Techniques for Functional Testing of Factors Associated with Arctic charr Polymorphism: phenotypic analysis in zebrafish and *in vitro* assays in cell culture.

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Techniques for Functional Testing of Factors Associated with Arctic charr Polymorphism: phenotypic analysis in zebrafish and *in vitro* assays in cell culture.

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Research project in biology for foreign students in the Faculty of Life and Environmental Sciences.

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Útdráttur

Sköpulag fæðuöflunarfæra í fiskum, kjálkar og tálknbogar, hafa áhrif á fæðuöflun og fæðuval. Fjölbrigðni í fæðuöflunarfærum opnar leiðir til nýtingar mismunandi fæðuvista og gerir mismunandi afbrigðum kleyft að lifa innan sama vistkerfis. Fjögur bleikjuafbrigði eru í Þingvallavatni, tvö þeirra eru bontnsækin (dvergbleikja og kuðungableikja) og tvö nærast í vatnsbolnum (murta og sílableikja). Þessi afbrigði eru frábrugðin hvert öðru með tilliti til hegðunar, útlits, lögunar og fæðu og munur er á genatjáningu og tímasetningu þroskunaratburða milli þeirra. Ferlar þróunar og aðlögunar sem leiða til myndunar fjölbreytileika innan sama kerfis eru mikið rannsakaðir um þessar mundir. Í þessu verkefni var markmiðið að setja upp og prófa kerfi sem hægt er að nota til að sannreyna niðurstöður sem fást úr rannsóknum á bleikjustofnum (en bleikjan hentar ekki sem tilraunalífvera). Annars vegar er um að ræða að nýta sebrafiska sem lifandi líkan fyrir svipgerðaráhrif breytileika í genatjáningu á höfuðbein fiska, og hins vegar notkun frumuræktа til að kanna samskipti stjórnpróteina og stjórnraða, sérstaklega með s.k. luciferasa prófi.

Í þessari fyrstu prufukeyrslu var virkni microRNA-199a á mögulegt bindiset í 3’UTR svæði *ets2* mRNA könnuð, sem og áhrif microRNA-199a á lögun höfuðbeina. Ekki tókst að ljúka frumuræktahlutanum að fullu. Settar voru upp ferskar frumuræktir úr fóstrum og mismunandi aðstæður prófaðar. Í hinum hluta verkefnisins var miR-199a hindra sprautað í fóstur sebrafiska og greindust marktæk áhrif á höfuðlag fiskana sem benda til þess að munur sem er á höfuðlagi milli bleikjuafbrigða í Þingvallavatni gæti stafað af breytileika sem hefur greinst í tjáningu miR-199a milli þessara afbrigða á fósturskeiði

Abstract

Trophic morphology in Teleosts, including the jaw and pharyngeal arches, determines feeding type and performance. Polymorphism in trophic morphology facilitates niche partitioning and the coexistence of morphs within ecosystems. In Lake Thingvallavatn, Iceland, Arctic charr displays four morphs, two benthic; small (SB) and large (LB), and two pelagic; piscivorous (PI) and planktivorous (PL). These ecomorphs show disparity in behaviour, morphology and feeding type, as well as variation in gene expression and heterochronic ontogenetic shifts. The evolutionary and adaptive processes that generate sympatric trophic polymorphism are the subject of much current research. In this project the overall aim was to set up and perform test-runs of experimental systems for evaluating results from gene expression analysis in Arctic charr morphs. These systems are 1) Zebrafish (*Danio rerio*) as an *in vivo* model the effects of variation in gene-expression on craniofacial morphogenesis in teleost fish; 2) luciferase assay in primary Arctic charr cell cultures and mammalian cell lines for functional analysis of regulatory interactions.

I tested the role of miRNA-199a in generating morphological differences by mimicking (phenocopying) morph phenotypes in zebrafish and set up conditions for primary cell cultures and transfection of a human cell line to validate the predicted miRNA-mRNA target pair miR-199a and *ets2.* A miR-199a inhibitor was injected into zebrafish embryos and a significant difference in craniofacial morphology was seen, using geometric morphometrics analysis. The results support the notion that the differences in trophic morphology between the Þingvallavatn morphs could, at least in part, be due to an underlying difference in miR-199a expression between the benthic and limnetic morphotypes at developmental stages.

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Abbreviations

PL	Planktivorous
PI	Piscivorous
SB	Small Benthic
LB	Large Benthic
AC	Aquaculture Stock
miRNA	Micro RNA
UTR	TUntranslated Region
mRNA	Messanger RNA
RISC	RNA induced silencing complex
LNA™	Locked Nucleic Acid
DNA	Deoxyribose Nucleic Acid
dpf	Days Post Fertilisation
ts	Theiler Stage
MC	Meckel's cartilages
EP	Ethmoid plate
CH	Ceratohyal
HS	Hysosymplectic
CB	Ceratobranchial
BB	Basibranchials
PCA	Principle Component Analysis
WISH	Whole Mount *in situ* Hybridisation
ets2	v-ets avian erythroblastosis virus E26 oncogene homolog 2
HEK	Human Embryonic Kidney
PFA	Paraformaldehyde
MEM	Minimum Essential Medium
dpf	Days Post Fertilisation
PFAGA	Paraformaldehyde/glutaraldehyde

PBT	Phospho-Buffered Saline Solution containing Tween
SSC	Saline-sodium citrate
Anti-DIG-AP	Anti-digoxigenin antibody linked to alkaline Phosphatese
NBT-BCIP phosphate	nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
DMSO	Dimethyl sulfoxide
BSA	Bovine serum albumin
T25	25 ml Culture Flask
SOB	Super Optimal Broth
LB	Lysogeny Broth
L15	Leibovitz Medium
FBS	Fetal Bovine Serum
PenStrep	*Penicillin streptomycin*
P/S	PBS containing PenStrep and Fungizone
SOC	Super Optimal Broth containing glucose
DMEM	Dulbecco‘s Modified Eagle Medium

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Firstly, I want to thank my supervisors Sigríður Rut Franzdóttir, Kalina Hristova Kapralova, and Zophonías Oddur Jónsson for giving the opportunity to work on this project, and showing me plenty of support and patience. The techniques and knowledge I have gained from this fascinating project are above and beyond my wildest expectations. I would also like to thank Kalina for all the help with the morphometric analysis as well as all the correction and suggestions in this report.

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1 Introduction

1.1 Trophic Morphology

Trophic morphology refers to anatomical features such as jaw shape and protrusion as well as gill rakers which provide an adaptive advantage at a particular trophic level and are therefore closely linked to feeding type (Morita & Suzuki, 1999; Muschick *et al.*, 2011). Many fish species, such as African cichlids (*Cichlidae spp.*), pumpkinseed sunfish (*Lepomis gibbosus*), sticklebacks and Arctic charr (*Salvelinus alpinus)*, show large fitness advantages from relatively subtle phenotypic variation in trophic morphology (Parsons, 2011). Craniofacial variation in morphology and function provides an interesting link between anatomical, biomechanical and developmental traits with fish ecology and aquatic community composition.

Phenotypic plasticity and changes also play an important role in the diversification of species through adaptive radiation (Muschick *et al.,* 2011). Comparisons of craniofacial morphology and resulting feeding types can indicate phylogenetic relationships due to clear evolutionary trajectories. The origins of such diversification may be due to an alleviation of local food competition within the greater population (Westneat, 2005). Ecomorphology related to feeding type is underpinned by efficiency of the craniofacial attributes of the predator to catch and consume prey. The critical moment of food acquisition provides the substrate for natural selection to act upon, thereby influencing gene expression and downstream modification of craniofacial morphology of the taxa.

1.2 Arctic charr

Arctic charr populations have the most northerly range of any freshwater fish (Klemesten *et al.,* 2003). As a habitat generalist the species is located in alpine, boreal, temperate, arctic and subarctic freshwater systems. The phenotypes and ecology of this species shows greater variation than most Salmonidae and fish in general (Klemesten *et al.,* 2003) and distinct coexisting morphs are common (Skúlason *et al.,* 1989). Examples of parallel evolution of trophic polymorphism in Arctic charr occur across the Nordic region, UK, and Canada (Klemesten *et al.,* 2003). Niche flexibility and life history associated habitat shifts are also common when interspecific competition and population densities are elevated.

In Iceland alone there are an estimated 1,000 populations (Skúlason *et al.,* 1989). Lake Thingvallavatn, Iceland provides a grand example of sympatric trophic specialisation due to phenotypic variation in Arctic charr (Jonsson & Jonsson, 2001). Heterogeneity in the biotic and abiotic features of the lentic ecosystem is associated with the trophic morphology of the four ecomorphs (Skúlason *et al.* 1989). Two morphotypes populate Thingvallavatn (Fig. 1): 1) limnetic; evenly protruding jaws and a relatively pointed snout, comprising of planktivorous (PL) and piscivorous (PI) charr, and 2) benthic; with a retracted lower jaw, and rounded snout, comprising of small (SB) and large (LB) charr (Snorrason *et al.,* 1989). The ecological partitioning of these morphs is due to habitat, trophic position and endoparasite community (Sandlund *et al.,* 1992).

Figure 1. *Comparison of limnetic and benthic Arctic charr morphology.* A limnetic (top) Arctic charr (*Salvelinus alpinus)* from aquaculture stock (AC) contrasted with a small benthic (bottom) (SB) from Lake Thingvallavatn, Iceland. miRNA-199a is highly expressed during embryonic development in the AC morph which is characterised by an evenly protruding jaw and a pointed snout shape, (similar phenotype to the naturally occurring limnetic morphs in in Thingvallavatn) whereas expression is low in SB morph which is characterised by a retracted lower jaw and a blunt snout shape. Source: Kapralova *et al.* 2014.

1.2.1 Evolution

The sequence for Arctic charr at Thingvallavatn were as follows; 1) colonisation of Arctic charr in Iceland followed the latest glacial retreat 10,000 years ago which allowed ecological opportunism, 2) populations settled in various microhabitats resulting in phenotypic shifts, 3) populations specialised according to the selection pressures and food resources in the respective microhabitats: populations in benthic habitats adapted shortened bodies and retracted lower jaws, whereas those in limnetic habitats developed fusiform bodies and terminal jaws, 4) gene flow between the ecomorphs was limited, although hybridisation was possible (Parsons, 2011).

