# Frequency and effects on survival of abnormal otoliths in hatchery-reared Atlantic salmon (*Salmo salar*)

Benedikte Austad


Master thesis

Center for Ecological and Evolutionary Synthesis
(CEES)
Department of Biosciences
Faculty of Mathematics and Natural Sciences
UNIVERSITETET I OSLO

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http://www.duo.uio.no

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# ABSTRACT

Saggital otoliths are inner-ear structures of all teleost fish with functional importance for hearing and balance. They usually consist of aragonite, a polymorph of calcium carbonate, but may also take the form partly or entirely of vaterite, a different polymorph of calcium carbonate. These vateritic otoliths are classified as abnormal and occur sporadically in wild fish, but are much more frequent in hatchery-reared fish. Abnormal otoliths have consequences for the inner-ear functions of fish directly, and may be a symptom of environmental stress, affecting survival indirectly.

The experiment was divided into two parts. The aim of the first part was to assess differences in frequency of abnormal otoliths and degree of abnormality (% vaterite) in the abnormal otoliths of different groups of hatchery-reared Atlantic salmon smolt. The groups differed in parental brood stock origin, number of generations in hatchery or mean annual temperature in hatchery. The aim of the second part was to determine whether abnormal otoliths affect survival of Atlantic salmon. Smolt from the corresponding groups were released for ocean migration two consecutive years and the otoliths of the returning adults were subsequently collected. Frequency of abnormal otoliths and degree of abnormality (% vaterite) in the abnormal otoliths of the adults were then compared to those found in the smolt. This formed the basis for evaluating the potential effects on survival.

In this experiment, larger smolt and increased number of generations in hatchery correlated with higher frequency of abnormal otoliths, indicating growth and population effects on the formation of abnormal otoliths. The returning adults had a lower frequency of abnormal otoliths, but they did occur in a number of the adults and in high coverage (% vaterite), indicating that abnormal otoliths are not detrimental to survival, but may have a significant negative effect.

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# 1. Introduction

## 1.1 The Atlantic salmon

Atlantic salmon (*Salmo salar*) have diverse life histories, most being anadromous, meaning they migrate between fresh water and salt water where they utilize the habitat best suited for the particular life stage; i.e. feeding or breeding (Jonsson and Jonsson 2011, Thorstad, Whoriskey et al. 2011, Thorstad, Whoriskey et al. 2012). For Atlantic salmon, the migration between freshwater habitat and ocean feeding grounds in the North Atlantic can be over 2000 km long (Jonsson and Jonsson 2011).

The salmon embryos incubate and hatch in the river gravel, emerge as fry and develop into parr. They remain as parr a few years before developing into smolt, ready to undergo the long ocean migration (Keenleyside and Yamamoto 1962, Hansen and Quinn 2011). In order to cope with the impending high salt levels in the seawater and other factors characteristic for the open ocean pelagic environment, the parr undergo a range of extensive changes known as smoltification. This smoltification involves morphological changes such as developing slimmer, silvery bodies and physiological changes such as increases in gill Na+K+ATPase activity, allowing for an increase in osmoregulatory ability necessary to tolerate high salinity water (Nichols, Edo et al. 2008, Jonsson and Jonsson 2011, Thorstad, Whoriskey et al. 2012).

Atlantic salmon spend from one to several years at sea to grow and mature before they migrate back to the site where they themselves originated to reproduce (Jonsson and Jonsson 2011). But due to the many threats to ocean survival, this is easier said than done. The initial introduction to the ocean for the post-smolt is one of the most vulnerable phases in the salmon's life because of high predation risk, and increasingly so if growth is weak and ocean temperatures are low (Friedland, Hansen et al. 2000). Marine mortality is generally high for Atlantic salmon and, usually, less than 10% survive from smolt

stage to returning adults (Jonsson and Jonsson 2004). In Norway, the ocean survival of Atlantic Salmon has been in decline the last decades. In the river Imsa, for example, the survival has gone from around 17% in the 1980s to between 1 and 4% recent years (Anon 2018). Furthermore, hatchery-reared salmon smolt examined in the river Imsa have even lower ocean survival rates than wild. This is the norm; hatchery-reared Atlantic salmon generally have lower ocean survival than their wild conspecifics**.** This may be because wild salmon live in natural conditions and are therefore exposed to threats and challenges that hatchery-reared salmon do not experience. They may thus develop anti-predator behavior and foraging skills the hatchery-reared salmon do not (Thorstad, Uglem et al. 2011). Additionally, hatchery-reared salmon have a much higher egg to smolt survival rate than wild, resulting in a significantly lower selection pressure (Jonsson, Jonsson et al. 2003).

The Atlantic salmon that survive their marine phase find their way back to their natal habitat with high precision (>90% on average), and low percentages straying to other rivers (Fleming 1996, Thorstad, Whoriskey et al. 2011). This site fidelity is an adaptive trait attributed to the knowledge that the distinct habitat is suitable for breeding and rearing of progeny, as the parents themselves successfully grew up there. Homing results in reproductive isolation, which in turn allows local adaptation to the specific natal habitat (Dittman and Quinn 1996, Fleming 1996). Therefore, different salmon populations differ both ecologically and genetically, and Atlantic salmon show high diversity in life history traits because they are so strongly influenced by environmental differences and the local conditions they experience (Thorstad, Whoriskey et al. 2011).

Exactly how salmon find their way back to their native river with such high precision is complex and somewhat of a mystery. However, there is established knowledge about contributing factors. Navigation most likely involves a combination of detection of celestial, chemical, oligotrophic and geomagnetic cues (Putman, Lohmann et al. 2013). The migration route may be learned by smolt on their outward migration through recognition of route-specific external

chemical and oligotrophic signals (Hansen and Quinn 2011, Jonsson and Jonsson 2011). It has also been found that juvenile salmon imprint on the magnetic field of the area where they initially entered the ocean and detect the same magnetic field upon return (Hansen and Quinn 2011, Putman, Lohmann et al. 2013).

## 1.2 The role of the otoliths

Otoliths are structures in the inner ear of all teleost fish. The inner ear serves three purposes: detection of angular and linear acceleration and the detection of sound. It generally consists of three semicircular canals and three otolithic organs forming three pouches containing otoliths (Schulz-Mirbach, Ladich et al. 2019). These structures are of great importance to the fish's sensation of gravity, ability to hear, mobility and balance (Reimer, Dempster et al. 2016). The largest of the three otoliths, sagittae, is popularly used when conducting studies of otoliths of teleost fish (Falini, Fermani et al. 2004).

Detection of sound is possible for fish when the inner ear is stimulated by acoustic particle motion (Schulz-Mirbach, Ladich et al. 2019). Because the tissue of the fish body has a similar density to the surrounding water, this particle motion is not detectable without otoliths (Popper and Hawkins 2018). Otoliths are calcite structures and have a much higher density than the water and surrounding tissue. Sound pressure causes a slower movement of the otolith than the soft tissue and creates relative motion between the otolith and the sensory hair cells (Schulz-Mirbach, Ladich et al. 2019). This is how teleost fish with the help of their otoliths can detect sound directly. Some teleosts detect sound indirectly as well, through sound pressure stimulating their gas-filled swim bladders, although still depending on their otoliths. These fish hear a broader range of sound frequencies and are hearing specialists. Salmonids are, however, hearing generalists; they only detect sound directly and do not get any "help" from their swim bladder. Salmonids are generally not seen as having particularly great senses of hearing (Hawkins and Johnstone 1978, Popper and Lu 2000, Schulz-Mirbach, Ladich et al. 2019).

Usually, sagittal otoliths consist of aragonite, a polymorph of calcium carbonate, and are chemically inert with high purity (Falini, Fermani et al. 2004, Schulz-Mirbach, Ladich et al. 2019). This means that the otolithic structure already produced does not change; it only grows with daily accretion of new, permanently retained material (Campana 1999, Schulz-Mirbach, Ladich et al. 2019). Sometimes, however, the sagittal otoliths acquire the formation partly or entirely of vaterite, a different, less dense polymorph of calcium carbonate. These otoliths are categorized as abnormal (Falini, Fermani et al. 2004, Schulz-Mirbach, Ladich et al. 2019). Salmonids are especially susceptible to the vaterite form, and the differences in properties of the polymorphs have consequences for the movement of the otoliths in the inner ear (Sweeting, Beamish et al. 2004).

The abnormal variations of otoliths do occur sporadically in fish in their natural habitats; however, studies indicate that the occurrence of the abnormal sagittal otoliths in farmed fish is much higher. Analyses on numerous mass exploited species indicate that abnormal otoliths occur in ~10 % of wild fish, but in ~ 50-80% of hatchery-reared fish (Oxman, Barnett-Johnson et al. 2007, Reimer, Dempster et al. 2016).

One of the consequences of this vaterite replacement in the sagittal otoliths are loss of hearing sensitivity across most of the known hearing range for salmonid fish (Oxman, Barnett-Johnson et al. 2007, Reimer, Dempster et al. 2016). Additionally, the more prominent the coverage of vaterite in the otoliths, the more severe the hearing impairment likely becomes, and the density differences between vaterite and aragonite may affect hearing directionality specifically (Reimer, Dempster et al. 2016). Furthermore, the formation of abnormal otoliths may be a symptom of stress and reduced survival on a larger scale.

## 1.3 The aim of the thesis

Given the importance of otoliths for the inner-ear functions of teleost fish and that abnormal otoliths are so common in hatchery-reared fish, a relevant question is how these may affect survival. This thesis' primary objective is to examine the otoliths of hatchery-reared Atlantic salmon smolt that have been released for ocean migration, and that may or may not return to their native river as adults, and to see if otolith abnormalities and ability to return have a correlation.

The aim of this thesis is to examine the following questions:

- **Are there differences between smolt groups (populations and/or temperature regimes in hatchery) in frequency of abnormal otoliths and/or degree of vaterite in the abnormal otoliths?**

- **Is there a higher frequency of abnormal otoliths and/or degree of vaterite in the abnormal otoliths in the smolt than those of the returning adults?**

# 2. Materials and methods

## 2.1 Overview

In the context of this thesis otoliths refers only to saggital otoliths. In 2016 and 2017 hatchery reared Atlantic salmon were released in the lower part of the river Imsa (N=6958 and N=8933, respectively). Fish from these releases were later recaptured as adults when ascending to the river Imsa. In addition, to allow comparing frequencies of abnormal otoliths a total of 1016 smolt of the 2016 cohort and 1047 of the 2017 cohort were euthanized and their otoliths analyzed to determine a baseline frequency of abnormal otoliths in smolt. The hatchery-fish used in the 2016 and 2017 release consisted of fish from different groups/populations (**Table 2.1**). The otoliths of all the returning adult fish were collected.

## 2.2 Experimental location

The experiment was conducted over two years at the NINA research station, Ims, located by the river Imsa in Rogaland in western Norway (58°50'N, 6°E). The river drains into the Høgsfjord estuary, is approximately 1 km long, and contains a small population of anadromous Atlantic salmon (Jonsson and Jonsson 2016). The salmon here migrate to the North Atlantic to feed in the ocean, and return as adults to spawn, usually after one year. The research station is located here with aims to acquire knowledge about the management of wild salmon populations (NINA 2019). There is no fishing in Imsa and the salmon population here has been monitored since 1975 (Jonsson and Jonsson 2016).

The river Imsa is equipped with at a Wolf trap (**Picture 2.1**) located 150 meters above the river estuary, catching all descending fish over ~ 10 cm long, and a box trap catching all ascending fish (Jonsson, Jonsson et al. 2017).

**Picture 2.1:** The Wolf trap located at the bottom part of Imsa (Photo: Knut Bergersen, NINA).

## 2.3 Experimental fish

The experimental fish consist of hatchery-reared salmon smolt. In total the fish in this experiment originate from three different populations and two different years of release: the river Imsa population (2016 and 2017), the river Lone population (2016 and 2017) and the river Figgjo population (not released) (**Table 2.1**). The groups also differ in number of generations in hatchery; the wild parental brood stock of the Lone populations dates back around 30 years, the smolt of the Imsa and Figgjo population are progeny of first generation hatchery-reared parents (personal communication, Knut Bergersen, NINA).

The rivers Imsa and Figgjo are located quite near each other (both 59° N), while Lone is located a bit further north (60°N). Lone is a grilse population, meaning the vast majority of the fish are mature after one winter in the ocean, while the Imsa and Figgjo populations are intermediate, meaning the majority of the fish are mature after one winter in the ocean but with a significant proportion of the fish maturing after two winters in the ocean. The fish of the Lone population are therefore naturally somewhat smaller at mature size (mean adult length±SD; 575±SD 46 mm) than those of Imsa and Figgjo (mean adult length 604±69 and 625±60 mm, respectively) (Hansen and Jonsson 1989, Jonsson, Jonsson et al. 2007).

