Norges miljø- og biovitenskapelige universitet

Masteroppgave 2021 60 stp
Fakultetet for biovitenskap

Tracking GnRH neurons *in vivo* following CRISPR knock out of Six6 in Atlantic salmon

Thomas Nesset
Master i molekylærbiologi

Abstract

Gonadotropin releasing hormone (GnRH) is a conserved neuroendocrine decapeptide, crucial for the onset of puberty and for the functioning of the hypothalamic-pituitary-gonadal axis (HPG). The homeodomain protein sine oculis related homeobox 6 (Six6) is necessary for GnRH neuronal development in mammals. We wanted to investigate the role of Six6 in GnRH neuronal development in the Atlantic salmon (*Salmo salar*), since the effects of Six6 during embryogenesis in Atlantic salmon is little studied. I sought to induce knockout of the Six6 gene *in vivo* in salmon embryos during the one cell stage using Clustered-Regularly-Interspaced-Short-Palindromic-Repeats (CRISPR) Cas9 and perform immunohistochemistry with GnRH specific antibodies on the knocked-out embryos as they developed to track changes in GnRH neuronal development. While the immunohistochemistry procedure failed to produce successful immunostaining of GnRH neurons, I did manage to knockout Six6 with a very high efficiency, helping establish a CRISPR protocol that can be used to knockout Six6 in Atlantic salmon.

Acknowledgements

First and foremost, I would like to thank my main supervisor, Guro Katrine Sandvik. Thank you for your knowledge, your great feedback, and especially for your unyielding optimism in the times when I myself had little to spare.

To Noman Reza, my co-supervisor who had the daunting task of taking me and another master student on as supervisor while still having so much work to do himself. Thank you for all of your patience for my questions and for being a rock during the first few hectic months of this experiment.

To my fellow master student Axmee Regmi, thanks for being someone to vent to when things were going wrong and for all of the good talks we had. Thank you for being a friend.

Thank you, Jacob Seilø Torgersen for all of your help with the confocal microscopy and thank you Romaine Fontaine, for helping with the procurement of antibodies.

Table of Contents

1 Introduction:

1.1 The Atlantic salmon and GnRH

The Atlantic Salmon (*Salmo salar)* is a staple of diet throughout much of the world and is Norway's most exported fish in both tonnage and value. Export of salmon has steadily increased over the past 20 years. With the increasing trend of nations moving towards more renewable energy sources, the traditional Norwegian way of life that is fishing may potentially have to make up much of the deficit that will rear its head once Norway cuts back on its extraction of fossil fuels.

With salmon being such an important source of food and revenue, understanding and investigating its physiological and underlying molecular mechanisms is a worthwhile endeavour.

After hatching, the Atlantic salmon spends its formative years (~1-6) in freshwater before migrating to the marine environment sometime in the spring. To move from a freshwater to the sea, it must undergo several physiological adaptations in order to survive and grow. (Fjelldal et al., 2018)

This process is known as smoltification or parr-smolt transformation. (Hoar 1988). After a growth period of 1-3 years, they migrate back to the river where they hatched for sexual maturation and spawning. (Klemetsen et al., 2003).

Of significant interest are the processes which leads to reproductive competence, i.e. puberty. Puberty can be defined as "the transformation from a sexually immature juvenile into a mature adult by providing the brain-pituitary-gonad (BPG) axis". (Weltzien et al., 2004)

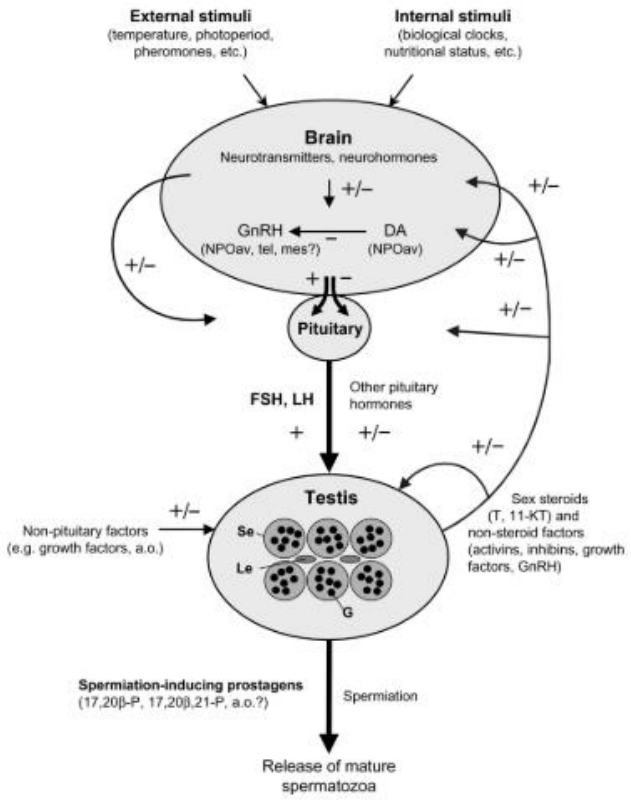

Figure 1: The BGP axis. GnRH is secreted from the GnRH neurons in the hypothalamus, and acts upon the pituitary to stimulate the release of LH and FSH. These then bind to receptors on the gonads to stimulate release of hormones like testosterone, oestrogen, and progesterone. (Xie et al., 2015, Weltzien et al., (2004). Adapted from Weltzien et al., (2004).

Essential to the forming of a functioning BGP axis are Gonadotropin Releasing Hormones (GnRH). It is the major hypothalamic neurohormone in the vertebrates brain and regulates reproduction in them all. (Okubo and Nagahama, 2008)

The gonadotropin-releasing hormones (GnRHs) are hormones that stimulate the production and release of two gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). When FSH and LH are released into circulation, they stimulate the gonads by binding to specific membrane receptors, most likely on testicular Sertoli and Leydig cells in males. (Weltzien et al., 2004) Gonads have two primary functions, they produce germ cells during spermatogenesis, and they produce sex steroids during steroidogenesis.

During steroidogenesis they also produce important growth factors that are involved in the regulation of reproduction. These factors either work directly on the gonadal tissues through

paracrine or autocrine fashion, or they are involved in negative/positive feedback loops on the hypothalamus and pituitary through endocrine action.

The discovery of GnRH in molluscs as well as protochordates, shows that it is an ancient and well-preserved decapeptide (Zhang et al., 2000) speaking to its critical importance in the development of a sexually mature animal. Furthermore, cloning of the genes encoding GnRHs as well as cDNA from different vertebrate species has uncovered that its overall organization is well preserved among the different forms of GnRH. (Table 1)

Old Name	New name	1	2	3	4	5	6	7	8	9	10	Reference
Mammalian GnRH (mGnRH)	GnRH 1	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH2	Matsuo et al. 1971
Chicken GnRH I (cGnRH-I)	GnRH 1	x	x	x	x	x	x	x	Gln	x	x	Miyamoto et al. 1982
Chicken GnRH II(cGnRH-II)	GnRH 2	x	x	x	x	His	x	Trp	Tyr	x	x	Miyamoto et al. 1984
Frog GnRH (fGnRH)	GnRH 1	x	x	x	x	x	x	x	Trp	x	x	Yoo et al. 2000
Seabream (sbGnRH)	GnRH 1	x	x	x	x	x	x	x	Ser	x	x	Powell et al. 1994
Catfish GnRH (cfGnRH)	GnRH 1	x	x	x	x	His	x	x	Asn	x	x	Ngamvongchon et al. 1992
Herring GnRH (hrGnRH)	GnRH 1	x	x	x	x	His	x	x	Ser	x	x	Carolsfeld et al. 2000
Salmon GnRH (sGnRH)	GnRH 3	x	x	x	x	x	x	Trp	Leu	x	x	Sherwood et al. 1983
Medaka GnRH (mdGnRH)	GnRH 1	x	x	x	x	Phe	x	x	Ser	x	x	Okubo et al. 2000
Whitefish GnRH (whGnRH)	GnRH 1	x	x	x	x	x	x	Met	Asn	x	x	Adams et al. 2002
Guinea pig GnRH (gpGnRH)	GnRH 1	x	Tyr	x	x	x	x	Val	x	x	x	Jimenez-Linan et al. 1997
Dogfish GnRH (dfGnRH)	?	x	x	x	x	His	x	Trp	Leu	x	x	Lovejoy et al. 1992
Lamprey GnRH-I (lGnRH-I)	?	x	x	x	x	His	Asp	Trp	Lys	x	x	Sherwood et al. 1986
Lamprey GnRH-II (lGnRH-II)	?	x	x	x	x	His	x	Trp	Phe	x	x	Kavanaugh et al. (2008)
Lamprey III (lGnRH-III)	?	x	x	Tyr	x	Leu	Glu	Trp	Lys	x	x	Sower et al. 1993

Table 1: Structure of the known gonadotropin-releasing hormone (GnRH) variants, with mammalian GnRH (mGnRH) as reference. Variants found in teleost fish marked in bold. x indicates regions of identity. All forms are composed of 10 amino acids with an N-terminal pyroglutamate and C terminal glycinamide. Adapted from Okubo and Nagahama (2008).

Vertebrates possess several different forms of GnRH, that are classified into three groups, those being GnRH1, GnRH2 and GnRH3.(Gaillard et al., 2018) Up until quite recently the convention was to name forms of GnRH after the originator species. In this convention, GnRH1 was called mGnRH, GnRH2 was called cGnRH and GnRH3 was called sGnRH after mammals, the chicken, and the salmon, respectively. To minimize the confusion for both author and reader, in this thesis only the numerical form of names will be given from this point on.

These different forms are a result of different genes that have most likely arisen due to early whole genome duplications in the vertebrate genome. So far GnRH3 has only been identified in teleost fish and was suspected to have arisen due to teleost specific genome duplication, however comparative genome analyses suggests that it is instead derived from earlier genome duplication in a vertebrate ancestor, and subsequently lost in the tetrapod lineage. (Okubo et al., 2002)

Each GnRH is encoded by its gene (Fig 2) as a precursor polypeptide called prepro-GnRH. This polypeptide includes a signal peptide, following by the decapeptide GnRH, a proteolytic cleavage site and a Gonadotropin associated peptide (GAP). The GnRH gene consists of four exons and three introns. In order, Exon 1 encodes 5` untranslated region (5`-UTR). Exon 2 encodes the N-terminus of prepro GnRH including signal peptide, the GnRH decapeptide itself, the protelytic cleavage site (Gly-Lys-Arg) and the N terminus of the GAP. Exon 3 encodes the middle segment of GAP and finally, exon 5 encodes the C-terminus of the GAP and the 3`UTR. (Adelman et al., 1986, Okubo and Nagahama, 2008)

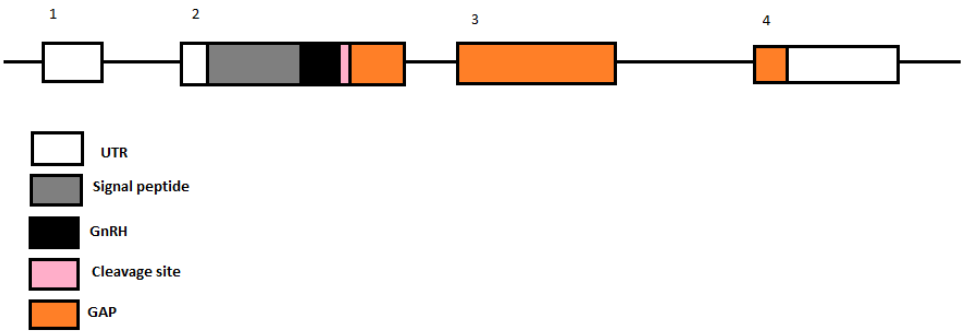

Figure 2: Diagram of the GnRH gene in vertebrates. Exons and introns represented by boxes and horizontal lines lines respectively. Exon numbers displayed. Figure adapted from Okubo and Nagahama, 2008.

GnRH is a product of an interconnected three-part system that constitute the mentioned BPG-axis, neuroendocrine neurons in preoptic regions of the brain form an interface between the central nervous system (CNS) and the endocrine system. Inhibitory and stimulatory inputs merge on these neurons and output GnRH (Weltzien et al., 2004). In mammalian brains, GnRH is released from nerve endings and pass through the hypophyseal portal system in pulses to stimulate synthesis of FSH and LH. Communication between the GnRH neurons in the hypothalamus and the pituitary occurs via vascular circulatory system called the median eminence, with both inhibitory and stimulating hormones converging on the capillary system of the ventral hypothalamus. (Biran et al., 2015).

This system is lacking in teleost fish (Whitlock et al., 2019) and the axons of the GnRH neurons instead project directly from the hypothalamus to the pituitary. (Fig 1 A/B) As opposed to mammals, GnRH release from the pituitary in teleost does not seem to be pulsatile in nature, or at least no clear indication of this has been found. (Kah and Dufour., 2011)

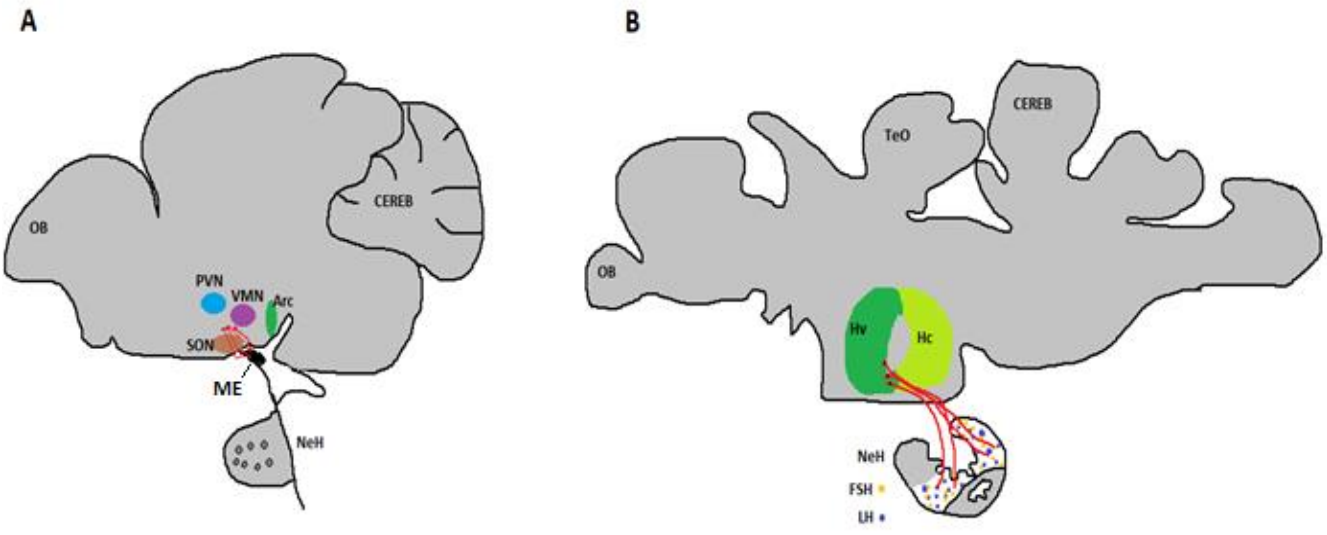

Figure 3: Schematic of a lateral view of mouse (A) and zebrafish (B) brain. In mammalian brains, the GnRH (red lines) cells extend to the median eminence which connects to the pituitary. In teleost fish (B) the GnRH cells make direct connections with the pituitary. Abbreviations: Arc, arcuate nucleus; Hv, ventral zone of periventricular hypothalamus; Hc, caudal zone of periventricular hypothalamus; preoptic area; ME, median eminence; NeH, neurohypophysis; OB, olfactory bulb; PVN, paraventricular nucleus; SON, supraoptic nucleus; TeO, tectum opticum; VMN, ventromedial nucleus. Authors own illustration, adapted from Whitlock.et al., 2019.