Speciation between these current ecomorphs within the superspecies; *S. alpinus* species complex, is predicted to be incipient (Jonsson & Jonsson, 2001). Neutral microsatellite marker analysis by Kapralova *et al.* (2011) revealed that genetic divergence is present,

however subtle, between the ecomorphs, which suggests initiation of reproductive isolation and eventually speciation (Kapralova *et al*. 2011). Gene flow has significantly decreased between the morphs and naturally occurring hybrids are rare. Parsons (2011) suggested that resource polymorphism provides an important mechanism for this. Competitive interactions between the juveniles of the pelagic and piscivorous morphs resulted in an ontogenetic niche shift and subsequent adult niche partitioning and body size variation. Such ontogenetic plasticity during embryonic development serves as an internal mechanism for adaptation proceeding speciation. Whereas, segregation of the small benthic and large benthic morphs occurred due to external mechanisms; adaption to various substrate types on the volcanic benthos (Parsons, 2011). Although there was some environmental influence, Arctic charr in Thingvallavatn display genetic polymorphism, as each morph is phenotypically stable, and any changes occur over generations (Adams, 1999).

1.2.2 Heterochrony

Heterochrony is the changes in temporal aspects of development between taxa and provides a rich source of novel variation. Even a miniscule perturbation at an early stage can reverberate into substantial differences in the entire body of the mature organism (Parsons, 2011). The divergence of Arctic charr morphs was contributed by heterochronic changes and is also supplemented by food resource differences during early development in combination with allometry, changes in body proportions during ontogenetic growth and development of an organism or during evolutionary changes (Parsons, 2011; Skúlason *et al.,* 1999). The embryonic forms of all the morphs is fairly similar, resembling the benthic phenotype with a subterminal mouth and a blunt snout (displaying paedomorphy), whereas this has not been retained in the piscivorous and planktivorous forms which exhibit phenotypes with a terminal mouth and pointed snout (Skúlason *et al.,* 1989).

1.3 microRNAs

As posttranscriptional regulators, microRNAs (miRNAs) present an additional level of control for gene expression (Haflidadóttir *et al.* 2010). The characteristic non-coding 3'UTR sequence of miRNAs is the binding site for messenger RNA (mRNA). The variation in the abundance of these binding sites allows for differential fine tuning of gene expression, often leading to subtle phenotypic changes (Ebert and Sharp 2012). miRNAs negatively affect protein function by identifying and binding to the 5' cap of the cis mRNA which corresponds to the miRNA's specific 3'UTR sequence, thereby preventing translation of the particular gene (Peterson *et al.* 2009). mRNA decay by deadenylation is also induced by the miRISC components (Karbiener *et al.* 2014). This 'incoherent feed-forward loop' provides the mechanism for precision adjustments of gene expression, compared to what is possible by affecting the transcription of genes directly (Peterson *et al.*, 2009).

miRNAs are highly conserved between taxa, however they display temporal and spatial variation in expression (Kapralova *et al.* 2014). As natural selection does not act directly upon miRNAs, they may introduce stability to developmental processes (Kapralova *et al.* 2014). Peterson *et al.* (2009) describes miRNAs with having the ability to buffer oscillations in gene expression. Redundancy is such that many miRNAs can be 'knocked out' without observable changes.

1.3.1 Target Analysis

There can be multiple compatible mRNAs to the 3'UTR sequence of a single miRNA, however few are biologically relevant (Karbiener *et al.* 2014). miRNA target analysis identifies direct miRNA-mRNA interactions and the effect this has on biological processes. In order to determine the link between an observable phenotype, the analogous miRNA

which produces it, and the directly targeted mRNA, a series of analyses are required; 1) bioinformatic tools, such as *In silico* techniques analyse miRNA-mRNA seed match and evolutionary conservation to predict direct miRNA-mRNA interactions, 2) reporter gene assays provide validation of this, 3) biological relevance is determined by loss- or gain-of-function experiments to identify direct miRNA targets by producing a phenocopy of the miRNA effect. Alternatively, this can also be achieved by disruption of miRNA-mRNA interactions.

1.3.2 miRNA Inhibition

In vivo Locked Nucleic Acid (LNA^{TM}) miRNA inhibitors are DNA analogues custom designed specifically for a miRNA target of interest (Exiqon, retrieved 2016). This form of oligonucleotide forms a methylene bridge between its 2'-oxygen of ribose and the 4'-carbon of the miRNA target analogue, locking it into a co-polymer and conferring thermal stability and nuclease resistance (Fluiter, 2003). *In vivo* delivery of the miRNA inhibitor is optimised due to its pharmacokinetic properties. The potent reduction in miRNA expression in a wide range of tissues by inhibitor knockdown enables functional analysis *in vivo.* Furthermore, the pharmocodynamic properties of this compound are enhanced by the highest affinity for target miRNA compared to all other DNA analogs, thereby minimising potential secondary effects (Braasch & Corey, 2001). The specific effect this has on miRNA expression will be to phenocopy a miRNA loss-of-function individual (mutant).

1.4 Whole Mount *in situ* Hybridisation

Whole mount *in situ* hybridisation (WISH) is an important tool for understanding gene activity, especially during embryonic development, by identifying specific targeted nucleic acid sequences in embryos and cells. This method can be applied to distinguish patterns of

spatial and temporal gene expression between taxa and even individuals that have been experimentally manipulated, thus providing insight into the role of a particular nucleic acid sequence (Thisse & Thisse, 2008).

1.4.1 WISH for miRNA-199a and *ets2*

As developmentally regulated nucleic acid sequences miRNAs and transcription factors are also suitable for analysis by WISH. miRNA-199a and *ets2* expression can be visualised even at low levels using WISH methods. In a study by Ligthart (2014) WISH staining showed expression of miRNA-199a and *ets2* in cells outside of the cartilage in the pharyngeal arches of Arctic charr embryos. This pattern was observed in both the PL and LB ecomorphs.

1.5 Zebrafish Model Species

Zebrafish (*Danio rerio*) is a practical genetic model and presents opportunities for research. The manageable size and high fecundity of this species makes it a very cost-effective biological tool. The transparent embryo is also useful for morphological analysis (Yelick & Schilling, 2002). Genetic and developmental research of zebrafish craniofacial morphology has produced a thorough reservoir of knowledge (Ahi, 2014).

1.5.1 Craniofacial Structure

The ontogeny of craniofacial development has been lineage-traced to reveal the mechanisms and gene expression that form these structures (Yelick & Schilling, 2002). Firstly, the jaw and pharyngeal arches are derived from the cranial neural crest cells which migrate from the rhombomeres and become segmented into each of the pharyngeal arch cartilages, including the anterior mandibular and hyoid arch structures, as well as the posterior branchial arches (Fig. 2) (Schilling *et al.*, 1996). The fates of the cranial neural crest cells are maintained by

community effects (Yelick & Schilling, 2002). Chondrification of the six different cartilage types, the most common hyaline, in the cranium begins two days post-fertilisation and is complete after five days, before replacement with cartilage bones, then eventually endochondral or perichondral bone.

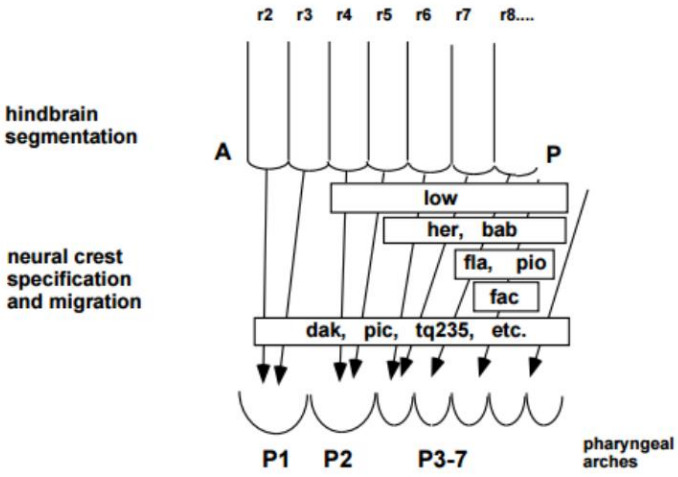

Figure 2. *Neural crest cell fate in pharyngeal arches.* Diagram representing the gene expression determining cell fate of neural crest cells in groups giving rise to the seven pharyngeal arches (P1-P7). Source: Schilling *et al.* 1996.

The major elements of the spanchnocranium are present and visible in the embryo of the zebrafish and the Arctic charr however there are some clear positional shifts and size differences (Fig. 3). The Meckel's cartilages show a more anterior position in relation to the Ethmoid plate (indicator of upper jaw position) in zebrafish. The Ceratohyal is also more distal from the Basibranchials and Ceratobranchials, compared to that of Arctic charr. In

both Arctic charr and Zebrafish seven branchial arches extend posterolaterally from the midline.

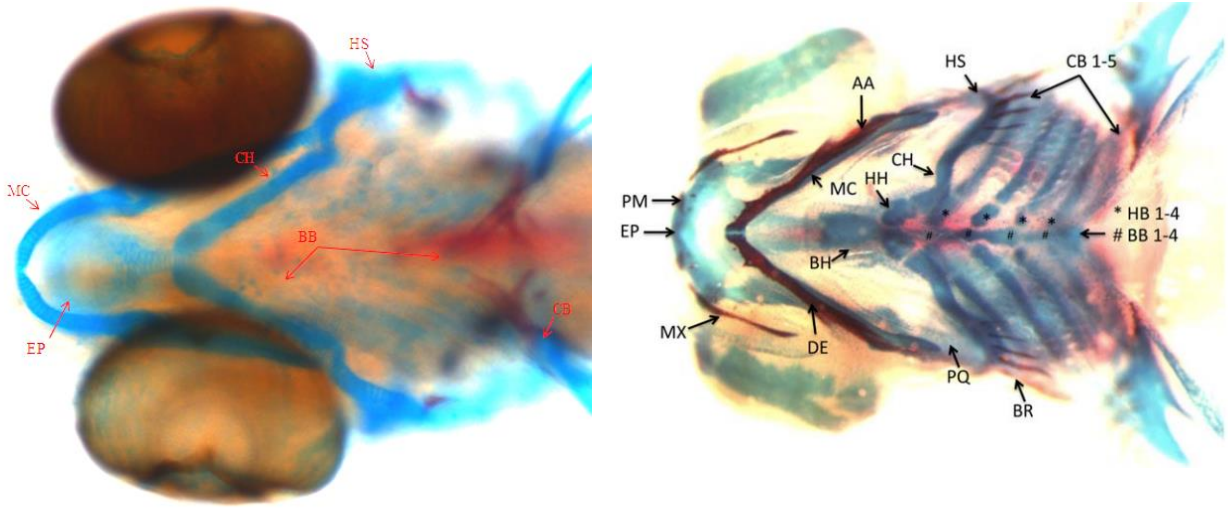

Figure 3. *Comparison of Zebrafish and Arctic charr spanchnocranium structure.* Major elements of the spanchnocranium structure of a 5 day post fertilization (5dpf) zebrafish (*Danio rerio*) larvae (left) and a stage 346ts Arctic charr (*Salvelinus alpinus)* larvae (right), Source: Kapralova *et al.* (2014). Cartilage (blue) and dental bone (red) of the following important craniofacial elements has formed; Meckel's cartilages (MC), Ethmoid plate (EP), Ceratohyal (CH), Hysosymplectic (HS), Ceratobranchial (CB), Basibranchials (BB).