Eggs of the Imsa population were incubated in either natural water temperatures following seasonal fluctuations from approximately 2-8°C in winter to around 20°C in summer (Imsa Cold), or in experimental water temperature conditions of approximately 7-8°C, not following seasonal fluctuations (Imsa Warm) (Jonsson, Jonsson et al. 2016, Jonsson and Jonsson 2018). The eggs incubated in experimental water temperatures, not following the natural fluctuations, experienced on average approximately 3°C warmer water temperatures (Jonsson and Jonsson 2018). Because of the importance of temperature in developmental stages one can expect a difference in size between the groups of smolt. The total lengths of the smolt were measured in this experiment. In total, there are four different groups analyzed as part of this experiment, differing in either temperature regime in hatchery and/or population of origin.

## 2.4 Experimental design

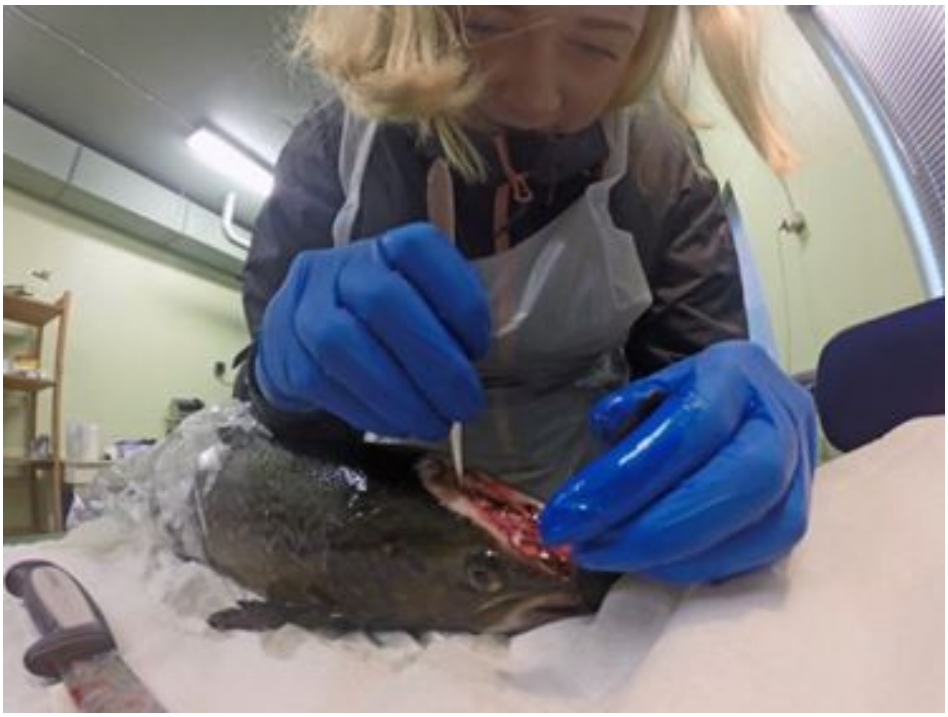
Juveniles were raised to smolt stage, and a number of random sampled smolt were selected for otolith extraction whereas a number of smolt were, for most of the groups, released in Imsa below the dam and allowed to migrate to sea (**summary in Table 2.1**). Two consecutive releases were made; the first in 2016 consisting of individuals tagged with Carlin tags (Carlin, 1955) and the second in 2017 consisting of individuals tagged with either Carlin tags or 12 mm Passive Integrated Transponder (PIT) tags. The variation in the number of smolt released or tags used is due to the fact that the fish were also part of other experiments not related to this one. The returning fish of these groups were subsequently recaptured in the box trap located above the river Imsa estuary. Smolt released in 2016 are expected to return in 2017 and 2018, whereas smolt released in 2017 are expected to return in 2018 and 2019, the 2019 not collected.

**Table 2.1:** No. of Atlantic salmon smolt and adults and no. of otoliths analyzed, and to which group and year of release they belong. "Cold" and "Warm" in group names refers to water temperature in hatchery, natural or experimental, respectively. If nothing else is stated the smolt were reared in natural temperature conditions. 1SW and 2SW refers to number of years the fish spent in the ocean; 1 or 2 years, respectively. No. of 2SW adults of the 2017 release is yet to be determined (TBD).

| **Group** | **No. of smolt analyzed** | **No. of otoliths analyzed** | | **No. of smolt released** | **No. of returning adults** | |
|---|---|---|---|---|---|---|
| ***2016*** | | **Smolt** | **Adult** | | **2017(1SW)** | **2018(2SW)** |
| Imsa Warm | 129 | 251 | 34 | 2981 | 12 | 5 |
| Imsa Cold | 5 | 10 | 12 | 1988 | 6 | 0 |
| Lone | 271 | 533 | 8 | 1989 | 2 | 2 |
| Figgjo | 676 | 1330 | -- | -- | -- | -- |
| ***2017*** | | | | | **2018(1SW)** | **2019(2SW)** |
| Imsa Warm | 350 | 684 | 254 | 4965 | 129 | TBD |
| Imsa Cold | 350 | 674 | 80 | 1992 | 41 | TBD |
| Lone | 347 | 671 | 10 | 1976 | 12 | TBD |
| **Total** | 2128 | 4153 | 418 | 15 922 | 202 | 7 |

## 2.5 Extraction of the otoliths

The analyzed fish were euthanized with anesthetic overdoses prior to retrieving the otoliths. The process of retrieving the otoliths was done using a knife to make a cut on the dorsal side of the fish, just in front of the gills, obliquely downwards to open up the head, find the otoliths and collect using forceps (**Picture 2.2, Figure 2.1**). The otoliths were then put in paper envelopes for storage; some were also wrapped in tissue paper for protection. They were not cleaned until they were taken out to be photographed because they were quite fragile, and it was easier to preserve them and protect them from breaking up with minimal handling in the extraction process.



**Picture 2.2:** Retrieving otoliths of an adult Atlantic salmon (Photo: Anders Foldvik, NINA).

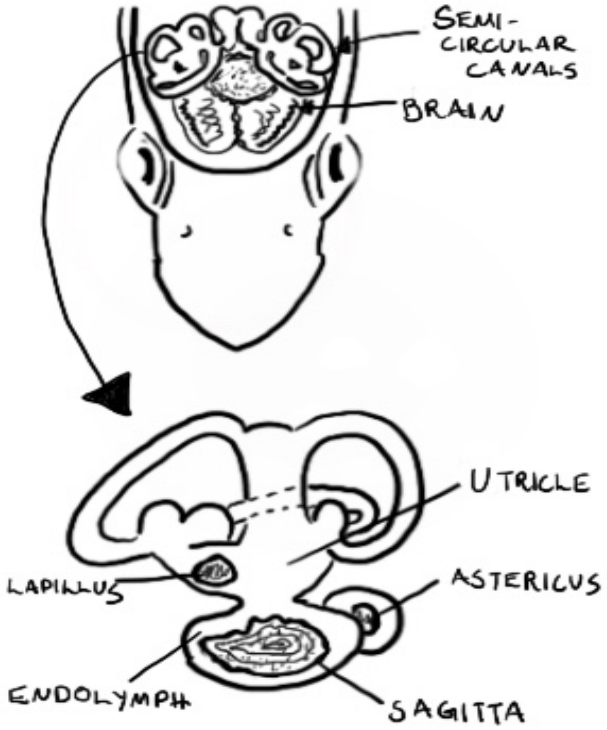
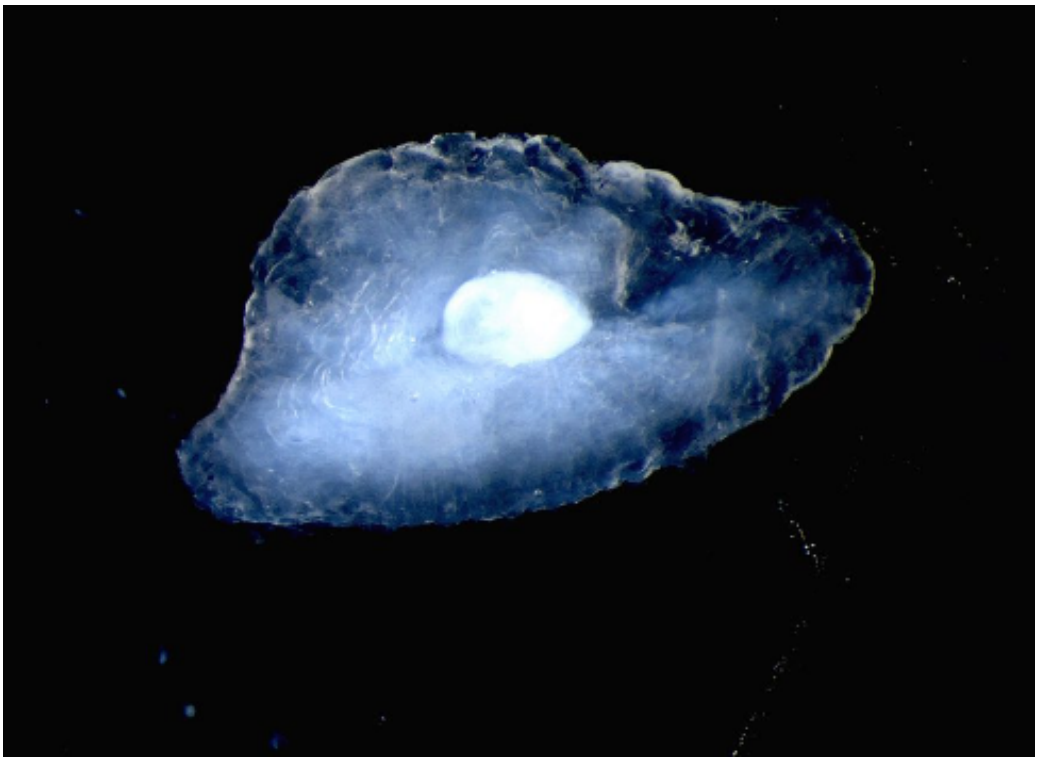



**Figure 2.1**: Schematic drawing showing the location of the sagittal otoliths (sagitta) in the inner ear of the Atlantic salmon, one on each side, that were extracted for analysis (drawn using the software Procreate®).

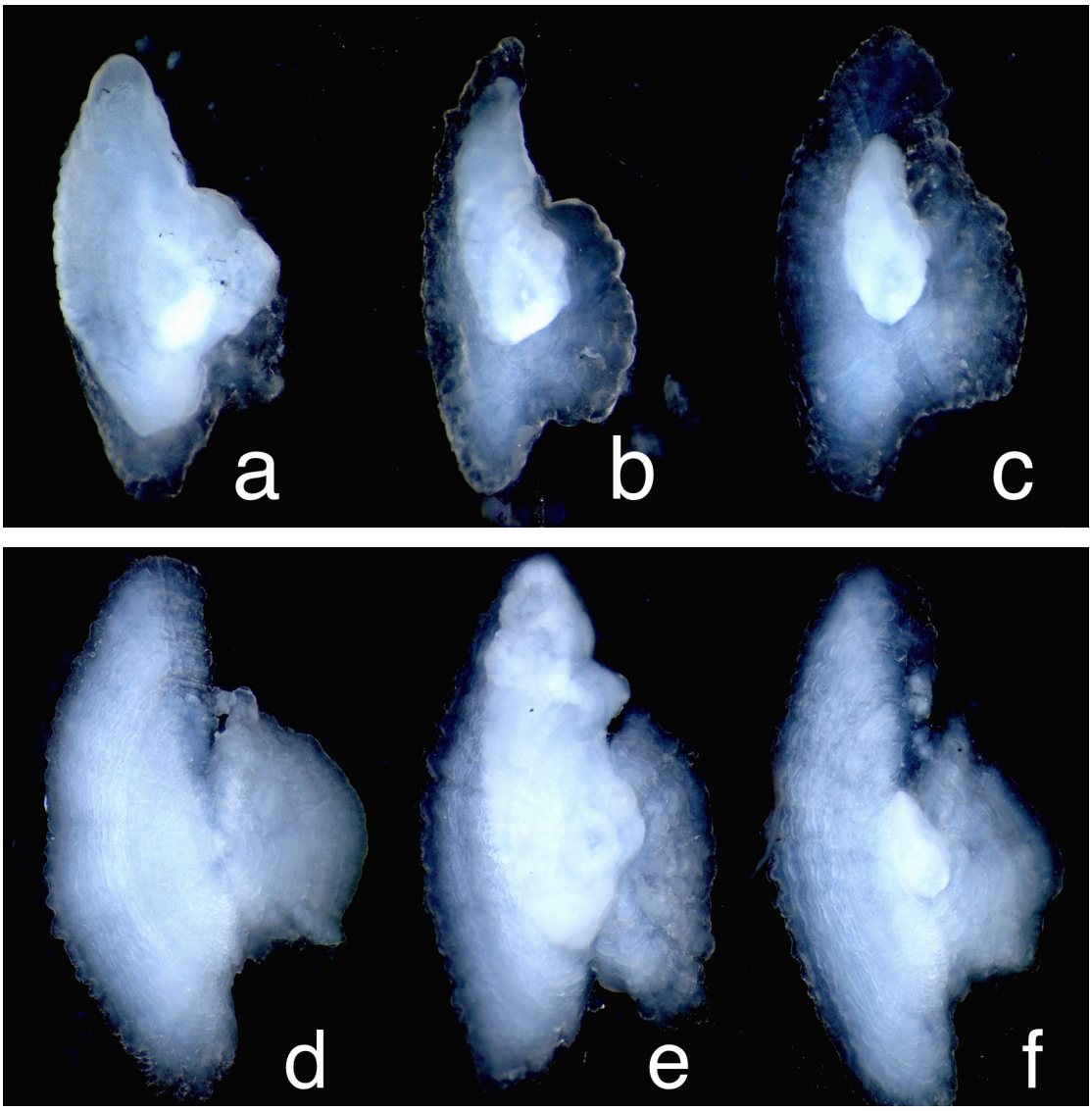
## 2.6 Categorizing the otoliths

Abnormal otoliths are in this context defined as otoliths partly or fully consisting of vaterite. Distinguishing between vaterite and aragonite is possible to do visually when looking at the otoliths in a stereomicroscope (**Picture 2.3**). Each otolith was photographed, in water, with a Leica DC300 digital camera connected to a Leica MZ8 stereomicroscope connected to a computer. The software IrfanView (Škiljan, 1996) was used to view, photograph and save the photographs. 2.5X zoom was sufficient magnification for most, except for a few otoliths from the adults that were quite large and less magnification was appropriate.