During vertebrate development, GnRH neurons arise from the nasal placode before migrating to the forebrain via the olfactory and vomeronasal nerves. Upon entry to the forebrain, GnRH neurons continue migrating caudoventrally towards the Pre-Optic Area (POA) and hypothalamus. Once in this position, the neurons extend axons to either the median eminence (tetrapods) or directly to the pituitary (teleosts) and mature into their adult phenotype as secretory neurons. (Wray, 2010)

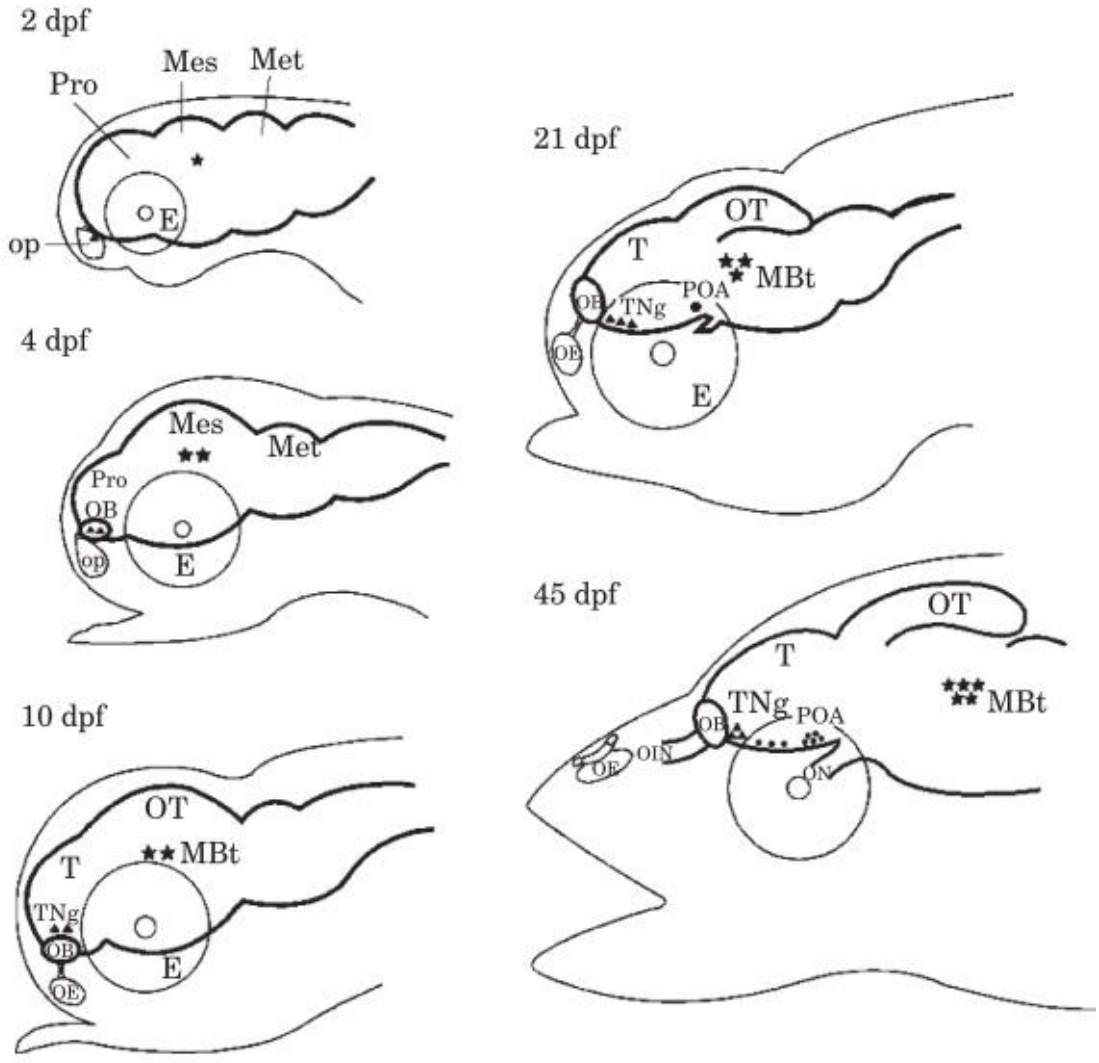

Figure 4: Schematic representing the ontogeny of three GnRH neurons in the hybrid striped bass. Midbrain GnRH2 system (star shape) appears in the midbrain of the hybrid striped bass at 2 days post-fertilization (dpf) and proliferate in this location throughout development. Forebrain GnRH3 neurons (triangle shape) emerge in the developing olfactory area and appear in the olfactory placodes (OP) at 32 hrs post fertilization (hpf) (shown at 2 dpf) and migrate to the forebrain, reaching their final destination along the terminal nerve ganglion (TNg) at 4-10 dpf. Forebrain GRnH1 neurons (circle shapes) appear at 21 dpf in the preoptic area (POA) and later as a continuum along the ventral telencephalon. E, eye; MBt, midbrain tegmentum; Mes, mesencephalon; Met,

metencephalon; OB, olfactory bulbs; OE, olfactory epithelium; OlN, olfactory nerve; OT, optic tectum; Pro, prosencephalon; T, telencephalon. Figure adapted from Yashuvi et al., 2006.

In all vertebrates hitherto studied, two or three molecular forms of GnRH are found, distributed in three distinct areas in the brain: 1. The midbrain tegmentum (MBT) 2. Along the terminal nerve (TN) 3. The hypothalamic area. (Yamamoto, 2003). The form of GnRH present in the hypothalamus is the main form responsible for hypophysiotropic action of gonadotropin release (Somoza et al., 2002).

In salmonids, only two forms of GnRH have been found, those being GnRH2 and GnRH3, since salmon only has two forms, it is believed that GnRH3 is the form acting as the true hypophysiotropic form. (Sherwood et al., 1983, Ando and Urano 2005).

1.2 Six6 and putative effects

Six6 is a homedomain transcription factor in the sine oculis homebox family, that is found across many different species, and is grouped into three subgroups, those being Six1/2, Six3/6 and Six4/5. The gene family is important in embryogenesis for many different structures. While Six1, Six2, Six4 and Six5 have broad expression patterns during embryogenesis Six3 and Six6 are localized to the brain and eye region during embryogenesis where they regulate brain and eye development. (Kawakami et al., 2000)

In the Atlantic salmon the Six6 gene has been associated with age and size at maturity (Barson et al., 2015) as well as spawning ecotype in the Atlantic salmon and the sockeye salmon (*Oncorhynchus nerka).*(Kurko et al., 2020).

It has a total length of 2776 bps with two exons and one intron, exon 1 is 863 bps long, exon 2 is 803 bps long and the intron is 1110 bps long. After translation it is a protein with 390 amino acids. In Atlantic salmon the Six6 gene associated with spawning, size and age at maturity, is located on chromosome 9,(Ref.#NC_027308.1)

The salmonid lineage has undergone a whole genome duplication (WGD)independently of the common teleost fish WGD events. (Macqueen and Johnston 2014). Because of these duplication event, there are several copies of the Six6 gene as well as two Six6-like genes located in different parts in the genome. There is a homologue located on chromosome 1 (Ref# NC_027300.1) but this version does not display the same association pattern with age and size at maturity that the one on chromosome 9 does.

The Six6-like genes are located on chromosome 7 (Ref# NC_027306.1) and on chromosome 18 (Ref# NC_027317.1). While none of these genes, like the Six6 homologue, are associated with age or size at maturity, they are mentioned here because when editing a multi-copy gene, the other copies may compensate for the knockout by changing their expression levels. The gRNA design must also be specific to the Six6 gene located on chromosome 9, to avoid editing any of the other copies.

It has been demonstrated that Six6 regulates maturation of gonadotropin-releasing-hormone (GnRH) neurons and expression of GnRH in the hypothalamus in mice, with female Six6 KO mice failing to go through the oestrous cycle normally as well as failing to ovulate and failing to produce litters. It is also believed to be involved in the survival and successful migration of GnRH neurons with Six6 KO mice having a reduction of up to 89% in GnRH neuronal population (Larder et al., 2011, Pandolfi et al., 2019).

1.3. Genome editing and CRISPR

Genome editing is a powerful molecular technique that has amazing potential for a multitude of applications from medicine to molecular biology, biotechnology, agriculture, and farming. In essence, genome editing is the process of changing an organism's DNA by, removing, adding or changing the genetic material at particular locations. One of, if not the most, efficient methods of genome-editing is induction of a double stranded break (DSB) in the DNA by using a DNA nuclease. The cellular repair processes after such an event often results in mutagenesis. In the preceding decades, targeted genome editing was usually performed either with zinc-finger nucleases (ZFN) or transcription activator-like effector nucleases. But since 2013 a new technology has arisen that has rapidly become the dominant genome editing tool in laboratories around the world, Clustered-Regularly-Interspaced-Short-Palindromic-Repeats (CRISPR). (Carroll, 2017).

CRISPR, is an innate defense mechanism that bacterium utilize against invading bacteriophages. Snippets of foreign genetic material are captured and incorporated into CRISPR-arrays. Transcription of these arrays create cRNAs that binds to Cas nucleases and this complex can then recognize and interfere with foreign genetic materials. (Knott and Doudna 2018).

Consequently, CRISPR does not require design of the nuclease itself, instead it is necessary to design sgRNA specific to the gene of interest. The CRISPR system is composed of two main components, a guide RNA (sgRNA) and a CRISPR-associated endonuclease (Cas

protein). The sgRNA is a short synthetic RNA oligonucleotide that contains a scaffold sequence for Cas binding and a designed nucleotide (~ 20 nucleotides) spacer that hybridizes with the intended genomic target. This is a very flexible system since the genomic target can be changed by changing the target sequence of the sgRNA. Once the sgRNA has been bound to Cas9, the Cas9 nuclease undergoes a conformational change that shifts it from an inert state to its active DNA-binding conformation. The genomic target can be any sequence of ca 20 nucleotides, provided it is unique when compared to the rest of the genome and adjacent to a Protospacer Adjacent Motif (PAM), the PAM is required for stable binding of Cas 9. (Knott and Doudna 2018).

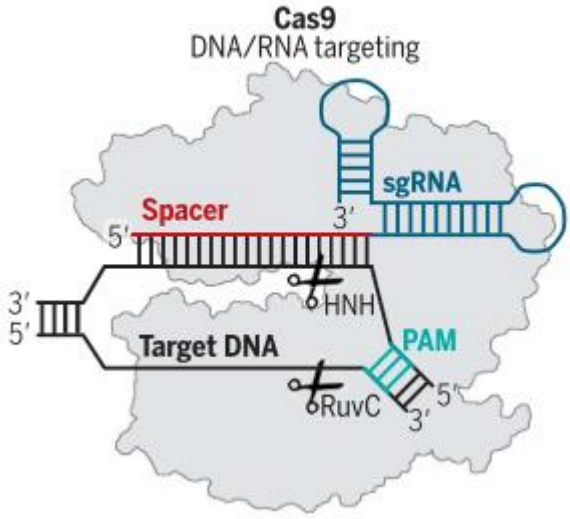

Figure 5: The CRISPR Cas9 system;
After binding to a DNA target, the seed sequence (not shown) at the 3`end of the sgRNA will anneal to target DNA in a 3`to 5`direction, provided sufficient homology. Upon binding the target DNA, Cas9 undergoes a second conformational change which enables the nuclease domains HNH and RuvC to cleave opposite strands of target DNA,resulting in a double stranded break (DSB). Adapted from Knott and Doudna (2018).

I will be using CRISPR to induce KO. CRISPR KO is the most basic application of CRISPR when one considers many of its broader aspects such as base editing, prime editing, or RNA manipulation. However, in fish CRISPR KO can be quite challenging.

1.4 CRISPR in fish

Before CRISPR arrived on the scene, genome editing was largely the domain of existing mammalian model species such as the mouse and rat. With CRISPR though, genome editing became more accessible for a wide range of species, including teleost fish.

Two model species of fish, the zebrafish *Danio rerio* and the medaka *Oryzias latipes* have seen a large body of work using the CRISPR technique, including KO, knock in, and base editing. (Watakabe et al., 2018, Fang et al., 2018, Hoshijima et al., 2019, Zhang et al., 2017) Successful genome editing using CRISPR has also been performed on other fish that are not

usually recognized as model species, such as the blind cave fish (*Astyanax mexicanus)*. (Klaassen et al., 2018).

The Atlantic Salmon is large and active fish and is thus expensive to feed and maintain. It takes a long time to reach maturity (~3 years for females) and it is therefore inconvenient and time consuming to study the F1 generation, so F0 generation is often studied. This requires a high degree of knock out success, as we can only use the individuals with both alleles knocked out.

Edvardsen et al., (2014) found that by knocking out the pigmentation genes tyrosinase (*tyr)* and solute carrier family 45 member 2 (*slc45a2*), they could achieve a 40% mutation induction for the *slc45a2* and 22% for *tyr*. According to the paper, this is the first successful use of CRISPR/Cas9 in a marine cold-water species, but more importantly, demonstrate that CRISPR is a method suitable for study of the F0 generation of the Atlantic salmon.

1.5 Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to detect, visualize, semi-quantification of the presence of antigens in tissues or cells. It can detect a broad range of antigens specific to anything from amino acids, proteins as well as pathogens and specific cells.

The method is based on the immunological reaction that occurs between the epitope of the antigen and the paratope of a given antibody, allowing for detection *in situ*. The method of visualization can be performed in different ways such as enzymatically catalysed colour staining or via fluorescence-based detection. (Matos et al., 2010)

The method can be broadly divided into two phases:

1. Preparation and fixation of tissue, blocking to avoid non-specific binding, primary antibody incubation and secondary antibody incubation.
2. Visualization and quantification of expression.

Fixation with a proper medium, such as 4% Paraformaldehyde (PFA) is essential in order to prevent autolysis and preserve tissue morphology while still maintaining antigenicity. (Im et al., 2019)

To prevent non-specific binding of antibody and tissue, blocking of these sites by a blocking agent is necessary to reduce background staining (BGS), or “noise”. These blocking agents

compete for the nonspecific binding sites in the tissue and serve to decrease non-specific binding when incubated either before or in conjunction with the immunohistochemical reagents.

Proper antibodies are critical in order to get good results which can be challenging when dealing with teleost fish due to their biodiversity. They are the largest infraclass in the class Actinopterygii (ray finned fishes) and make up roughly half of the extant vertebrate species. They are also remarkably diverse in morphology, ecology and behaviour. There is evidence that during early evolution of the ray finned fish lineage a round of tetraploidization/rediploidization took place and hundreds of paralogues from this event have been maintained for millions of years into modern times. (Volff, 2005)

1.6 Confocal Microscopy

With conventional microscopes a section of tissue is placed on the objective glass and the entire tissue is illuminated by light and visualized and although it is at its britgtest and most intense at the focal point of the objective lens, since the whole tissue sample is illuminated, there can be some background “noise”, compromising the quality of images. Confocal microscopes the beam of incoming light (excitation beam) is focused through the objective lens on a very small spot inside the tissue and can be connected to a computer to produce virtual images of very high quality with fine details and can be used to reconstruct 3-D images of tissues. (Elliott., 2020).

Another thing to consider is using whole mount or sections when viewing the tissue(s). While whole mount presents a better representation of the entire tissue as it exists *in vivo* such as neurological connections in the brain, distribution of nerve fibres and shape and appearance of cells. A single three-dimensional image can be more informative than many tissue sections. The downside is that due to the opaque nature of tissues, proper clearing is necessary and if not successfully achieved the light from the microscope will not penetrate properly, and any fluorescence will go unnoticed. Sections provide thin slices of tissue. These sections provide an easier method of detecting fluorescence but creating a 3d model of the differing sections can be a problem. (Kagayama and Sasano 1999)

The main aim of this thesis was to investigate if there was any change in GnRH neuron number or migration in the brain of the Atlantic salmon after CRISPR induced knockout (KO) of Six6.