1.6 Luciferase Assay

In vitro assays to determine regulatory interactions function by identifying *cis*-acting regulatory elements, such as transregulatory factors which modulate transcription initiation, and enable an in-depth understanding of the expression of a particular gene or network of genes at a cellular level (von der Ahe, 1991). Genetic reporter assays, such as Luciferase reporter technology, are adaptable tools that indicate gene expression at a cellular level coupled with gene expression or phenotype, thereby determining the biological relevance of a gene. Bioluminescence caused by the interaction of luciferase enzyme and a luminescent

substrate is highly sensitive and useful for low levels of target gene expression. The target gene is cloned upstream of the firefly luciferase gene (*luc*) and cotransfected, with a *Renilla* control vector, into cells. The luciferase and *Renilla* detection reagents measure luciferase activity and determine the quantitative presence of the reporter when the target gene is expressed (Allard and Kopish, 2008).

1.7 Arctic charr Primary Cultures

Primary cultures are derived from the cells, tissues or organs of the organism of interest (King *et al.*, 2013). These are useful for maximising and cultivating biological material for further investigations, such as chromosome analysis and luciferase assays.

1.7.1 Salmoninae Protocol

Previous investigations for production of Arctic charr primary cultures have not been recorded in the literature. However, protocol for designed for other closely related Salmoninae, a taxonomic subfamily within Salmonidae, might be suitable with the inclusion of necessary modifications. Within Salmoninae, *Salvelinus* and *Oncorhynchus* were among the last genera to diverge (19-16 million years ago) from a monophyletic group which also included *Salmo*, *Parahucho* and *Parasalmo* (Shedko *et al.* 2012). The relatively close relatedness of these genera suggests that primary cell cultures are likely to need similar procedures and conditions. Protocol to produce reliable monolayer cultures in Pacific salmon (*Oncorhynchus spp.*) and Steelhead trout (*Oncorhynchus* mykiss) has been described by Fryer *et al.* (1965). This involved dissociation of adult and embryonic tissues using trypsin and dilution of the cell suspension to obtain optimal cell concentration of 1.5 million cells per ml for seeding. Highest cell yields were gained from cultures using Minimum Essential Medium (MEM) and 20% calf serum. The cultures were incubated at 18 or 23^{o}C in a

revolving rack. The pH of the cultures remained relatively stable and media replacements were only necessary after the first 48 hours, then on 4 day intervals thereafter. Viable cell populations were observed after 3.5 days.

1.8 Aims

This investigation is part of a project aimed at understanding the molecular and developmental mechanisms producing trophic polymorphism in Arctic charr in Lake Thingvallavatn.

The overall aim of this particular investigation was to set up and test methods functional analysis of findings relating to differential gene expression between Arctic charr ecomorphs, starting with the validation of the predicted miRNA-mRNA target pair miR-199a and ets2. To this purpose an *in vivo* and an *in vitro* experimental system were established.

The investigation was divided into the following sub-aims:

1. Analyse the expression and role of miR-199a in craniofacial development using zebrafish as a model

This section of the investigation aimed to determine whether miRNA-199a plays a role in craniofacial morphogenesis. The results would verify whether zebrafish are effective model species for functionally testing associative evidence from Arctic charr, as multiple factors make such experiments difficult to perform in the charr. A whole mount *in situ* hybridisation was conducted on zebrafish larvae to localise the expression of miRNA-199a in the head. It was hypothesised that staining miRNA-199a would show similar expression patterns to Arctic charr and staining would occur around the arches, jaw and nasal cavity, because

miRNA-199a is associated with chondrification of the craniofacial elements during embryonic development (Kapralova, 2014).

Furthermore, phenotypic analysis aimed to determine if reduced levels of miRNA-199a caused a retracted lower jaw. It was hypothesised that alteration of miRNA-199a levels affects the protrusion of the lower jaw (a null hypothesis that miRNA-199a does not affect jaw morphology). The biological relevance of miRNA-199a for trophic morphology (jaw size and shape) was determined by inhibiting miRNA-199a. Geometric morphometric analyses of the splanchnocranium were used to quantify the differences in morphology.

2. Set up a standardized method for establishing primary cell cultures from Arctic charr embryos

To establish an optimal procedure for setting up primary cultures from Arctic charr embryos, the application of protocol produced for Pacific salmon and Steelhead trout by Fryer *et al.* (1965) was evaluated. Various culture conditions were altered and the observational results regarding cell adherence and growth was analysed to determine the optimal conditions for Arctic charr. It was hypothesised that Arctic charr primary cultures would require similar conditions to other Salmoninae.

3. Set up a luciferase assay in primary cells and mammalian cell lines to study miRNA/mRNA interactions.

This section of the investigation aimed to set up a luciferase assay to functionally validate a predicted conserved miRNA-199a binding site in the Arctic charr *ets2* 3'UTR. It was hypothesised that the the activity of a luciferase-*ets2-3'UTR* reporter is affected by the abundance of miRNA-199a (a null hypothesis that *ets2* levels (luciferase levels) are not affected by miRNA-199a).

2 Materials and Methods

2.1 Phenotypic Analysis in Zebrafish

2.1.1 Sampling and Injecting

Eggs were collected from recently spawned zebrafish from the zebrafish core facility located at The University of Reykjavik. A nanoinjector (custom-made) and modified capillary was used to introduce 2.3 nanolitres of LNA^{TM} miRNA inhibitor, diluted from the stock 5x (1ng/injection) and 10x (0.5ng/injection) into the egg yolk before the first division occurred. The untreated control sample organisms were not injected.

2.1.2 Staining and Photography

A total of 57 specimens; 32 untreated control and 25 treated with inhibitor, were stained with alcian blue to show cartilage and alizarin red to show ossified bone development and stored in 50% glycerol (Walker & Kimmel, 2007). Protocol by Thisse & Thisse (2008) was used to capture high-resolution photographs of each specimen. Stained individuals were mounted in a drop of glycerol on a microscope slide between two bridges made of one layer of microscope slide and a coverslip was placed on top. The body of the individual was orientated using the coverslip to accurately and symmetrically position the head with its ventral side visible, by gently shifting the coverslip, hence rolling the fish. A Leica (MZ10) stereomicroscope at an arbitrary magnification of 100x was used to photograph the head.

2.1.3 Morphometric Analysis

The craniofacial shape, viewed from the ventral side was characterised using 12 landmarks (Fig. 4.) positioned on anatomical features such as the ethmoid plate (indicative of the position of the upper jaw) and lower jaw, as well as the hyoid arches (Fig. 3). The landmarks mathematically defined the shape by enabling the analysis of all possible pairwise distances and angles between each landmark and were digitised using software tps.DIG (Rohlf, 2006). General Procrustes analysis was then undertaken in software MorphoJ (Klingenberg, 2011), which ensured the standardisation of the number, position, orientation and scale for all equivalent landmarks in order to be comparable and for any variation to be attributed exclusively to variation in shape. Generalized Procrustes superimposition scales to unit centroid size 1.0, shifts and rotates landmarks according to the best fit, or minimum sum of squares distances between equivalent landmarks.

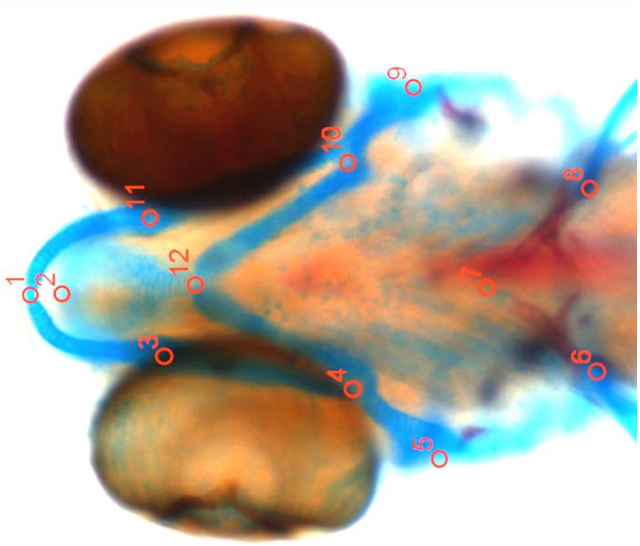

Figure 4. *Landmark positions.* A ventral view of the head of a 5 days post fertilization (5dpf) zebrafish larvae (*Danio rerio*). The 12 landmarks (4 pairs of bilateral and 4 mid-line landmarks) used in this study characterize the craniofacial shape and are indicated by red circles. The descriptions of each landmark are as follows; 1) symphysis of the left and right Meckel's cartilages, 2) anterior tip of the ethmoid plate, 3/11) articulation of Meckel's cartilages, 4/10) distal ends of the ceratohyals, 5/9) hyosymplectic, 6/8) distal ends of the ceratobranchials 5, 7) distal basibranchial 5, and 12) symphysis of the left and right ceratohyal.

2.1.4 Statistical Analysis

The Procrustes distance is a useful calculation to determine the distance between shapes, specifically the distances between corresponding landmarks of two arrangements after Procrustes superimposition. The centroid is the average *x* and *y* coordinates of all the landmarks and is the exact centre of the landmark arrangement. Centroid size is measured in the same units as the landmarks and calculates the size for landmark arrangements. Thus, the number and dispersal of landmarks around the centroid is quantified by the centroid size (Dahi & Naes, 2004).

Multivariate principal component analysis (PCA) was used to analyse the overall variation in the dataset. The two priori (observation groups) were separated by Discriminant Function Analysis, the reliability of which was evaluated by leave-one-out cross validation.

