Master thesis in Biotechnology
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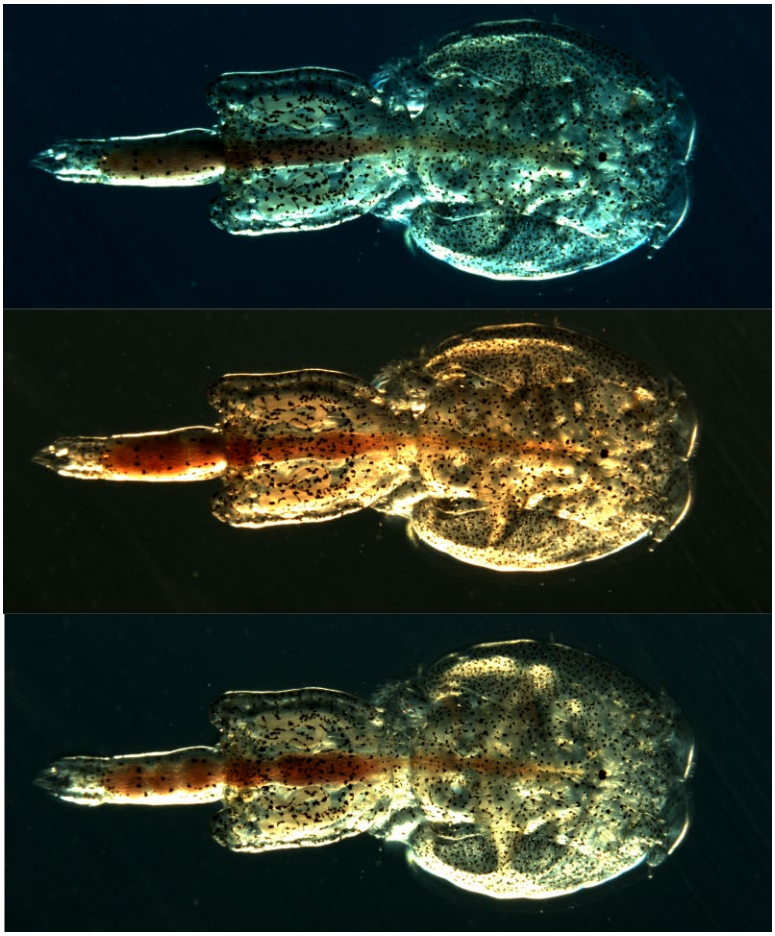
Protocol for stable germ-line mutagenesis facilitated by CRISPR/Cas9 RNP complex via peptide nanoparticle microinjection in Salmon lice (*Lepeophtheirus salmonis*)



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Abstract:

Aquaculture is a rising economical industry since its inception, that is positioned as sustainable and reliable food source for continuously expanding human population. Salmon farming produces nutrition rich in omega−3 and omega−6 fatty acids, vital components of human diet that considerably increases income in countries where salmon is farmed. As in agriculture, the cultivated product is prone to infestations that rapidly spread in densely populated environment. Salmon lice (*Lepeophtheirus salmonis*), a parasite flourishing on salmon, is one of the major threats to the industry that does not have a complete solution. In order to expand understating of the problem modern technologies such as CRISPR should be applied to study gene functions based on loss of function experiments. Current approach of CRISPR delivery in marine organisms is based solely on embryonic microinjection and represents complexity for small organisms and laboratories where necessary equipment is absent. Here Cas9 protein, known as CRISPR was applied in combination with branched amphiphilic peptide nano capsules in attempt to establish a laboratory protocol for germ line knock-out experiments. In presented study 70 individuals both male and female lice in pre-adult stage were injected with Cas9/BAPC solution for germline knock-out, 20 were injected for somatic knock-out. BAPC delivery vector has not shown any mutation on target site in combination of GFP labelled Cas9 RNP complexes. Drawbacks of presented approach and alternative vectors are discussed within report with outline on future perspectives of CRISPR technology in Salmon lice.

Acknowledgment:

Sincerely grateful to Atle.M.Bones for wonderful opportunity to participate in Salmon Lice Task Force research and challenging but exciting project. Special gratitude towards Prashanna Guragain for cooperation, teamwork and support along the experiment. I am also thankful to Per Winge for design of sgRNA, Torfinn Sparstad for suggestions in HRM optimisation, Anna S. Båtnes and Stine Østerhus for maintenance and monitoring at SeaLab facility, Narve Opsahl for assistance in image analysis.

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Abbreviations:

Cas9 Associated Protein 9

CRISPR Clustered Regularly Interspaced Short Palindromic Repeat

crRNA CRISPR RNA

DSB Double Strand Break

NHEJ Non-Homologous End Joining

PAM Protospacer Adjacent Motif

pre-crRNA precursor CRISPR RNA

sgRNA single-guide RNA

CT cephalothorax

GS genital segment

AB abdominal segment

NP nanoparticle

DNA Deoxyribonucleic acid

BAPC Branched Amphiphilic Peptide Capsules

LOF Loss-of-function

RNP Ribonucleoprotein

Trp Tryptophan

HRM High Resolution Melt analysis

WT wild-type

GFP Green Fluorescent Protein

Introduction:

Background:

Human population is projected to increase to 11.2 billion by the end of the century, and the buying power is expected to continue to increase (UN Economic & Social Affairs, 2019). These increases will drive the need for human food, and scarcity is expected to be a great challenge for humans in the coming century (FAO, 2020a). The cultivable land needed for agriculture is decreasing and the future of food production is likely in the marine environment. At least one-third of agricultural output is currently lost due to damage caused by pests and diseases (Oerke et al., 2012). Arthropods, by far the largest invertebrate phylum that include approximately eighty percent of all animal species inhabiting the earth (Ghafor, 2020; Liang et al., 2013), cause a main problem. Agricultural losses due to arthropods are enormous (Bradshaw et al., 2016), and major attention has been paid to the study of terrestrial arthropods. Comparatively, the marine arthropods and parasites have received less attention. Salmon lice (*Lepeophtheirus salmonis*) are a major threat to both farmed and wild Atlantic salmon in the North Atlantic region (MJ, 2006; Torrissen et al., 2013). Parasite is capable of traveling large distances across the ocean via ocean currents which ultimately results in farm-to-farm infestation spread. When present in high numbers on the fish causes secondary infections that are frequently lethal, decreasing total yield on the farm, in addition violating fish welfare act. Current knowledge of underlying biology of salmon lice is limited and does not allow to find immediate solution. Salmon lice limit the further growth of the salmon aquaculture industry. During last decades great scientific effort has been dedicated to solve salmon lice problem, however the challenge remains unsolved (Guragain et al., 2021). Fortunately, 21st Century has brought tools to expand the knowledge frontiers based on genetics of the parasite, possibly leading to discoveries of new vaccine targets or technologies to control infestations. CIRSPR technology has been established in some marine organisms as embryonic microinjection: in zebrafish (Irion et al., 2014), in corals (Cleves et al., 2018) and squids (Crawford et al., 2020). However, small size marine organisms and requirements of automated equipment for precision of such injection are limiting expansion of CRISPR technology into marine biology. Here first trial of CRISP/Cas9 RNP complexed with peptide nanocapsules was performed for induction of genetic mutation in Salmon lice.

Salmon lice:

Salmon louse (*Lepeophtheirus salmoni*s) is a salmonid specific hematophagous parasite specialized in host fish of the three genera *Salmo, Salvelinus and Oncorhynchus* (Wootten et al., 1982). Salmon lice

have a significant effect on salmonids during the marine life cycle as they feed on mucus, blood and epidermal tissues (Costello, 2006). When present on fish in high numbers it causes physiological distress, growth reduction, immune system suppression and osmoregulation imbalance (Costello, 2006; Johnson et al., 2004) representing a threat to fish welfare and decrease in production efficiency in salmon farms. Life cycle of the parasite begins with planktonic stage (nauplii I) and followed by seven other stages separated by molt (Goater et al., 2013) (Fig.1). The cycle begins when the first free-living larvae is hatched from egg-strings attached to adult females. Further, all larvae stages nauplii I, nauplii II, copepodite stage can travel long distances through the ocean currents until finding a suitable host (Brooks, 2005).