Since this has never been done before in my group, I had to set up and test different methods. Therefore, my project was divided in several secondary aims:

I) To knock-out the Six6 gene with CRISPR gene editing in Atlantic salmon embryos at the one-cell stage

II) To monitor the edited embryos and track mortality in the different groups

III) To genotype the injected embryos to detect knock-out individuals

IV) To test out protocols for clearing of whole-mount embryos

V) To test out protocols and GnRH antibodies for fluorescent whole-mount immunohistochemistry

2.0 Material and methods.

2.1 Design and production of gRNAs

Using Chop Chop (https://chopchop.cbu.uib.no/), three sgRNA candidates were selected for CRISPR induced KO of Six6 and screened against salmon genome assembly to avoid off-target effects. The program also checks for potential sites where the RNA could be self-complementary, which could lead to the RNA forming a hairpin loop and negatively affect binding to the target sequence.

To easily recognize knockouts and avoid study of mosaic animals, slc45a2 was included in the CRISPR construct. *slc45a* is involved in pigmentation and KO of this gene results in albino animals. The effects of *slc45a* and its gRNA were described by Edvardsen et al., (2014).

Once the target site had been selected three pairs of DNA oligos were ordered for Six6 and one for slc45a, with the following sequences:

Table 2: gRNA oligo sequences

Oligo	Forward	Reverse	Length
gRNA 1 oligo	taggGACAACCCCTGCTGTAGCC	aaacGGCTACAGCAGGGGTTGTC	23
gRNA 2 oligo	taggCTTTGCTCGACAAACTTGCCTCG	aaacCGAGGCAAGTTTGTCGAGCAAAG	27
gRNA 4 oligo	taggAAGTGATAGAGATAGCTG	aaacCAGCTATCTCTATCACTT	22
slc45a gRNA	taggGGAACAGGCCGATAAGAC	aaacGTCTTATCGGCCTGTTCC	22

In order to ligate the oligos to the pt7-gRNA vector, two adapters are included in the 5' end (lowercase letters) of all oligos to create a duplex with four nucleotide overhangs at both ends after annealing. These overhangs are compatible with BsmBI sites in pT7-gRNA. (Addgene #46759)

The pt7-gRNA vector was digested with BsmBI restriction enzyme to insert the gRNA sequence, as already mentioned this restriction site is compatible with the adapters on the gRNA oligos. 1µg(10.6 µl) of the vector was digested with 1µl BsmBI(equivalent to 10 units) in a reaction with 5µl of 10X NEBuffer and 33.5 µl of H2O, to a total reaction volume of 50µl. This reaction volume was then incubated at 55 °C for 1 minutes. After this digestion reaction, Zymo DNA Clean and Concentrator - 25 was used (Zymo) to remove any leftover enzyme. The digested products were then ran on gel to confirm digestion.

1 µl of each forward and reverse oligo of each gRNA were annealed in T4 Ligase Buffer (NEB, Massachusetts, USA) by incubating at 95 °C for 5 minutes followed by cooling to 25 °C for 45 minutes, and subsequently diluted in a 1:200 ratio of EB buffer.

1 µl of annealed oligonucleotides were ligated into 50ng (1.9 µl) of BsmBI digested pT-7-gRNA using 2µl T4 DNA ligase (NEB) and 5.1µl H2O to a total volume of 10µl. The mixture was then left at room temperature for 1 hour.

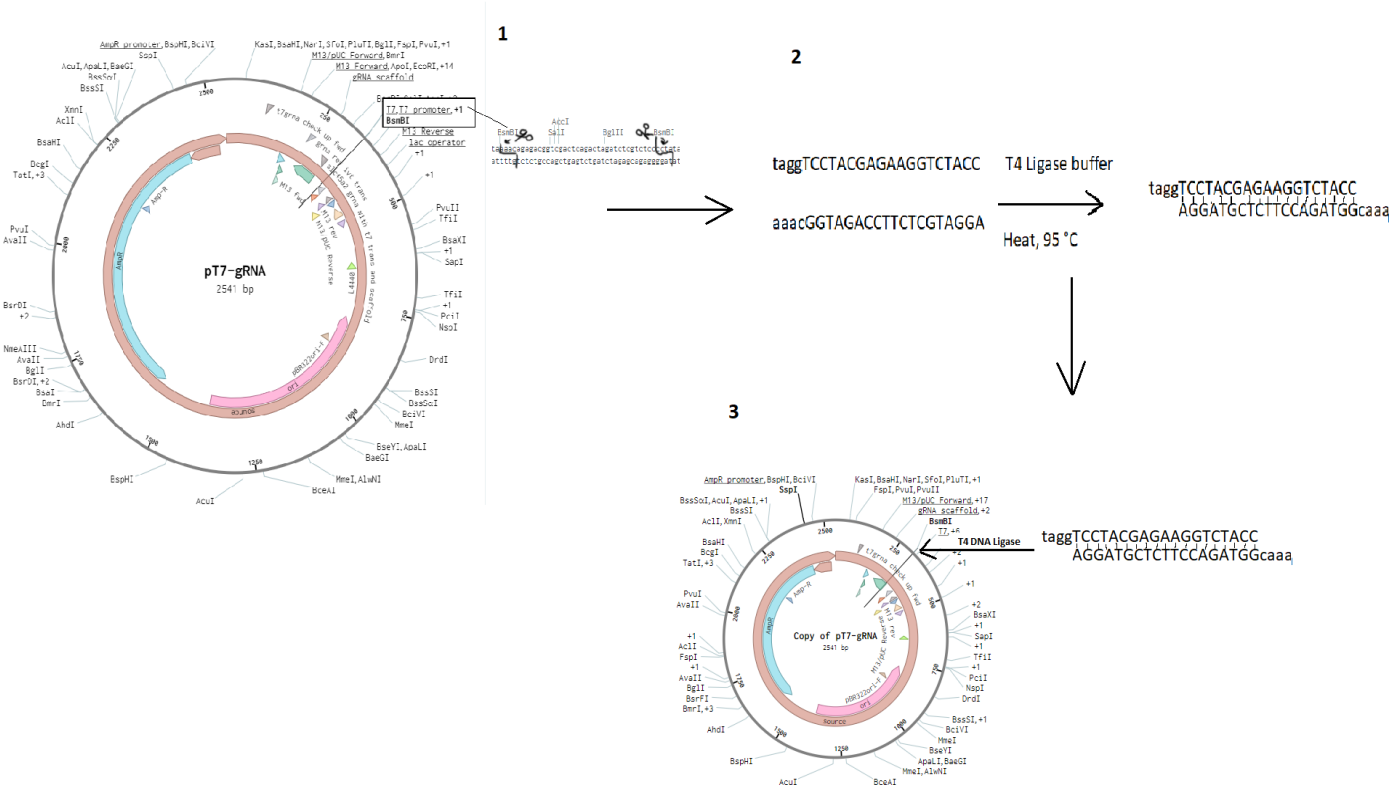

Figure 6: Creating recombinant pT7-gRNA. **1:** BsmBI cuts the plasmid at the restriction site, creating sticky overhangs. **2:** Oligos (gRNA1oligos in this example) are annealed together, creating a heteroduplex with overhangs compatible with the cut site in pT7-gRNA. **3:** The heteroduplex is inserted into the plasmid, catalysed by T4 Ligase.

The plasmids were then transformed into competent DH5α cells (*E.coli*) by first thawing the competent cells on ice, followed by pipetting 2µl of the prepared plasmids to the tube containing the cells and mixed well. The cells were then kept on ice of 30 minutes, followed by immersing them in a water bath set to 42 °C for 30 seconds, this heat shock destabilizes the bacterial membrane and increases permeability allowing entry of the plasmid. Immediately after, the cells were transferred to ice and kept for 5 minutes. 250µl of S.O.C medium (Invitrogen) was added to the cells and the mixture was incubated at 37°C at 250

rpm for 1 hour. 50µl and 100µl of mixture was then plated on LB agar plates containing 100µg/ml Ampicillin and incubated overnight at 37°C.

2.2 Colony PCR

To validate the presence of the insert, random colonies from the LB agar plate were selected for colony Polymerase Chain Reaction (PCR), using Invitriogen II Hot-Start Green PCR Master Mix (Thermofisher). Each reaction was made up to 20ul, containing 10ul of Platinum II Green PCR Master Mix (2X) 1ul of forward and reverse primer, 4ul of ddH2O and 4 ul Platinum GC enhancer. The PCR reaction was then run under the following conditions:

1. 94 C for 2 minutes
2. 94 C for 2 minutes
3. 54 degrees for 15 seconds
4. 68 C for 30 seconds
5. Cycle to step 2(x32 cycles)
6. 4 C hold

Primers used are given in table 2, for each gRNA the forward gRNA oligonucleotide was used as forward primer, while pT7_gRNA_check_rev primer was used as a universal reverse primer.

PCR products were then run on 1% agarose gel and colonies with clearly defined bands with desired length were inoculated in 5 ml LB broth containing 100µg/ml ampicillin before incubation overnight at 37 C on 250 rpm. 1:1 glycerol stock was then prepared using 50% glycerol and stored at -80 C. Plasmids were extracted with Zymo Research plasmid miniprep kit (Zymo research) and sent for sequencing (GATC LightRun, Eurofins Genomics).

Table 3: PCR and plasmid sequencing primers

PCR	**Forward**	**Reverse**
g1	TAGGGACAACCCCTGCTGTAGCC	TTCGCTATTACGCCAGC
g2	TAGGTAAAGTTCTCGGAAATTCC	TTCGCTATTACGCCAGC
g4	TAGGAAGTGATAGAGATAGCTG	TTCGCTATTACGCCAGC
Plasmids	**Forward**	**Reverse**

g1	TTCGCTATTACGCCAGC	AAACGGCTACAGCAGGGGTTGTC
g2	TTCGCTATTACGCCAGC	AAACGGAATTTCCGAGAACTTTA
g4	TTCGCTATTACGCCAGC	AAACGGAATTTCCGAGAACTTTA

2.3 Transcription of Cas9 mRNA and gRNA *in vitro*

To produce Cas9 nuclease mRNA, pTST3-nCas9n (Addgene #46757) was linearized with XbaI (NEB) and gel purified using QIAquick Gel Extraction kit (Qiagen). Cas9 mRNA was produced using the mMessage mMachine T3 kit (Ambion) and cleaned up with the RNAeasy mini kit (Qiagen) gRNAs pT7-gRNA was linearized using BamHI (NEB) and purified with DNA Clean and Concentrator - 25 (Zymo Research). gRNAs were transcribed with T7 Megascript kit (Ambion) and mirVANA miRNA Isolation kit (Invitrogen) used to purify gRNAs. To check integrity of synthesized Cas9 mRNA and gRNA the Agilent 600 Nano kit and Agilent 2100 Bioanalyzer were used (Agilent Technologies). Further, to verify the presence of desired gRNA sequence, all gRNAs were sequenced using the t7gRNA check-up primer, TTCGCTATTACGCCAGC

2.4 Microinjection of salmon eggs

Atlantic Salmon eggs were fertilized in filtered fresh water containing 0.5mM reduced glutathione and incubated at 4°C for 5 hours until the first cell was visible.

Eggs were placed on a petri dish and held on concavities and injected with a mixture of 50 ng/µl gRNA each for *slc45a* and *Six6* along with 150 ng/µl (total volume of 20 µl) of Cas9 mRNA using the Eppendorf Femtojet 4x, each group was separated by respective gRNA, (1,2 and 4). The negative control group fertilized the same day was not injected with anything and were not exposed to the same temperature change from lying in the petri dish like the injected groups were.

After injection, eggs were kept in fridges with a water temp of ~ 6 degrees and kept for 69 days. After 5 weeks, 6 eggs from each gRNA group were dechorionated and the embryos were treated for DNA extraction and sent for sequencing to identify any KO's.

Keeping the eggs in our own fridge was undesirable, as it was hard to properly set the water temp to our desired setting, and it fluctuated between 4-8 °C regularly.

Therefore, a new batch of CRISPR and Cas9 was prepared, but this time all three gRNAs were pooled together and co-injected with the Cas9. This mixture was made up to 10 µl, according to the following table. The gRNA was isolated using RNeasy kit (Qiagen)

Table 4: Injection mix for second batch of CRISPR injections

Reagent	**Amount**	**Final concentration**
Cas9	1.91 µl	150 ng/ul
Six6 g1	0,62 µl	50 ng/ul
Six6 g2	2,98 µl	50 ng/ul
Six6 g4	0,34 µl	50 ng/ul
Slc45a gRNA	0,49 µl	50 ng/ul
H2O	3,66 µl	NA
Total	**10 µl**	

The second batch of injected eggs were injected and delivered to NMBU Fish Research Center on 04.02.21. Here, the eggs were placed in floating baskets and temperature properly monitored. Like the previous batch, negative control was not injected with anything.

Table 5: Differences between first and second injection batches

Method	**First batch injected 03.10.20**	**Second batch injected 04.02.21**
Egg storage	Kept in fridge in lab	Kept at fish lab at NMBU
gRNA Six6	50 ng/ul of one gRNA in separate injection mixes	50 ng/ul of all gRNA for one single injection mix
gRNA *slc45a*	50 ng/ul in injection mix	50 ng/ul in injection mix

2.5 Sample preparation for GnRH immunohistochemistry

Salmon embryos from the second batch collected 03.03.21, (28 days post fertilization (dpf) 210-degree days (dd) 22.03.21 (47 dpf, 342dd) and 19.04.21 (82dpf 615 dd) were fixed in 4% PFA and PBST and left for 4 hours. This is well suited to preserve tissue morphology and arrest post-mortem degradation. However, it can lead to loss of immunoreactivity due to masking or damaging antibody binding sites. (Rickert and Maliniak., 1989)

It is important to use an optimal antibody titre for this step, in order to achieve optimal specific binding with the least amount of BGS. The dilution of the antibody is important here as antibodies have differing affinities. A high-affinity antibody is prone to react faster, and stain more intensely compared to a lower affinity antibody given identical incubation times. Correct dilution therefore is therefore of high importance. (Gown., 2016)

Putative Six6 KO`s along with a negative control group were collected. Except for the first group, the eggs were decoronated and the embryos retrieved. Following this they were dehydrated by immersion in EtOH/PBST solution in ascending concentration, as follows:

- 15 min in 25% EtOH in PBST
- 15 min in 50% EtOH in PBST
- 15 min in 75% EtOH in PBST
- 15 min in 96% EtOH in PBST

Embryos were then put in 100% MetOH and stored at -20 C.

2.6 Protocol R&D

Since there were no clear-cut immunohistochemistry protocols in salmon readily available, it was necessary to perform some tests to decide on the best possible method. During preparation for immunohistochemistry several different protocols for blocking and tissue clearing were attempted and in fact created in the lab.

Blocking protocol 1, provided by Romaine Fontaine:

Salmon embryos were rehydrated in reverse order, ie:

- 15 min in 96% EtOH in PBST
- 15 min in 75% EtOH in PBST
- 15 min in 50% EtOH in PBST
- 15 min in 25% EtOH in PBST

Embryos were then washed 10 times in PBST for 10 minutes.

2.6.1 Preparation of whole mount tissue samples

During rehydration in 96% EtOH, salmon embryos were decapitated roughly 1 mm behind the neck area, and incubated in a blocking solution. 15ml of blocking solution was prepared with the following reagents:

Table 6: Blocking protocol with Normal Goat Serum reagents

Reagent	**Amount**
Normal Goat Serum (NGS)	750 ul
Triton X	450 ul
DMSO	112 ul
PBSt	13.68 ml
Total	**15 ml**

Blocking protocol 2.

Same rehydration and decapitation protocol as above

Table 7: Blocking protocol with dried milk reagents

Reagent	**Amount**
Dried powdered milk	0.45 g (450 ul)
Triton x	450 ul
PBSt	14.1 ml
Total	**15 ml**

2.6.2 Clearing agent protocol:

To make the tissues more transparent and to possibly avoid having to section the embryos it was necessary to perform tissue clearing, three different protocols were tried:

Clearing Agent one, Glycerol:

The simplest protocol, 50ml of 20%, 40% and 80% Glycerol in PBSt solution were made, and the tissue sample was immersed in 20% for 1 hours, 40% for 1 hour and 80% for 2 days.