**Picture 2.3:** Example of an abnormal otolith with visually distinguishable aragonite (white opaque area in the middle) and vaterite (glass-like and almost transparent area around the middle). This otolith is sampled from a 2017 154 mm long smolt of the Lone population.

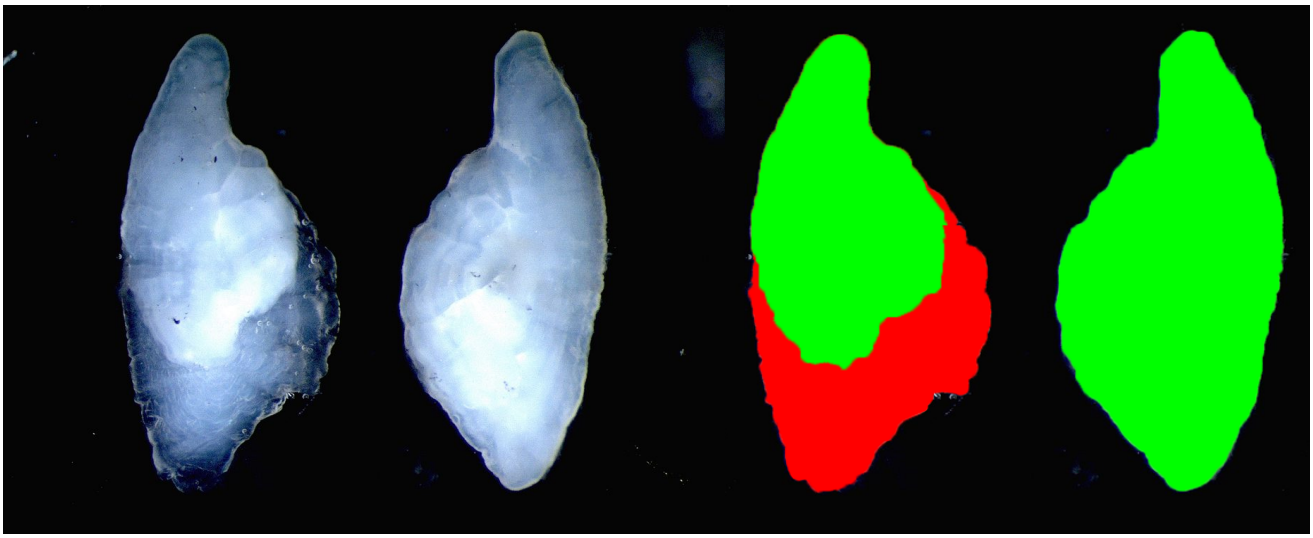
There was large variation in how much of the otolith area that consisted of vaterite (**Picture 2.4**). For that reason it was necessary to further analyze these in order to get a more precise picture or a "degree of abnormalness" for each of these.




**Picture 2.4:** Variation among abnormal otoliths. Top row (a,b,c) show abnormal otoliths from three different smolt from the 2017 groups; a = Lone (161 mm), b = Lone (137 mm), c = Imsa Warm (198 mm). Bottom row (d,e,f) show abnormal otoliths from three different adults; d = 2017 Imsa Cold, e = 2016 Lone, f = 2017 Imsa Warm.

On each abnormal otolith the part consisting of vaterite was quantified by outlining the part or parts consisting of vaterite and the part or parts consisting of aragonite using Adobe™ Photoshop™. The outlining was done using the Quick Selection tool, which automatically outlines structures on the photograph; this automatic outlining was then overseen and adjusted in areas where the tool had done an insufficient job. The aragonite was then colored in green and vaterite colored red in Photoshop, the colors having no significant purpose other than allowing a script to distinguish between the two (**Picture 2.5**).

The amount of red and green pixels were then quantified and the percentage of the total area of the otolith (no. of green pixels + no. red pixels) that consisted of vaterite was in that way measured. The pixels in the transition between the two more or less equally consisted of both colors and were decidedly divided by two. This quantification was done using a script in Python (Rossum & Jake, 2001), which reads the pixel data from the pictures as RGBA-channels; red, green, blue and alpha (transparent) (**see script in Appendix A**). Each of the abnormal otoliths was, using this method, assigned a number ranging between >0 and 1, or a percentage of vaterite. The otoliths with the value of 0 had no visible vaterite and consisted only of aragonite, categorizing them as normal. The categorization of the individual otoliths was thus done in two different ways; normal/abnormal and degree of vaterite (% vaterite).



**Picture 2.5**: Example of the analysis process in Photoshop. **Left**: The picture displays two otoliths sampled from a 2017 179 mm long Lone smolt. The right otolith with no visible vaterite, the left otolith with a clearly visible vaterite part. **Right:** The same picture showing how the otolith was colored with red and green in order to quantify to what degree the otolith was abnormal.

## 2.7 Statistics

All figures and statistical analyses were performed in R studio version 1.1.423 (RStudio 2016). Linear models were checked for normality and homoscedasticity using diagnostic plots to check the model fit.

### *2.7.1 Effect of size and group on frequency of abnormal otoliths and on degree of abnormality (% vaterite) in smolt*

The response variable (Value) in the first analysis refers to the occurrence of abnormal otoliths (at least one abnormal otolith = 1, no abnormal otoliths = 0). Variation in value was tested using logistic models with group (Imsa Cold, Imsa Warm, Lone, Figgjo) and length (mm) as main effects, in addition to interaction effects of group and length (Group x Lengths). This was done for data of both years (2016 and 2017). Individuals where one otolith was unavailable, or group identity was missing (unknown) were excluded from the analysis. The full model used for both years was:

Value ~ Group x Length

As no interaction was found between group and length for either year, a simplified model was used for analysis of both years:

Value ~ Group + Length

Generalized linear models were used by implementing the glm() function with a binomial distribution and a logit-link. The ggplot2-package (Wickham 2016) in R was used to visualize the models. McFadden's R squared was calculated to determine model fit. McFadden's R squared is a pseudo-R squared developed for logistic regressions, where the higher Mcfadden's R squared indicates greater model likelihood (Veall and Zimmermann 1994).

In the next analysis the estimated mean proportion of vaterite for the two otoliths was used as response variable. The values thereby rank from >0 to 1, since all fish with two normal otoliths were excluded. Variation in degree of vaterite in the abnormal otoliths was tested using linear models with group (Imsa Cold, Imsa Warm, Lone, Figgjo) and length (mm) as main effects, in addition to interaction effects of group and length (Group x Length). Individuals where one otolith was unavailable, or information on group or length was missing, were excluded from the analysis. The full model used for both years:

Value ~ Group x Length

As the interaction was significant for the 2016 analysis the full model was used, but as there was no significant interaction found in the 2017 analysis, the model used was:

Value ~ Group + Length

To test for the effects of the explanatory variables (Group and Length) a linear model was used by the function lm(). The ggplot2 package in R was used to visualize the models.

As the linear models (2016 and 2017) did not meet assumptions of normality and homoscedasticity based on both diagnostic plots and the ols_test_normality() function from the olsrr-package (Hebbali, 2018), which provides four different normality test statistics (Shapiro Wilk, Kolmogorv Smirnov, Cramer von Mises and Anderson Darling), the linear model results could not be used to make conclusions about the effect of length and group. To improve normality attempts to transform the response variable was made using log-, square root- and Box-Cox transformations. As transformation did not improve normality to a satisfactory level the variable of group was removed and linear models were instead created individually for each group using the response variable (Value) and explanatory variable (Length):

Value ~ Length

Each of these models were individually checked for normality by diagnostic plots and OLS-tests, and log-, square root- and Box-Cox- transformations were implemented in attempts to improve on normality where these assumptions were not met. Transformation failed for most of the individual group models. Therefore, length and value data was plotted without regression line and group differences were assessed using ANOVA and post hoc Tukey tests in order to determine significant differences between groups.

### *2.7.1 Comparing otoliths of smolt and adults*

Differences in the proportion of abnormal otoliths between the smolt and the adults where the sample size was large enough was tested using $\chi 2$-tests. This was done for the grand total of all groups, for the total of the two years separately and according to group/year individually. Testing the degree of vaterite in the abnormal otoliths of the smolt against the degree of vaterite in the abnormal otoliths of the adults Wilcoxon rank sum tests were used to determine difference in means. This was done for the total of the two years separately and according to group/year individually. The ggplot2 package in R was used to visualize the data.

Additionally, in order to determine whether the sampled smolt were representative for the released smolt and that the sampling had occurred randomly without any significant length bias, the average lengths of the sampled smolt were compared to the average lengths of the released smolt (**see Table 1.B in Appendix B**). The abnormal otolith frequencies of the released smolt were estimated by plotting their average lengths with the regression lines of the sampled smolt corresponding with their respective groups and year (**see Figure 1.B and 2.B in Appendix B**). The slopes differed for each group in the logistic models, so the effect of length differed somewhat. And as there was also a slight difference in mean lengths of the sampled and the released smolt, the frequencies of abnormal otoliths varied marginally for certain groups. The

estimated frequencies of the released smolt were in turn compared to the observed frequencies of abnormal otoliths of the adults again by performing $\chi 2$-tests (**see Table 2.B in Appendix B**).

# 3. Results

## 3.1 Overview

Based on the data collected in this experiment abnormal otoliths are common in hatchery-reared smolt. For most of the smolt groups the frequency of abnormal otoliths was relatively high, the lowest frequency found being 11% and the highest over 60%, and in total 42% of the smolt had at least one abnormal otolith (**Table 3.1**).

**Table 3.1**: The total amount of individual fish included in the experiment (N) and in how many of these abnormal otoliths occur (N*) and to which group, year (2016, 2017) and stage (smolt, adult) they belong. The percent of the total in which abnormal otoliths occur is also added; the higher percent of each row is presented in bold.

| ***2016*** | **Smolt** | | **Adult** | |
|---|---|---|---|---|
| Imsa Warm | N = 120<br>N*= 47 | **39%** | N = 17<br>N* = 2 | 12% |
| Imsa Cold | N = 5<br>N*= 2 | 40% | N = 6<br>N*= 3 | **50%** |
| Lone | N = 259<br>N*= 166 | **64%** | N= 4<br>N*= 2 | 50% |
| Figgjo | N = 652<br>N*= 334 | 51% | -- | -- |
| **Total** | N = 1036<br>N*= 549 | **53%** | N = 27<br>N*=7 | 23% |
| ***2017*** | **Smolt** | | **Adult** | |
| Imsa Warm | N = 334<br>N* = 49 | 15% | N = 121<br>N* = 25 | **21%** |
| Imsa Cold | N = 324<br>N*= 38 | 11% | N = 40<br>N*= 10 | **25%** |
| Lone | N = 324<br>N*= 207 | **64%** | N = 10<br>N*= 5 | 50% |
| **Total** | N = 982<br>N*= 294 | **30%** | N = 171<br>N*= 40 | 23% |
| ***Grand total*** | N = 2018<br>N*= 843 | **42%** | N = 198<br>N*= 47 | 24% |

## 3.2 Effect of size and group on occurrence of at least one abnormal otolith in smolt

In the context of this subchapter, the value is either 0 (0 of 2 otoliths are abnormal) or 1 (1 or 2 of 2 otoliths are abnormal). For both years logistic regression models showed a significant length effect, slightly less significant for the 2017 model and a better model fit (**Figure 3.1, Figure 3.2, Table 3.2**).