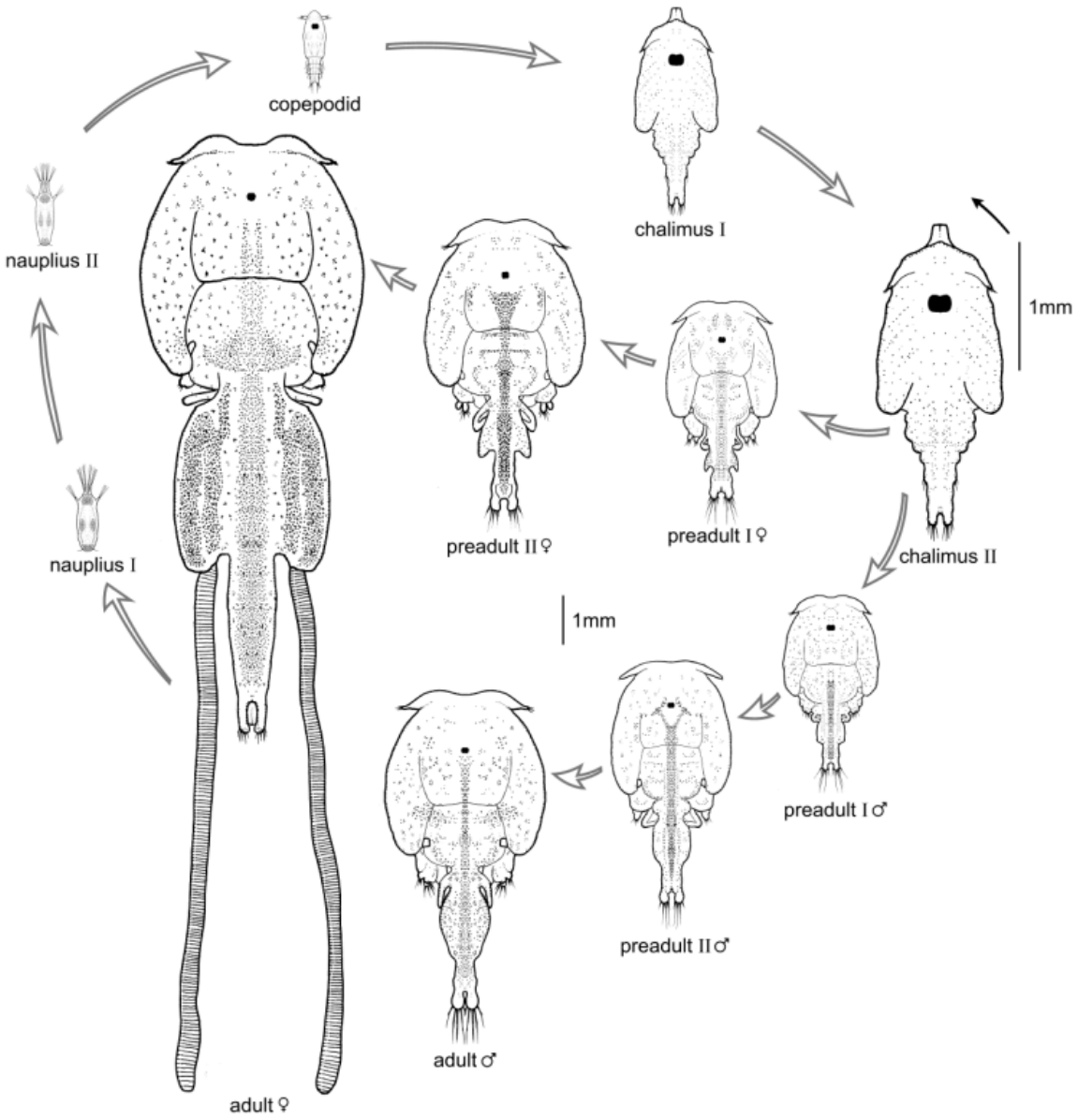


Fig.1: Salmon lice life cycle. Salmon lice have eight developmental stages. It starts development from planktonic nauplii I, II, and develops further into infectious copepodite, immobile chalimus I, II, and adult louse. Illustration: "SLRC Lepeophtheirus salmonis life cycle" by Sea Lice Research Centre is licensed under a Creative Commons AttributionShareAlike 4.0 International License. (Erna Irene Heggland, 2020)

After host settlement the infectious copepodite develops into adult lice separated by moult in every developmental stage. Growth rate of salmon louse is temperature dependent and spans from 432 to 271-degree days (Hamre et al., 2019). As soon as louse reaches the adult stage, it is capable of sexual reproduction where females can produce 11 sets of egg strings from single fertilization (Heuch et al., 2000). Fully developed salmon lice are divided into three segments, where reproductive organs in both sexes are located in cephalothorax (CT), adjacent to the brain region (Fig.2).

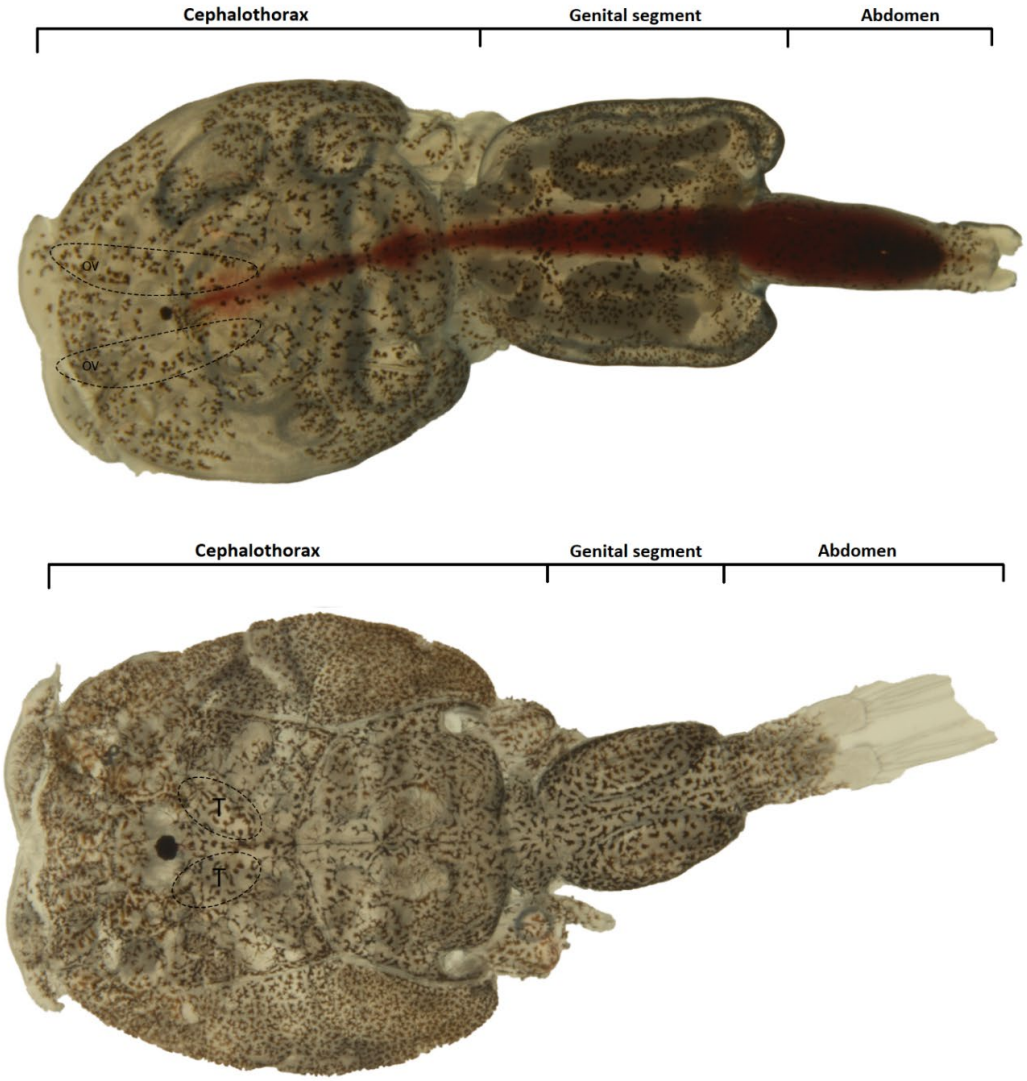


Fig.2: **Morphology of salmon lice.** Gross morphology of adult louse is divided into 3 segments cephalothorax (CT), abdomen (AB) and genital segment (GS). Reproductive organs for both sexes are located within CT segment (OV – ovary, T – testicle) adjacent to the brain region and coalescent eyes.

Loss-of-function approach:

Loss-of-function (LOF) approach has been successfully applied across various fields and has gained remarkable insights in genetic functions and interactions. LOF experiments target DNA, RNA or protein to ablate its function and are generally divided into knock-down (RNAi) and knock-out (CRISPR) experiments (Housden et al., 2016). During recent years protocol for RNAi was well established in salmon lice (Eichner et al., 2014), and was applied in studies of critical components in ferritin, chitin, vitellogenesis (Borchel et al., 2019; Braden et al., 2020; Heggland et al., 2020) and other vital pathways (ref review). RNAi method has its own limitations as it does not allow to observe phenotype along life cycle progression and provides limited understanding of complete gene function and off-target effects without null mutant control (Zimmer et al., 2019). Therefore, other genetic tools like CRISPR/Cas9 protein complex might be a valuable method in extension of genetic studies in salmon lice. CRISPR/Cas9 system discovered as a bacteria adaptive immune system (type II), was not only used in genome editing for the study of fundamental research, but also for the possibility of applied research in genetic engineering of economically important organisms. CRISPR system is comprised of two main components - the Cas9 protein and the sgRNA protein complex, binding of which to double stranded DNA results in double strand breaks (DSBs) (Fig.3).