2.2 Whole Mount *in situ* Hybridisation

Embryo Pre-fixation

Zebrafish embryos were fixed in 4% paraformaldehyde (PFA) for 30 mins before being washed in Phospho-Buffered Saline Solution containing Tween (PBT). Then the embryos were dehydrated in increasing concentrations of methanol (25%, 50%, 75% and 100%) diluted in PBT for storage at -20°C.

Probe Hybridisation

These were rehydrated in decreasing concentrations of methanol (75%, 50%, and 25%) diluted in PBT before being washed in pure PBT. The embryos were permeabilised using Proteinase K diluted 1:2000 in PBT for 30 mins before being washed in pure PBT. Post-fixation using PFAGA (Paraformaldehyde/glutaraldehyde) and PBT washes followed. The

embryos were pre-hybridised with Hybridisation buffer, then hybridised with Hybridisation buffer containing a miRNA-199a probe at 64°C overnight.

Washing, Blocking and Anti-body Reaction

Non-specifically bound RNAs were washed from the embryos with SSC (Saline-sodium citrate) salt solutions (2xSSC, 2xSSC/0.1% Tween, and 0.2%SSC/0.1% Tween) at 64°C, then PBT at room temperature. The embryos were rocked in freshly prepared Blocking solution (400 µl BSA (Bovine serum albumin), 500 µl Sheep serum, 100 µl DMSO (Dimethyl sulfoxide) and 9 ml PBT) for two hours at room temperature, before being incubated at 4°C overnight with Anti-digoxigenin antibody linked to alkaline Phosphatese (anti-DIP-AP) diluted 1:2000 in Blocking solution under constant rocking.

Colour Reaction

The antibody was removed with successive PBT washes. Then the freshly prepared Colouration buffer (2 ml 1M $MgCL_2$, 4 ml 1M Tris pH 9.5, 4 ml 1M NaCl, 50 µl Levasmisole, 4 ml 10% Tween 20 and 25.95 ml milliQ water) was added before nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT-BCIP) diluted 20 µl/ml in Colouration buffer was added to the embryos which were stored in the dark at 4°C. When staining was observed, the Colouration buffer was removed with PBT washes, and the embryos were fixed in 4% PFA and stored in PBT at 4°C.

2.3 Luciferase Assay Preparation

2.3.1 Cell culture

HEK293T cells were dissociated using 100 µl of 0.5% trypsin in 1 ml of PBS. The cell suspension was centrifuged at 1,700 rpm for 3 mins. The supernatant was discarded and the seed pellet was resuspended in 1 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and gently pipetted to mix. The concentrated cell suspension was distributed evenly and re-seeded into three T25 flasks containing 4 ml of DMEM containing 10% FBS. The cells were cultured at 37°C in a cell incubator with a CO_2 concentration of 5% and a humidity of 98%. The process was repeated every 2-3 days.

2.3.2 Cryopreservation

Cell passage protocol was undertaken and the cell pellet was resuspended in 9 ml of DMEM. Dimethylsulfoxide (DMSO) was mixed with 10% FBS. These were mixed 1:1, distributed into cryotubes, and stored at -80°C overnight before being transferred to -150°C. To thaw, the melted liquid was centrifuged and the seed pellet was resuspended in DMEM and 10% FBS.

2.3.3 Cell Counting & Seed Concentration

A hemacytometer was used to measure cell concentration with the following formula; Number of cells × (Counted area (mm^2) ÷ chamber depth (mm)) = cells per 1µl. The appropriate volume of cell suspension was seeded in each 200 µl well (96 well plate) to achieve a desired cell density; 500, 1000, 2000, 5000, 10000, 15000, and 20000.

2.3.4 Plasmid Preparation

E. coli bacteria was transformed with the plasmids: PIS0 (Yekta *et al.*, 2004), PIS0 ets2 3'UTR C1 (Ásrún M. Óttarsdóttir, BS project), and a Renilla luciferase control plasmid diluted 1:100 with milliQ water, before heat shocking and incubating in Super Optimal Broth containing 20 mM glucose (SOC) for one hour. The cell suspensions were centrifuged and the cell pellet resuspended. 80 µl of each plasmid transformed cell suspension, as well as PIS0 ets2 QCH 2.2-2, were cultured on Luria Broth agar plates and incubated at 37°C overnight. A single colony of each was swabbed and placed in Luria Broth (LB) medium containing 10µl AMP (100mg/ml) and rocked at 37°C. Plasmids were isolated using a Nucleospin(R) kit and protocol (Macherey-Nagel). DNA concentration was measured using a nanodrop (ND-1000).

Multiple attempts to grow PIS0 transformed *E.coli* failed. Cultivation was successful for PIS0 ets2 3'UTR C1 and PIS0 ets2 QCH 2.2-2 inoculated *E. coli*. PI50 ets2 3'UTR C1 and PIS0 ets2 QCH 2.2-2 both yielded high plasmid concentrations, whereas *Renilla* yielded a relatively low concentration (Table. 5).

Table 5. *Plasmid Concentration.* DNA concentration of plasmid solutions; PI50 ets2 3'UTR C1, PI50 ets2 QCH 2.2-2 and *Renilla* after plasmid isolation from *E. coli* cultures.

Sample	DNA concentration (ng/µl)
PI50 ets2 3'UTR C1	718.09
PI50 ets2 QCH 2.2-2	565.88
Renilla	122.54

2.3.5 Test Transfection

HEK293T cells were seeded at densities of 1,000 cells per well. DMEM containing 10% FBS was added to reach a total volume of 360 µl per well (96 well plate) and the plates were incubated at 37°C overnight. Lipofectamine 3000 and the plasmid DNA were diluted in separate PCR strip wells and all conditions follow the layout shown in Table. 1. The Lipofectamine mastermix contained either 1.8 µl or 0.3 µl Lipofectamine in 120 µl and 5 µl of Opti-MEM respectively (1:67 and 1:17). 5.15 µl of each was distributed into the appropriate wells. The DNA dilution mix contained 130 µl Opti-MEM and 5.2 µl P3000 Reagent. 10 µl of was distributed into each well, with the exception of the control wells, which received 10.4 µl. The appropriate volume of the DNA plasmids PLVTHM (223.7 ng/ µl), PI50 (112.1 ng/ µl), and miRinh-FAM were added to the DNA dilution mix. The DNA mix was added to the Lipofectamine mix with minimal pipette mixing. This was incubated at room temperature for 5 mins before the entire volume was added to the HEK293T cells (70-90% confluent) and cultured at 37^{o} C. The culture was monitored for fluorescence after 24, 48 and 72 hours.

Table 1. *Test Transfection Layout.* Volume of Lipofectamine 3000 (1.8 µl and 0.3 µl diluted in 120 µl and 5 µl Opti-MEM respectively), and DNA plasmids (PLVTHM (223.7 ng/ µl), PI50 (112.1 ng/ µl), and miRinh-FAM) contained in P3000 (diluted in 10.4 µl Opti-MEM) added to confluent Human Embryonic Kidney (HEK) 293T cells in wells of 96 well tray for a test transfection.

	1	**2**
A	1.8 µl Lipofectamine 5.2 µl P3000 0.45 µl of 10 fold dilution of PLVTHM	1.8 µl Lipofectamine 5.2 µl P3000 0.9 of 10 fold dilution of PI50
B	1.8 µl Lipofectamine 5.2 µl P3000 2.25 µl of 10 fold dilution of PLVTHM	1.8 µl Lipofectamine 5.2 µl P3000 0.45 µl PI50
C	1.8 µl Lipofectamine 5.2 µl P3000 0.45 µl PLVTHM	1.8 µl Lipofectamine 5.2 µl P3000 0.9 µl PI50
D	1.8 µl Lipofectamine 5.2 µl P3000 0.9 µl PLVTHM	1.8 µl Lipofectamine 5.2 µl P3000 1.8 µl PI50
E	0.3 µl Lipofectamine 5.2 µl P3000 0.45 µl PLVTHM	0.3 µl Lipofectamine 5.2 µl P3000 0.9 µl PI50
F	1.8 µl Lipofectamine 0.1 µl miRinh-FAM	5.2 µl P3000 CONTROL
G	1.8 µl Lipofectamine 0.5 µl miRinh-FAM	0.3 µl Lipofectamine 0.5 µl miRinh-FAM
H	1.8 µl Lipofectamine 1 µl miRinh-FAM	5.2 µl P3000 CONTROL

The test transfection yielded no results. The HEK293T cells showed no change 24 and 48 hours after the test transfection. The experiment was deemed unsuccessful.

2.4 Arctic charr Primary Cultures

2.4.1 Antibiotic Wash

Embryos were washed and shredded in P/S wash, consisting of equal amounts of 400 µl/ml *Penicillin streptomycin* (PenStrep) and 10 µl/ml Fungizone in phosphate-buffered saline solution (PBS), for a minimum of three five-minute washes. All procedures were undertaken

in a sterile cell culture hood. All surfaces and equipment was wiped with 70% ethanol before entering the culture hood and on a regular basis.

2.4.2 Single Cell Culture

Embryonic tissues were dissociated by adding 0.25% trypsin in PBS and rocking at 500 rpm and 11°C for 30 mins. The supernatant was collected and the process repeated until all the tissue was dissolved. The cell suspension was centrifuged at 1700 rpm for 3 mins and the trypsin was removed. The seed pellet was resuspended in 1 ml of Leibovitz medium (L-15) supplemented with 0.3 gm/L L-glutamine containing 10 % fetal bovine serum (FBS).

2.4.3 Explants

Embryonic tissues were shredded in PS wash using a sterile scalpel.

2.4.4 Seeding Cells

Cells were seeded in various medium, antibiotic and density conditions (Table. 2). Control wells were also included which did not contain $NaHCO_2$ or antibiotics. The trays were incubated at 12°C.

Table 2. *Media Condition Treatments.* Various media treatments tested in this investigation to determine the optimal media conditions for *in vitro* Arctic charr (*Salvelinus alpinus*) embryonic cell monolayer cultures.

Seed type	**Medium type**	**$NaHCO_3$ Concentration (mM)**	**Antibiotic Treatment**	**FBS Concentration (%)**
Single cell suspension	L15	10	PenStrep + Fungizone	5
Explants	MEM	20	PenStrep	10
		30	Fungizone	20

2.4.5 Additional Media

Additional media was added to the cultures after 24 hours of incubation. Media was replaced every 3-5 days following seeding. Media was gently pipetted out and was replaced with the same treatment conditions. The wells were checked using a microscope and often showed a general reduction in abundance and density.