Clearing agent two, TDE (2,2-thiodiethanol):

50 ml of 20%, 40% and 60% TDE in PBSt were prepared and the tissue immersed in 20% for 30 minutes, 40% for 30 minutes and in 60% for 2 days.

Clearing agent three, Qbic1/2

This clearing agent had to be assembled before use and was set up as two different solutions:

Table 8: Qbic 1 reagents

Reagent	**Amount**
N,N,N,N Tetrakis	12.5ml
Triton x	7.5ml
Urea	12.5 g
H2O	17.5ml
Total	**50ml**

Table 9: Qbic 2 reagents

Reagent	**Amount**
Triethanolamine	5ml
Triton x	50µl
Urea	12.5g
H2O	32.45ml
Total	**50ml**

The tissue sample was left in Qbic 1 at 4 degrees for 3 days, and then immersed in Qbic 2 solution for 1 day.

2.7 Sectioning

Tissue samples were glued onto the base of the specimen tube, using a small amount of glue, and positioned using forceps, adjusting the orientation as straight as possible for transverse sections. The tube was then filled with 4% freshly made agarose gel in MQ-water. After the gel had been added, the gel was quickset by placing the specimen tube in the provided chilling block that had been precooled in ice.

300 um sections were then cut using the VF-310-0Z compresstome (Precisionary Instrument) with speed set to 4 and oscillation set to 7.

2.8 Immunostaining

The sections of tissues of each embryo were immersed in a blocking solution and left overnight at 4 degrees with agitation. The following day, the blocking solution was pipetted out and incubated in a solution composed of blocking agent and primary antibody. This was also left overnight at 4 degrees with agitation. Next, the tissue sections were washed 10 times for 10 minutes in PBST and then incubated in a solution composed of the secondary antibody diluted at a 1/1000 ratio of PBST overnight at 4 degrees with mild agitation. For all tissue samples the dry-milk blocking agent was used, except for one extra positive control group which was blocked with the NGS serum. Only one could be made due to supply issues

Table 10: Overview of different primary/secondary antibodies and their concentrations.

Primary antibody	**Primary antibody dilution**	**Blocking agent**	**Secondary antibody**	**Secondary antibody dilution**
Zn-12-s (Pos control)	1/1000	Powdered milk	Donkey-Mouse	1/1000
Zn-12-s (Pos control)	1/1000	NGS	Donkey-Mouse	1/1000

SgRB	1/500	Powdered milk	Goat-Rabbit	1/1000
Sal-Gap	1/1000	Powdered milk	Goat-Rabbit	1/1000
Sb-GAP	1/1000	Powdered milk	Goat-Rabbit	1/1000
ChII GAP	1/1000	Powdered milk	Goat-Rabbit	1/1000

Description of antibodies tested

Zn 12-s:

Zn-12-s which served as the positive control, is a neuronal cell surface marker. This antibody was used to confirm that blocking and immunostaining protocol were effective as well as to determine which blocking agent would prove the most effective. It has previously been used to label neurons and map the organization of the hindbrain in Zebrafish embryo. (Trevarrow et al., 1990).

Gonadotropin Associated Polypeptide Antibodies (SAL-GAP, Sb-GAP, CHII-GAP)

Since the actual decapeptide is so short and show a large degree of homology between the variants, it is a challenge to make them specific. Therefore, these antibodies do not associate directly with GnRH, but instead with the GAP of the respective GnRHs. These antibodies were donated to us from a collaborating lab, Finn-Arne Weltzien´s lab at the VET faculty (NMBU). There was not much information on the tubes, but they probably originated from Olivier Kah´s lab in France.

The naming convention of these antibodies goes back to the old form of naming:

Sal-GAP: Salmon Gonadotropin Associated Polypeptide

CHII-GAP: Chicken II Gonadotropin Associated Polypeptide

SB-GAP: Seabream Gonadotropin Associated Polypeptide

Sigma Rabbit (Sg-Rb)

This antibody was also donated by Finn-Arne Weltzien's lab. Unlike the GAP antibodies this one associates with the mGnRH. While salmon does not have the mGnRH it has still been used before with success in other non-mammalian species such as the European lancelet (Chambery et al., 2009)

DAPI labelling:

100 ul of 100x stock DAPI **(**4′,6-diamidino-2-phenylindole), was diluted with 400ul H2O and 500 ul glycerol to create working solution. 10ul of this was then added to 1ml of PBST and the sections were immersed in this solution and left for 4 hours with agitation, covered in aluminium foil to limit light exposure. DAPI penetrates the tissues and stains cell nuclei, it emits fluorescence when binding to double stranded DNA.

2.8 Mounting samples on slides

The brain sections from the different embryos were placed in a petridish and covered with PBST to prevent dehydration. Using a forceps, the sections were placed on microscope slides. Filter paper was applied to absorb excess PBST before adding Vectashield mounting medium (Vector labs), the slides were then covered with microscope slide cover slips. The slips were carefulle placed to try to eliminate bubbles forming in the mounting medium.

Slides were kept in a box covered with aluminium foil to prevent photobleaching.

2.9 Confocal microscopy

The mounted tissue samples were viewed on a confocal laser scanning microscope (LSM 980). As mentioned in the introduction, this microscope allows for visual acquisition of differing layers depending on fluorophore present rather than illuminating the entire tissue sample like with conventional microscopy.

Lasers with different wavelength corresponding to the two present fluorophores were used: 405 nm for DAPI and Alexa Fluor 488 nm for the positive control and GnRH samples. Tissue samples were viewed using 10x (air) zoom and then with digital zoom in set to 50x for all samples with the exception of the dry milk positive control where the 20x (air) was used.

All images were then processed with Fiji (ImageJ) to produce the final images.

3 Results:

3.1 Bioanalyzer readouts

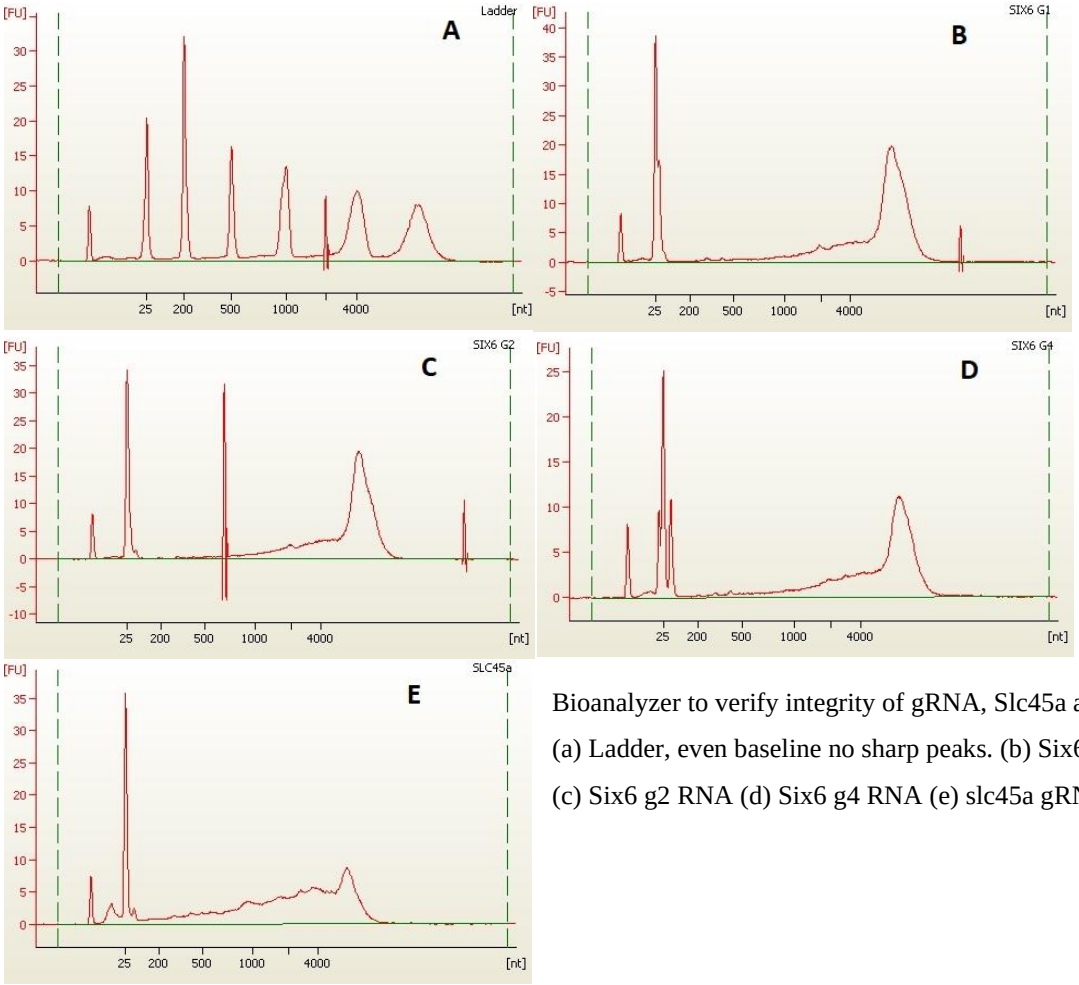

Figure 7: Bioanalyzer to verify integrity of gRNA, Slc45a and Cas 9. (a) Ladder, even baseline no sharp peaks. (b) Six6 g1 RNA. (c) Six6 g2 RNA (d) Six6 g4 RNA (e) slc45a gRNA

The gRNAs bioanalyzed here were mixed with the Cas9 enzyme before injection occured. Fig 7 (a) Ladder is good with even baseline and no sharp peaks. (b) Six6 g1 RNA has a small peak at the start this is likely small RNA fragments which can happen with phenol extraction. Second peak is the gRNA itself and the large peak on the right is the Cas9 RNA. All gRNAs

follow this pattern, with the exception of Six6 g2 which has a peak at ≈700 bp. Six6 g4 has some additional peaks which could signify some RNA degradation, but should still be usable.

3.2 Mortality of salmon embryos/eggs from first injected batch

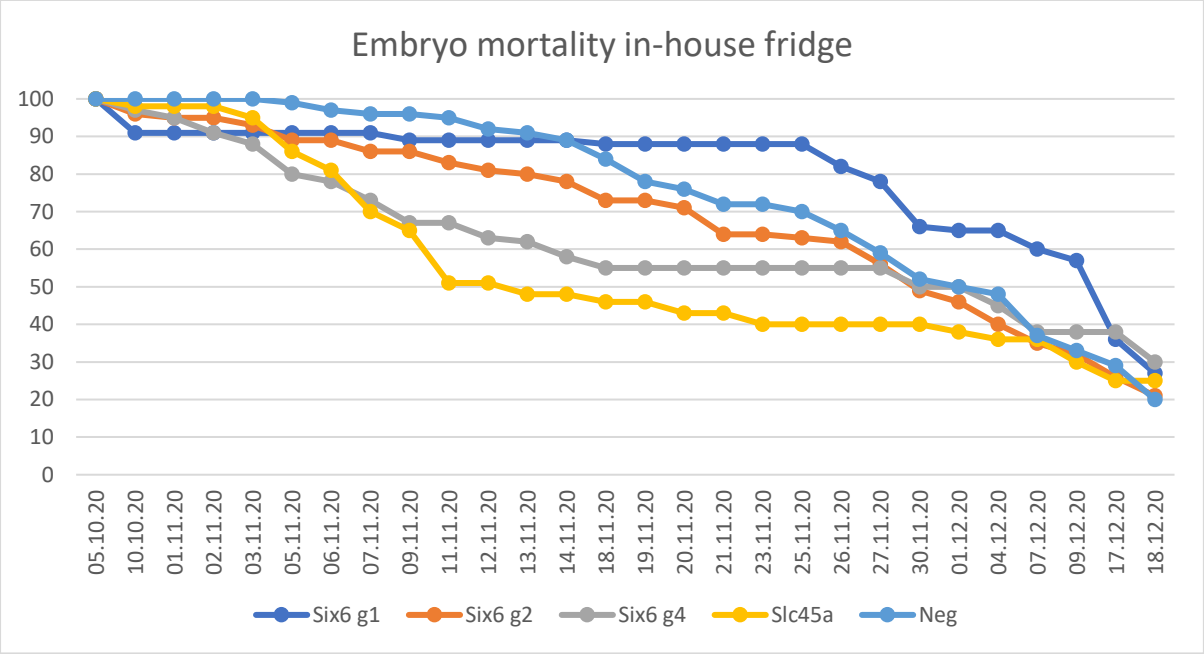

Fig 8: Mortality in negative control and the first batch of embryos injected with a single gRNA, slc45a and Cas9.

Quite high rate of mortality for all groups was observed. Fig 8 shows that Six6 g1 lost embryos at a 73% death rate. Six6 g2 lost embryos a 79% death rate, and Six6 g4 lost embryos at a 70% death rate. Slc45a, 80% death rate. Negative control also lost 80%. While Six6 g2, g4 slc45a, and negative control group lost embryos at a relatively even rate, the Six6 g1 embryos died at an even rate at first and then had a large spike in mortality towards the end of the period.

3.3 Mortality of salmon embryos/eggs from second injection batch

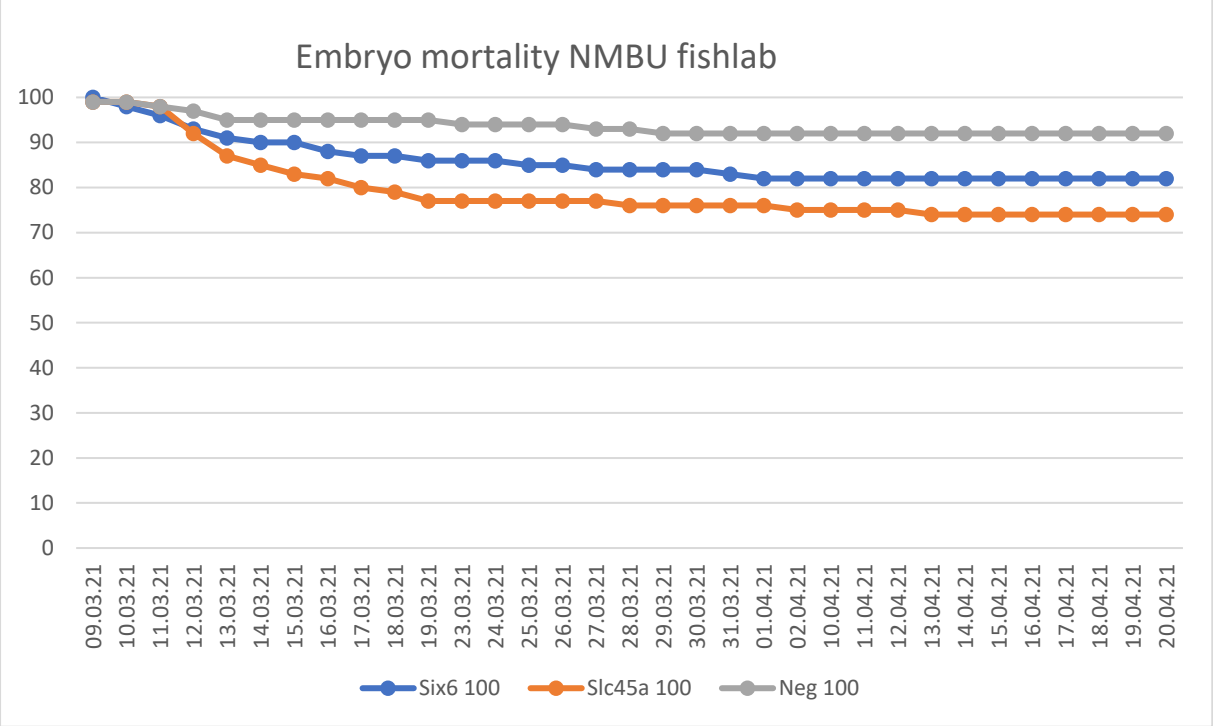

Fig 9: Mortality in salmon embryos kept at the NMBU fish lab as a percentage. Six6 mortality rate 18% Negative control group 8%, Slc45a 26%

The second batch of injections wherein the different Six6 gRNAs were pooled prior to injection suffered a far less dramatic rate of mortality than the first batch. As can be seen in Fig 9 the Six6 injected group suffered a 18% mortality rate, slc45a suffered a 26% death rate while in negative control group, only 5% of embryos died. The proper containment and monitoring afforded by the NMBU fish lab compared to the in-house fridges likely impacted the mortality rate. Average water temperature over the period was 7.5 °C.

