboard
> Diplost <- read.table (file = "clipboard", sep = "\t", header = TRUE, dec = ",")
> View(Diplost)
> Diplost
   Fish Treatment Parasites Activity LogActivity
1    28      High       128   747.10        2.87
2    30      High        72   798.90        2.90
3     2      High        69   475.88        2.68
4    37      High        59  1152.39        3.06
5    13      High        57   878.63        2.94
6    18      High        57  1006.63        3.00
7    24       Low        20  1667.76        3.22
8     4       Low        16  2189.43        3.34
9    14       Low        16  1327.00        3.12
10    9       Low        15  1427.84        3.15
11   11       Low        15  1061.54        3.03
12   35       Low         8  1730.11        3.24
> # make a model
> M1 <- lme(Activity ~ Treatment, random = ~1|Fish, method = "ML", data = Diplost, na.action = "na.omit")
> # fitted vs standardised residuals
> plot (M1)
> plot (M1, resid(., type = "p") ~ fitted(.) | Treatment,  abline = 0, grid = FALSE)
> # qqplots
> qqPlot (resid (M1), grid = FALSE, envelope = .95, line = "quartiles")
 4 11
 8 11
> qqPlot (resid (M1), grid = FALSE, groups = Diplost$Treatment, envelope =.95, line = "quartiles")
> t.test(Activity ~ Treatment, data = Diplost)

        Welch Two Sample t-test

data:  Activity by Treatment
t = -3.9145, df = 8.1625, p-value = 0.004281
alternative hypothesis: true difference in means between group High and group Low is not equal to 0
95 percent confidence interval:
 -1149.072  -298.978
sample estimates:
mean in group High  mean in group Low
           843.255           1567.280

> r.squaredGLMM (M1)
          R2m       R2c
[1,] 0.6256943 0.9538527
```

Supplementary figure 2: R-script from the statistical analysis of neuronal activity. Containing the data used to generate the LME, residual and Q-Q plots as well as the t-test.

Appendix G. List of the most up- and downregulated DEGs from Takvatn

Supplementary table 4: List of the five most up- and downregulated differentially expressed genes in arctic char with low vs. high parasite infection from Takvatn. Entrez ID refers to gene ID in the NCBI database. Log2FC is the log 2 transformed fold change of the up7down regulation.

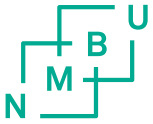
Entrez ID	Log2FC	Adjusted p	Gene_symbol	Description
111971164	9,31	2.2e-04	LOC111971164	doublesex- and mab-3-related transcription factor A2-like
111975324	7,61	6.2e-03	dmrta2	DMRT-like family A2
111972551	4,72	1.0e-02	LOC111972551	pro-opiomelanocortin B
111982708	4,72	1.6e-03	LOC111982708	somatolactin
112074680	4,51	4.1e-03	LOC112074680	aldehyde dehydrogenase family 1 member A3-like
112076875	-2,87	3.0e-02	LOC112076875	uncharacterized LOC112076875
111980326	-2,92	2.5e-05	LOC111980326	transmembrane protein 104
111971075	-3,14	3.6e-02	LOC111971075	integrin alpha-4-like
111951932	-4,52	1.4e-05	LOC111951932	VIP peptides-like
111952333	-5,48	9.7e-03	LOC111952333	interleukin-6 receptor subunit beta-like

Appendix H. List of DE genes in the GnRH signaling pathway

Supplementary table 5: List of the differentially expressed genes in the GnRH signaling pathway. Entrez ID refers to gene ID in the NCBI database. Log2FC is the log 2 transformed fold change of the up7down regulation.

DE genes in the GnRH signalling pathway

Pathway ID	Log2FC	Gene symbol	Entrez ID (NCBI)	Gene description
salp04912	3.1	lhb	111968033	luteinizing hormone subunit beta
	2.68	gnrh3	111951966	gonadotropin-releasing hormone 3
	1.55	LOC111979299	111979299	matrix metalloproteinase-14
	1.41	LOC111972787	111972787	melanoma receptor tyrosine-protein kinase
	1.16	LOC111956947	111956947	epidermal growth factor receptor-like
	0.46	LOC111955034	111955034	cell division control protein 42 homolog
	-0.37	calm3a	111949612	calmodulin 3a (phosphorylase kinase, delta)
	-0.47	LOC111951418	111951418	mitogen-activated protein kinase 11
	-0.53	LOC111974605	111974605	guanine nucleotide-binding protein G(q) subunit alpha
	-0.56	pld2	111950576	phospholipase D2
	-0.62	grb2a	112067730	growth factor receptor-bound protein 2a
	-0.67	map2k2b	111956577	mitogen-activated protein kinase kinase 2b
	-0.75	mapk14a	111976697	mitogen-activated protein kinase 14a
	-0.75	LOC111957031	111957031	son of sevenless homolog 1
	-0.75	elk1	112069764	ETS transcription factor ELK1
	-0.76	camk2b1	111974573	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta 1
	-0.77	adcy2	111954801	adenylate cyclase 2
	-0.78	LOC111953718	111953718	guanine nucleotide-binding protein subunit alpha-11
	-0.87	LOC111960905	111960905	calcium/calmodulin-dependent protein kinase type II subunit beta-like
	-0.88	LOC112077218	112077218	cAMP-dependent protein kinase catalytic subunit alpha
	-1	LOC111977149	111977149	adenylate cyclase type 5-like
	-1.02	gna11b	111972124	guanine nucleotide binding protein (G protein), alpha 11b (Gq class)
	-1.23	LOC111968570	111968570	protein kinase C delta type



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