3 Results

3.1 Phenotypic Analysis

3.1.1 Observational Results Illustrated Phenotypic Variation of Jaw Protrusion Between Inhibitor Treated and Untreated Control Specimens.

miRNA-199a shows differential expression between the Arctic charr morphs during embryonic development at stages when cartilages of the craniofacial structures are being formed. We used zebrafish as a model to address the question whether differences in miRNA-199a-3p levels can influence craniofacial morphology. After injection of single-cell embryos with an miRNA-199a inhibitor at two different concentrations, observable phenotypic differences were seen in splanchnocranial structures between the miRNA-199a inhibitor and untreated control specimens in five day old larvae (Fig. 5). The inhibitor treated specimens showed relatively equal upper and lower jaw protrusion (terminal jaw phenotype), whereas the untreated control specimens showed greater protrusion in the lower jaw (superior jaw phenotype). Disparity in jaw protrusion was less obvious between the treatment groups (different concentration of the inhibitor) .

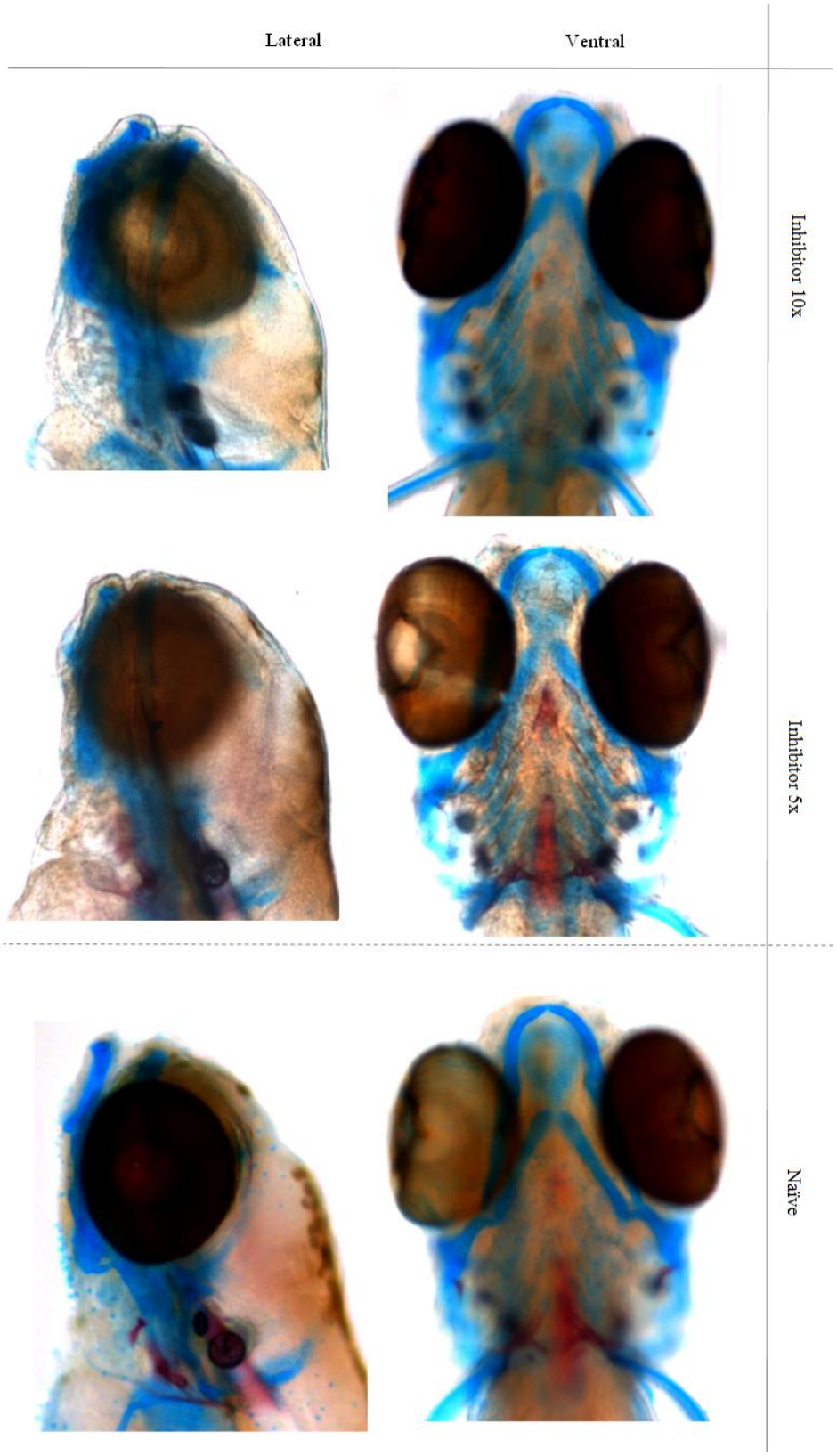

Figure 5. *Phenotypic expression observations.* Ventral and lateral views of 5 day post fertilization (5dpf) zebrafish (*Danio rerio*) larvae comparing 3 treatments; Inhibitor 10x (injected with 10x concentrate inhibitor), Inhibitor 5x (injected with 10x concentrate inhibitor) and untreated control (penetrated but no inhibitor inserted).

3.1.2 Geometric Morphometrics Analysis Showed Variation of Jaw Protrusion Between Inhibitor Treated and Untreated Control Specimens.

We next performed geometric morphometrics analysis to test our hypothesis that miRNA-199a levels affect jaw length, with reduced levels causing a benthic-like phenotype (retracted lower jaw, ethmoid plate anteriorly shifted)

Principle Component Analysis comparing the landmark positions of the untreated control and inhibitor treated samples showed that the miRNA treated samples and the non-injected (untreated control) samples separate along the third principle component (PC3, 13% of the variation). PC3 represents the disparity between the symphysis of the Meckel's cartilages and the ethmoid plate, and anterior-posterior shift of articulation Meckel's cartilages, symphysis ceratohyals, and hysosymplectic, as well as ceratobranchial and hysoymplectic width (Fig. 6). There was a significant difference between the mean Procrustes distance of the untreated control and inhibitor treated samples at 0.0510 mm (T-square, 0.02).

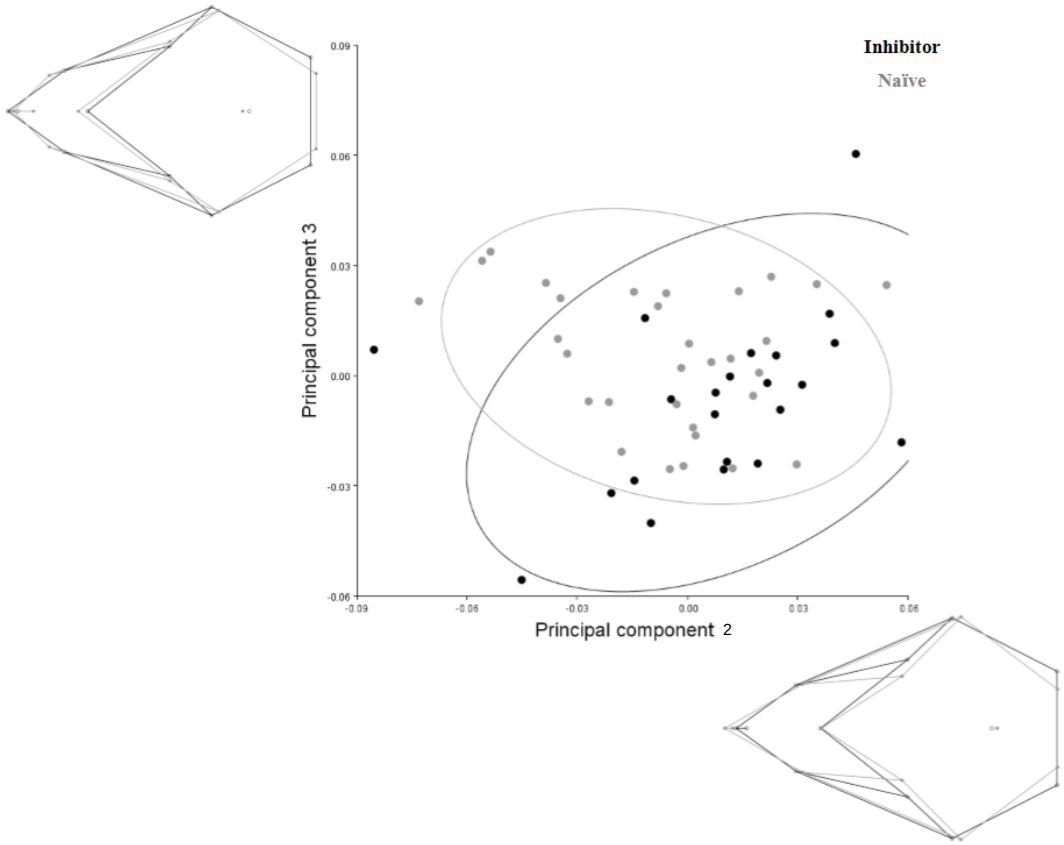

Figure 6. *Treatment principle component analysis.* Scatter plot of the PC2 and PC3 scores for the entire dataset (untreated control and inhibitor treated samples) of Procrustes distances. Confidence ellipses are set to 90%. The scale factor represents a change in Procrustes distance and it is set to 0.1. Wireframes depict shape changes associated with the two Principal Components shown in each graph (PC2 & PC3). In the wire frames, the extreme negative value is shown in grey and the extreme positive values in black.

3.1.3 Geometric Morphometrics Analysis Showed Variation of Jaw Protrusion Between Inhibitor Concentration Treatment Levels.

If different concentration of inhibitor were injected into the yolk of zebrafish eggs, then it was expected that the zebrafish injected with higher concentrations would exhibit lower degree of lower jaw retraction compared to zebrafish injected with lower concentrations, because the influence of the miRNA-199a inhibitor on craniofacial phenotype is expected to be concentration-sensitive up to a saturation point.

Discriminant Functional Analysis showed that 100% of the specimens were classified correctly under the true group; 5x dilution and 10x dilution of the miRNA Inhibitor (Fig. 7). The leave-one-out cross-validation of the discriminant functional analysis showed that 68.8% of the 05x specimens were classified correctly, whereas 50% of the 10x dilution were classified correctly (Table. 3). There was not a significant difference between the mean Procrustes distance of the 5x and 10x dilution treated samples at 0.038 mm (T-square, 0.06). Principle Component Analysis comparing the landmark positions of the 5x and 10x dilutions for inhibitor treated samples showed that PC1, representing ceratohyal and ceratobranchial width as well as the position of the hyosymplectic along the anterior-posterior axis, and PC2, representing this and the added element of disparity between the symphysis of the Meckel's cartilages and the ethmoid plate, collectively accounted for 62.2% of the variance between the two most extreme forms. The two treatment samples separate along the third component (PC3).