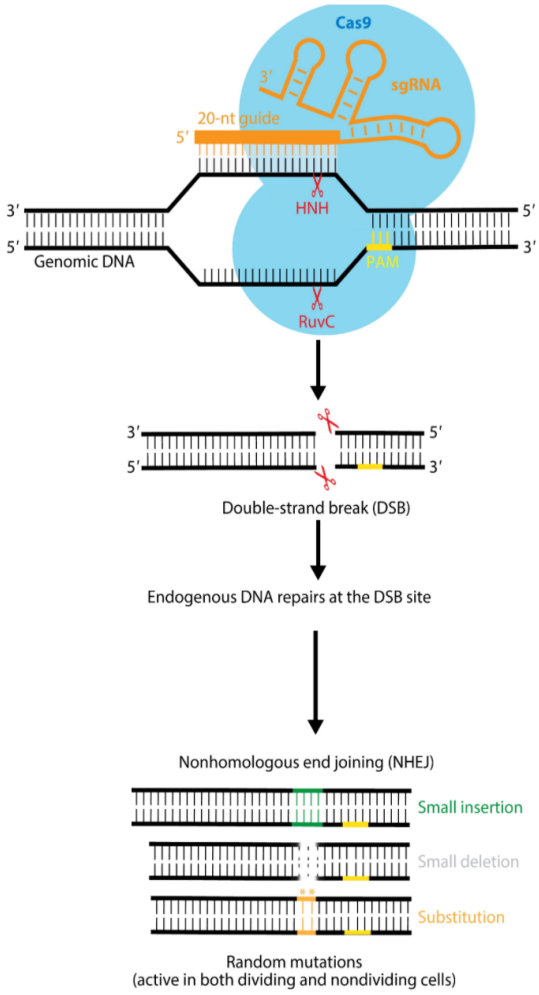


Fig.3: CRISPR\Cas9 complex mode of action: sgRNA locates target site by binding to complementary sequence of 20 nucleotides adjacent to PAM (NNG) and activates nuclease domains HNH and RuvC initiates cleavage that results in DSB. DSBs inside the cell are usually repaired by NHEJ pathway which often causes indels. Adopted from (Jiang and Doudna, 2017)

Single-guided RNA (sgRNA) consists of custom 20 nucleotides adjacent to the PAM (5'-NGG-3' for spCas9) site, that defines complementary DNA sequence it can bind to. Composition of sgRNA for optimal target binding has been already identified and should be 40-80% of GC to stabilise RNA-DNA duplex and span 17-24 bp to minimize off-targets (Zhang et al., 2016). When the complex is bound the action of two nuclease domains RuvC and HNH that induce DSB often lead to error prone NHEJ repair pathway (Fig.3). CRISPR knock-out strategy is based on errors after DNA repair that may lead to out of frame mutation in the exonic regions of the gene rendering transcripts non-functional (Jinek et al., 2012). These mutations are the basis of LOF genetic studies that allow to observe the impact of null mutation on subsequent biomolecular changes and phenotype. Moreover, this method allows to identify differences between single and double knock-out mutants if such are present. Nowadays, advances in commercial manufacturing facilitate production of pure spCas9 protein and its mutant derivatives on large scale, which allows application of CRISPR as in vitro Ribonucleoprotein (RNP) complex in genetic studies. Cas9/sgRNA RNPs has been shown as promising method as it is a straightforward and rapid CRISPR gene editing approach with less off-target effects and lower immunogenicity in comparison to plasmid-based systems (Fajrial et al., 2020; Kouranova et al., 2016; Ramakrishna et al., 2014; Wei et al., 2020).

Transfection:

Transfection is a delivery procedure of foreign material such as proteins or nucleic acids into the cells in order to induce specific changes. Transfection have been used to induce pluripotent stem cell (iPS cell), small interference RNA (siRNA) knock-down experiments, and production of human tissue plasminogen activator in immortalized Chinese hamster ovary (CHO) cells for therapeutic purposes, and CRISPR knock-out experiments (Kim and Eberwine, 2010). At the present day there is a wide variety of transfection methods available which usually are clustered into three groups: biological (virus-mediated), chemical (polymeric, lipid particles) and physical (injection, biolistic, electroporation, laser irradiation, sonoporation, and magnetic nanoparticles). Recently developed new chemical approach, termed BAPC-assisted CRISPR delivery, involves the use of Branched Amphiphilic Peptide Capsules (BAPC) BAPtofect™ (Phoreus™ Biotechnology, Inc. Olathe, Kansas, US). These peptide nanoparticles consist of equal proportions of two branched peptide sequences, bis (FLIVI)-K-KKKK and bis (FLIVIGSII)-

KKKKK, and when added together to solution with specific ionic concentration, self-assemble to form bi-layer capsules (Fig.4). Fully formed capsules are capable of delivering nucleic acids, small proteins, and solutes and range from 10 to 500nm in size (Wessel et al., 2019). BAPC has been shown to be taken up by cells, likely though nonselective internalisation process (Sukthankar et al., 2014). Additionally, BAPC have been successfully applied in germline mutagenesis in terrestrial arthropods without agents for endosomal escape, jewel wasp *Nasonia vitripennis* for CRISPR/Cas9 RNP delivery (Chaverra-Rodriguez et al., 2020). These findings provide potential for further application of BAPC technology for CRISPR/Cas9 mediated generation of stable germline mutations in marine arthropods.

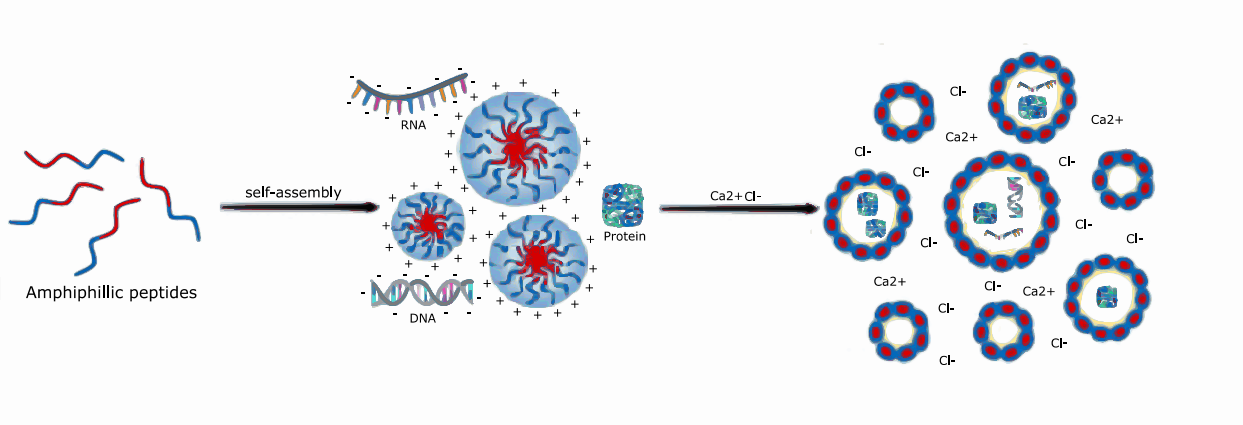


Fig.4 BAPC Mechanism. Amphiphilic peptides such as bis (FLIVI)-K-KKKK and bis (FLIVIGSII)-KKKKK are capable of self-aggregation into spherical micelles. Positive charge around protrusions of micelles facilitates binding of negatively charged nucleic acids and protein residues (Avila et al., 2016). Addition of salts to the solution changes micelle formation to the "locked" state capable of transporting material (Wessel et al., 2019). Adopted from (Du et al., 2019).

Kynurenine pathway:

Tryptophan (Trp) is an amino acid that is involved in synthesis of proteins and methoxyindoles (serotonin and melatonin). Non-protein pathway of TRY is based on cleavage of the indole ring and formation of kynurenine (KYN) (Oxenkrug, 2007) (Fig. 5). Almost 99% of the ingested tryptophan is unused for protein synthesis thus catabolised in the KYN pathway (Davis and Liu, 2015).

This pathway leads to the synthesis of ommatins and ommins that represent either dimers or oligomers of kynurenine derivatives, compounds responsible for dark-colored and yellow-brown or purple pigments in arthropods (Dontsov et al., 2020a). Studies shown that decrease in products of KYN pathway is associated with loss of coloration in albino strain of a terrestrial isopod *Armadillidium vulgare*

(Hasegawa et al., 2000) and crab spiders *Misumena vatia* (Riou and Christidès, 2010). Ommatins and ommins are grouped up as ommochromes components that in addition to pigmentation, perform important functions as antioxidant and light absorption agent in insect eyes (Dontsov et al., 2020b). Ommochromes are also involved in pigmentation of cephalopods produced by specialized cells chromatophores have been shown critically dependent on the KYN pathway with emphasis on Tryptophan-2,3-dioxygenase (TDO) activity (Crawford et al., 2020). TDO is heme-containing protein and an important enzyme catalysing the initial step in KYN pathway. This enzyme has received a lot of attention in neuroscience and immunology as KYN pathway has linked serotonin deficiency to immunosuppression in humans (Yu et al., 2016) and even to increased lifespan of *Drosophila melanogaster* (Oxenkrug, 2010). TDO is encoded by gene referred to as *vermillion* in *D. melanogaster*, required for production of brown pigment in the eye (Fridell and Searles, 1991). TDO is evolutionarily conserved across species, which means that studies may provide translational sentiment. From studies based on *C. elegans* it is known that TDO is mainly present as homodimers that can form tetramers to be enzymatically active (Ren and Correia, 2000), opening possibility of different phenotypes in mono and bi-allelic knock-out models.