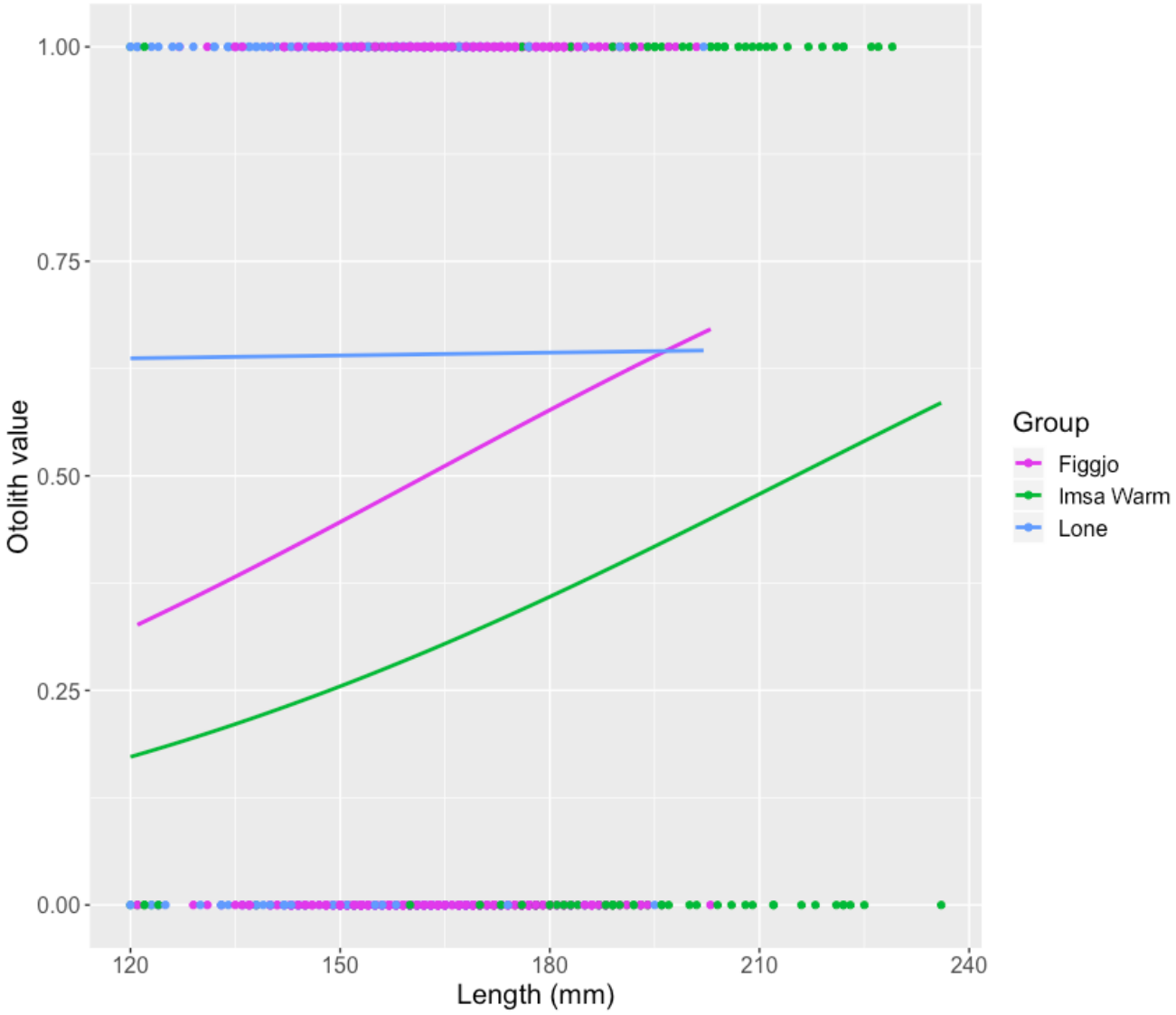



**Figure 3.1:** 2016 smolt: Logistic model of the effect of the explanatory variable, length, (mm) on the dependent variable, otolith value (0 or 1), for the three groups (Figgjo, Imsa Warm and Lone).

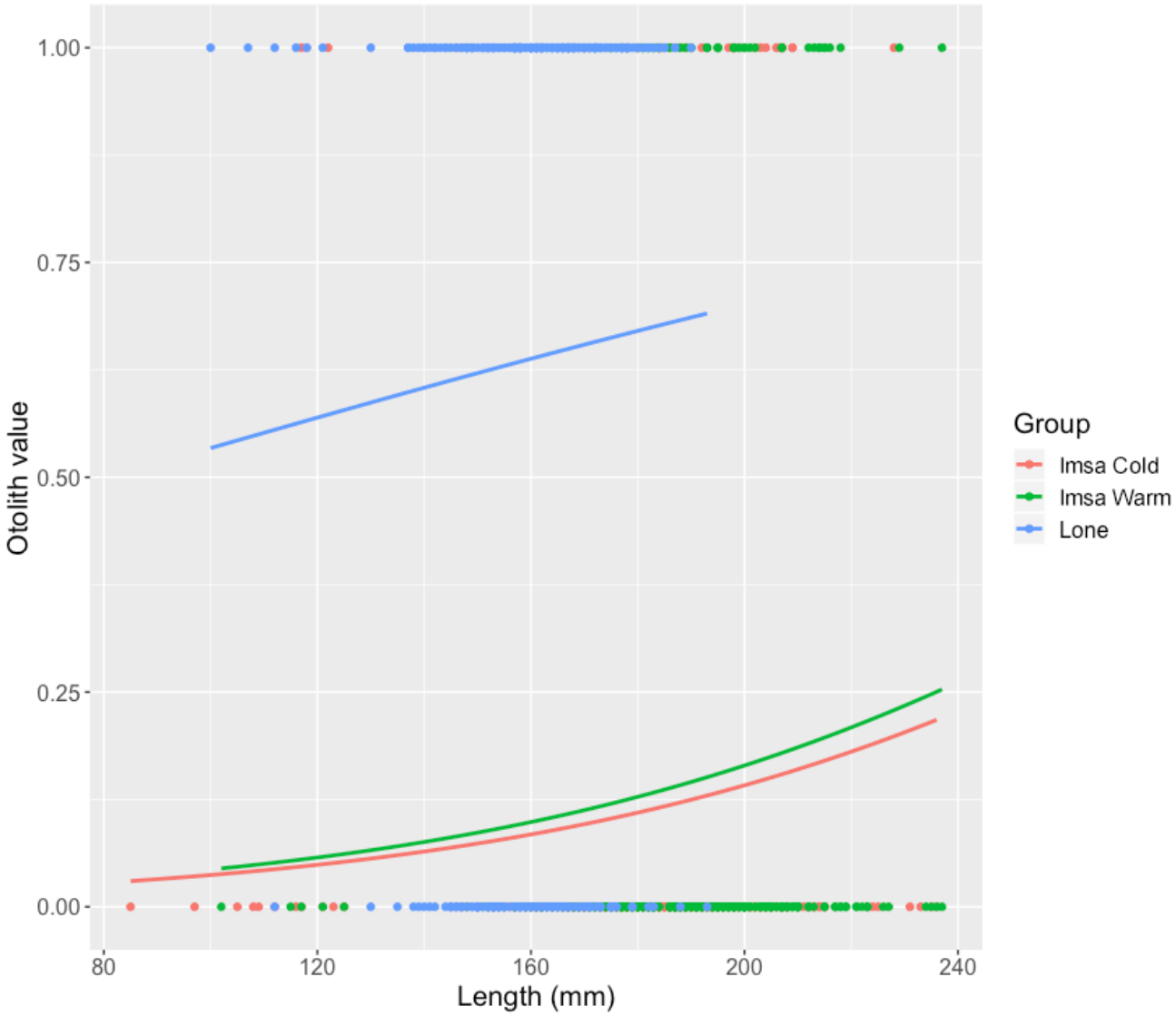



**Figure 3.2:** 2017 smolt: Logistic model of the effect of the explanatory variable, length, (mm) on the dependent variable, otolith value (0 or 1), for the three groups (Imsa Cold, Imsa Warm and Lone).

**Table 3.2**: Estimates for the generalized linear models used to test for variance in otolith value (0 or 1) against the groups (Figgjo, Imsa Warm, Imsa Cold, Lone) and smolt length (mm). Significant p-values are represented in bold.

| ***2016*** | **Estimate** | **Std. Error** | **z-value** | **p-value** |
|---|---|---|---|---|
| Intercept (Figgjo) | -2.26 | 0.67 | -3.21 | 0.001 |
| Imsa Warm | -0.80 | 0.23 | -3.51 | **<0.001** |
| Lone | 0.67 | 0.16 | 4.22 | **<0.001** |
| Length | 0.01 | 0.01 | 3.31 | **0.001** |
| McFadden's R squared: | 2.4% | | | |
| ***2017*** | | | | |
| Intercept (Imsa Cold) | -4.26 | 0.94 | -4.51 | <0.001 |
| Imsa Warm | 0.19 | 0.23 | 0.81 | 0.420 |
| Lone | 2.88 | 0.25 | 11.68 | **<0.001** |
| Length | 0.01 | 0.005 | 2.44 | **0.015** |
| McFadden's R squared: | 22.4% | | | |

The 2016 Tukey test showed that there was a significant difference in frequency of abnormal otoliths between all the groups, the most significant difference being between the Lone and the Imsa Warm group (p-value<0.001). The 2017 Tukey test showed that there was a significant difference in frequency of abnormal otoliths between the Lone group and the others with a (p-value <0.001), but no significant difference between the Imsa Warm and Imsa Cold groups.

## 3.3 Effect of size and group on degree of abnormality (% vaterite) in otoliths of smolt

The following analyses only include individuals that had at least one abnormal otolith. The average proportion of vaterite in these otoliths was calculated and used as the response variable in the context of this subchapter.

The average proportion of vaterite (±SD) in abnormal otoliths for the 2016 smolt were 0.43±0.22 for the Figgjo group, 0.44±0.24 for the Lone group and 0.76±0.32 for the Imsa Warm group (**Figure 3.4**). The Tukey test showed no significant difference between Lone and Figgjo and thus equally significant differences between Imsa Warm and the other groups ($P < 0.001$). The average proportion of vaterite (±SD) for the 2017 smolt groups were generally somewhat lower; 0.29±0.17 for the Imsa Warm group, 0.27±0.17 for the Imsa Cold group and 0.38±0.17 for the Lone group (**Figure 3.5**). The Tukey tests showed significant differences between the Lone group and Imsa Warm and Imsa Cold; the Imsa Cold and Imsa Warm groups were not significantly different.

As the full linear models and simplified models for the whole dataset with both length and group as explanatory variables did not meet assumptions of normality, linear regression was performed for each group of both years individually.

For the linear regression models of the 2016 smolt groups, neither the Imsa Warm nor the Lone groups met normality assumptions, and attempts to transform the response variable to achieve normality were unsuccessful. For the Imsa Cold group, square root transformation of the response variable was successful in achieving assumptions of normality, and the output of the linear regression gave a non-significant effect of length for this group (p-value = 0.67), however and the model only explained 0.5% of the variation based on R-squared. Similarly, the linear regression models for the 2017 smolt groups did not meet assumptions of normality neither before nor after transformations

were performed. There was thus not found an effect of length on degree of vaterite in this experiment.

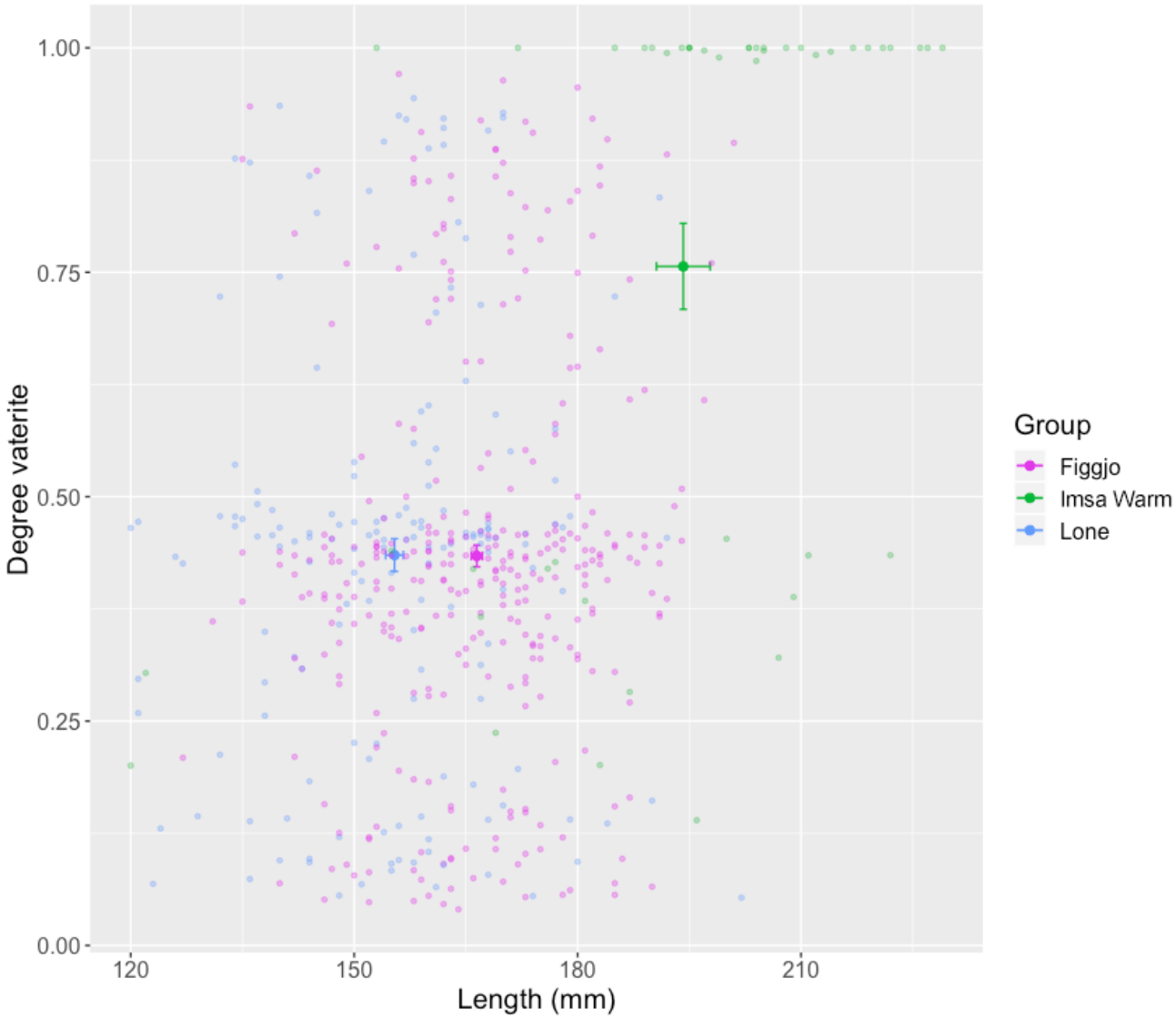



**Figure 3.4:** 2016 smolt: Length (mm) plotted against degree of vaterite in abnormal otoliths for the three smolt groups (Figgjo, Imsa Warm and Lone). The slightly larger, highlighted points represent mean value and mean length for each group. Standard error lines are shown vertically from points for degree of vaterite and horizontally for length.