3.4 Tissue clearing

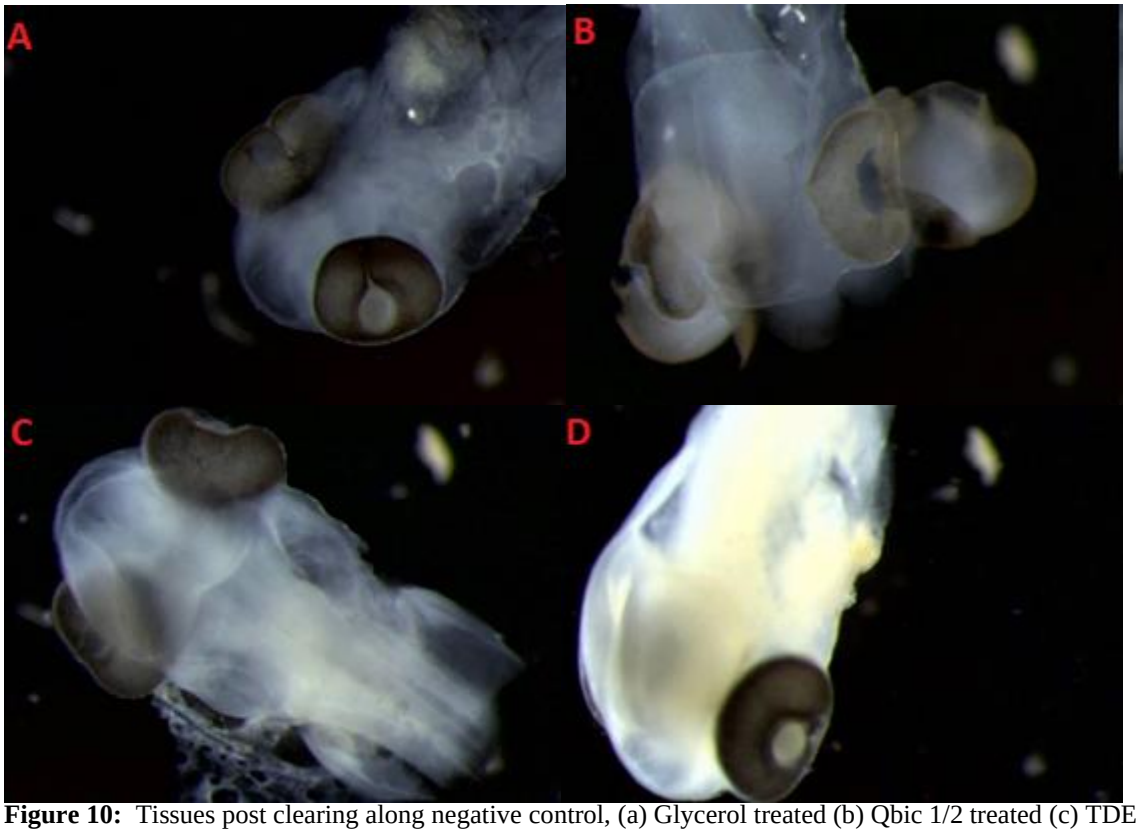

Figure 10: Tissues post clearing along negative control, (a) Glycerol treated (b) Qbic 1/2 treated (c) TDE treated (d) Negative control

While both (a) Glycerol and (b) Qbic 1/2 treated tissues had good clearing, Qbic treatment caused some morphological damage with the eyes becoming swollen and dislodging from their sockets. (c) TDE clearing was the most effective, when compared to (d) negative control.

3.5 Sequencing results

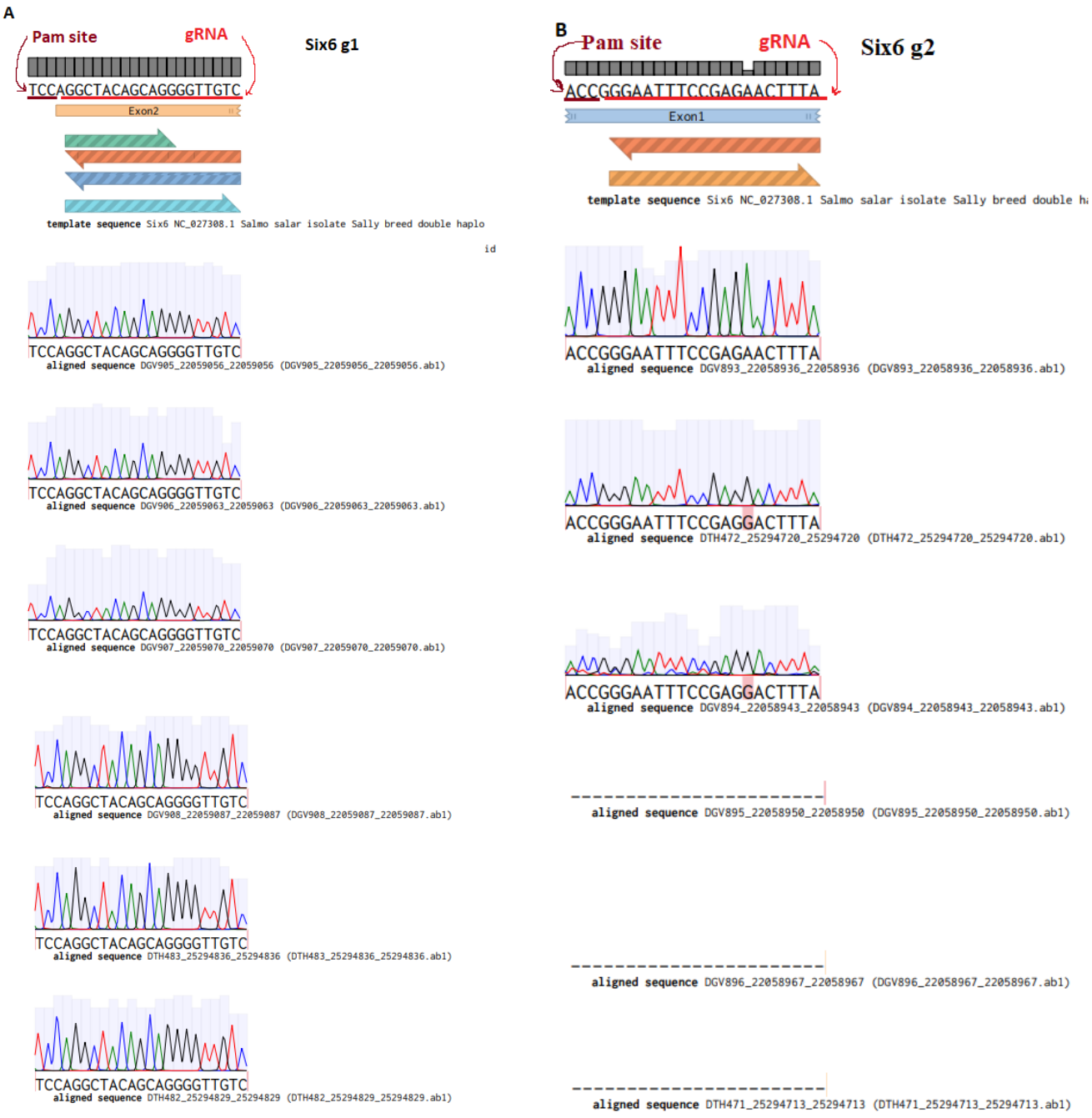

Fig 11: Sequencing from the first batch of injected embryos with chromatogram taken from benchling. (a) Six6 g1 (b) Six 6 g2.

Six6 g1 injected embryos (a) show complete homology to wild type (WT) sequence and no mismatches indicating that knockout has failed in these embryos. Six6 g2 (b) injected display some mismatches in two of the sequences, which could be due to knockout, final three sequences have failed. Six6 g4 injected embryos were also sequenced, but the samples failed and only returned blank sequences, the same as seen in the last three Six6 g2 samples.

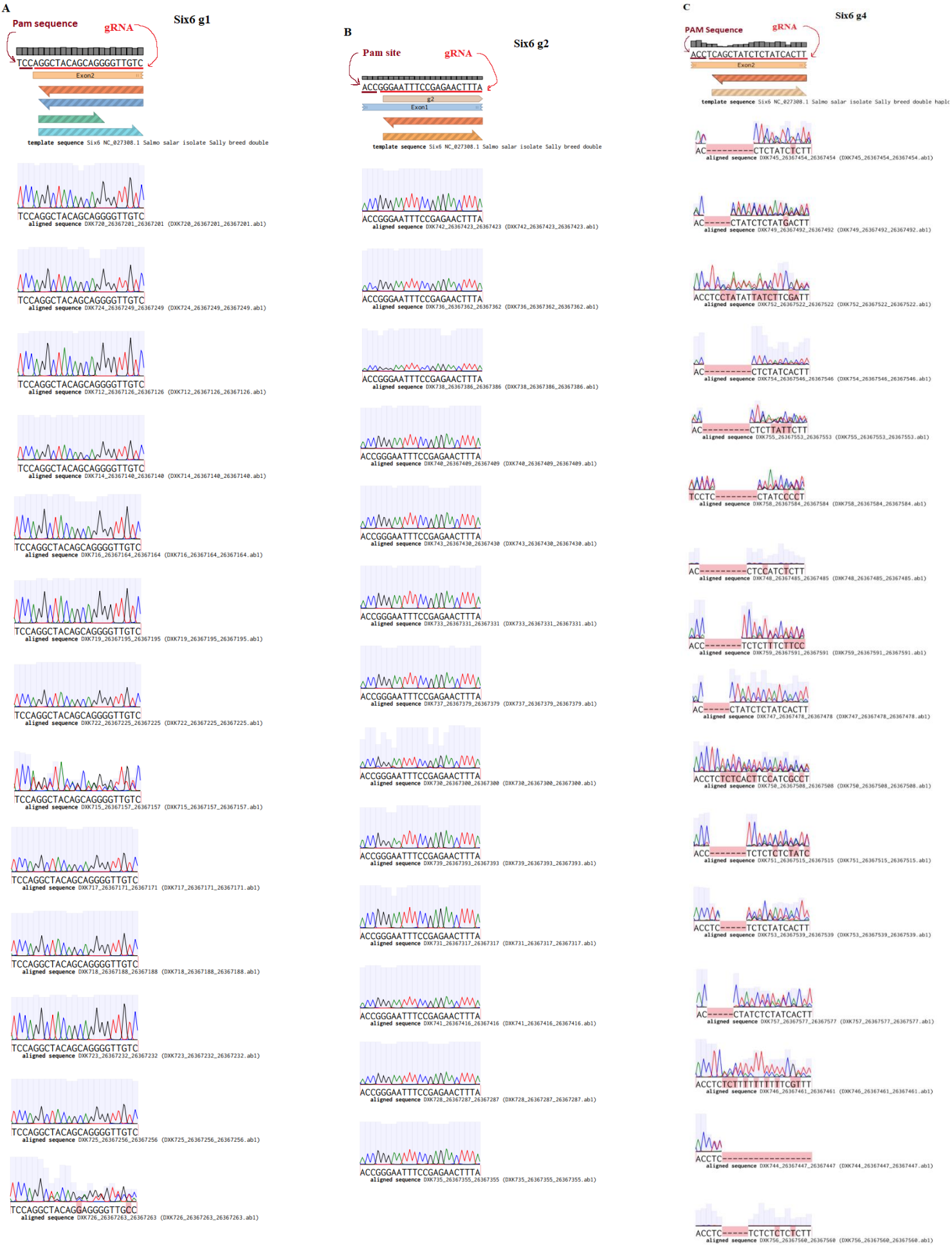
A
Six6 g1
Pam sequence
gRNA
TCCAGGCTACAGCAGGGGTTGTC
Exon2
template sequence Six6 NC_027308.1 Salmo salar isolate Sally breed double
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK720_26367201_26367201 (DXK720_26367201_26367201.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK724_26367249_26367249 (DXK724_26367249_26367249.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK712_26367126_26367126 (DXK712_26367126_26367126.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK714_26367140_26367140 (DXK714_26367140_26367140.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK716_26367164_26367164 (DXK716_26367164_26367164.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK719_26367195_26367195 (DXK719_26367195_26367195.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK722_26367225_26367225 (DXK722_26367225_26367225.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK715_26367157_26367157 (DXK715_26367157_26367157.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK717_26367171_26367171 (DXK717_26367171_26367171.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK718_26367188_26367188 (DXK718_26367188_26367188.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK723_26367232_26367232 (DXK723_26367232_26367232.ab1)
TCCAGGCTACAGCAGGGGTTGTC
aligned sequence DXK725_26367256_26367256 (DXK725_26367256_26367256.ab1)
TCCAGGCTACAGGAGGGGTTGCC
aligned sequence DXK726_26367263_26367263 (DXK726_26367263_26367263.ab1)
B
Six6 g2
Pam site
gRNA
ACCGGGAATTTCCGAGAACTTTA
g2
Exon1
template sequence Six6 NC_027308.1 Salmo salar isolate Sally breed double
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK742_26367423_26367423 (DXK742_26367423_26367423.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK736_26367362_26367362 (DXK736_26367362_26367362.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK738_26367386_26367386 (DXK738_26367386_26367386.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK740_26367409_26367409 (DXK740_26367409_26367409.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK743_26367430_26367430 (DXK743_26367430_26367430.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK733_26367331_26367331 (DXK733_26367331_26367331.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK737_26367379_26367379 (DXK737_26367379_26367379.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK730_26367300_26367300 (DXK730_26367300_26367300.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK739_26367393_26367393 (DXK739_26367393_26367393.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK731_26367317_26367317 (DXK731_26367317_26367317.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK741_26367416_26367416 (DXK741_26367416_26367416.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK728_26367287_26367287 (DXK728_26367287_26367287.ab1)
ACCGGGAATTTCCGAGAACTTTA
aligned sequence DXK735_26367355_26367355 (DXK735_26367355_26367355.ab1)
C
Six6 g4
PAM Sequence
gRNA
ACCTCAGCTATCTCTATCACTT
Exon2
template sequence Six6 NC_027308.1 Salmo salar isolate Sally breed double haplc
AC---------CTCTATCTCTT
aligned sequence DXK745_26367454_26367454 (DXK745_26367454_26367454.ab1)
AC-----CTATCTCTATGACTT
aligned sequence DXK749_26367492_26367492 (DXK749_26367492_26367492.ab1)
ACCTCCTATATTATCTTCGATT
aligned sequence DXK752_26367522_26367522 (DXK752_26367522_26367522.ab1)
AC---------CTCTATCACTT
aligned sequence DXK754_26367546_26367546 (DXK754_26367546_26367546.ab1)
AC---------CTCTTATTCTT
aligned sequence DXK755_26367553_26367553 (DXK755_26367553_26367553.ab1)
TCCTC--------CTATCGCCT
aligned sequence DXK758_26367584_26367584 (DXK758_26367584_26367584.ab1)
AC---------CTCCATCTCTT
aligned sequence DXK748_26367485_26367485 (DXK748_26367485_26367485.ab1)
ACC-------TCTCTTCTTCC
aligned sequence DXK759_26367591_26367591 (DXK759_26367591_26367591.ab1)
AC-----CTATCTCTATCACTT
aligned sequence DXK747_26367478_26367478 (DXK747_26367478_26367478.ab1)
ACCTCTCTCACTTCCATCGCCT
aligned sequence DXK750_26367508_26367508 (DXK750_26367508_26367508.ab1)
ACC-------TCTCTCTCTATC
aligned sequence DXK751_26367515_26367515 (DXK751_26367515_26367515.ab1)
ACCTC-----TCTCTATCACTT
aligned sequence DXK753_26367539_26367539 (DXK753_26367539_26367539.ab1)
AC-----CTATCTCTATCACTT
aligned sequence DXK757_26367577_26367577 (DXK757_26367577_26367577.ab1)
ACCTCTCTTTTTTTTTCGTTT
aligned sequence DXK746_26367461_26367461 (DXK746_26367461_26367461.ab1)
ACCTC----------
aligned sequence DXK744_26367447_26367447 (DXK744_26367447_26367447.ab1)
ACCTC-----TCTCTCTCTCTT
aligned sequence DXK756_26367560_26367560 (DXK756_26367560_26367560.ab1)

Fig 12: Sequencing results for the second injection batch with chromatogram for the different Six6 gRNA targets taken from benchling. (a) Six 6 g1 (b) Six 6 g2 (c) Six6 g4

(a) Six6 g1 sequencing indicates little deviation from WT sequence, with the exception of the final sequence where there are some mismatches, this could indicate indel formation in one of the alleles (b) Six6 g2 had no indels or mismatches at all, and all sequences display complete homology to target sequences indicating no knockout of target. (c) Six6 g4, all sequences either contain indels or have large sections of mismatches, indicating successful knockout of target sequence.