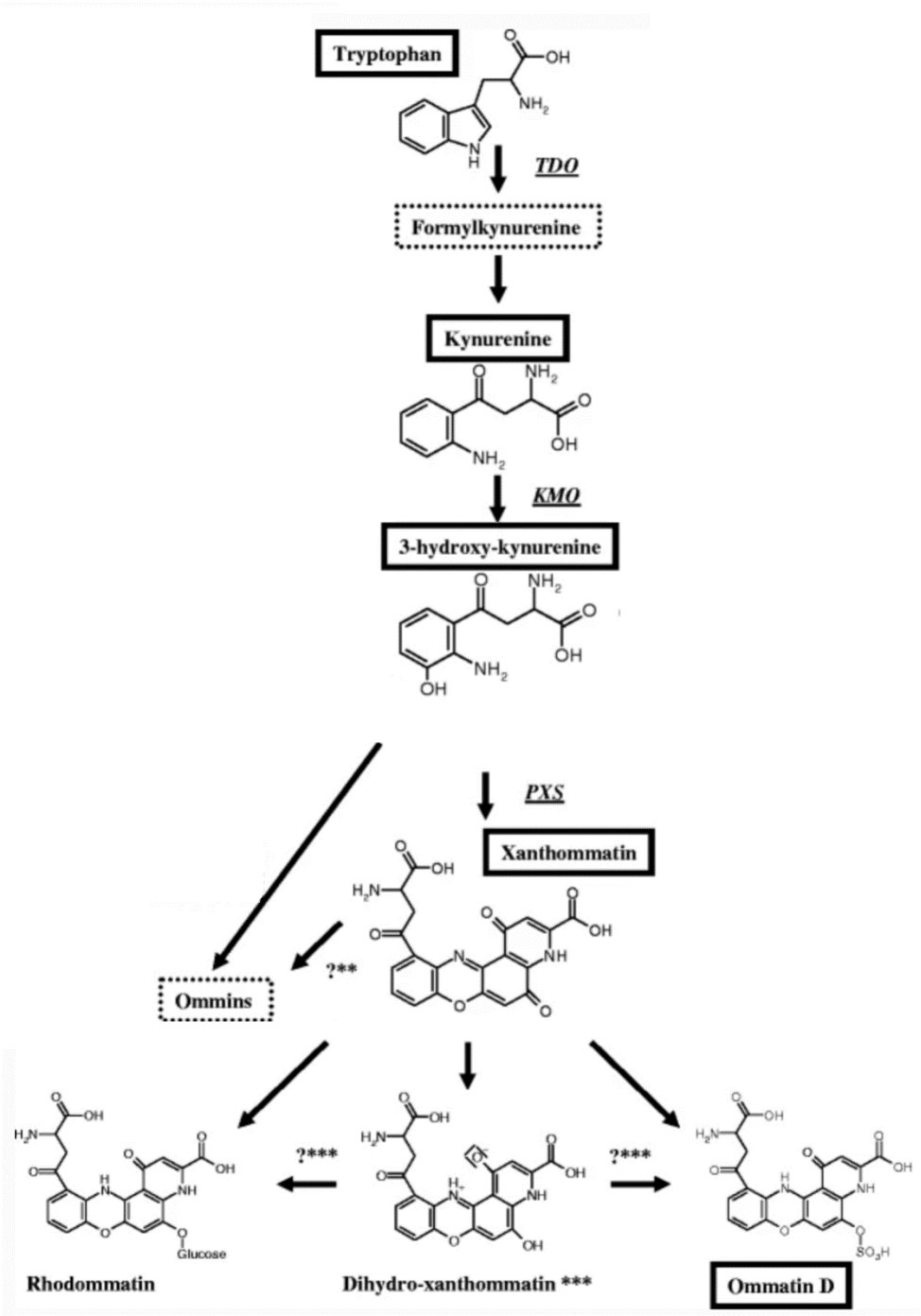


Fig 5. Kynurenine pathway. Initiation of kynurenine pathway begins with cleavage of indole ring of Tryptophan, which forms N-formylkynurenine (Searles and Voelker, 1986). Xantommatins are producing yellow/brown colours, Ommatin D red, Ommins are responsible for purple/yellow colours (Figon and Casas, 2019). Adopted from (Riou and Christidès, 2010)

Scope of Research:

The aim of this experiment is to establish CRISPR/Cas9 knock-out protocol for stable germ line mutation in order to generate mutant salmon lice strain. Two exons of TDO enzyme have been selected as a knock-out targets for its putative impact on pigmentation of eye and body in *Lepeophtheirus salmoni*s that could serve as a selection marker. The importance of ommochromes synthesis has not been yet described in salmon lice and could be presented on the basis of the LOF experiment. As Trp is an initial substrate for both melatonin and kynurenine pathways, ablation of *TDO* gene could result in increased melatonin synthesis that might change coloration pattern. Absence of dark pigments in the eye of salmon lice might change the colour to red, and decrease of the antioxidant protection might consequently disrupt the function of the organ.

Materials and methods:

Gene analysis and PAM site selection:

Genetic region of the TDO enzyme was derived from supercontig (accession LSalAtl2s1080). *TDO* is encoded in 6 exonic regions embedded in total of 8kb of DNA. *TDO* is highly expressed throughout lifecycle of salmon lice in various tissues and life stages underlining its importance (Fig.5a). Exon one, two have been used in successful embryonic knock-out studying in squids and three in *C.elegans.* Here In order to increase chances of successful knock-out two different sgRNA binding sites (PAM) were located by in-house script, targeting exon one and two, separated 2.5kb apart (fig.5b)

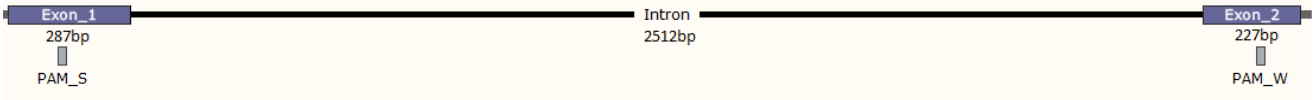


Fig. 5 *TDO* gene and its expression. 5b -Binding site of the sgRNA have been selected for two exons depicted PAM_S and further referred as S target site and PAM_W as W. Sequencing data for analysis was derived from local salmon lice genomic database.

Microinjection solution:

Rehydrated BAPC was mixed with sgRNA and incubated on ice for 30min. Solution was vortexed for 30 sec every 10 min. Afterwards Cas9 protein was added for further incubation at the room temperature for 20 min. Next, CaCl2 solution was added to the mixture thoroughly vortexed and incubated for 20 min at room temperature. Final concentrations for solution - gRNA (2000ng/µl), spCas9 (1000 ng/µl), BAPC (500 ng/µl), CaCl2 (94 ng/µl).

Microinjection:

Microinjection was carried out by two people – one operating microinjection needle and another controlling the aspiratory tube assembly. Needle punctured the CT site in close vicinity to ovaries (fig.2), afterwards pressure was carefully applied on aspiratory tube forcing the solution into the tissue.

Germline knock-out:

The fish housed in local SeaLab facility at 12°C and were infected with 500 copepodites (*LsGulen*). After two weeks the salmon lice were collected and injected microinjection solution at preadult stage, according to growth rate based on temperature at 12°C (Hamre et al., 2019). Post injection lice were

kept at 4°C for 24 hours and afterwards were manually placed back on fish. Two separate tanks were used for two different PAM sites and one tank for control group. Each flow-through tank housed 5 fish, at constant temperature at 12°C, with respect to the fish welfare conditions (Kristiansen et al., 2020). After two weeks egg strings produced by female lice were collected and incubated in flow-through incubator at 10°C. When hatched, samples were analysed under microscope for phenotypical changes and further subjected to DNA extraction.

Somatic knock-out:

For somatic knock-out 15 adult specimens were collected (both male and female) injected with microinjection solution in CT or GS segment and stored at 4 °C. After 24h injection was verified by fluorescent microscopy. After 72h injected segment was separated from the rest of the body and subjected to DNA extraction.

RNP in vitro cleavage:

PCR amplicon for S region was purified using column-based purification according to manufacturer protocol (ReliaPrep™ DNA Clean-Up and Concentration System, Promega, USA). CRISPR/Cas9 and sgRNA was incubated at 25 °C in 30 mM HEPES, 150 mM KCL, pH 7.5 for 20 min. 5µg was combined with 1µl of CRISPR/Cas9 RNP complex, 1 µl (200 mM HEPES 1M NaCl, 50 mM MgCl2 ,1mM EDTA pH 6.5) and H20 up to 10 µl. Solution was incubated for 90 min at 37 °C. Afterwards 1 µl of Proteinase K (20 mg/mL) was added and incubated for 10 min at 56 °C. DNA fragments were further visualised by agarose gel electrophoresis (2%).

DNA extraction:

Samples were fixated in 80% ethanol and stored at -20 °C prior to extraction. DNA extraction was carried using DNAeasy kit according to manufacturer protocol (QIAGEN, Valencia, CA). Quality of DNA was verified by NanoDrop™ 2000, and diluted to working concentration of 20ng/µl.

PCR amplification:

PCR reaction (94 °C for 30 sec, 59 °C for 20 sec, 74 °C for 30 sec) was performed in 20 µl volume (0.1 µM of each primer, 1.5 mM MgCl2, 1 µM dNTP, 40ng of DNA, 1 U of Dream Taq polymerase (Thermo Fisher

Scientific, Inc., Waltham, MA). Amplicon for S region was amplified using primers (PAM_S_F1, PAM_S_R1) and primers for W (PAM_W_F1, PAM_W_R1) region.

HRM:

HRM was performed on 1:100000 dilution of primary PCR product with PAM_S_F2, PAM_S_R2 with annealing temperature at 59 °C, PAM_W_F2, PAM_W_R2 at 63 °C for secondary PCR. Products were subjected to melting at 95°C for 60°C sec 4.4°C/s ramp, 40°C for 60s, 65°C for 1 sec at 2.2°C/s ramp and 95°C for 1s. Procedure was performed on LC96 (Roche Light Cycler 96, 96-well plate platform)

Fluorescent microscopy:

Samples after injection were subjected to confocal microscopy. Images have been taken using 488/522 lasers on Leica TCS SP8 confocal microscope (x5, x20 magnification).