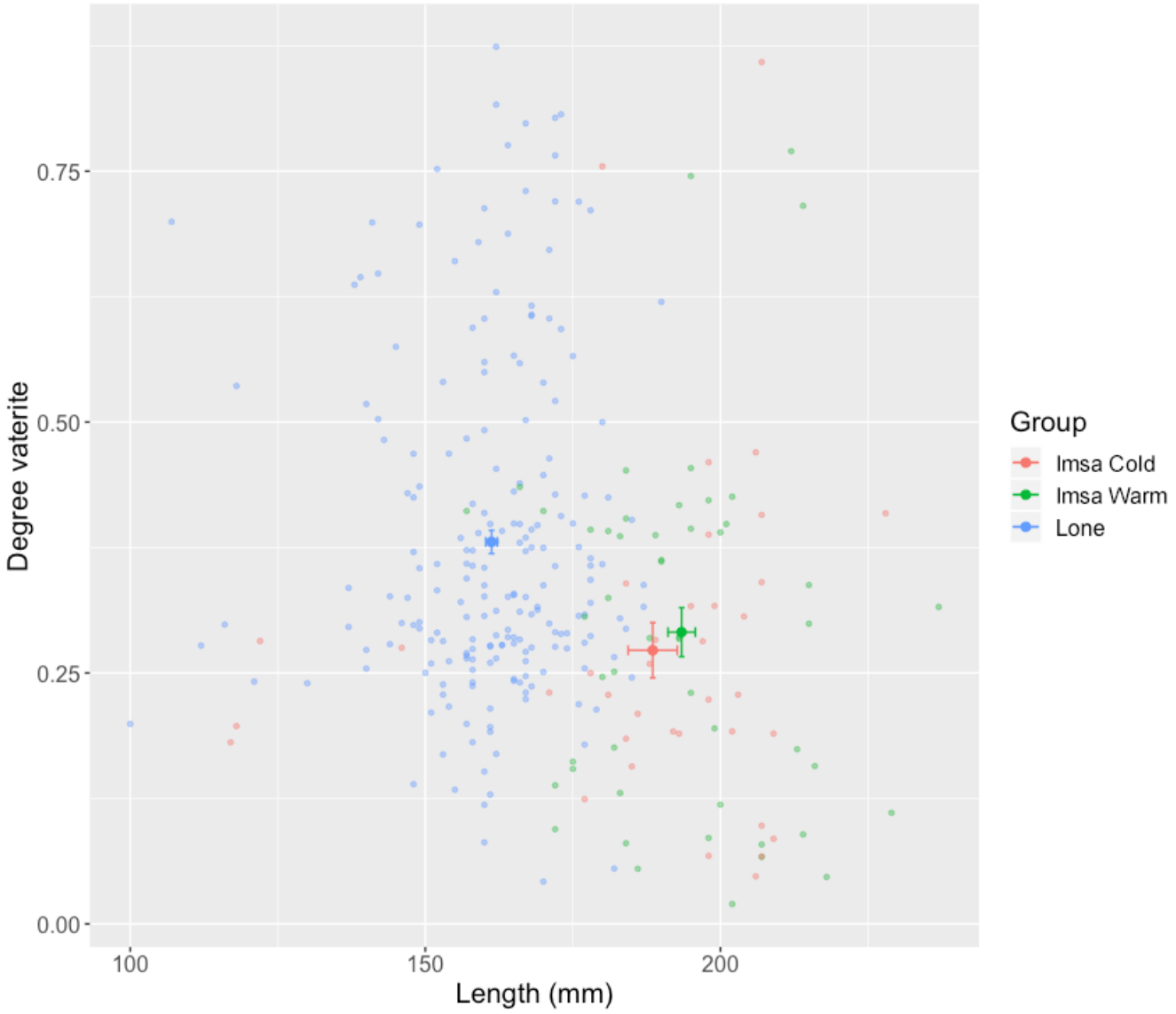



**Figure 3.5:** 2017 smolt**:** Length (mm) plotted against degree of vaterite in abnormal otoliths for the three smolt groups (Imsa Cold, Imsa Warm and Lone). The larger, highlighted points represent mean value and mean length for each group. Standard error lines are shown vertically from points for degree of vaterite and horizontally for length.

## 3.4 Comparing frequency of abnormal otolith between smolt and returning adults

In the following analyses the frequency of at least one abnormal otolith in smolt was compared with the frequency in the returning adults. The Figgjo group of which no fish were released is thereby excluded. Comparing the grand total of smolt during both years and in all groups analyzed with the grand total of returning adults the result showed that the smolt had a significantly larger frequency of abnormal otoliths (37%) than the adults (24%) ($\chi2=13.8$, $p<0.001$).

Similarly, the same comparison for the two years separately also showed a higher frequency of abnormal otoliths in the smolt, however this difference was not significant for the 2017 smolt. When comparing adults and smolt individually according to year and groups the results varied. 2016 Imsa Warm smolt had a significantly larger proportion of abnormal otoliths than their adult counterpart. There was not found a significant difference between the 2017 Imsa Warm smolt and adults. The 2016 Lone group and Imsa Cold group had sample sizes that were too small to reasonably compare statistical significance of difference, this was also the case for the 2017 Lone group (**summary in Table 3.3**).

**Table 3.3:** Results of $\chi2$-tests performed for difference in frequency of abnormal otoliths between smolt and returning adults. The empty cells indicate that the sample sizes were too small to perform reliable $\chi2$-tests. Significant p-values are presented in bold.

| ***2016*** | $\chi2$ | p-value |
|---|---|---|
| Imsa Warm | 4.87 | **0.03** |
| Imsa Cold | **--** | **--** |
| Lone | -- | -- |
| **Total** | 9.18 | **0.002** |
| ***2017*** | | |
| Imsa Warm | 2.34 | 0.13 |
| Imsa Cold | 5.48 | **0.02** |
| Lone | **--** | **--** |
| **Total** | 3.03 | 0.08 |
| ***Grand total*** | 13.81 | **0.0002** |

## 3.5 Calculating the frequency of abnormal otoliths in the released smolt

In the previous subchapters only information about non-released sampled smolt was used, which are the representative sample of the smolt that were released. For obvious reasons we do not have the information about the otoliths of the smolt that were actually released, only the information about the length of these. Based on the average lengths of the smolt that were analyzed and the lengths of the smolt that were released, an estimation was done to estimate the frequency of abnormal otoliths that most likely occurred in the released smolt, and in that way how many individuals of the released smolt had abnormal otoliths (**see Table 1.B, Figure 1.B, Figure 2.B in Appendix B**). These new estimated frequencies did not differ much from what was found for the analyzed smolt, and when performing new $\chi 2$-tests in comparing frequencies of abnormal otoliths between the smolt and returning adults the results did not in general indicate different results from the previous (**see Table 2.B in Appendix B**).

## 3.6 Comparing degree of abnormality (% vaterite) in abnormal otoliths of smolt and returning adults

In total, there was no significant difference in the average degree of vaterite in the abnormal otoliths between the smolt and the adults for either year (**Figure 3.6, Table 3.4**). When comparing the groups individually, only the 2017 Lone group showed a significant difference; the adults had a higher degree of vaterite in their abnormal otoliths (**Figure 3.7, Table 3.4**)

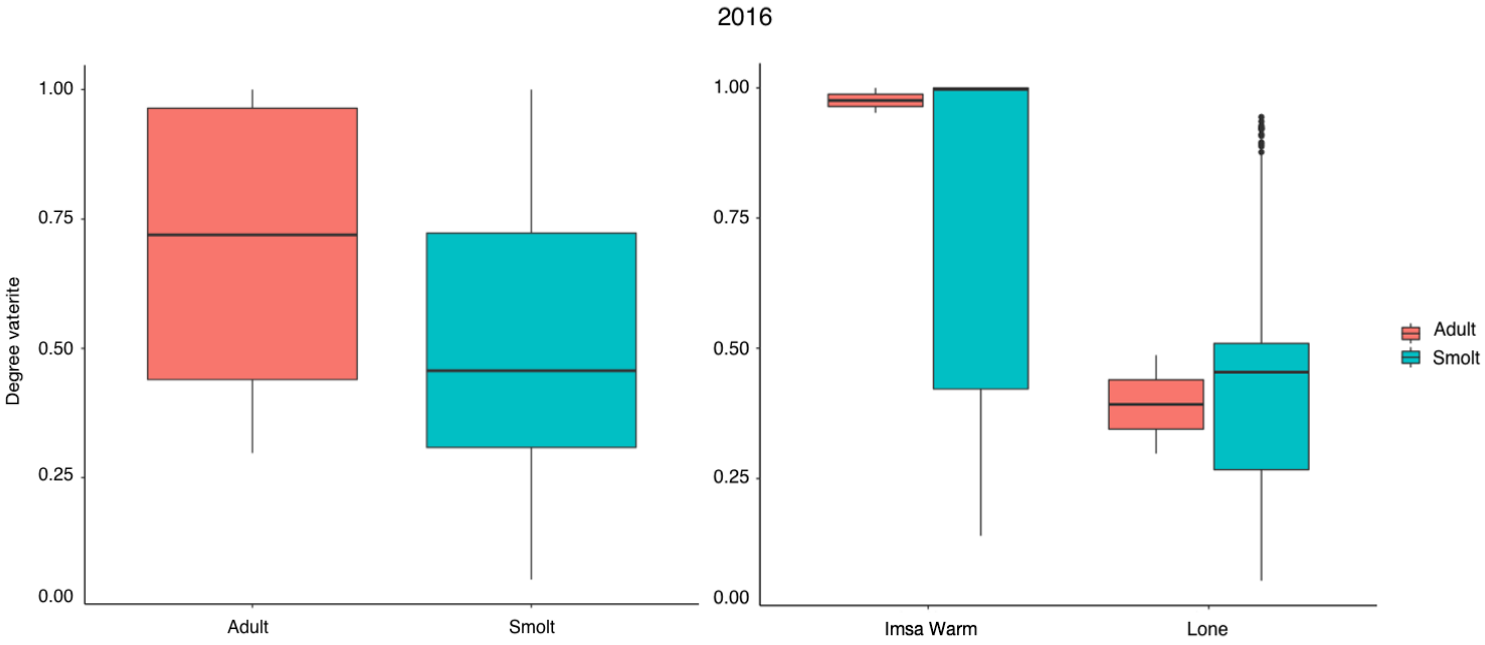



**Figure 3.6: Left side:** Boxplots showing mean degree of vaterite in abnormal otoliths of total 2016 adults and smolt. **Right side:** Boxplots showing mean degree of vaterite in abnormal otoliths of the different 2016 groups as smolt and adults.