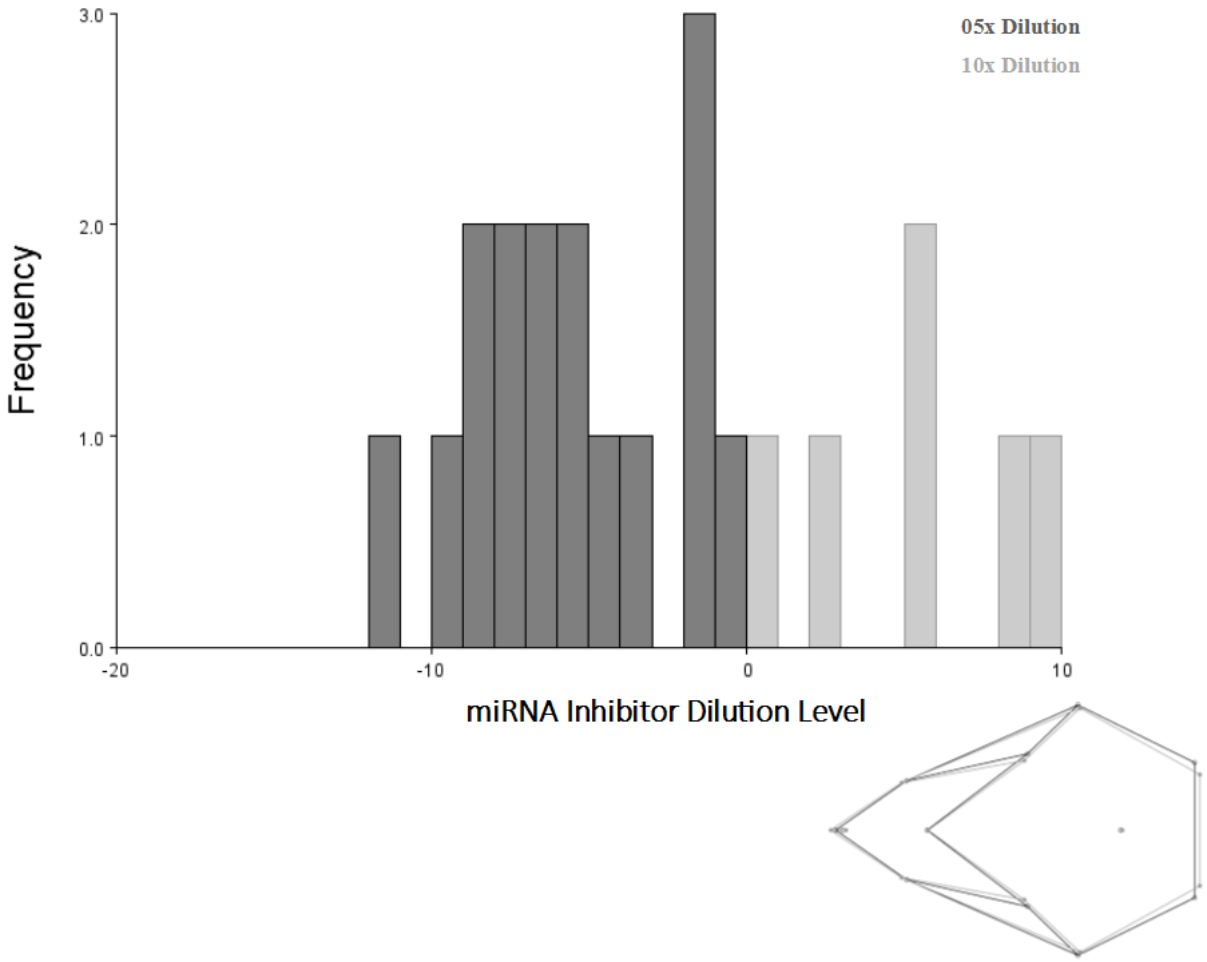

Figure 7. *Concentration Discriminant functional analysis.* Histogram of discriminant scores of the concentration levels within miRNA Inhibitor treated sample (dilution levels 5x and 10x). Permutation number is set to 1000. Wireframes depict mean shape associated with the two concentration groups shown in the graph.

Table 3. *Inhibitor Concentration Classification/ Misclassification.* Classification/Misclassification table for discriminant function and cross-validation of the 05x and 10x dilution of miRNA Inhibitor prioris. The permutation number was set to 1000.

	Classification/ Misclassification Allocation			
Priori (dilution)	**Discriminant Function**		**Cross- validation**	
	05x	10x	05x	10x
05x	16	0	11	5
10x	0	6	3	3

3.2 Whole Mount *in situ* Hybridisation

Initially, WISH was performed on 5dpf zebrafish larvae using a diluted probe, due to human error and no staining occurred and the experiment was deemed unsuccessful (Table. 4). Later, the *in situ* hybridisation was repeated with 3dpf zebrafish and again staining did not occur. Finally, WISH was repeated with 3dpf zebrafish using the correct probe concentration and supervision during pivotal procedures in the protocol. However, staining did not occur and was again deemed unsuccessful.

Table 4. *WISH Staining Results.* Whole mount *in situ* hybridisation staining results for 3 and 5 day post fertilization (dpf) zebrafish (*Danio rerio*) larvae treated with various probe concentrations.

Age (dpf)	Probe Concentration	Staining
5	Diluted	None
3	Diluted	,, ,,
3	Correct	,, ,,

3.3 Luciferase Assay Preparation

3.3.1 Plasmid propagation and test transfection

As a genetic reporter, luciferase is an effective tool to investigate transcriptional regulation in cell cultures. The American firefly *Renilla reniformis* luciferase is commonly used. It is cloned upstream or downstream of regulatory sequences under study (in this case upstream of the 3'UTR of *ets2*, containing a conserved predicted miRNA-199a binding site) and luciferase activity is then used to monitor gene expression (transcriptional activation or degradation of mRNA transcripts) (Brasier *et al.,* 1989). Gene tranfection of reporter constructs into cells is performed using a transfection agent. The plasmid vector is transduced into the cells of interest which are then grown in cell-specific growth media in

24 well plates. A luminometer reader measures the intensity of luminescence from the firefly luciferase and *Renilla* luciferase, which is used as a passive reference (Tannous, 2009).

A luciferase assay for validating the effect of miRNA-199a-3p on *ets2* in human cells was prepared in this investigation, and involved plasmid vector propagation and testing conditions for the transfection of Human Embryonic Kidney (HEK) 293T cells using Lipofectamine 2000, a DNA and RNA transfection agent. Plasmid propagation in E. coli cultures was successful for PIS0 ets2 3'UTR C1 (plasmid containing the *ets2* 3'UTR from Arctic charr), PIS0 ets2 QCH 2.2-2 (derivative of the same plasmid with a mutated recognition site for miRNA-199a-3p), and a *Renilla* luciferase plasmid*;* however this could not be achieved for the original PIS0 plasmid, which is an essential control in these expreiments. The test transfection was performed using GFP expression vectors (to directly visualize transfection efficiency) and fluorescently labelled miRNA-199a-3p inhibitors (in an attempt to visualize successful transfection of small RNAs into cells). Conditions tested are shown in table 1. No positive cells could be found when the cultures were examined under a UV microscope after 24, 48 and 72 hours.

3.4 Arctic charr Primary Cell Cultures

Primary cultures are derived from the cells, tissues or organs of the organism of interest. These are useful for maximising and cultivating biological material for further investigations, such as chromosome analysis and luciferase assays. Protocol to produce reliable monolayer cultures in Arctic charr has not yet been described. However, protocol for Pacific salmon and Steelhead trout has been described by Fryer *et al.* (1965). If the various conditions, procedures and materials described in the *In vitro* embryonic cell monolayer culture protocol for Pacific Salmon and Steelhead Trout by Fryer *et al.* (1965)

were modified and used for Arctic charr embryonic tissue, it was expected that an optimal procedure would be ascertained, because Arctic charr, Pacific Salmon and Steelhead Trout all belong to a single subfamily Salmoninae within the family Salmonidae and consequently have many genetic and biological similarities. Variations of the procedures and materials in this paper were implemented to determine the optimal protocol for preparation of reliable primary monolayer cultures from Arctic charr embryonic tissue.

The observational results indicated that the optimal primary culture conditions for Arctic charr are L15 medium containing 10% FBS and MEM containing 20% FBS. Explant derived cultures also yielded larger and more robust cells. Antibiotic wash of the explants is necessary to prevent contamination and antibiotics (Penicillin/Streptomycin) should be added to the media, however the fungicide Fungizone disrupted cell growth. Arctic charr primary cultures proved to be extremely vulnerable to contamination, resistant to adherence and consistently showed a decline in size and viability after seven days. Multiple factors including, temperature, cell longevity and mortality, were attributed to this lack of long-term, stable culture.

3.4.1 Test Cultures Compared Seed Type & FBS Concentrations.

In order to determine whether explants or single cell cultures were more effective for Arctic charr both types were tested and compared. Furthermore, the test cultures were subjected to various FBS concentrations to determine the optimal serum conditions that stimulate the most effective cell adherence and growth in Arctic charr primary cultures.

Explants yielded larger adhering cells but were more vulnerable to contamination for all serum concentration levels tested (Table. 6). L15 containing 10% FBS appeared to give higher levels of cell adherence.

Table 6. *Seed Type & Concentration Observations.* Modifications of fetal bovine serum (FBS) concentration (5, 10, and 20%) in Leibovitz medium supplemented with 0.3 gm/L L-glutamine **(**L15) media and seed type (explant and single cell) for Arctic charr embryonic single cell culture.