Hairloop primer screening:

Reaction of total 20µl contained 1.8 mg/ml BSA, and 1 mM dNTP, 4 µL 5× Phusion HF buffer (New England Biolabs), 1 µM of each primer (PAM_S_F1 and PAM_S_HL_R for S, PAM_W_F1 and PAM_W_HL_R for W), 0.15 µL Phusion High-Fidelity DNA Polymerase (New England Biolabs), 50 ng genomic DNA. Settings for the PCR program: 98°C – 1 min, then 40 cycles of: 98°C – 10 s, 59.5°C – 30 s, 72°C – 30 s, then 72°C – 10 min then cooled to 4°C until collection. Protocol adopted from (Kroll et al., 2021)

Results:

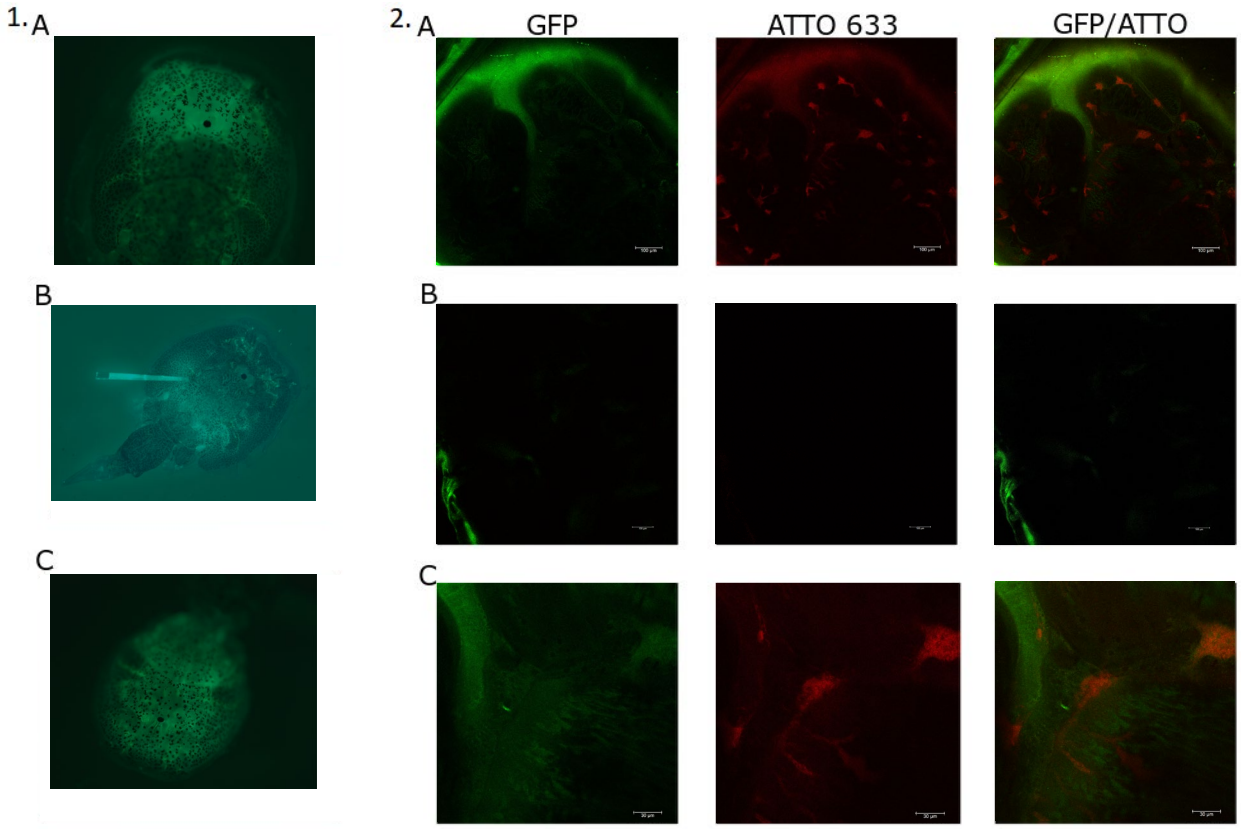


Fig.6 Fluorescent imaging of Salmon lice. 1(A, B, C) – Lice images under epi-fluorescent microscope during injection (GFP). CT images under confocal laser microscope obtained 24h post injection. 2.A – 5x magnification of louse injected with CRISPR/Cas9 RNP solution. 2.B - Negative control. 2.C – 20x magnification of louse injected with CRISPR/Cas9 RNP solution.

Total 90 specimens both male and female lice in pre-adult stage were injected with microinjection solution, 35 for each PAM site, 20 were injected with mock transfection. Epifluorescent microscope was used to verify the success of an injection (Fig.6.1). The solution rapidly spread throughout CT region highlighting internal tissues and organs, where discrepancies between GFP and ATTO florescence are visible (Fig.6.2). Lice for negative control (injected without sgRNA) has been stored for 5 days at 4 °C after injection and have shown 35% survival rate, whereas lice injected for both PAM sites after two weeks have combined 16% of survival rate (data not shown). Although under microscope phenotypes resembling the expected knock-out were identified (Appendix 2), first and second cohorts hatched from egg strings, that had two weeks difference were analysed by HRM have shown that all sequences identical to WT (Fig.7). Further Sanger sequencing of the samples have confirmed WT for both S and W target sites in all analysed samples. In addition, hairloop primer screening was used for somatic knock-out samples where no amplification has been found (data not shown). In order to verify induction of

DSB at the target site Cas9 RNP complex was tested in vitro and have shown the expected fragmentation of the S amplicon (fig.8).

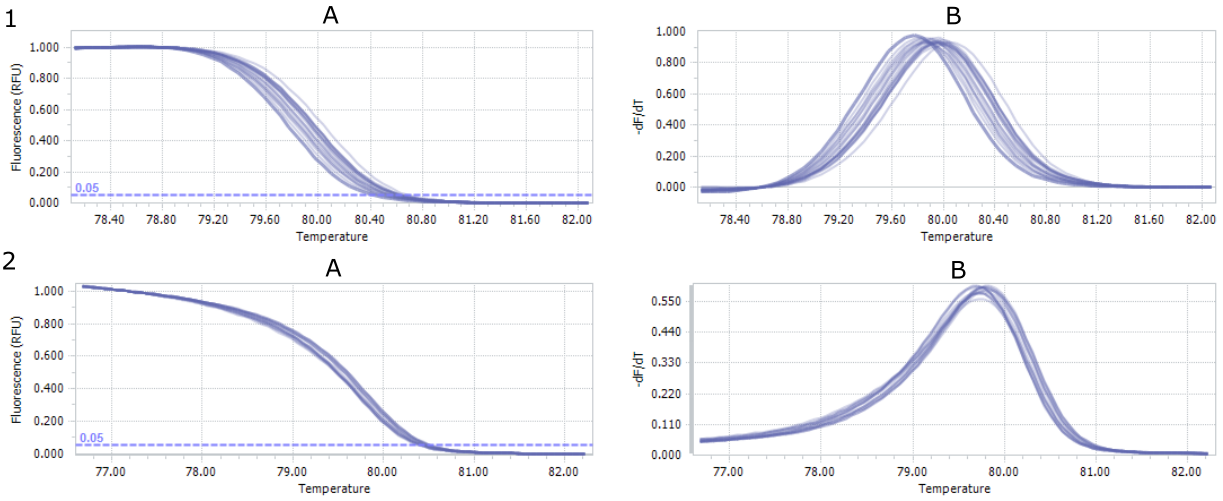


Fig.7 HRM analysis of S and W PAM amplicons. 1 – W PAM parents and offspring samples, 2 – S PAM, where A – normalised melting curves, B – Normalised melting peaks. Both hatched samples (germ-line knock-out) and somatic knock-out samples are included.

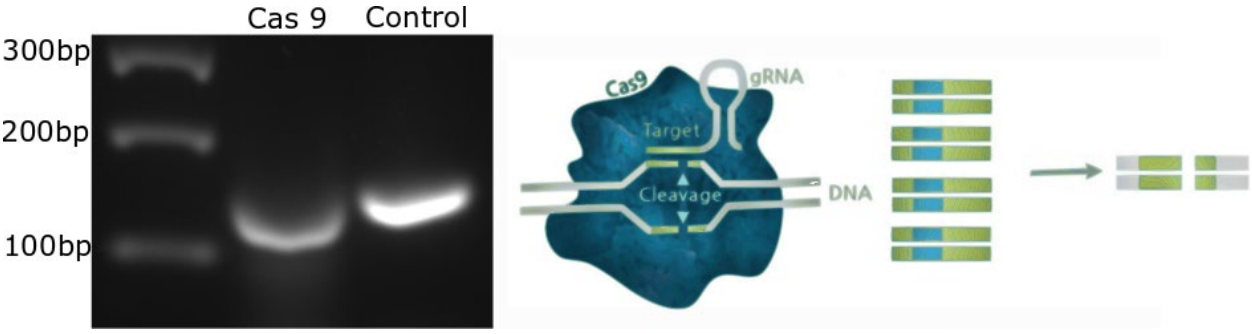


Fig.8 In vitro RNP cleavage. PCR amplicon of S target site (147bp) is displayed as control, cas9/sgRNA mixture targeting S site mix with amplicon displayed as Cas9. Cleavage of Cas9 results in 100bp and 47bp fragments.