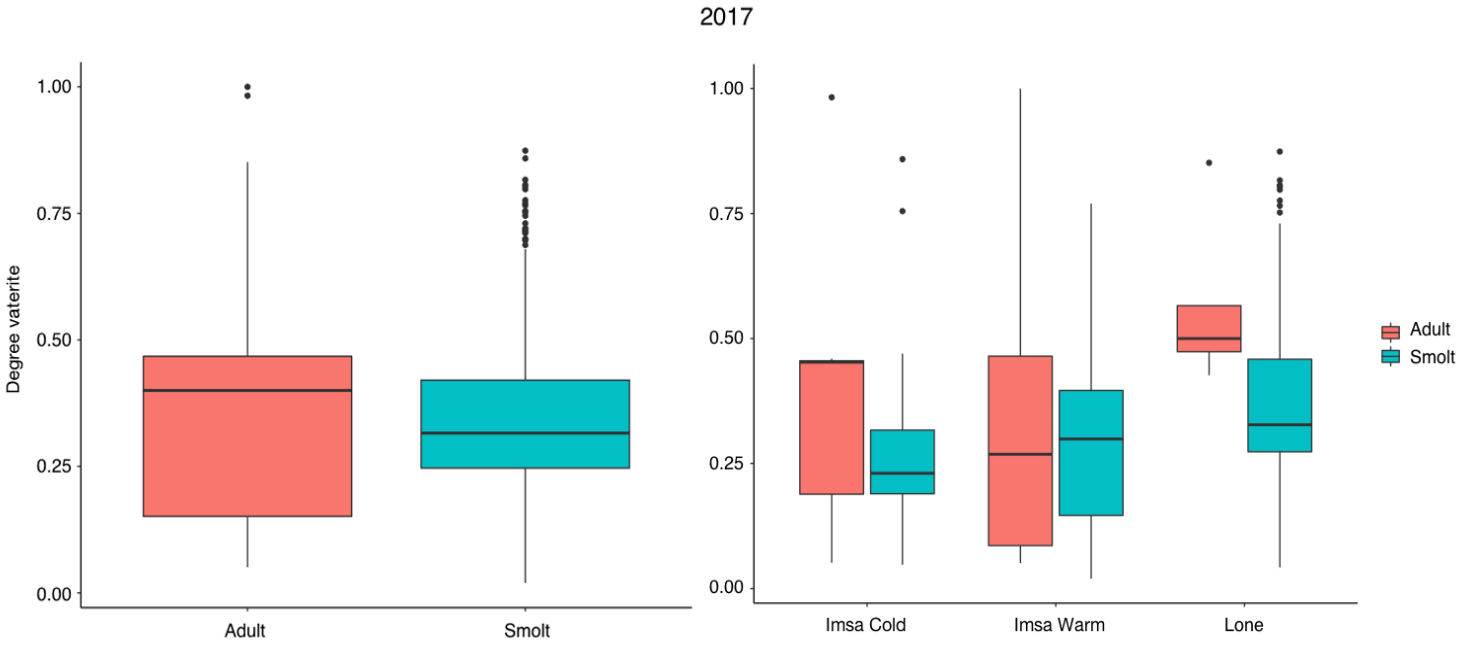



**Figure 3.7: Left side:** Boxplots showing average degree of vaterite in abnormal otoliths of total 2017 adults and smolt. **Right side:** Boxplots showing average degree of vaterite in abnormal otoliths of the different 2017 groups as smolt and adults.

**Table 3.4:** Summary of Wilcoxon rank sum tests conducted to test for difference in degree of vaterite in the abnormal otoliths between smolt and adults of the different groups and years. Significant p-values are presented in bold.

| **Wilcoxon rank sum test** | | |
|---|---|---|
| ***2016*** | **W** | **p-value** |
| Imsa Warm | 52 | 0.76 |
| Lone | 168 | 0.99 |
| **Total** | 576 | 0.23 |
| ***2017*** | | |
| Imsa Warm | 690 | 0.78 |
| Imsa Cold | 247 | 0.29 |
| Lone | 845 | **0.02** |
| **Total** | 11060 | 0.55 |

# 4. Discussion

In this experiment the otoliths of smolt from different groups were analyzed, in order to determine frequency of abnormal otoliths and the degree of vaterite (% vaterite) in the abnormal otoliths. These groups differed in parental brood stock origin, number of generations in hatchery or mean annual temperature in hatchery. Smolt originating from the same groups were released for ocean migration in 2016 and 2017 (except Figgjo), and some returned as adults in 2017 and 2018 (a few are also expected to return in the fall of 2019). The otoliths were collected from the returning adults, and frequency and degree of abnormality (% vaterite) was determined for these as well, in order to compare these to the smolt groups. These comparisons formed the basis for evaluating the potential effects of abnormal otoliths on survival.

## 4.1 Differences between smolt groups

The smolt analyzed in this experiment showed large variations in both frequency of abnormal otoliths and the degree of vaterite in the abnormal otoliths between groups. Generally there was a high frequency of abnormal otoliths. And although no wild smolt were analyzed in this experiment, we know that abnormal otoliths are more common in hatchery-reared fish than wild (Reimer, Dempster et al. 2017). This raises the question of what specifically causes this change in crystalline structure. The change from aragonite to vaterite happens under extreme stress due to variation in the environment (Falini, Fermani et al. 2004). These may be stresses from hunger, temperature, density, many of which are enhanced in hatcheries (Oxman, Barnett-Johnson et al. 2007, Reimer, Dempster et al. 2017).

Fish metabolisms are ectothermic of nature; the formation of otoliths may be sensitive to environmental changes and the production of vaterite may be influenced by increases in metabolic rate (Oxman 2012, Sweeting, Beamish et al. 2004). Hatchery conditions are often designed to speed up growth through

commercial feed, light conditions and temperature regimes. Recent studies have indicated that rapid growth may be the primary and universal cause of abnormal otoliths. This may possibly due to change in the composition of otolith membrane proteins, or lower [Ca2+]/[CO3 2-] ratio in the endolymph which favors the formation of vaterite (Reimer, Dempster et al. 2017). Norwegian salmon yearling raised in hatcheries showed increasing frequency of abnormal otoliths as well as degree of vaterite replacement with increasing size (Reimer, Dempster et al. 2016). Therefore the environmental factors fish in hatcheries are subjected to may be most important in the formation of vaterite otoliths, and more so than genetic control (Gauldie 1986, Reimer, Dempster et al. 2017).

In this experiment, the Lone group stood out in having significantly higher frequency of abnormal otoliths than the other groups consistently both years they were analyzed. This despite being reared under the same conditions as the Imsa Cold and Figgjo groups, and therefore the hatchery-condition-induced higher frequency of abnormal otoliths should be equally high for these three groups, if the conditions in the hatchery are all that matter. However, the Lone population had a much longer hatchery ancestry than the other groups, so this population most likely has accumulated non-beneficial genetic mutations as a result of low selection pressure in hatchery over generations. For wild Atlantic salmon, survival from egg to smolt is only around 1.7% on average, and may be 20 times higher for hatchery-reared conspecifics (Araki, Berejikian et al. 2008, Jonsson and Jonsson 2011, Glover, Solberg et al. 2017). The higher frequency of abnormal otoliths for the Lone population may indicate a population effect. The formation of vaterite may thus be linked to both environmental factors and genetic irregularities, which may be accumulated in the Lone population over generations (Sweeting, Beamish et al. 2004).

The Figgjo and Imsa populations are ancestrally much closer to their wild parental brood stock than the Lone population, in that they are progeny of first generation hatchery-reared parents. Despite this, Figgjo (only analyzed in 2016) had a significantly higher frequency of abnormal otoliths than the 2016 Imsa Warm group. This is surprising because the Imsa Warm experienced warmer

water than Figgjo. The difference may perhaps indicate an effect of genetic variation between the populations on the formation of vateritic otoliths. This effect may be especially relevant for salmonids because of the large genetic variation between populations due to local adaptation (Oxman 2012).

The Imsa Warm and Imsa Cold groups were essentially the same population and differed only in mean annual temperature regimes in hatchery, resulting in faster embryogenesis of the Imsa Warm group than the Imsa Cold group (Jonsson and Jonsson 2018). Average low/high water temperature and fast growth is linked to the formation of vateritic otoliths, and studies show 50-60% (depending on light conditions) higher frequency of abnormal otoliths in salmon eggs reared in 13°C than those reared in 6°C (Gauldie 1986, Reimer, Dempster et al. 2017). Both Imsa Warm and Imsa Cold in this experiment were analyzed in 2017; they also differed in mean total lengths, suggesting that the Imsa Warm group grew faster, as length is a proxy for growth rate in this experiment. Indeed, the percent abnormal otolith frequency was higher for the Imsa Warm group than the Imsa Cold group (14.67% and 11%, respectively).

Furthermore, Lone and Imsa Warm are between-year comparable, and both groups showed slightly longer average length and higher frequency of abnormal otoliths in 2016 than in 2017. Additionally, the results of the logistic regression models of both 2016 and 2017 indicated that length had a significant positive effect on the probability of smolt having at least one abnormal otolith. And although these models were somewhat weak in explaining variation based on McFadden's R squared, all of these results combined may reinforce the notion that rapid growth results in higher frequency of abnormal otoliths as previously suggested, and may also explain some of the observed differences between the smolt groups (Reimer, Dempster et al. 2017).

In summary; in terms of frequency of abnormal otoliths observed in this experiment, there are between-group and within-group (between year) differences that may be attributed to temperature induced higher growth rate or population effects. This may support the notion that the observed variation could

be genetically controlled to a certain degree. As phenotypes emerge from interactions between genes and environment during development, the differences that are observed between the groups may be results of different responses to the environment by different populations (Jonsson and Jonsson 2014).

What about the degree of vaterite (% vaterite) in the abnormal otoliths? The average vaterite coverage for the groups that were between-year comparable, Lone and Imsa Warm, was larger in 2016 than in 2017, again suggesting some effect of faster growth. When comparing the Imsa Warm and Imsa Cold groups of the 2017 analysis there was no statistically significant difference, although Imsa Warm had a slightly higher average degree of vaterite in their abnormal otoliths. Linear models testing the relationship between degree of vaterite and length was only obtained for the Imsa Cold group, where length had a non-significant effect.

The Imsa Warm group of 2016 did, however, stand out in having a much higher degree of vaterite in their abnormal otoliths, compared both to the other groups and within-group between years. The large coverage was especially prominent in the larger smolt. Building a linear model that met assumptions of normality for the 2016 Imsa Warm group was still difficult to accomplish, probably because of the relatively small sample size and the extreme values (many had two completely vaterized otoliths, giving them mean of maximum value 1). Otoliths develop from before hatching until death, so the switch from aragonite to vaterite in the case of the 2016 Imsa Warm group most likely happened early in the embryogenesis for this group (Campana 1999).

## 4.2 Comparing smolt and adults

In total, there was a higher frequency of abnormal otoliths in smolt than in the returning adults of this experiment (37% and 24%, respectively). This result was consistent separately for groups and years (where sample sizes were large enough to conduct χ2-tests), with the exception of the 2017 Imsa Cold group. In general this indicates that individuals with abnormal otoliths may have been less capable of returning. There are two possible reasons why an individual did not return; it did not survive its marine phase or it was unable to navigate to its native river. This will first be discussed in terms of the specific functions of the otoliths and the consequences vateritic otoliths may have for survival and navigation.

Abnormal otoliths causes reduced hearing sensitivity for Atlantic salmon. The primary issue with hearing loss is predator avoidance. Underwater predators and prey produce sound in the infrasound range; below 20 Hz. Juvenile Atlantic salmon show awareness responses at 5-10 Hz, and avoidance responses at around 10 Hz, and hearing at these frequencies may indeed be impaired by vaterite (Knudsen, Enger et al. 1992, Reimer, Dempster et al. 2016). However, this potential hearing loss is not completely detrimental to Atlantic salmon because abnormal otoliths occurred in a number of the returning adults with high coverage (% vaterite) in this experiment. This is established knowledge because abnormal otoliths have been observed in adults before, and so it has been hypothesized that at least the loss of hearing sensitivity from abnormal otoliths may in some way be compensated for in fish (Oxman, Barnett-Johnson et al. 2007).

One way fish may compensate for hearing impairment is through schooling. Atlantic salmon do react to predators by schooling, and the schooling behavior mode in general supersedes territoriality when seaward migration begins. This perhaps makes them, at least in part, less vulnerable to the potentially detrimental effects of hearing loss caused by abnormal otoliths (Pavlov and

Kasumyan 2000, Oxman, Barnett-Johnson et al. 2007, Handeland, Järvi et al. 2011). However, this may only be the case in the initial post-smolt phase in the estuary, and whether schooling behavior could compensate for hearing loss in the high sea is less clear. Predation on Atlantic salmon in the open ocean in general is also a mystery, but studies indicate that early marine mortality is an important determinant on return rate (Ward and Hvidsten 2011). In other words, the negative effects of hearing loss could theoretically be negligible because mortality is high in the initial marine phase for everyone anyway. And if predation is less important in the open ocean, the effects of hearing loss after the early phase in the estuary may be less severe.

Furthermore, as Atlantic salmon are hearing generalists, and have poorer hearing than many other teleosts with a narrower sound frequency span to begin with, they may not be as highly reliant on hearing in the first place. That being said, salmon do detect and react to waterborne sounds of predators, so hearing is by no means trivial (Hawkins and Johnstone 1978).