3.6 Confirming albino embryos

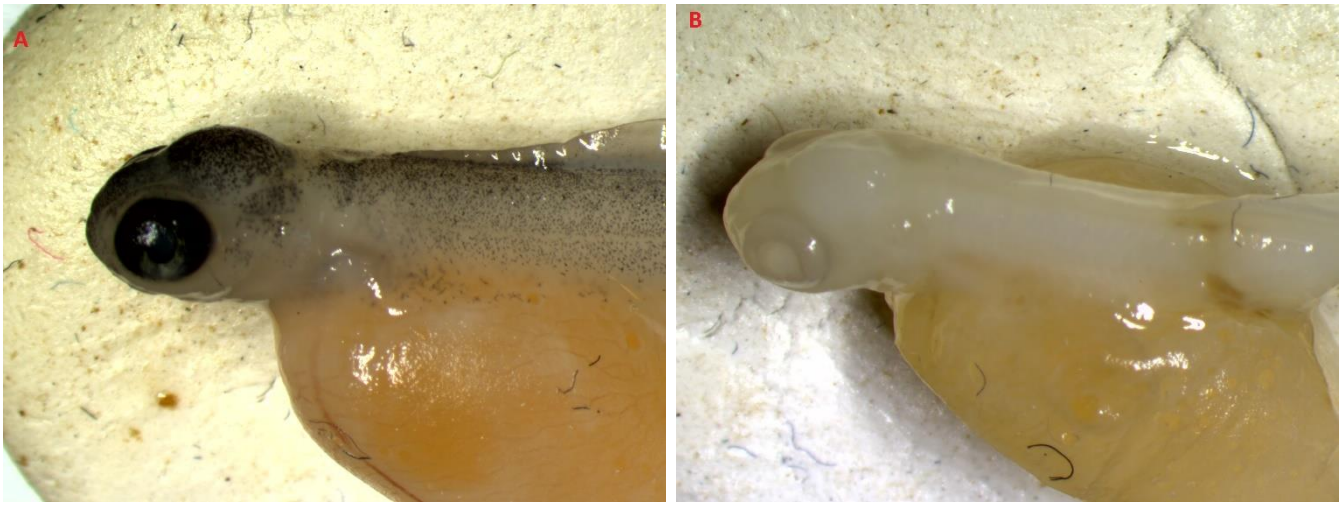

Fig 13: Comparison between embryo from the negative control group (a) and Six6 injected of second batch (b), injected embryo displays significant albinism.

Slc45a was included in the injection mix in order to quickly visually identify embryos where Cas9/CRISPR knockout had been effective. As seen in Fig 13 (b), clear lack of pigmentation in epidermis and in the eye of the embryo indicate successful KO of slc45a.

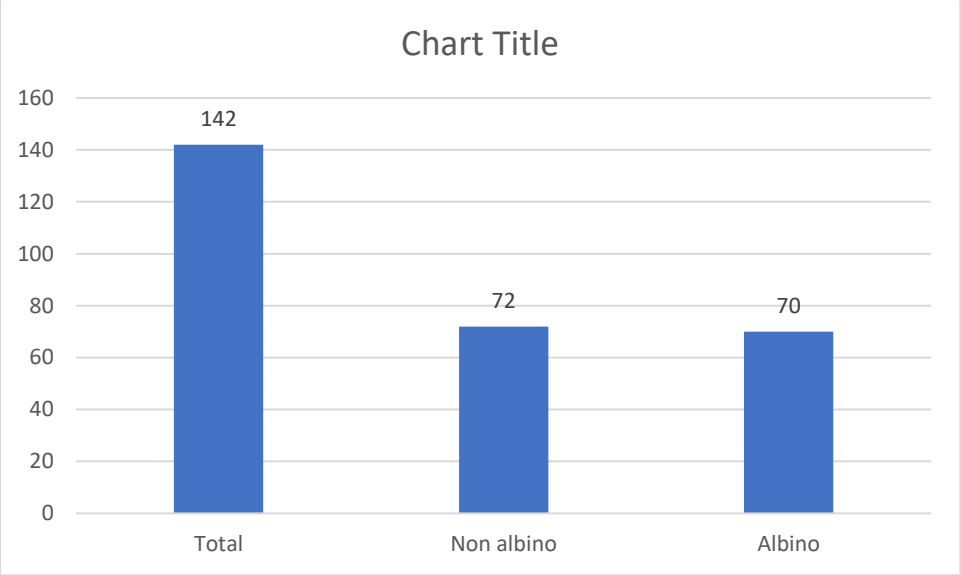

Fig 14: Number of albino and non-albino embryos compared to the total number of embryos as of 17.04.21.

Out of the 142 surviving embryos injected with the Six6 injection mix, 70 were albino, a total of 49%.

3.7 Confocal imaging

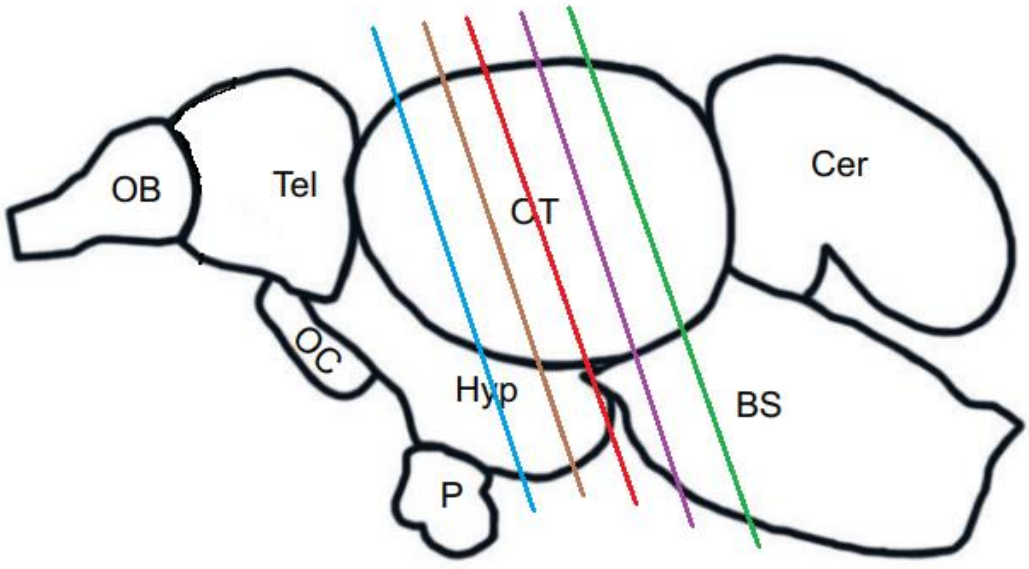

Fig 15: Sagittal view of the Atlantic salmon brain with approximate location sections. Blue;Pos control milk Red; sb-GAP, Brown:Sg-Rb, Green: Sal-GAP+ POS control NGS Purple: CHII. Abbreviations: BS; brainstem; Cer, cerebellum; Hyp, hypothalamus; OB, olfactory bulb; OC, optic chiasm; OT, optic tectum; P, pituitary; Tel, telencephalon. Figure adapted from Vindas et al., (2017)

It should be noted here that the majority of the sections procured suffered from morphological damage during sectioning. The sections presented here are the ones that were sufficiently undamaged to give decent results.

3.7. 1 Dry milk blocking positive control

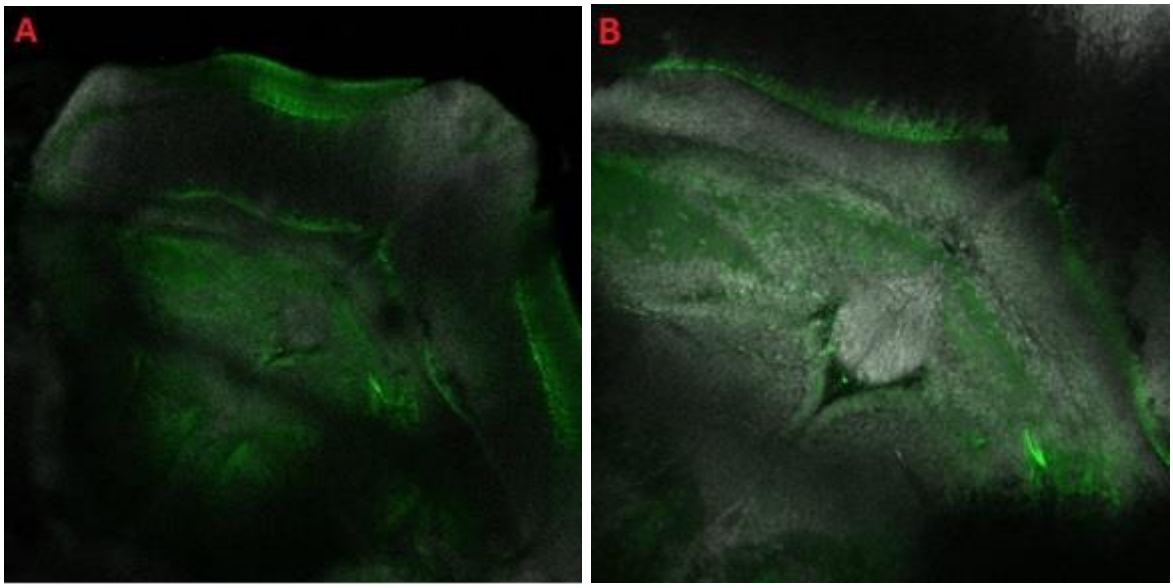

Fig 16: (a) Positive control transverse section 300µm 10x zoom. (b) Transverse section 300µm 20x zoom. 82dpf. Good staining of cell membranes

Positive control seems successful with good immunostaining of neuronal cell membranes (green fluoresence) as well as nuclear staining with DAPI (gray), long slender immunofluorescent streaks are likely axons.

3.7.2 NGS blocking positive control

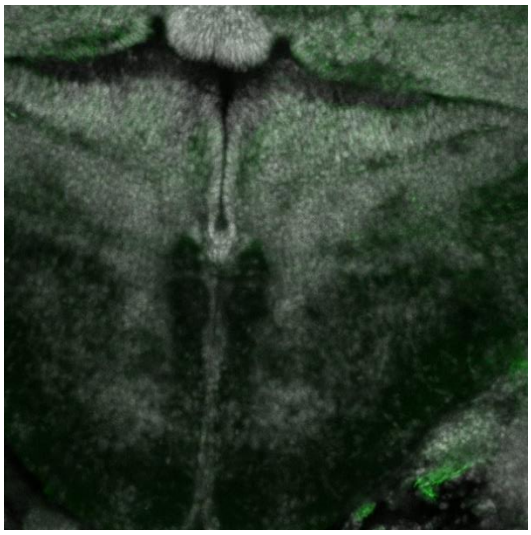

Fig 17: Positive control transverse section 82dpf 300µm 10x zoom. 82dpf Less effective staining of cell membranes.

Weak staining of cellular memranes or at least less visible under current parameters.

3.7.3 Sb-GAP

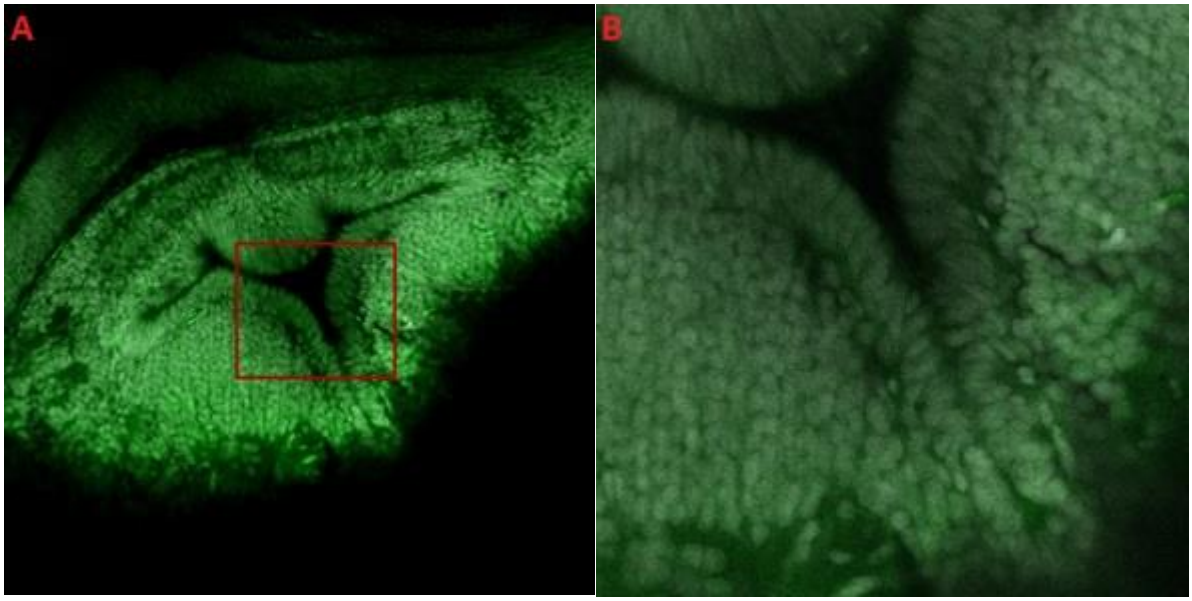

Fig 18: Transverse section of tissue incubated with SB-GAP antibody(a) 300µm section 10x zoom (b) 300µm section with digital zoom at 50x; 82dpf limited view due to air bubble formation.