Discussion:

Overall microinjection trials have resulted in high death rate in pre-adult stage. The main cause of this was specimen size, as the injection was performed manually, frequently piercing of CT leading to internal injuries and osmotic disbalance. The lack of volume control randomised injection volume per individual, where excessive volume caused swelling of the CT. For improvement, microinjection step could be performed on automated microinjection arm controlled by x, y, z joystick, which would increase precision and control the injected volume, hence increase survival rate. In addition, injection can be also performed on adults which should also result in higher survival rate, however, might decrease efficacy of germ line knock-out. Application of BAPC vector has not shown any positive knock-out delivery (Fig.7). As Cas9 RNP complex quality has been confirmed by in vitro cleavage (Fig.8), it leads to assumption that BAPC nanoparticles encapsulation might be affected by higher salt concentration in haemolymph of marine arthropods when compared to previous trials in terrestrial *Nasonia vitripennis* (Chaverra-Rodriguez et al., 2020). BAPCs have shown to be capable of delivery cytochrome c and RNase A, however EGFP delivery that aggregates in water was not delivered to cells (Sukthankar et al., 2014). Discrepancies between green and red fluorescence support the hypothesis that BAPC might aggregate into larger structures in high salt concentration and/or EGFP tandem attachment to Cas9 protein that prohibits complexation and restricts further dissemination and cellular uptake, leading to clumping in vessels (Fig.6.2C) (Sukthankar et al., 2014). During this experiment extracted DNA material was thoroughly analysed by 3 different methods, all of which have confirmed that samples contain only non-mutated DNA. As the delivery vector have not shown promised efficacy, this protocol requires alternative transfection method in order to be established. Alternatives such as cationic lipid nanoparticles that have shown high transfection efficiency in terrestrial insects, although variable in different species (Cheng et al., 2011), targeted delivery similar to P2C, ovary associated protein tag (Chaverra-Rodriguez et al., 2018), addition of cell penetrating peptides (Shen et al., 2018) or graphene-oxide carriers (Yue et al., 2018), are possible options. In addition, lack of available cell line for laboratory experiments, especially transfection trials complicate establishment for *in vivo* protocols. It will be important to develop a cell line derived from salmon lice for future advances in salmon lice research.

Conclusion:

Genotyping of injected samples have shown no difference from wild-type, offspring of injected lice have been confirmed to be identical to wild-type for both sgRNAs injections. BAPC mediated transfection has shown no effect when combined with CRISPR/Cas9 RNP complex in both somatic and germ-line knock-out experiments. Further establishment of the protocol requires alternative methods of delivery.

Future perspectives:

Current need in healthcare of efficient non-toxic methods supported by financial funding is driving scientific research beyond borders leading to new scientific discoveries. In addition, numerous modifications of CRISPR proteins developed continuously (Nidhi et al., 2021). Successful establishment of delivery vector as in the presented experiment would open new possibilities for application of Cas9 and other Cas proteins, which extends the possibilities of genetic studies *in vivo* in marine organisms. CRISPR associated technologies such as gene drive (Brossard et al., 2019), that is speculated to eradicate malaria are promising as well for pest control infestation on salmon farms. Moreover, generation of double knock-out models facilitated by CRISPR allows to obtain data about genetic interaction between genes based on observed phenotype (Castañeda-Zegarra et al., 2020). Our knowledge about ectoparasites is quite limited, therefore application of CRISPR systems in Salmon lice could provide valuable insights that might transcend beyond aquaculture.

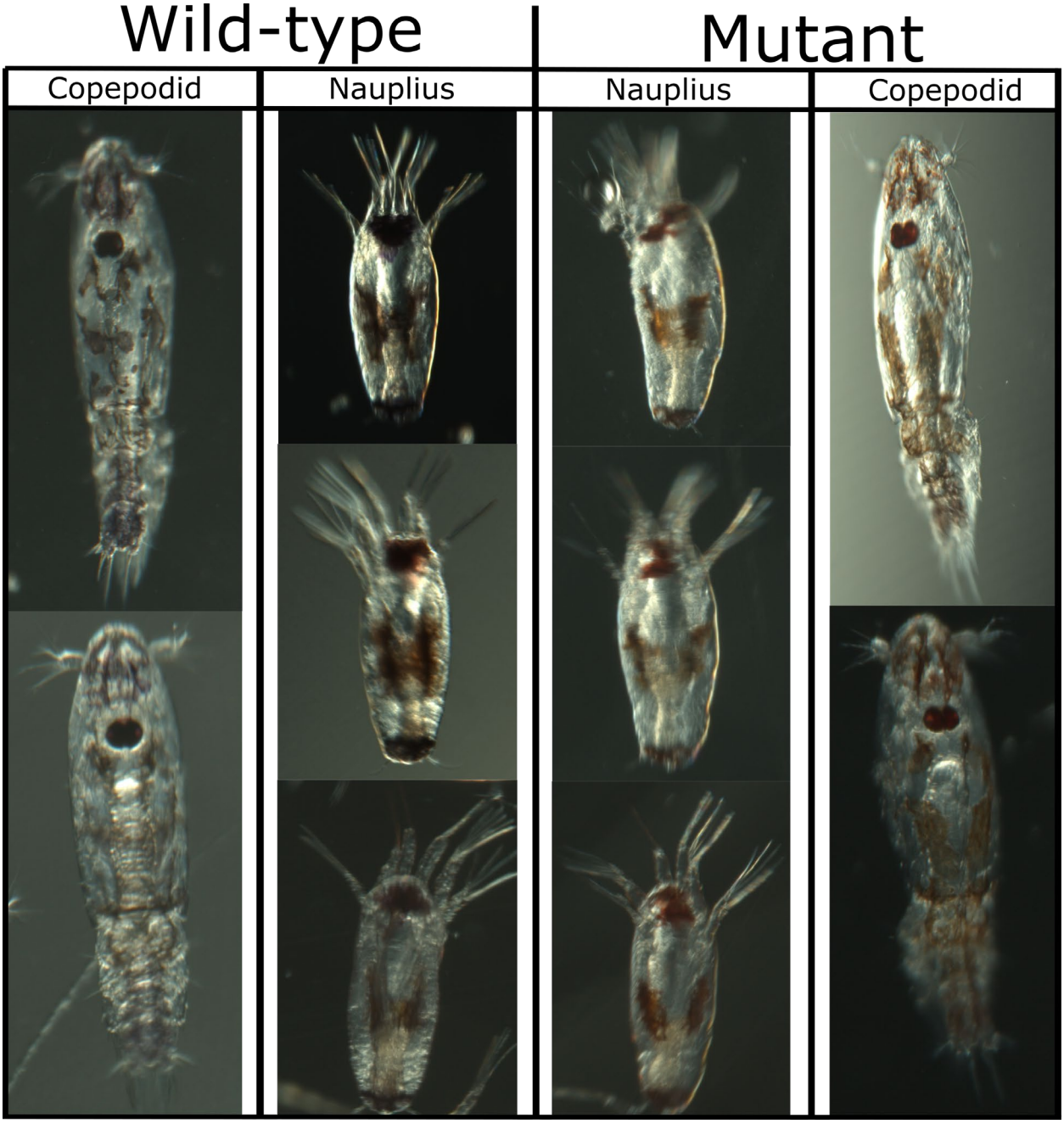
Appendices:

Appendix 1:

Reagent type	Tag	Content	Source
sgRNA	PAM_W	5’ GCUGGAGAGAACACCUGGAC 3’	Sigma-Aldrich
sgRNA	PAM_S	5’ CACGACUCUAUAGGGUAGUG 3’	Sigma-Aldrich
Primer	PAM_S_F1	5’ GGACATAAACTCCGTGATTG 3’	Sigma-Aldrich
Primer	PAM_S_R1	5’ GGATCCTAATACGACTCACTATAGG 3’	Sigma-Aldrich
Primer	PAM_S_F2	5’ GAGAAACGGATTTAGTGGAT 3’	Sigma-Aldrich
Primer	PAM_S_R2	5’ GTTTCAAGGAGAGTAATGGAGT 3’	Sigma-Aldrich
Primer	PAM_W_F1	5’ CAAGTGCCATTATACGGAAGT 3’	Sigma-Aldrich
Primer	PAM_W_R1	5’ CCACAAAAGGCCTTGTGTGTA 3’	Sigma-Aldrich
Primer	PAM_W_F2	5’ GGAACTTCAAGATGTGGAAAC 3’	Sigma-Aldrich
Primer	PAM_W_R2	5’ GTTACGTCAATGCTATCCTTT 3’	Sigma-Aldrich
Headloop Primer	PAM_S_HL_R	5’ CTGAGATATCCCATCACTCC CAGATGGAGACATTGTTTCAAG 3’	Invitrogen
Headloop Primer	PAM_W_HL_R	5 ‘CCTCTCTTGTGGACCTGACC TACTGTCTCCTTATACTTGTTC 3’	Invitrogen
Cas9 protein	eSpCas9-GFP	Cas9+NLS+GFP	Sigma-Aldrich
BAPC	Peptide nanoparticle	BAPtofect-25	Phoreusbiotech