Treatment	**2 days**	**5 days**	**7 days**	**14 days**
Explants L15 FBS 5%	Cells adhering and dispersing from explant. Larger cells.	Fungal contamination surrounding the explant. Some small cells remain at the peripheries. TERMINATED.	/	/
Explants L15 FBS 10%	" "	Cells adhering well. High concentrations of small cells, well dispersed. Cell size reduced.	Cells adhering and increasing in size.	Cells dead
Explants L15 FBS 20%	" "	Lower concentrations, fairly sparse. Smaller cells.	" "	" "
Single Cell L15 FBS 5%	High concentration of cells in small aggregations and or single cells. Smaller cells.	" "	Fibroblas-like clusters, large spread.	" "
Single Cell L15 FBS 10%	Lower concentrations, fairly sparse. Smaller cells.	" "	Lower abundance and density.	" "
Single Cell L15 FBS 20%	" "	" "	" "	" "

3.4.2 Test Cultures Compared Media Types, FBS & $NaHCO_2$ Concentrations.

In order to determine whether L15 or MEM media were more effective for Arctic charr both types were tested and compared. Furthermore, the test cultures were subjected to various FBS concentrations to determine the optimal serum conditions that stimulate the most effective cell adherence and growth in Arctic charr primary cultures. Different concentrations of $NaHCO_2$ were also added to determine if is enhances survival or whether it is indeed necessary for buffering, as cells were grown under atmospheric conditions and not at elevated CO_2 levels and the L15 medium is made for such culture conditions.

All wells yielded populations of small adhering cells after two days (Table. 7). After 5 days the condition and density of the cells in 10mM and 20mM $NaCO_2$ deteriorated, whereas those in the control, and 30mM $NaCO_2$ showed high cell densities and were subcultured to prevent overcrowding. Overall, 5 % FBS in both media types yielded the highest cell densities. After 7 days cell size decreased drastically and adherence was minimal until cell death occurred.

Table 7. *Media Type, FBS & $NaCO_2$ Concentration Observations.* Modifications of fetal bovine serum (FBS) serum concentration (5, 10, and 20%) and $NaHCO_2$ (0, 10, 20, 30 mM) in Leibovitz medium supplemented with 0.3 gm/L L-glutamine (L15) and Minimum Essential Medium (MEM) media and for Arctic charr embryonic single cell culture. Each well contained 0.083 ml of single cell suspension, 0.05 ml Fungizone and 0.1 ml *Penicillin streptomycin* (PenStrep).

Treatment			**Observations**			
Media Type	**FBS (%)**	**$NaHCO_3$ (mM)**	**2 days**	**5 days**	**7 days**	**14 days**
L15	5	control	Cells adhering. Low density. Small cell size.	High cell density. Subcultured.	Cell size shrinking	Cells dead
		10	" "	Low cell density. Small cells.	" "	" "
		20	" "	" "	" "	" "
		30	" "	High cell density. Subcultured.	" "	" "
	10	control	" "	" "	" "	" "
		10	" "	Low cell density. Small cells.	" "	" "
		20	" "	" "	" "	" "
		30	" "	High cell density. Subcultured.	" "	" "
	20	control	" "	" "	" "	" "
		10	" "	Low cell density. Small cells.	" "	" "
		20	" "	" "	" "	" "
		30	" "	High cell density. Subcultured.	" "	" "
MEM	5	control	" "	" "	" "	" "
		10	" "	Low cell density. Small cells.	" "	" "
		20	" "	" "	" "	" "
		30	" "	High cell density. Subcultured.	" "	" "
	10	control	" "	" "	" "	" "
		10	" "	Low cell density. Small cells.	" "	" "
		20	" "	" "	" "	" "
		30	" "	High cell density. Subcultured.	" "	" "
	20	control	" "	" "	" "	" "
		10	" "	Low cell density. Small cells.	" "	" "
		20	" "	" "	" "	" "
		30	" "	High cell density. Subcultured.	" "	" "

3.4.3 Test Cultures Compared Media & Antibiotic Types.

In order to determine whether L15 or MEM media were more effective for Arctic charr both types were again tested and compared in conjunction with different antibiotics (Penicillin/Streptomycin and Fungizone).

Initially, after 2 days all wells yielded fairly large adhering cells (Table. 8). High and moderate cell abundance and growth was observed for the control and Pen/Strep antibiotic treatment wells respectively after 5 days. However, lower abundance and growth was observed for all treatments containing Fungizone after 5 days. Overall, L15 + 10% serum and MEM + 20% FBS seemed most optimal, with L15 yielding slightly larger cells than MEM. Contamination caused the termination of several wells after 7 days, which mainly occurred in the control treatments and adjacent wells. The remaining control wells showed high cell adherence and size whereas all other treatments showed a decreased in adherence and cell size. Soon after all wells had either become contaminated or cell condition had deteriorated such that they were deemed dead.

Table 8. *Media & Antibiotic Type Observations.* Modifications of antibiotic treatment (Pen/Strep + Fungizone, Pen/Strep, Fungizone, and Control) and fetal bovine serum (FBS) serum concentration (5, 10, and 20%) in Leibovitz medium supplemented with 0.3 gm/L L-glutamine (L15) and Minimum Essential Medium MEM media and for Aquaculture Arctic charr embryonic explant cell culture. Each well contained 1-2 mm^2 of embryonic explant, 0.05 ml Fungizone and/or 0.1 ml *Penicillin streptomycin* (Pen/Strep).

		Treatment	**Observations**			
Media Type	**FBS (%)**	**Antibiotics**	**2 days**	**5 days**	**7 days**	**14 days**
L15	5	Pen/Strep + Fungizone	Cells adhering. Fairly large cells	Moderate Growth	Cells Shrinking	Cells dead
		Pen/Strep	" "	Moderate Growth	Cells Shrinking	" "
		Fungizone	" "	Less growth	Contamination	/
		Control	" "	High Growth	Contamination	/
	10	Pen/Strep + Fungizone	" "	Moderate Growth	Cells Shrinking	Cells dead
		Pen/Strep	" "	Moderate Growth	Cells Shrinking	" "
		Fungizone	" "	Less growth	Less growth	" "
		Control	" "	High Growth	High Growth	" "
	20	Pen/Strep + Fungizone	" "	Moderate Growth	Cells Shrinking	" "
		Pen/Strep	" "	Moderate Growth	Cells Shrinking	" "
		Fungizone	" "	Low Growth	Contamination	/
		Control	" "	High Growth	Contamination	/
MEM	5	Pen/Strep + Fungizone	" "	Moderate Growth	Cells Shrinking	Cells Dead
		Pen/Strep	" "	Moderate Growth	Cells Shrinking	" "
		Fungizone	" "	Low Growth	Low Growth	" "
		Control	" "	High Growth	High Growth	" "
	10	Pen/Strep + Fungizone	" "	Moderate Growth	Cells Shrinking	" "
		Pen/Strep	" "	Moderate Growth	Cells Shrinking	" "
		Fungizone	" "	Low Growth	Low Growth	" "
		Control	" "	High Growth	High Growth	" "
	20	Pen/Strep + Fungizone	" "	Moderate Growth	Contamination	/
		Pen/Strep	" "	Moderate Growth	Contamination	/
		Fungizone	" "	Low Growth	Contamination	/
		Control	" "	High Growth	Contamination	/

4 Discussion

4.1 Phenotypic Analysis

4.1.1 Geometric Morphometrics Analysis Showed Variation of Jaw Protrusion Between Inhibitor Treated and Untreated Control Specimens.

In accordance with the prediction miR-199a inhibitor injected zebrafish showed phenotypes that mimic the benthic craniofacial morphology, the results of this investigation demonstrated that the inhibitor treated zebrafish has a more retracted lower jaw and an anteriorly shifted ethmoid plate, compared to the untreated control (non-injected). Thus, the prediction that lower expression of miRNA-199a is associated with increased lower jaw retraction (benthivorous ecomorph phenotype) in Arctic charr was supported by the findings.

miRNA-199a has been implicated in playing a role in the trophic polymorphism between benthic and limnetic Arctic charr morphs due to the downstream targets of miRNA-199a and variation in temporal and spatial expression between the morphs during embryonic development. Kapralova *et al.* (2014) identified 72 miRNAs with differential expression between the SB and AC morphs, at multiple developmental stages, that are involved in developmental processes such as skeletogenesis and myogenesis. miRNA-199a is highly expressed in the AC morph during embryonic development, however expression is low in the SB morph. During development the expression level of miRNA-199a is initially elevated before decreasing as cartilage and bone form, suggesting that it plays an important role in

early chondrogenesis (Chen *et al.*, 2004). This expression occurs in the whole craniofacial structure, concentrated mainly in the Meckel's cartilage, hyoid arch, ethmoid plate and gill arches in Arctic charr, as well as the epithelia surrounding the cartilage of the pharyngeal arches, oral cavity and pectoral fins in zebrafish (Skúladóttir, 2014).

Computational approaches by Skúladóttir (2014) revealed the putative target of miRNA-199a to include *ets2* as a binding site and target gene in both the conservation and candidate gene approach, due to sharing similar expression pattern during development in zebrafish and therefore also a likely target in Arctic charr. *Ets2* only has one binding site which is analogous to miRNA-199a and is conserved between Arctic charr and zebrafish. Furthermore, WISH (Whole mount *in situ* hybridisation) staining and cryosectioning by Ligthart (2014) showed that the patterns of miRNA-199a and *ets2* expression were both found in cells outside of the cartilage within the pharyngeal arches during embryonic development in LB and PL Arctic charr. The link between miRNA-199a and *ets2* is speculative, and requires confirmation by a luciferase assay test, but their expression occurred in the same direction, thus, supporting that *ets2* is a probable target of miRNA-199a.

The transcription factor *ets2* is likely the regulator of a gene network described by Ahi *et al.* (2014). This network was found to be differentially expressed during the head development of contrasting Arctic charr morphs (benthic and limnetic morphs from Thingvallavatn). *Ets2* shows high levels of expression during cartilage development of the craniofacial structures (Maroulakou et al. 1994). The benthic and limnetic morphotypes show differences in the transcriptional dynamics of *ets2* (Ahi *et al.*, 2014). Developing cartilage, such as skull precursor cells, show high levels of *ets2* expression and overexpression results in skeletal abnormalities in mice (Sumarsono *et al.,* 1996). The decrease in miRNA-199a concentration

by treating zebrafish embryos with miR-199a inhibitor can alter the craniofacial structure through its direct target *ets2*. However, more studies are needed to test this

The craniofacial morphology shows ecological convergence and is a useful indicator of feeding type between many fish species (Agostinho & Hahn, 2001; Hulsey *et al.* 2005; Qi, 2002;). In particular, the lower jaw morphology and mouth position are closely related to food resource type (Qi, 2002). Benthic morphs typically show a more subterminal mouth to exploit benthic dwelling aquatic arthropods (Morita & Suzuki, 1999). SB and LB ecomorphs in Thingvallavatn have jaw morphologies that exemplify this, whereas the PL and PI ecomorphs display jaw morphologies typical of pelagic species (Snorrason *et al.,* 1989). Disruptive selection, such as observed in Arctic charr, favours one phenotypic extreme, hence one type of prey, at the expense of another. This trade-off in feeding performance allows niche partitioning and decreases intra-specific competition thereby providing a mechanism for coexistence (Qi, 2002). Thus it can be speculated that variation in the concentration of miRNA-199a during development may contribute to the craniofacial specialisation of the Arctic charr ecomorphs, as well as the retention of such polymorphism in Thingvallavatn.