In terms of homeward navigation abnormal otoliths may also have an effect because of the importance otoliths have for gravity sensation and linear acceleration in the water column (Reimer, Dempster et al. 2016). Theoretically otolith irregularities may have an impact on swimming performance and perhaps also navigational efficiency. But again, it can be concluded in this experiment with absolute certainty that homeward migration is at least possible for Atlantic salmon with completely vateritic otoliths. The effects are thus not completely detrimental but could possibly be substantial and perhaps also prolong the homeward migration process. Atlantic salmon may navigate homeward utilizing multiple sensory organs that in combination allow for high precision homing, possibly by mapping the area around them. The inner ear is the most important fish sensory organ for detection of distant sources, so the functions of the otoliths may be important in navigation (Popper and Lu 2000).

Jonsson and Jonsson in 2018 found that eggs incubated in warmer than natural water returned from the ocean later in the season than those incubated in

natural water temperatures. They attributed this to be a phenotypically plastic response to temperature during embryogenesis, and showed that Atlantic salmon are adaptive to potential warmer water as a result of climate change in the future (Jonsson and Jonsson 2018). The fish incubated in warmer water in Jonsson and Jonsson's experiment probably also had a higher frequency of abnormal otoliths than those that were incubated in natural water. So, if indeed abnormal otoliths, to a degree may impact the effectiveness of navigation, the findings of Jonsson and Jonsson be perhaps be linked to abnormal otoliths. This is of course mere speculation and there is no conclusive evidence of this from the results of this experiment.

Additionally, if navigational ability is compromised by inner-ear abnormalities, a compensatory mechanism for fish to still successfully migrate home could be to do so with help of others. Berdahl et al. in 2016 proposed a hypothesis of collective navigation from the ocean in anadromous salmonids. The basic theory being a "wisdom of the crowd" form of navigation where salmon aggregate in the ocean and migrate together with higher precision than the individual. But again, whether the Atlantic salmon of this experiment actually display such behavior is unclear, and this navigational hypothesis is suggested to be most relevant for salmon species with larger propensities for social behavior than the Atlantic salmon (Berdahl, Westley et al. 2016). Yet, one of the articles referenced to support this theory was the findings of Jonsson et al. from 2003 that showed that hatchery-reared Atlantic salmon stray to a higher degree than wild, possibly due to genetic components and environmental conditioning (Jonsson, Jonsson et al. 2003). This finding was relevant because a stronger relationship between homing and run size for hatchery-reared than wild was observed, possibly due to hatchery-reared Atlantic salmon being more inclined to socially migrate because they are used to living in high densities. However, if in fact abnormal otoliths affect precision of navigation, it could be speculated that also these observations also may be attributed to otolith irregularities; because if hatchery-reared salmon are less capable of navigating correctly due to their abnormal otoliths are they also more inclined to migrate in numbers?

Additionally, a factor that could potentially play a large role in explaining and interpreting the findings of this experiment is the data on asymmetry of the otoliths. Asymmetry was not accounted for in this experiment. Asymmetry in otolith mass, through for example a fish having one normal and one abnormal otolith, could cause abnormal swimming, reduce sound localization and generally reduce performance of the fish (Gagliano, Depczynski et al. 2007). Asymmetry may in that way stand out as one of the more important contributors of a potential reduced navigational ability and lower survival. Although asymmetry was not quantified in this experiment, it was observed in at least one of the adults with high coverage of vaterite in one otolith and no visible vaterite in the other.

The trend in total indicates almost twice as high frequency of abnormal otoliths in the smolt than in the adults, which supports the notion that the survival in the ocean or navigational ability may indeed be impacted by the occurrence of abnormal otoliths for Atlantic salmon. However, there are other factors involved that may complicate the picture that will be discussed in the following subchapters.

There were large variations between the abnormal otoliths in both the smolt and the adults; from almost completely consisting of aragonite, to completely vateritic (**see Picture 2.4-d**). Studies indicate that hearing impairment increases with degree of vaterite (Reimer, Dempster et al. 2016). So was there a higher degree of vaterite replacement in the abnormal otoliths of the smolt than the adults? In this experiment there generally wasn't many significant differences, and the results varied. The only significant difference found was between Lone smolt and Lone adults, where the adults actually had a higher degree of vaterite in their abnormal otoliths. This may perhaps indicate that the returning adults that did in fact have abnormal otoliths and still returned depended on the compensatory factors previously mentioned, regardless of how vateritic their otoliths were.

## 4.3 Smolt frequency of abnormal otoliths and adult return rate

Up until this point only the abnormal otoliths frequencies in the smolt and the returning adults have been discussed, but what about the actual return rates of adults? The frequency of abnormal otoliths in smolt groups and the corresponding adult return rates were plotted against each other according to groups and years (**Figure 4.1**), and there does, indeed, look as though there is a negative correlation between frequency of abnormal otoliths and return rate. However numerous factors complicate this picture.

The Lone population had lower return rates (0.2% and 0.5%) and higher frequencies of abnormal otoliths consistently both years compared to the other groups. But is there a true correlation between the two? One important factor to take into consideration here is that the Lone population is not genetically native to the Imsa River, as is the case for the Imsa population. Hatchery-reared fish perform poorer in the wild, and studies also suggest that non-local hatchery-reared salmonids perform even worse, which indicates a favoring of local genotypes (Bams 1976, Araki, Berejikian et al. 2008). The relatively lower return rate of the Lone population may simply be a case of this population being genetically non-native to the Imsa River. This despite the fact that studies indicate that Atlantic salmon return to the rivers they migrated from as juveniles no matter their genetic origin, and that the cues they pick up on outward migrations are the ones that determine return (Hansen and Jonsson 1994).

A perhaps more important factor contributing to the low return rates for the Lone population is most likely the fact that these fish stem from numerous generations of hatchery-reared parents, and their wild ancestral brood stock dates back to the late 1980s. The Imsa population differs from the Lone population in that they are much closer to their wild ancestors. Therefore, the Lone population have acquired lower fitness over generations, and may be less conditioned for the unforgiving challenges of the ocean environment, possibly due to genetic adaptation to captivity (Christie, Ford et al. 2014). In other words; when it comes to return the stakes are against the Lone population both in terms

of locality and their long hatchery background, so their low return rates are difficult to attribute to the high frequency of abnormal otoliths alone, although the two may be connected.

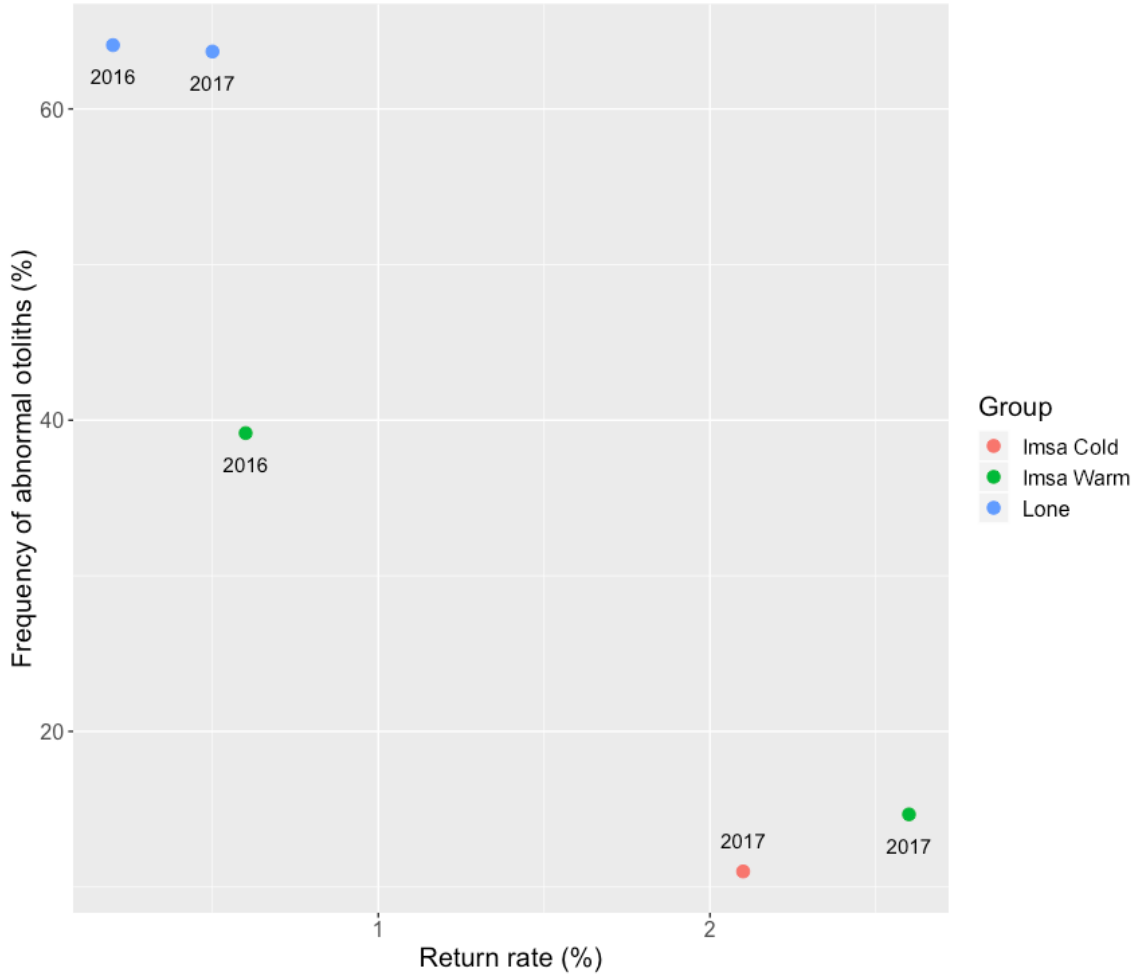



**Fig. 4.1:** Frequency (%) of abnormal otoliths for the smolt of each released group/year plotted against adult return rate (%) of the corresponding group/year. Note that only data of the 2017 Imsa Cold group is included, due to very few smolt of this group analyzed in 2016.

More interesting though, is the between-year pattern of negative correlation between frequency of abnormal otoliths and return rate seemingly present for the Imsa Warm group, with return rates of 0.6% and 2.6%. But one factor complicates this picture further: the discrepancy of the way in which these were tagged. In 2017 2985 of the Imsa Warm group (Tot. N=4965) were PIT-tagged, while the rest were Carlin-tagged. In 2016 all the Imsa Warm fish were Carlin-tagged. Meaning the higher return rate/survival may, at least in part, be attributed to lower tag-mortality of the 2017 Imsa Warm group. Studies have shown that Carlin-tags results in less activity and longer migration periods than

PIT tags, and survival of Arctic charr (*Salvelinus aplinus*) indicate that Carlin-tags may double the mortality compared to those tagged with interior tags. However, this tagging method may interfere with fish behavior and increase post-smolt mortality (Strand, Finstad et al. 2002, Huusko, Huusko et al. 2016). The majority of the Imsa Warm fish that returned in 2018 were indeed PIT-tagged.

The figure (**Figure 4.1**), although speculative, is interesting. But the figure is not complete; as previously stated, more fish of the 2017 release are expected to return in the fall of 2019 and therefore couldn't be included as a part of this thesis. However, if the number of 2SW fish from the 2016 release is any indication (Tot. N=9), the 2019 numbers will most likely be low, and the figure probably won't change drastically.

A general take-away from this subchapter is that return rates are low for all groups. Here also lies the main issue in determining the effect abnormal otoliths may have had; very few returned in general. This is not unique for the salmon of this experiment, nor unique only for hatchery-reared fish, although return rates of wild salmon are higher than for hatchery-reared salmon. The current decline of wild salmon populations is attributed to low growth rates, reduced sea age at maturity (less multi sea winter individuals) and low marine survival (Jonsson and Jonsson 2004). Marine mortality may directly be increased as a result of suboptimal temperature conditions due to climate change or farmed salmon escapees compromising the genetic integrity of wild salmon, as well as infestation of sea lice, which is more severe when survival is low to begin with (Chaput 2012, Vollset, Barlaup et al. 2019). Furthermore, 1SW salmon have higher return rates than multi sea winter, and a high initial post-smolt growth rate is important as predation is often limited by size, for that reason decreasing growth in the freshwater phase may also contribute to the sea mortality trends (Gregory, Ibbotson et al. 2019). Hatchery fish differ in marine growth from wild; behaviorally due to their limited foraging capability as a result of being fed, and although they may be larger as smolt this somatic growth does not necessarily correlate directly with development (Vollset, Barlaup et al. 2019). The smolt of this experiment were larger than they would have been in natural conditions,

but may still be somewhat underdeveloped and less capable of coping with the pelagic environment. Because there are many extrinsic and intrinsic factors that may be affecting the hatchery-reared Atlantic salmon survival of this experiment, it is difficult to directly correlate their low return rates to them having normal or abnormal otoliths. On the other hand, in general the adults of this experiment that returned had a lower frequency of abnormal otoliths than the released smolt, so there is an indication that otolith abnormalities may have an effect on survival of Atlantic salmon.