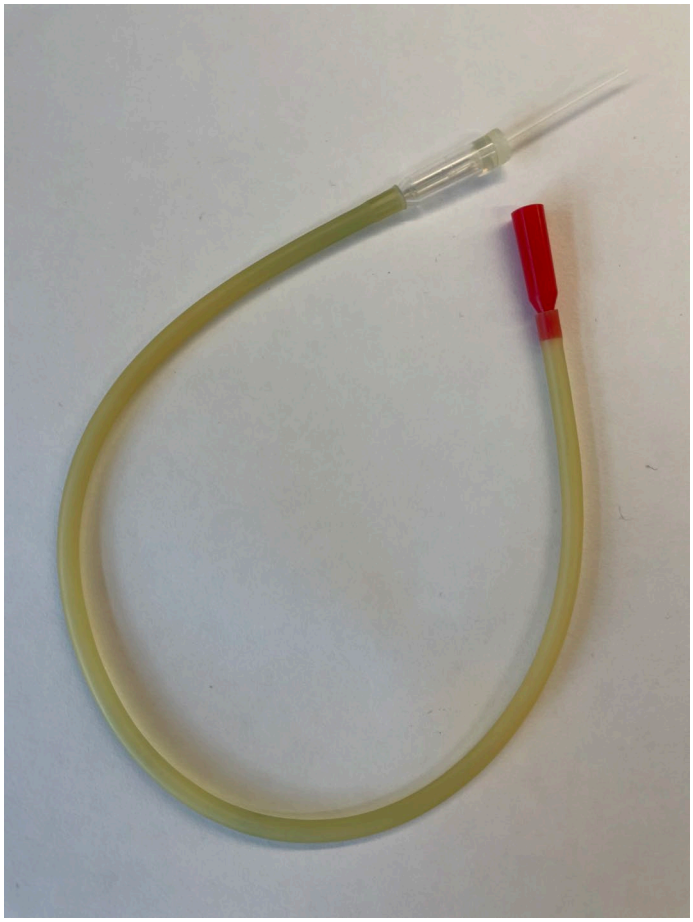
Appendix 1 – Key resource table, a collection of reagents applied in experiment.

Appendix 2:



Appendix 2 – Snapshot table of salmon lice under Zeiss SYCOP 3. Left side samples hatched from control group, right side – samples injected with W PAM RNP solution that resembled the expected mutant phenotype.

Appendix 3:



Appendix 3 – Image example of aspiratory tube assembly with microinjection needle attached. Injection solution is loaded into needle by capillary action and forced out by applying pressure on the tube.

References:

Brooks, K.M., 2005. The Effects of Water Temperature, Salinity, and Currents on the Survival and Distribution of the Infective Copepodid Stage of Sea Lice (Lepeophtheirus *Salmonis*) Originating on Atlantic Salmon Farms in the Broughton Archipelago of British Columbia, Canada. Reviews in Fisheries Science 13. https://doi.org/10.1080/10641260500207109

Chaverra-Rodriguez, D., Dalla Benetta, E., Heu, C.C., Rasgon, J.L., Ferree, P.M., Akbari, O.S., 2020. Germline mutagenesis of *Nasonia vitripennis* through ovarian delivery of <scp>CRISPR-Cas9</scp> ribonucleoprotein. Insect Molecular Biology 29, 569–577. https://doi.org/10.1111/imb.12663

Costello, M.J., 2006. Ecology of sea lice parasitic on farmed and wild fish. Trends in Parasitology 22, 475–483. https://doi.org/10.1016/j.pt.2006.08.006

Crawford, K., Diaz Quiroz, J.F., Koenig, K.M., Ahuja, N., Albertin, C.B., Rosenthal, J.J.C., 2020. Highly Efficient Knockout of a Squid Pigmentation Gene. Current Biology 30, 3484-3490.e4. https://doi.org/10.1016/j.cub.2020.06.099

Davis, I., Liu, A., 2015. What is the tryptophan kynurenine pathway and why is it important to neurotherapeutics? Expert Review of Neurotherapeutics. https://doi.org/10.1586/14737175.2015.1049999

Dontsov, A.E., Sakina, N.L., Yakovleva, M.A., Bastrakov, A.I., Bastrakova, I.G., Zagorinsky, A.A., Ushakova, N.A., Feldman, T.B., Ostrovsky, M.A., 2020a. Ommochromes from the Compound Eyes of Insects: Physicochemical Properties and Antioxidant Activity. Biochemistry (Moscow) 85, 668–678. https://doi.org/10.1134/S0006297920060048

Dontsov, A.E., Sakina, N.L., Yakovleva, M.A., Bastrakov, A.I., Bastrakova, I.G., Zagorinsky, A.A., Ushakova, N.A., Feldman, T.B., Ostrovsky, M.A., 2020b. Ommochromes from the Compound Eyes of Insects: Physicochemical Properties and Antioxidant Activity. Biochemistry (Moscow) 85, 668–678. https://doi.org/10.1134/S0006297920060048

Eichner, C., Nilsen, F., Grotmol, S., Dalvin, S., 2014. A method for stable gene knock-down by RNA interference in larvae of the salmon louse (Lepeophtheirus salmonis). Experimental Parasitology 140. https://doi.org/10.1016/j.exppara.2014.03.014

Erna Irene Heggland, 2020. Characterization of genes and proteins involved in parasite blood-feeding. Bergen, Norway.

Goater, T.M., Goater, C.P., Esch, G.W., 2013. Parasitism. Cambridge University Press. https://doi.org/10.1017/CBO9781139047876

Hamre, L., Bui, S., Oppedal, F., Skern-Mauritzen, R., Dalvin, S., 2019. Development of the salmon louse Lepeophtheirus salmonis parasitic stages in temperatures ranging from 3 to 24°C. Aquaculture Environment Interactions 11. https://doi.org/10.3354/aei00320

Hasegawa, Y., Negishi, S., Naito, J., Ikeda, R., Hasegawa, H., Nagamura, Y., Ishiguro, I., 2000. Ommochrome genesis in an albino strain of a terrestrial ISOPOD. Advances in Experimental Medicine and Biology 467, 645–648. https://doi.org/10.1007/978-1-4615-4709-9_82

Heuch, P.A., Nordhagen, J.R., Schram, T.A., 2000. Egg production in the salmon louse [*Lepeophtheirus salmonis* (Krøyer)] in relation to origin and water temperature. Aquaculture Research 31. https://doi.org/10.1046/j.1365-2109.2000.00512.x

Housden, B.E., Muhar, M., Gemberling, M., Gersbach, C.A., Stainier, D.Y.R., Seydoux, G., Mohr, S.E., Zuber, J., Perrimon, N., 2016. Loss-of-function genetic tools for animal models: cross-species and cross-platform differences. Nature Reviews Genetics. https://doi.org/10.1038/nrg.2016.118

Iversen, A., Hermansen, Ø., Nystøyl, R., Hess, E.J., Rolland, K.H., Garshol, L.D., Marthinussen, A., 2019. Kostnadsutvikling og forståelse av drivkrefter i norsk lakseoppdrett. Faglig sluttrapport, Nofima rapportserie.

Jiang, F., Doudna, J.A., 2017. CRISPR–Cas9 Structures and Mechanisms. Annual Review of Biophysics 46. https://doi.org/10.1146/annurev-biophys-062215-010822

Kim, T.K., Eberwine, J.H., 2010. Mammalian cell transfection: The present and the future. Analytical and Bioanalytical Chemistry 397, 3173–3178. https://doi.org/10.1007/s00216-010-3821-6

Oerke, E.-C., 2006. Crop losses to pests. The Journal of Agricultural Science 144. https://doi.org/10.1017/S0021859605005708

Oxenkrug, G.F., 2010. The extended life span of Drosophila melanogaster eye-color (white and vermilion) mutants with impaired formation of kynurenine. Journal of Neural Transmission 117, 23–26. https://doi.org/10.1007/s00702-009-0341-7

Oxenkrug, G.F., 2007. Genetic and hormonal regulation of tryptophan-kynurenine metabolism: Implications for vascular cognitive impairment, major depressive disorder, and aging, in: Annals of the New York Academy of Sciences. Blackwell Publishing Inc., pp. 35–49. https://doi.org/10.1196/annals.1403.003

Riou, M., Christidès, J.P., 2010. Cryptic Color Change in a Crab Spider (Misumena vatia): Identification and Quantification of Precursors and Ommochrome Pigments by HPLC. Journal of Chemical Ecology 36, 412–423. https://doi.org/10.1007/s10886-010-9765-7

Searles, L.L., Voelker, R.A., 1986. Molecular characterization of the Drosophila vermilion locus and its suppressible alleles. Proceedings of the National Academy of Sciences of the United States of America 83, 404–408. https://doi.org/10.1073/pnas.83.2.404

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O.T., Nilsen, F., Horsberg, T.E., Jackson, D., 2013. Salmon lice - impact on wild salmonids and salmon aquaculture. Journal of Fish Diseases 36, 171–194. https://doi.org/10.1111/jfd.12061

UN Economic & Social Affairs, 2019. World Population Prospects. Department of Economic and Social Affairs.

Wessel, E.M., Tomich, J.M., Todd, R.B., 2019. Biodegradable Drug-Delivery Peptide Nanocapsules. ACS Omega 4, 20059–20063. https://doi.org/10.1021/acsomega.9b03245

Wootten, R., Smith, J.W., Needham, E.A., 1982. Aspects of the biology of the parasitic copepods *Lepeophtheirus salmonis* and *Caligus elongatus* on farmed salmonids, and their treatment. Proceedings of the Royal Society of Edinburgh. Section B. Biological Sciences 81. https://doi.org/10.1017/S0269727000003389

Yu, C.P., Pan, Z.Z., Luo, D.Y., 2016. TDO as a therapeutic target in brain diseases. Metabolic Brain Disease. https://doi.org/10.1007/s11011-016-9824-z

Zhang, J.-P., Li, X.-L., Neises, A., Chen, W., Hu, L.-P., Ji, G.-Z., Yu, J.-Y., Xu, J., Yuan, W.-P., Cheng, T., Zhang, X.-B., 2016. Different Effects of sgRNA Length on CRISPR-mediated Gene Knockout Efficiency. Scientific Reports 6. https://doi.org/10.1038/srep28566

Zhang, Z.-Q., 2013. Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness (Addenda 2013). Zootaxa 3703, 1–82.

Zimmer, A.M., Pan, Y.K., Chandrapalan, T., Kwong, R.W.M., Perry, S.F., 2019. Loss-of-function approaches in comparative physiology: is there a future for knockdown experiments in the era of genome editing? The Journal of Experimental Biology 222. https://doi.org/10.1242/jeb.175737

Avila, L.A., Aps, L.R.M.M., Ploscariu, N., Sukthankar, P., Guo, R., Wilkinson, K.E., Games, P., Szoszkiewicz, R., Alves, R.P.S., Diniz, M.O., Fang, Y., Ferreira, L.C.S., Tomich, J.M., 2016. Gene delivery and immunomodulatory effects of plasmid DNA associated with Branched Amphiphilic Peptide Capsules. Journal of Controlled Release 241, 15–24. https://doi.org/10.1016/j.jconrel.2016.08.042

Borchel, A., Kongshaug, H., Nilsen, F., 2019. Identification and description of the key molecular components of the egg strings of the salmon louse (Lepeophtheirus salmonis). Genes 10, 1004. https://doi.org/10.3390/genes10121004

Braden, L., Michaud, D., Igboeli, O.O., Dondrup, M., Hamre, L., Dalvin, S., Purcell, S.L., Kongshaug, H., Eichner, C., Nilsen, F., Fast, M.D., 2020. Identification of critical enzymes in the salmon louse chitin synthesis pathway as revealed by RNA interference-mediated abrogation of infectivity. International Journal for Parasitology 50, 873–889. https://doi.org/10.1016/j.ijpara.2020.06.007

Bradshaw, C.J.A., Leroy, B., Bellard, C., Roiz, D., Albert, C., Fournier, A., Barbet-Massin, M., Salles, J.-M., Simard, F., Courchamp, F., 2016. Massive yet grossly underestimated global costs of invasive insects. Nature Communications 2016 7:1 7, 1–8. https://doi.org/10.1038/ncomms12986

Brossard, D., Belluck, P., Gould, F., Wirz, C.D., 2019. Promises and perils of gene drives: Navigating the communication of complex, post-normal science. Proceedings of the National Academy of Sciences 116, 7692–7697. https://doi.org/10.1073/PNAS.1805874115

Castañeda-Zegarra, S., Fernandez-Berrocal, M., Tkachov, M., Yao, R., Upfold, N.L.E., Oksenych, V., 2020. Genetic interaction between the non-homologous end-joining factors during B and T lymphocyte development: In vivo mouse models. Scandinavian Journal of Immunology 92, e12936. https://doi.org/10.1111/SJI.12936

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y., Peterson, D.R., Kim, D., McKeand, S., Rasgon, J.L., 2018. Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. Nature Communications 9. https://doi.org/10.1038/S41467-018-05425-9

Cheng, G., Liu, L., Wang, P., Zhang, Y., Zhao, Y.O., Colpitts, T.M., Feitosa, F., Anderson, J.F., Fikrig, E., 2011. An in vivo transfection approach elucidates a role for Aedes aegypti Thioester-Containing proteins in flaviviral infection. PLoS ONE 6. https://doi.org/10.1371/JOURNAL.PONE.0022786

Cleves, P.A., Strader, M.E., Bay, L.K., Pringle, J.R., Matz, M. v., 2018. CRISPR/Cas9-mediated genome editing in a reef-building coral. Proceedings of the National Academy of Sciences 115, 5235–5240. https://doi.org/10.1073/PNAS.1722151115

Crawford, K., Diaz Quiroz, J.F., Koenig, K.M., Ahuja, N., Albertin, C.B., Rosenthal, J.J.C., 2020. Highly Efficient Knockout of a Squid Pigmentation Gene. Current Biology 30, 3484-3490.e4. https://doi.org/10.1016/J.CUB.2020.06.099

Du, Z., Li, Q., Li, J., Su, E., Liu, X., Wan, Z., Yang, X., 2019. Self-Assembled Egg Yolk Peptide Micellar Nanoparticles as a Versatile Emulsifier for Food-Grade Oil-in-Water Pickering Nanoemulsions. Journal of Agricultural and Food Chemistry 67, 11728–11740. https://doi.org/10.1021/acs.jafc.9b04595

Figon, F., Casas, J., 2019. Ommochromes in invertebrates: biochemistry and cell biology. Biological Reviews 94, 156–183. https://doi.org/10.1111/brv.12441

Fridell, Y.W.C., Searles, L.L., 1991. Vermilion as a small selectable marker gene for Drosophila transformation. Nucleic Acids Research 19, 5082. https://doi.org/10.1093/nar/19.18.5082

Ghafor, I.M., 2020. Crustacean. Crustacea. https://doi.org/10.5772/INTECHOPEN.89730

Guragain, P., Tkachov, M., Båtnes, A.S., Olsen, Y., Winge, P., Bones, A.M., 2021. Principles and Methods of Counteracting Harmful Salmon–Arthropod Interactions in Salmon Farming: Addressing Possibilities, Limitations, and Future Options. Frontiers in Marine Science 0, 965. https://doi.org/10.3389/FMARS.2021.701793

Hamre, L., Bui, S., Oppedal, F., Skern-Mauritzen, R., Dalvin, S., 2019. Development of the salmon louse Lepeophtheirus salmonis parasitic stages in temperatures ranging from 3 to 24°C. Aquaculture Environment Interactions 11. https://doi.org/10.3354/aei00320

Heggland, E.I., Dondrup, M., Nilsen, F., Eichner, C., 2020. Host gill attachment causes blood-feeding by the salmon louse (Lepeophtheirus salmonis) chalimus larvae and alters parasite development and transcriptome. Parasites and Vectors 13, 225. https://doi.org/10.1186/s13071-020-04096-0

Irion, U., Krauss, J., Nüsslein-Volhard, C., 2014. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. Development 141, 4827–4830. https://doi.org/10.1242/DEV.115584

Kristiansen, T.S., Madaro, A., Stien, L.H., Bracke, M.B.M., Noble, C., 2020. Theoretical basis and principles for welfare assessment of farmed fish, in: Fish Physiology. Elsevier Inc., pp. 193–236. https://doi.org/10.1016/bs.fp.2020.09.006

Kroll, F., Powell, G.T., Ghosh, M., Gestri, G., Antinucci, P., Hearn, T.J., Tunbak, H., Lim, S., Dennis, H.W., Fernandez, J.M., Whitmore, D., Dreosti, E., Wilson, S.W., Hoffman, E.J., Rihel, J., 2021. A simple and effective f0 knockout method for rapid screening of behaviour and other complex phenotypes. eLife 10, 1–34. https://doi.org/10.7554/ELIFE.59683

Liang, X., Zhang, L., Natarajan, S.K., Becker, D.F., 2013. Proline Mechanisms of Stress Survival. https://home.liebertpub.com/ars 19, 998–1011. https://doi.org/10.1089/ARS.2012.5074

Nidhi, S., Anand, U., Oleksak, P., Tripathi, P., Lal, J.A., Thomas, G., Kuca, K., Tripathi, V., 2021. Novel CRISPR–Cas Systems: An Updated Review of the Current Achievements, Applications, and Future Research

Perspectives. International Journal of Molecular Sciences 2021, Vol. 22, Page 3327 22, 3327. https://doi.org/10.3390/IJMS22073327

Ren, S., Correia, M.A., 2000. Heme: A regulator of rat hepatic tryptophan 2,3-dioxygenase? Archives of Biochemistry and Biophysics 377, 195–203. https://doi.org/10.1006/abbi.2000.1755

Shen, Y., Cohen, J.L., Nicoloro, S.M., Kelly, M., Yenilmez, B., Henriques, F., Tsagkaraki, E., Edwards, Y.J.K., Hu, X., Friedline, R.H., Kim, J.K., Czech, M.P., 2018. CRISPR-delivery particles targeting nuclear receptor–interacting protein 1 (Nrip1) in adipose cells to enhance energy expenditure. Journal of Biological Chemistry 293, 17291–17305. https://doi.org/10.1074/JBC.RA118.004554

Sukthankar, P., Avila, L.A., Whitaker, S.K., Iwamoto, T., Morgenstern, A., Apostolidis, C., Liu, K., Hanzlik, R.P., Dadachova, E., Tomich, J.M., 2014. Branched amphiphilic peptide capsules: Cellular uptake and retention of encapsulated solutes. Biochimica et Biophysica Acta - Biomembranes 1838, 2296–2305. https://doi.org/10.1016/j.bbamem.2014.02.005

Wessel, E.M., Tomich, J.M., Todd, R.B., 2019. Biodegradable Drug-Delivery Peptide Nanocapsules. ACS Omega 4, 20059–20063. https://doi.org/10.1021/acsomega.9b03245

Yue, H., Zhou, X., Cheng, M., Xing, D., 2018. Graphene oxide-mediated Cas9/sgRNA delivery for efficient genome editing. Nanoscale 10, 1063–1071. https://doi.org/10.1039/C7NR07999K