DAPI staining can be seen, but green fluorescence overpowers this quite a lot. Certain points of higher intensity fluorescence, but presence of GnRH neurons cannot be confirmed. Low visibility due to air bubble formation (black part) in the vectashield medium, despite efforts to counteract this, as well as some tissue damage

3.7.4 SgRB

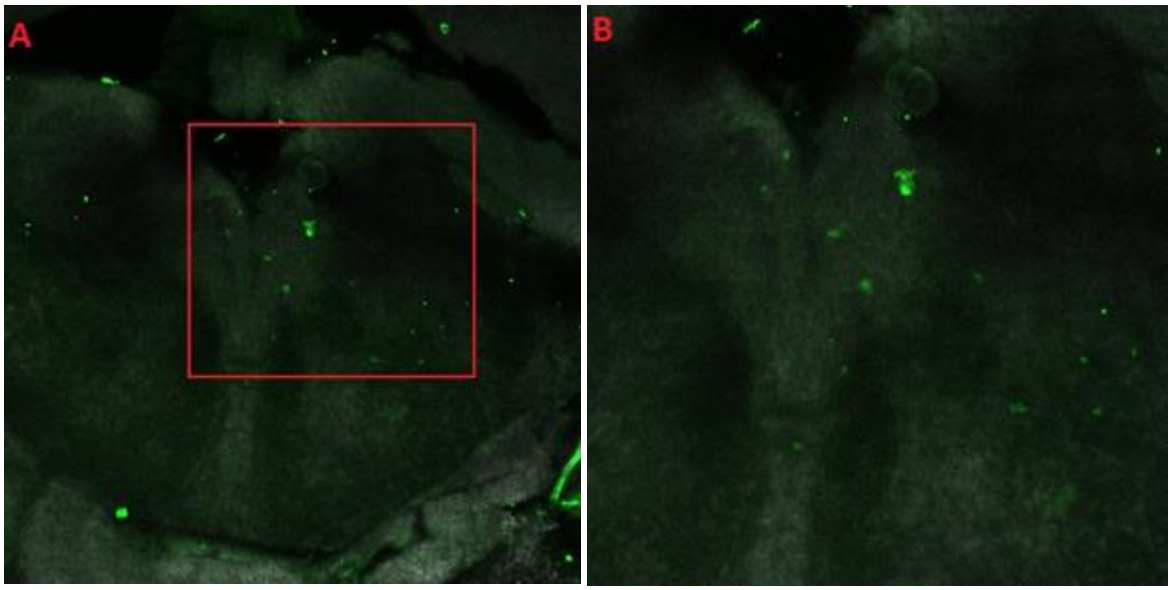

Fig 19: Transverse section of tissue incubated with Sg-RB antibody. (a) 300µm section 10x zoom (b) 300µm section with digital zoom at 50x. 82dpf. Several points of strong green fluorescence can be observed.

While several points of strong fluorescence can be seen in (a) and (b), their scattered nature indicates that these points are more than likely artefacts or unspecific bindings and not true positives of GnRH neurons.

3.7.5 Sal GAP

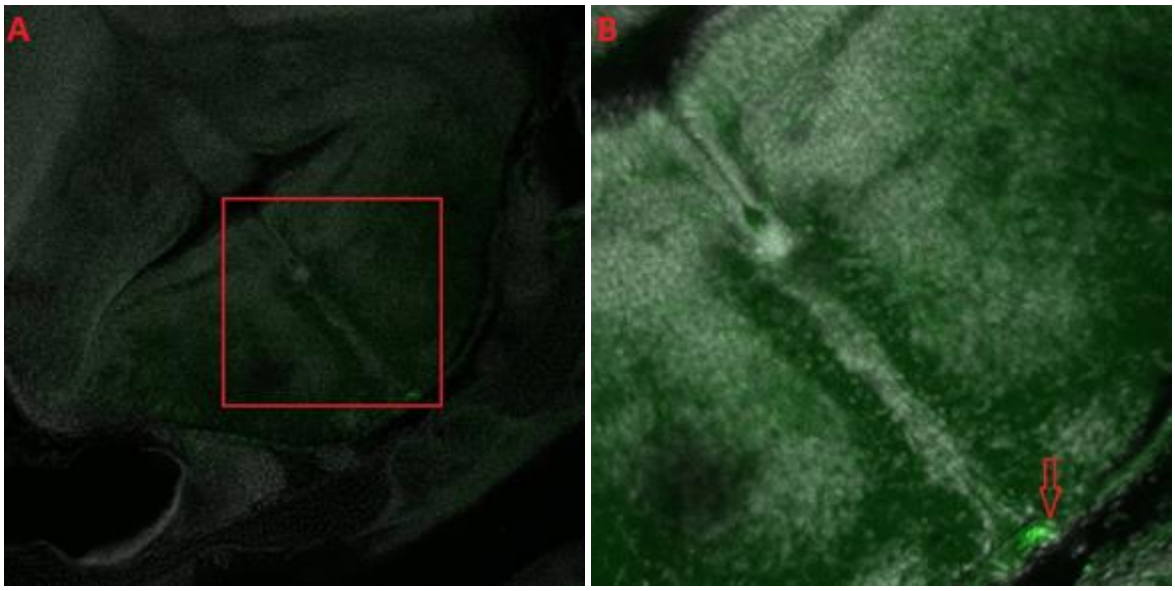

Fig 20: Transverse sections incubated with Sal-GAP antibody **(**a) 300µm section 10x zoom (b) 300µm section with digital zoom at 50x. 82dpf. Presence of a single point of strong fluorescence could indicate GnRH neurons.

These sections only display a single point of strong fluorescence which seems to originate from the hypothalamic region. Possible GnRH neuron cluster, but hard to say for sure due to the size and shape.

3.7.6 CHII

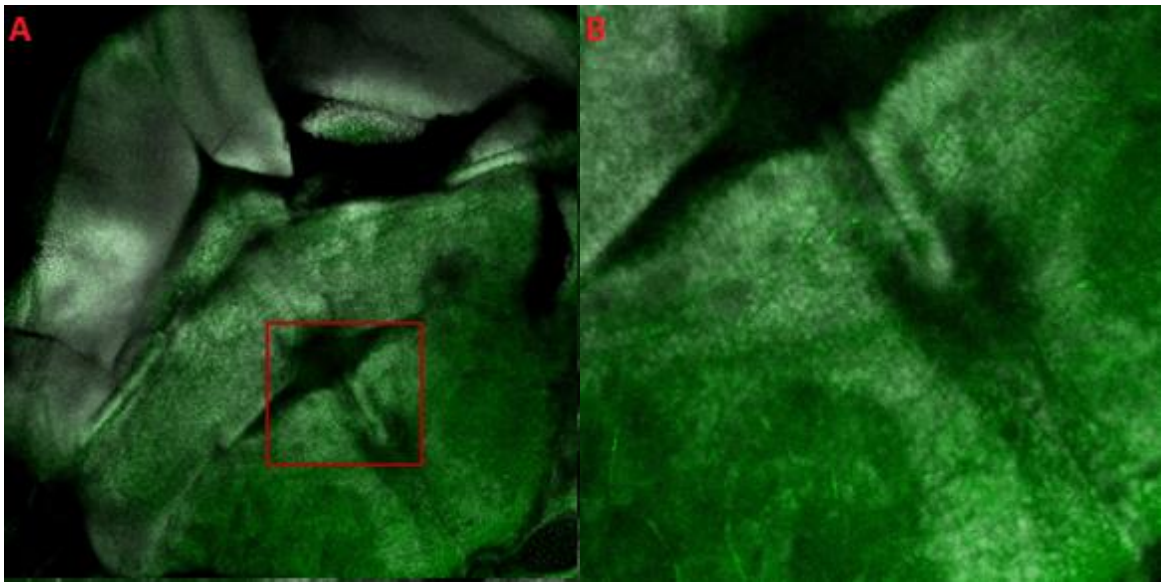

Fig 21: Transverse section of tissue incubated with CHII-GAP. **(**a) 300µm section 10x zoom (b) 300µm section with digital zoom at 50x. 82dpf. In (b) some streaks of higher intensity fluorescence can be detected.

Some points of stronger fluorescence detected as streaks in (b) when viewed with 50x zoom, but unlikely to be GnRH neurons due to both location and shape.

4 Discussion

4.1 Six6 gRNA,knockout and sequencing

The foremost objective of this thesis was to KO the Six6 gene in the Atlantic salmon brain.

The gRNA bioanalysis in Fig7 indicates good quality of gRNA. The ladder analysis displays an even baseline with no sharp peaks and matches well with the reference provided by the

manufacturer. The Six6 g2 RNA in Fig 7 C has a peak that could indicate some genomic DNA contamination and Six6 g4 in Fig 7 D seems like it has some degradation of RNA but still applicable.

While the first round of sequencing observed in Fig 11 B may indicate some degree of knockout, the sparse number of sequences left us to conclude that the knockout had been unsuccessful in this batch of embryos. It was hypothesized that Six6 gene might be essential in salmon embryo during development and that this was the reason for the very high mortality observed in Fig 8. However, Six6 knockout has been successfully performed in the mouse (Pandolfi et al., 2019, Xie et al., 2015) with no lethal outcomes, rather knockout of Six3 is what proves lethal (Pandolfi et al., 2019). The death rate of the negative control group is also comparative to the Six 6 groups and thus the high mortality rate in these embryos is more likely due to environmental factors, such as low oxygen flow, which greatly influences survival rates in the Atlantic salmon (Greig et al., 2007) This is especially likely, when comparing the mortality in the first batch to the second injection batch (Fig9) which was stored at the NMBU fish lab and had much lower mortality across all groups. Six6 and slc45a had similar mortality rates at 18% and 26% respectively while negative control only had an 8% death rate. The mortality rate similarity in the slc45a and Six6 group is likely to be a consequence of the damage inflicted during injection.

The sequencing results in Fig 12C however, is shows the knockout of Six6. Every sequence apart from the third, has indel formations in the 1-5bp region proximal to the PAM site. The sequence without any indels a may also be a knockout embryo. Since the PCR products sent for sequencing were performed on single individuals, the number of mismatches and the low peaks of the chromatogram indicate that one allele has been altered and this is what's causing the mismatches. If this is the case, the Six6 g4 RNA has an efficiency at or close to 100%.

Six6 g1 in Fig12A has some mismatches that could indicate knockout but if this is the case its efficiency is rather low. Fig 12C Six6 g2, displays complete homology to WT sequence and is most likely not a candidate for knockout.

The most likely reason for why Six6g 2 failed is simply that the gRNA failed to properly hybridize to the target sequence post injection.

The sequences in Fig12 have been trimmed down to save space, a bigger overview can be found in the appendix.

To counteract problems with faulty gRNA in future, buying gRNA from a reputable vendor should be considered. This can eliminate a potential source of error during gRNA synthesis for example. While the synthesis process is a learning opportunity, it is also prone to errors and RNase contamination which could lead to faulty gRNA.

For the KO itself, KO in cell culture may also be advantageous rather than going directly to live microinjections. Microinjections *in vivo* is very powerful to observe effects of gene knockout in a living organism and is very instructive when trying to understand how CRISPR/Cas9 operates in a living system. However, there is a risk that time may be wasted while one waits for the organism to mature if the knockout is unsuccessful. For some animals like medaka this may be fine, but for slow developing organism like the Atlantic salmon, cell culture knockout beforehand can allow for confirmation that the CRISPR/Cas9 and gRNA is working before starting experiments proper.

4.2 Clearing, immunohistochemistry, and confocal imaging of salmon brains

All clearing agents used in the tissue clearing for whole mount yielded good clearing of the tissues for use in whole mount. As observed in Fig10 A, TDE clearing agent was the most successful in decreasing tissue opacity. Fig10 B Qbic1/2 was successful in tissue clearing but caused some morphological damage to the tissue, specifically the eyes. The glycerol treatment observed in Fig 10 C also worked well, and is the easiest to achieve with only glycerol and no additional required to perform.

Blocking and immunostaining for the zn-12-s positive control worked for both tissue samples, but the better result was achieved with the dry milk powder block as can be seen in the differences between Fig 16 and 17. Fig 16 has good fluorescence and is similar to the results in Trevarrow et al., (1990) Metcalfe et al., (1990) as well as Nelson et al., (2019).

As for the GnRH antibodies, none of the images seem to contain any information that can be conclusively verified as true positives of GnRH neurons. However, as mentioned the microtome damaged the tissues and thus left only a select few tissue samples viable for microscopy, unfortunately the sections most susceptible to damage proved to be the early sections which would contain the olfactory placode, olfactory bulbs and telencephalon.

Fig 15 shows the approximate locations in the brain where the sections are taken and the viable sections are all from the midbrain/hindbrain and posterior forebrain region. Unfortunately, this means that the sections are taken too posteriorly for GnRH3 to be detected. Bailhache et al., (1994) mentions that GnRH3 (sGnRH) neurons in the Atlantic salmon has been found in the telencephalon, preoptic area and in the olfactory bulbs, with the highest density of GnRH3 neurons found in the junction between the telencephalon and olfactory bulbs. The neurons found were fusiform or bipolar in shape, and oriented rostro caudally. This does not match any of the results achieved, and therefore none of the fluorescent signals are likely to be GnRH neurons.

According to Vickers et al., 2004 GnRH2 can be located in the midbrain of the *Coregonus clupeaformis* another salmonid, but the section in Fig 21 does not display any signals that can be wholly confirmed as GnRH neurons. While there are streaks of higher fluorescence, their shape do not conform to the bipolar shape that are expected from GnRH neurons. (Roa and Tena-Sempere 2018)

While no GnRH neurons can be conclusively verified from these results, it's not possible to draw a conclusion as to whether the antibodies work or don't work. Since all sections here differ from areas in the brain where the GnRH type of interest would be, future experiments need to be done to verify whether or not these antibodies are effective in Atlantic salmon.

Given the time I would have made sure to get proper sections of the relevant parts of the brain. While some adjustment of the microtome setting may produce better results, such as lowering the oscillation, other sectioning methods could be considered. For instance, paraffin embedded sectioning, the paraffin provides a harder casing for the tissue and may keep the tissue sample from giving way as it contacts the blade.

Another thing to consider would be to take steps to unmask the epitopes of the antigens, such as heat induced epitope retrieval using a citrate buffer or enzyme treatment such as with Proteinase K.

For the immunostaining, dried powdered milk blocking can be recommended as this is cheap, readily available and yielded good results

5 References:

1. Adams, B. A., Vickers, E. D., Warby, C., Park, M., Fischer, W. H., Grey Craig, A., Rivier, J. E., & Sherwood, N. M. (2002). Three Forms of Gonadotropin-Releasing Hormone, Including a Novel Form, in a Basal Salmonid, Coregonus clupeaformis1. *Biology of Reproduction*, *67*(1), 232-239. https://doi.org/10.1095/biolreprod67.1.232

2. Adelman, J. P., Mason, A. J., Hayflick, J. S., & Seeburg, P. H. (1986). Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proc Natl Acad Sci U S A*, *83*(1), 179-183. https://doi.org/10.1073/pnas.83.1.179

3. Ando, H., & Urano, A. (2005). Molecular regulation of gonadotropin secretion by gonadotropin-releasing hormone in salmonid fishes. *Zoolog Sci*, *22*(4), 379-389. https://doi.org/10.2108/zsj.22.379

4. Barson, N. J., Aykanat, T., Hindar, K., Baranski, M., Bolstad, G. H., Fiske, P., Jacq, C., Jensen, A. J., Johnston, S. E., Karlsson, S., Kent, M., Moen, T., Niemelä, E., Nome, T., Næsje, T. F., Orell, P., Romakkaniemi, A., Sægrov, H., Urdal, K., Erkinaro, J., Lien, S., & Primmer, C. R. (2015). Sex-dependent dominance at a single locus maintains variation in age at maturity in salmon. *Nature*, *528*(7582), 405-408. https://doi.org/10.1038/nature16062

5. 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), 444-454. https://doi.org/10.1002/cne.903470310

6. Biran, J., Tahor, M., Wircer, E., & Levkowitz, G. (2015). Role of developmental factors in hypothalamic function [Review]. *Frontiers in Neuroanatomy*, *9*(47). https://doi.org/10.3389/fnana.2015.00047

7. Carolsfeld, J., Powell, J. F. F., Park, M., Fischer, W. H., Craig, A. G., Chang, J. P., Rivier, J. E., & Sherwood, N. M. (2000). Primary Structure and Function of

Three Gonadotropin-Releasing Hormones, Including a Novel Form, from an Ancient Teleost, Herring1. *Endocrinology*, *141*(2), 505-512. https://doi.org/10.1210/endo.141.2.7300

8. Carroll, D. (2017). Genome Editing: Past, Present, and Future. *The Yale journal of biology and medicine*, *90*(4), 653-659. https://pubmed.ncbi.nlm.nih.gov/29259529 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5733845/

9. Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics*, *188*(4), 773-782. https://doi.org/10.1534/genetics.111.131433