4.1.2 Geometric Morphometrics Analysis Showed Variation of Jaw Protrusion Between Inhibitor Concentration Treatment Levels.

Different levels of miRNA inhibitor yielded observable phenotypic differences, regarding the degree to which craniofacial morphologies mimicked the benthic ecomorph. However, the sample size was not sufficient to yield a significant difference between these samples, although the t-values were close to significant for 95% confidence level. Thus, the prediction

that zebrafish injected with higher concentrations would exhibit higher degrees of lower jaw retraction compared to zebrafish injected with lower concentrations, was not confirmed by the findings of this investigation. The hypothesis that the influence of the miRNA-199a inhibitor on craniofacial phenotype is concentration-sensitive requires further investigation with larger samples sizes.

Contrary to this Robertson *et al.* (2010) showed the effect of miRNA inhibitors has a dose-dependent impact on miRNA expression, which is dependent on the nucleotide matching of the inhibitor with that of the target site of the miRNA. Consequently, the concentration of a particular miRNA can be exogenously controlled by manipulating its analogous mRNA target or the miRNA itself using miRNA inhibitors (Orom *et al.,* 2006). Furthermore, the influence of miRNAs on mRNA, transcription factor, protein and gene targets modulation is known to be concentration-dependent (Wu *et al.,* 2013). miRNAs indirectly adjust gene expression in thousands of genes which serves as a protein fine-tuning system which meets the demand of particular cells at any point in time (Bartel & Rajewsky, 2008). Dosage is positively correlated with penetration rate and phenotypic severity (Bedell *et al.,* 2011).

The results of this investigation revealed no significant effect of dose on phenotype severity and were not consistent with findings in the literature (Bartel & Rajewsky, Bedell *et al.,* 2011; Wu *et al.,* 2013) and can be attributed to a multiple factors. Firstly, the penetration of miRNA inhibitor may not have been exhaustive. Differences in miRNA inhibitor concentration injected in the yolk may not translate to differences in miRNA inhibitor concentration that actually penetrate into the cells. Furthermore, the different doses of miRNA inhibitor injected may not have been distinct enough to elicit significant differences in phenotype. There is some possibility that this miRNA inhibitor shows non dose dependent effect on phenotype. The most likely explanation for this unexpected outcome was that a

lack of sufficient sample size reduced the power of the findings resulting in a non-significant t-value. It is expected that in further investigations, in which this has been rectified, the results would yield significant differences in the severity of phenotype determined by miRNA inhibitor concentration.

4.1.3 Limitations & Future

Accurate phenotypic analysis is often difficult due to the collateral effects of RNA interference, due to the effects miRNA can have on gene expression (Leong, 2012). Knockdown of miRNA function may not necessarily result in any observed developmental modifications (Kloosterman *et al.,* 2007). This can be due to the very subtle effect miRNAs can have on gene expression which may not translate into an observable phenotype. Alternatively, redundancies caused by overlapping in miRNA targets may compensate for the knockout of the function of any one miRNA. Such mechanisms instil robustness and stability in the gene expression. The miRNA inhibitor used in this investigation was associated with lower non-target miRNA knockdown rates and toxicity, compared to other antisense oligonucleotides (Braasch & Corey, 2001; Fluiter *et al.,* 2010), however some caution should be exercised when analysing the results.

The use of a model species also brings about limitations because miRNAs have been shown to target different mRNAs between different species and even cell types (Nakamura *et al.,* 2011). The impact this has on the validity of the findings of this investigation in minimised as *Ets2* only has one binding site which is analogous to miRNA-199a and is conserved between Arctic charr and zebrafish (Skúladóttir, 2014). Furthermore, zebrafish is widely regarded as a representative model species for many vertebrates and the major elements of

the splanchnocranium are present in the embryo of the zebrafish and the Arctic charr, however there are some clear positional shifts and size differences.

Phenotype varies between individuals due to the environmental effects or variation within the gene pool. The zebrafish were reared in constant environmental conditions within the zebrafish core facility and were samples were synchronised, in order to reduce these lurking variables. Furthermore, the sample size needed to be large enough to account for random variation in size of body parts. The overall sample size was sufficient to yield significant results, however the treatment level groups were not adequate. In further research larger sample sizes should be used to increase the power of the statistical analysis.

Further investigations should also be aimed to investigate the dose-response curve of this miRNA inhibitor by titrating its concentration, thereby establishing a dose range from which phenotype is no longer induced or observable to a threshold before non-specific effects occur (Eisen & Smith, 2008).

4.2 Whole Mount *in situ* Hybridisation

4.2.1 Proteinase K Function

The hypothesis for this section of the investigation was neither supported, nor disproved by the findings of this investigation due to the non-success of the WISH experiment. Initially, the first two WISH experiments were deemed non-successful due to a highly diluted probe caused by human error. This was remediated in a third repetition of WISH, all solutions were remade fresh and extra supervision was present to ensure accuracy in pivotal procedures, however staining did not occur and the experiment was again deemed non-successful. It was speculated the cause of this was a non-functioning proteinase K compound. Proteinase K is

a serine protease with a wide array of targets enabling rapid action and inactivation of endogenous RNases and DNases (Liu *et al.,* 2011). This treatment also ensures effective probe penetration essential for WISH because it breaks down structural barriers and removes cellular debris, as well as inactivating these nucleases that could potentially degrade the DNA or RNA of interest. It was likely that effective probe penetration for miRNA-199a did not occur due to the persistence of cellular debris and nucleases without competent proteinase K treatment.

4.2.2 Non-expression

Despite the speculated non-functioning proteinase K compound it was also possible the WISH would not have worked on the 5 dpf zebrafish because chondrification of the six different cartilage types, the most common hyaline, in the cranium is complete after five days (Yelick & Schilling, 2002), therefore miRNA-199a would no longer be expressed in the head. However, chondrification begins after 2 dpf, therefore staining would have been expected for the WISH repetitions using 3 dpf zebrafish.

4.2.3 Limitations and Future

Thisse & Thisse (2008) noted that the penetrative capabilities of RNA probes was reduced in zebrafish after 2 dpf (days past fertilisation) and later developmental stages. It is plausible that the WISH in this investigation yielded unsuccessful results due to the older age of the larvae used. In future investigations WISH should be performed on histological sections or earlier larval stages. The quality of all materials used should also be ensured.

4.3 Luciferase Assay Preparation

4.3.1 Plasmid Transduction and Concentration

The plasmid yield of PIS0 ets2 3'UTR C1 and PIS0 ets2 QCH 2.2-2 may have been greater than that of *Renilla* due to lurking variables such as differences in the quality of the initially inoculated plasmid or it's the efficiency of its transformation. Dower *et al.* (2008) showed that *E.coli* can be transformed highly efficiently although the yield of transformants varies greatly according to many factors including DNA concentration, cell concentration, temperature and the incubation period of the cells. It is possible that any one of these factors may have varied to cause a reduced plasmid yield of *Renilla.*

The inability to cultivate PIS0 inoculated *E.coli* suggests that a substance within the DNA compound may have been lethal to the bacterial cells. As a result no colonies could form when subjected to transformation with this particular batch of PIS0.

4.3.2 Test Transfection

The lack of success for the test transfection in this investigation can possibly be attributed to the DNA complexes adhering to the walls of the pipette tip during extraction and mixing. As a result the DNA concentration was diluted and no observations resulted. Other potential causes might be too little DNA or damaged plasmids. The transfection reagent used was new. Repeating the test is necessary before drawing any conclusions from these initial results.

4.3.3 Limitations and Future

In order to improve transformation efficiency of *E.coli* electroporation, subjecting the cells to a brief intense electrical field, in combination with heat shock in future investigations. The PIS0 should be replaced with a new batch to ensure its quality.

Future test transfection protocol should include the use of specialised pipette tips that prevent the adherence of DNA complexes such as Axygen® Maxymum Recovery® pipette tips.

4.4 Arctic charr Primary Cultures

An optimal *In vitro* embryonic cell monolayer culture protocol was not established for Arctic charr and the prediction that modification of protocol for Pacific Salmon and Steelhead Trout by Fryer *et al.* (1965) could be applied was not consistent with the findings of this investigation.

4.4.1 Optimal Media Conditions

The most optimal media type was L15 containing 10% FBS. This was speculated to be because 10% FBS represents an intermediate between a concentration too low to stimulate cell growth and a concentration with a potency that interferes with cell adherence. Lowering FBS after one week can increase yields. Explant seed types yielded larger adhering cells compared to single cell suspensions and was attributed to a lack of cell shock that may occur due to trypsin digestion to generate a single cell suspension. NaCO2 was not a necessary addition the media. The highest growth and adherence was noted for the antibiotic control treatment, despite increased vulnerability to contamination. This suggests that the presence of antibiotics in the medium may limit or disrupt cellular processes. Disruption appeared to

be particularly potent in Fungizone treated wells. It appeared that Fungizone prevented cell growth and cell adhering, whereas in combination with Pen/Strep the impact of this was less.

4.4.2 Limitations and Future

Despite the fact that no stable long term cultures were produced in this investigation these findings can be used to influence further research to find the optimal protocol for *In vitro* embryonic cell monolayer cultures for Arctic charr. Issues arose after 7-14 days for the cultures which suggests a short lifespan for the cells and a lack of cellular division. If the longevity and vulnerability to contamination was addressed this would increase the prospects for successful Arctic charr primary cultures in the future.

Despite the close evolutionary relatedness of *Salvelinus* and *Oncorhynchus* in the Salmoninae subfamily, it appears that the cellular processes in embryonic cells between these genera are too distinct to produce a protocol that successfully yields *In vitro* embryonic cell monolayer cultures for both. Arctic charr populations have the most northerly range of any freshwater fish (Klemesten *et al.,* 2003), therefore cellular processes may have adapted to these cooler and harder environmental conditions which renders the embryonic cells unsuitable for *in vitro* embryonic cell monolayer culture using the protocol base generated by Fryer *et al.* (1965) or for cultures in general. In the future modification should be made from protocol designed for fresh water Salmonidae species with higher latitudinal ranges.

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