## 4.4 Weaknesses and future perspectives

Ideally, this experiment would be designed in such a way that the smolt were all tagged in the same way and released in equal numbers both years and for all groups. Similarly, the number of smolt analyzed should ideally also have been the same and equal for each group. Here lies the weakness of this experiment. However, as these experimental fish were parts of other experiments not related to this, it is positive that they can be used to acquire knowledge and be researched in multiple areas.

In the future it would be interesting to conduct this experiment over more than two years to collect empirical data to determine the strength of the results with collectively larger sample sizes. Additionally, if there was a way to analyze the otoliths of an individual fish without having to kill it, experiments could possibly also answer the questions raised of whether the poorer performance of a hatchery-reared Atlantic salmon may be specifically attributed to their abnormal otoliths or to one of the many other factors that makes it different from a wild Atlantic salmon.

# 5. Conclusion

In this experiment, groups of hatchery-reared Atlantic salmon smolt varied in frequency of abnormal otoliths depending on growth rate, population and number of generations in hatchery. Faster growing smolt had an increased chance of having at least one abnormal otolith, which was also the case for smolt belonging to the group with the most generations in hatchery, based on logistic models. Significant length or group effects on the degree of vaterite (% vaterite) in the abnormal otoliths was not found, although there was a slight indication that the vaterite coverage was larger for groups that had grown faster. These results reinforce prior studies suggesting that fast growth causes abnormal otoliths in Atlantic salmon. A number of returning adults had abnormal otoliths with high vaterite coverage; abnormal otoliths were not completely detrimental. However, in total, there was a significantly lower frequency of abnormal otoliths in the adults than in the smolt, which was also the case for most of the groups separately. This may indicate a negative effect of abnormal otoliths on survival of Atlantic salmon.

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# Appendix A

The simplified script used to analyze pictures of otoliths in Python.

```python
#!/usr/bin/python

from __future__ import division

import os
import numpy as np
from scipy.misc import imread

def analyze(image):
  red = np.where((image[:, :, 0] > 0) & (image[:, :, 1] == 0) & (image[:, :, 3] > 0))
  green = np.where((image[:, :, 1] > 1) & (image[:, :, 0] == 0) & (image[:, :, 3] > 0))
  redAndGreen = np.where((image[:, :, 0] > 0) & (image[:, :, 1] > 0) & (image[:, :, 3] > 0))

  vaterite = len(image[red])
  aragonite = len(image[green])
  joined = len(image[redAndGreen])

  proportion = (vaterite + joined / 2) / (vaterite + aragonite + joined)

  return [
    vaterite,
    aragonite,
    joined,
    proportion
  ]

def main(imgName):
  imagePath = os.path.dirname(os.path.realpath(__file__)) + '/' + imgName
  image = imread(imagePath).astype(np.float32)

  vaterite, aragonite, joined, proportion = analyze(image)

  # print(vaterite)
  # print(aragonite)
  # print(joined)
  # print(proportion)

  id = imagePath.split('/').pop().replace('.png', '')

  file = open('result.txt', 'w')
  file.write('{:06d},{:10.16f}'.format(int(id), proportion))
  file.close()

if __name__ == "__main__":
    main('1.png')
```

# Appendix B

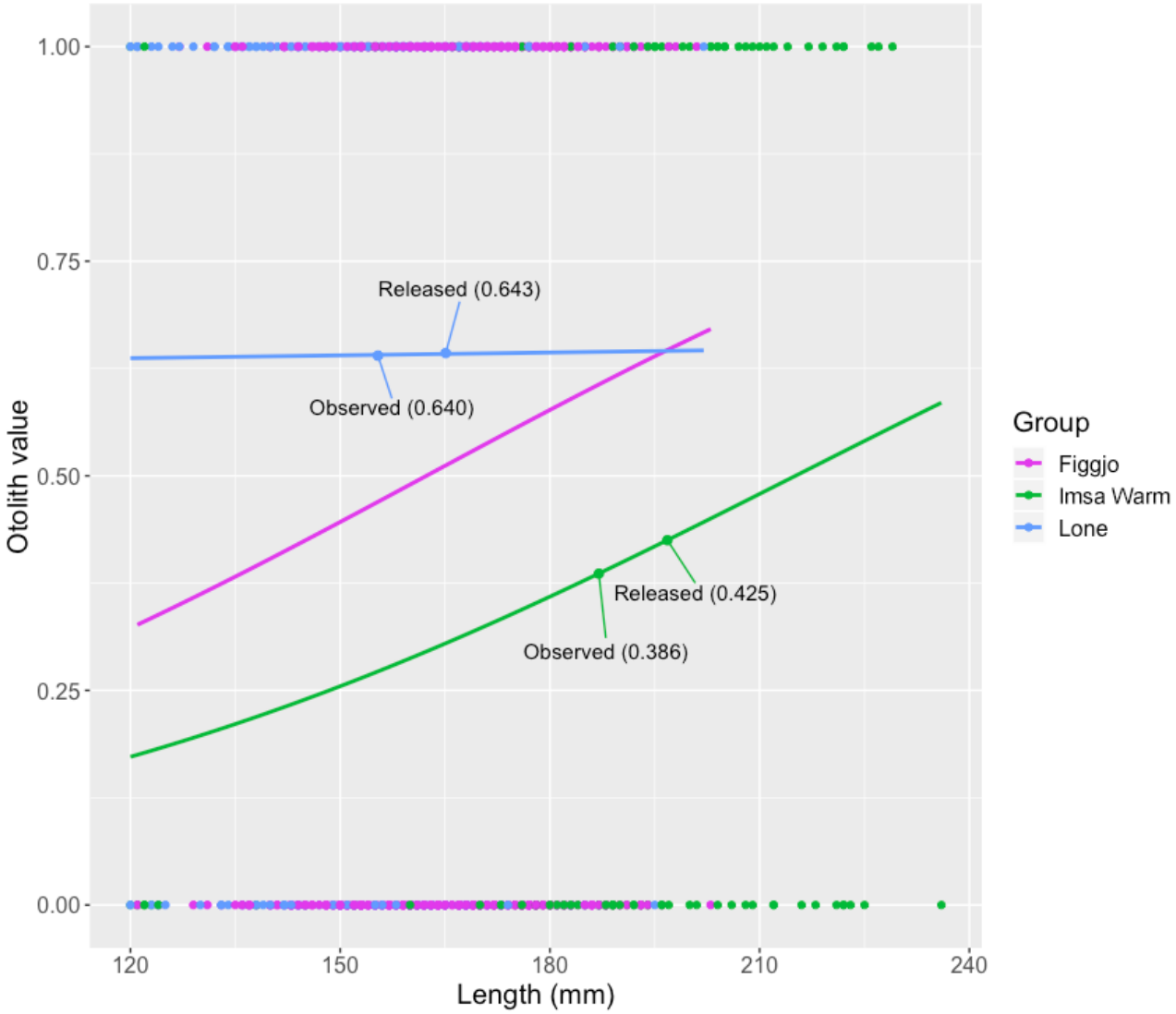


**Figure 1.B:** The mean frequencies of abnormal otoliths of smolt that were analyzed (Observed), and the estimated frequencies of the released smolt based on their mean length (Released) for the 2016 smolt groups.

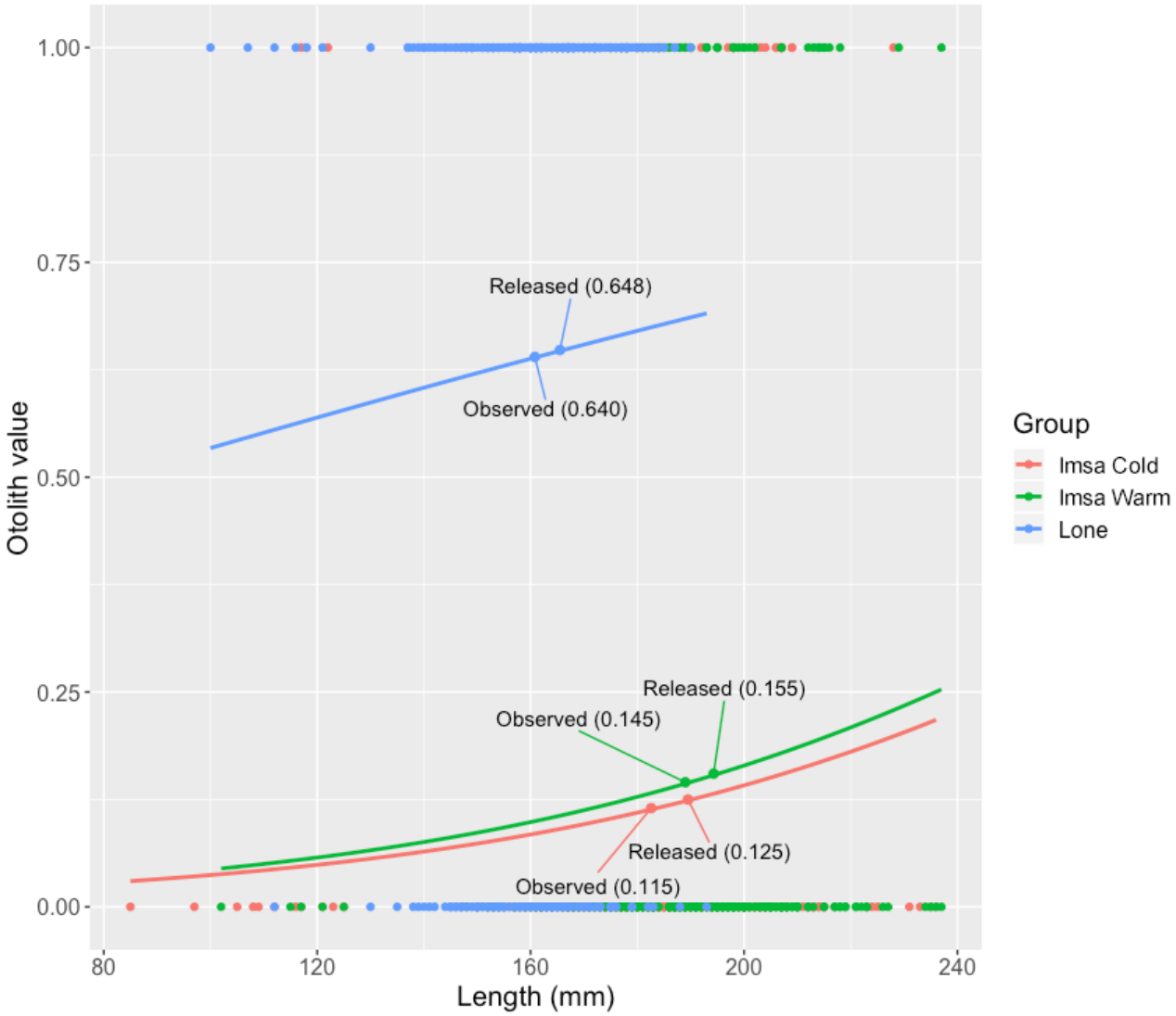


**Figure 2.B:** The mean frequencies of abnormal otoliths of smolt that were analyzed (Observed), and the estimated frequencies of the released smolt based on their mean length (Released) for the 2017 smolt groups.

**Table 1.B:** Table showing mean lengths ± SD (mm) of the smolt analyzed and mean lengths ± SD (mm) of the smolt released. Frequencies of the released smolt are estimated based on slopes of the logistic regression models.

| ***2016*** | **Imsa Warm** | **Imsa Cold** | **Lone** |
|---|---|---|---|
| Mean length (Observed) | 187.0±20.06 | 140.8 mm | 155.4±15.16 |
| 95% CI | 183.4-190.6 | | 152.5-156.3 |
| Mean length (Released) | 196.8±23.49 | 180.5±17.35 | 165.7±14.79 |
| 95% CI | 195.9-197.6 | 179.7-181.3 | 165.1-166.4 |
| Frequency (Observed) | 39% | 29% | 64% |
| Frequency (Released) | 43% | -- | 64% |
| ***2017*** | **Imsa Warm** | **Imsa Cold** | **Lone** |
| Mean length (Observed) | 189 ±19.06 | 182.6±22.51 | 160.8±13.42 |
| 95% CI | 186.9-191.0 | 180.1-185.1 | 159.3-162.3 |
| Mean length (Released) | 194.3 ±20.32 | 189.5±22.39 | 165.5±22.39 |
| 95% CI | 193.7- 194.9 | 188.6-190.4 | 164.5-166.5 |
| Frequency (Observed) | 15% | 12% | 64% |
| Frequency (Released) | 16% | 13% | 65% |

**Table 2.B:** Results of $\chi 2$-tests performed for difference in the estimated frequency of abnormal otoliths of the released smolt and the returning adults. Significant p-values are presented in bold.

| ***2016*** | $\chi 2$ | p-value |
|---|---|---|
| Lone | -- | -- -- |
| Imsa Warm | 6.73 | **0.009** |
| Imsa Cold | **--** | **--** |
| **Total** | 6.78 | **0.008** |
| ***2017*** | | |
| Lone | **--** | **--** |
| Imsa Warm | 2.95 | 0.08 |
| Imsa Cold | 5.48 | **0.02** |
| **Total** | 0.09 | 0.75 |
| ***Grand total*** | 408.64 | **<0.0001** |