10. Chambery, A., Parente, A., Topo, E., Garcia-Fernàndez, J., & D'Aniello, S. (2009). Characterization and putative role of a type I gonadotropin-releasing hormone in the cephalochordate amphioxus. *Endocrinology*, *150*(2), 812-820. https://doi.org/10.1210/en.2008-1066

11. Edvardsen, R. B., Leininger, S., Kleppe, L., Skaftnesmo, K. O., & Wargelius, A. (2014). Targeted Mutagenesis in Atlantic Salmon (Salmo salar L.) Using the CRISPR/Cas9 System Induces Complete Knockout Individuals in the F0 Generation. *PLOS ONE*, *9*(9), e108622. https://doi.org/10.1371/journal.pone.0108622

12. Elliott, A. D. (2020). Confocal Microscopy: Principles and Modern Practices. *Curr Protoc Cytom*, *92*(1), e68. https://doi.org/10.1002/cpcy.68

13. Fang, J., Chen, T., Pan, Q., & Wang, Q. (2018). Generation of albino medaka (Oryzias latipes) by CRISPR/Cas9. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *330*(4), 242-246. https://doi.org/https://doi.org/10.1002/jez.b.22808

14. Fjelldal, P. G., Schulz, R., Nilsen, T. O., Andersson, E., Norberg, B., & Hansen, T. J. (2018). Sexual maturation and smoltification in domesticated Atlantic salmon (Salmo salar L.) – is there a developmental conflict? *Physiological Reports*, *6*(17), e13809. https://doi.org/https://doi.org/10.14814/phy2.13809

15. Gaillard, A.-L., Tay, B.-H., Pérez Sirkin, D. I., Lafont, A.-G., De Flori, C., Vissio, P. G., Mazan, S., Dufour, S., Venkatesh, B., & Tostivint, H. (2018). Characterization of Gonadotropin-Releasing Hormone (GnRH) Genes From Cartilaginous Fish: Evolutionary Perspectives [Original Research]. *Frontiers in Neuroscience*, *12*(607). https://doi.org/10.3389/fnins.2018.00607

16. Greig, S., Sear, D., & Carling, P. (2007). A field-based assessment of oxygen supply to incubating Atlantic salmon (Salmo salar) embryos. *Hydrological Processes*, *21*(22), 3087-3100. https://doi.org/https://doi.org/10.1002/hyp.6635

17. Gown, A. M. (2016). Diagnostic Immunohistochemistry: What Can Go Wrong and How to Prevent It. *Arch Pathol Lab Med*, *140*(9), 893-898. https://doi.org/10.5858/arpa.2016-0119-RA

18. Hoshijima, K., Jurynec, M. J., Klatt Shaw, D., Jacobi, A. M., Behlke, M. A., & Grunwald, D. J. (2019). Highly Efficient CRISPR-Cas9-Based Methods for Generating Deletion Mutations and F0 Embryos that Lack Gene Function in Zebrafish. *Developmental Cell*, *51*(5), 645-657.e644. https://doi.org/https://doi.org/10.1016/j.devcel.2019.10.004

19. Im, K., Mareninov, S., Diaz, M. F. P., & Yong, W. H. (2019). An Introduction to Performing Immunofluorescence Staining. *Methods Mol Biol*, *1897*, 299-311. https://doi.org/10.1007/978-1-4939-8935-5_26

20. Jimenez-Liñan, M., Rubin, B. S., & King, J. C. (1997). Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. *Endocrinology*, *138*(10), 4123-4130. https://doi.org/10.1210/endo.138.10.5454

21. Kagayama, M., & Sasano, Y. (1999). [5] Mounting techniques for confocal microscopy. In *Methods in Enzymology* (Vol. 307, pp. 79-84). Academic Press. https://doi.org/https://doi.org/10.1016/S0076-6879(99)07007-X

22. Kah, O., & Dufour, S. (2010). Conserved and Divergent Features of Reproductive Neuroendocrinology in Teleost Fishes. *Hormones and Reproduction of Vertebrates - Volume 1, 1*. https://doi.org/10.1016/B978-0-12-375009-9.10002-5

23. Kawakami, K., Sato, S., Ozaki, H., & Ikeda, K. (2000). Six family genes--structure and function as transcription factors and their roles in development. *Bioessays*, *22*(7), 616-626. https://doi.org/10.1002/1521-1878(200007)22:7<616::Aid-bies4>3.0.Co;2-r

24. Klaassen, H., Wang, Y., Adamski, K., Rohner, N., & Kowalko, J. E. (2018). CRISPR mutagenesis confirms the role of oca2 in melanin pigmentation in Astyanax mexicanus. *Developmental Biology*, *441*(2), 313-318. https://doi.org/https://doi.org/10.1016/j.ydbio.2018.03.014

25. Klemetsen, A., Amundsen, P.-A., Dempson, J. B., Jonsson, B., Jonsson, N., O'Connell, M. F., & 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. https://doi.org/https://doi.org/10.1034/j.1600-0633.2003.00010.x

26. Knott, G. J., & Doudna, J. A. (2018). CRISPR-Cas guides the future of genetic engineering. *Science*, *361*(6405), 866-869. https://doi.org/10.1126/science.aat5011

27. Kurko, J., Debes, P. V., House, A. H., Aykanat, T., Erkinaro, J., & Primmer, C. R. (2020). Transcription Profiles of Age-at-Maturity-Associated Genes Suggest Cell Fate Commitment Regulation as a Key Factor in the Atlantic Salmon Maturation Process. *G3 (Bethesda)*, *10*(1), 235-246. https://doi.org/10.1534/g3.119.400882

28. Lovejoy, D. A., Fischer, W. H., Ngamvongchon, S., Craig, A. G., Nahorniak, C. S., Peter, R. E., Rivier, J. E., & Sherwood, N. M. (1992). Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc Natl Acad Sci U S A*, *89*(14), 6373-6377. https://doi.org/10.1073/pnas.89.14.6373

29. Macqueen, D. J., & Johnston, I. A. (2014). A well-constrained estimate for the timing of the salmonid whole genome duplication reveals major decoupling from species diversification. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1778), 20132881. https://doi.org/doi:10.1098/rspb.2013.2881

30. Masood, S., Wasielewski, R., Mengel, M., Nolte, M., & Werner, M. (2003). Influence of Fixation, Antibody Clones, and Signal Amplification on Steroid Receptor Analysis. *The Breast Journal*, *4*, 33-40. https://doi.org/10.1046/j.1524-4741.1998.410033.x

31. Matos, L. L. d., Trufelli, D. C., de Matos, M. G. L., & da Silva Pinhal, M. A. (2010). Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomarker insights*, *5*, 9-20. https://doi.org/10.4137/bmi.s2185

32. Matsuo, H., Baba, Y., Nair, R. M., Arimura, A., & Schally, A. V. (1971). Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun*, *43*(6), 1334-1339. https://doi.org/10.1016/s0006-291x(71)80019-0

33. Metcalfe, W. K., Myers, P. Z., Trevarrow, B., Bass, M. B., & Kimmel, C. B. (1990). Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. *Development*, *110*(2), 491-504.

34. Mishra, P., & Kanwar, S. (2016). Ribonucleases and their Applications. *Journal of Advanced Biotechnology and Bioengineering*, *4*. https://doi.org/10.12970/2311-1755.2016.04.01.3

35. Miyamoto, K., Hasegawa, Y., Minegishi, T., Nomura, M., Takahashi, Y., Igarashi, M., Kangawa, K., & Matsuo, H. (1982). Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. *Biochem Biophys Res Commun*, *107*(3), 820-827. https://doi.org/10.1016/0006-291x(82)90596-4

36. Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., & Matsuo, H. (1984). Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proc Natl Acad Sci U S A*, *81*(12), 3874-3878. https://doi.org/10.1073/pnas.81.12.3874

37. Nelson, H. M., Coffing, G. C., Chilson, S., Hester, K., Carrillo, C., Ostreicher, S., Tomamichel, W., Hanlon, S., Burns, A. R., & Lafontant, P. J. (2019). Structure, development, and functional morphology of the cement gland of the giant danio, Devario malabaricus. *Developmental Dynamics*, *248*(11), 1155-1174. https://doi.org/https://doi.org/10.1002/dvdy.88

38. Ngamvongchon, S., Sherwood, N. M., Warby, C. M., & Rivier, J. E. (1992). Gonadotropin-releasing hormone from thai catfish: chromatographic and physiological studies. *Gen Comp Endocrinol*, *87*(2), 266-274. https://doi.org/10.1016/0016-6480(92)90031-e

39. Okubo, K., & Nagahama, Y. (2008). Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. *Acta Physiol (Oxf)*, *193*(1), 3-15. https://doi.org/10.1111/j.1748-1716.2008.01832.x

40. Okubo, K., Amano, M., Yoshiura, Y., Suetake, H., & Aida, K. (2000). A Novel Form of Gonadotropin-Releasing Hormone in the Medaka, Oryzias latipes. *Biochemical and Biophysical Research Communications*, *276*(1), 298-303. https://doi.org/https://doi.org/10.1006/bbrc.2000.3476

41. Pandolfi, E. C., Tonsfeldt, K. J., Hoffmann, H. M., & Mellon, P. L. (2019). Deletion of the Homeodomain Protein Six6 From GnRH Neurons Decreases GnRH Gene Expression, Resulting in Infertility. *Endocrinology*, *160*(9), 2151-2164. https://doi.org/10.1210/en.2019-00113

42. Powell, J. F., Fischer, W. H., Park, M., Craig, A. G., Rivier, J. E., White, S. A., Francis, R. C., Fernald, R. D., Licht, P., Warby, C., & et al. (1995). Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain of cichlid and pumpkinseed fish. *Regul Pept*, *57*(1), 43-53. https://doi.org/10.1016/0167-0115(95)00014-3

43. Roa, J., & Tena-Sempere, M. (2018). Unique Features of a Unique Cell: The Wonder World of GnRH Neurons. *Endocrinology*, *159*(12), 3895-3896. https://doi.org/10.1210/en.2018-00870

44. Rickert, R. R., & Maliniak, R. M. (1989). Intralaboratory quality assurance of immunohistochemical procedures. Recommended practices for daily application. *Arch Pathol Lab Med*, *113*(6), 673-679.

45. Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J., & Vale, W. (1983). Characterization of a teleost gonadotropin-releasing hormone. *Proc Natl Acad Sci U S A*, *80*(9), 2794-2798. https://doi.org/10.1073/pnas.80.9.2794

46. Sherwood, N. M., Sower, S. A., Marshak, D. R., Fraser, B. A., & Brownstein, M. J. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J Biol Chem*, *261*(11), 4812-4819.

47. Somoza, G. M., Miranda, L. A., Strobl-Mazzulla, P., & Guilgur, L. G. (2002). Gonadotropin-releasing hormone (GnRH): from fish to mammalian brains. *Cell Mol Neurobiol*, *22*(5-6), 589-609. https://doi.org/10.1023/a:1021888420271

48. Sower, S. A., Chiang, Y. C., Lovas, S., & Conlon, J. M. (1993). Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology*, *132*(3), 1125-1131. https://doi.org/10.1210/endo.132.3.8440174

49. Trevarrow, B., Marks, D. L., & Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron*, *4*(5), 669-679. https://doi.org/10.1016/0896-6273(90)90194-k

50. Veldman, M. B., & Lin, S. (2008). Zebrafish as a Developmental Model Organism for Pediatric Research. *Pediatric Research*, *64*(5), 470-476. https://doi.org/10.1203/PDR.0b013e318186e609

51. Vickers, E. D., Laberge, F., Adams, B. A., Hara, T. J., & Sherwood, N. M. (2004). Cloning and Localization of Three Forms of Gonadotropin-Releasing Hormone, Including the Novel Whitefish Form, in a Salmonid, Coregonus clupeaformis1. *Biology of Reproduction*, *70*(4), 1136-1146. https://doi.org/10.1095/biolreprod.103.023846

52. Vindas, M., Gorissen, M., Höglund, E., Flik, G., Tronci, V., Damsgård, B., Thörnqvist, P.-O., Nilsen, T., Winberg, S., Øverli, Ø., & Ebbesson, L. (2017). How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. *The Journal of Experimental Biology*, *220*, jeb.153213. https://doi.org/10.1242/jeb.153213

53. Volff, J. N. (2005). Genome evolution and biodiversity in teleost fish. *Heredity*, *94*(3), 280-294. https://doi.org/10.1038/sj.hdy.6800635

54. Watakabe, I., Hashimoto, H., Kimura, Y., Yokoi, S., Naruse, K., & Higashijima, S.-i. (2018). Highly efficient generation of knock-in transgenic medaka by

CRISPR/Cas9-mediated genome engineering. *Zoological Letters*, *4*(1), 3. https://doi.org/10.1186/s40851-017-0086-3

55. 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). *Comp Biochem Physiol A Mol Integr Physiol*, *137*(3), 447-477. https://doi.org/10.1016/j.cbpb.2003.11.007

56. Whitlock, K. E., Postlethwait, J., & Ewer, J. (2019). Neuroendocrinology of reproduction: Is gonadotropin-releasing hormone (GnRH) dispensable? *Front Neuroendocrinol*, *53*, 100738. https://doi.org/10.1016/j.yfrne.2019.02.002

57. Wray, S. (2010). From nose to brain: development of gonadotrophin-releasing hormone-1 neurones. *Journal of neuroendocrinology*, *22*(7), 743-753. https://doi.org/10.1111/j.1365-2826.2010.02034.x

58. Xie, H., Hoffmann, H. M., Meadows, J. D., Mayo, S. L., Trang, C., Leming, S. S., Maruggi, C., Davis, S. W., Larder, R., & Mellon, P. L. (2015). Homeodomain Proteins SIX3 and SIX6 Regulate Gonadotrope-specific Genes During Pituitary Development. *Molecular Endocrinology*, *29*(6), 842-855. https://doi.org/10.1210/me.2014-1279

59. Yamamoto, N. (2003). Three gonadotropin-releasing hormone neuronal groups with special reference to teleosts. *Anat Sci Int*, *78*(3), 139-155. https://doi.org/10.1046/j.0022-7722.2003.00051.x

60. Yashuvi, Y., Klenke, U., Palevitch, O., Abraham, E., Zohar, Y., & Gothilf, Y. (2006). Ontogeny of gonadotropin-releasing hormone (GnRH) neurons in hybrid striped bass Morone sp.: whole-mount in situ hybridization analysis. *Journal of Fish Biology*, *69*(sa), 20-30. https://doi.org/https://doi.org/10.1111/j.1095-8649.2006.01074.x

61. Yoo, M. S., Kang, H. M., Choi, H. S., Kim, J. W., Troskie, B. E., Millar, R. P., & Kwon, H. B. (2000). Molecular cloning, distribution and pharmacological characterization of a novel gonadotropin-releasing hormone ([Trp8] GnRH) in frog brain. *Molecular and Cellular Endocrinology*, *164*(1), 197-204. https://doi.org/https://doi.org/10.1016/S0303-7207(00)00221-5

62. Zhang, L., Wayne, N. L., Sherwood, N. M., Postigo, H. R., & Tsai, P.-S. (2000). Biological and Immunological Characterization of Multiple GnRH in an Opisthobranch Mollusk, Aplysia californica. *General and Comparative Endocrinology*, *118*(1), 77-89. https://doi.org/https://doi.org/10.1006/gcen.2000.7457

63. Zhang, Y., Qin, W., Lu, X., Xu, J., Huang, H., Bai, H., Li, S., & Lin, S. (2017). Programmable base editing of zebrafish genome using a modified CRISPR-Cas9 system. *Nature Communications*, *8*(1), 118. https://doi.org/10.1038/s41467-017-00175-6

64. Zheng, T., Hou, Y., Zhang, P., Zhang, Z., Xu, Y., Zhang, L., Niu, L., Yang, Y., Liang, D., Yi, F., Peng, W., Feng, W., Yang, Y., Chen, J., Zhu, Y. Y., Zhang, L.-H., & Du, Q. (2017). Profiling single-guide RNA specificity reveals a mismatch sensitive core sequence. *Scientific Reports*, *7*(1), 40638. https://doi.org/10.1038/srep40638

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway