Embryo Development Under Context of Hybridization and a Review of Regional Patterns in Hybrid Frequency of Atlantic Salmon and Brown Trout

by

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ABSTRACT

Reproductive isolating mechanisms that maintain the integrity of species sometimes fail, resulting in hybridization. Such isolating mechanisms occur pre- and post-gamete release, and influence nuances of sperm phenotype that may affect offspring development. Hybridization between congeneric Atlantic salmon and brown trout occurs in their native range of sympatry in Europe, and via human introductions of brown trout in North America. It is known in other hybrid systems that pre-zygotic post-copulatory isolation via cryptic female choice perhaps mediated by ovarian fluid can bias fertilization towards conspecific sperm. Anthropogenic hybridization of these species in the invaded range of North America is not well understood. This provides an opportunity to understand mechanisms of isolation between the species. Therefore, I explored 1) developmental characteristics of offspring sired from sperm exposed to conspecific and heterospecific ovarian fluid and 2) developmental characteristics of pure salmon, pure trout, and bidirectional hybrids in two fluctuating temperature regimes. Ovarian fluid had no effect on the development of offspring, however, hybridization did. Hatch success of hybrids was high, supporting previous work. Although effect sizes were small, hybridization generally caused earlier hatching for each female, and hybrids produced with salmon eggs suffered the fastest rate of mortality shortly after hatching relative to hybrids of trout eggs. This is important, for previous work reported hybrids produced from trout eggs die shortly after hatching.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

Reproduction often represents the merger of two genomes, and evolutionarily can be considered as the ultimate goal of life (Fleming, 1996). Although it may seem fine-tuned and rather straightforward, for sexual reproduction to succeed and offspring to exist for most vertebrates that choose their mate(s), several processes must align and function between a female and male. First, individuals have to be able to interact, and reproduction timing must overlap. Second, rituals such as courtship must succeed, and genitalia have to be compatible/function. Third, post-copulatory pre-zygotic isolating mechanisms such as sperm competition and cryptic female choice must allow fertilization to happen. Finally, developing embryos must be viable and survive to hatching/birth. These are the processes responsible for maintaining the boundaries and integrity of species, by preventing interbreeding. The breakdown in these reproductive isolating mechanisms can result in hybridization.

The evolutionary outcomes of hybridization vary widely, but all have an influence on speciation (Malukiewicz et al., 2015; McQuillan et al., 2018). Whether hybridization is viewed as novel, adaptive, or disruptive is a continuous debate, and commonly depends on the context or discipline (Genovart, 2009). Fortunately, two recognized categories of hybridization exist that help classify this phenomenon. Natural hybridization is defined as the secondary contact between two populations that have evolved separately over a long period of time (Genovart, 2009). Generally, botanists are concerned and interested in this category of hybridization, because plants naturally interbreed frequently (Neri et al., 2018: Stebins, 1950). By contrast, there is anthropogenic hybridization, which is caused by species introductions, habitat alteration, and climate change (Hegarty, 2012). This category is of most concern to conservation biologists (Rhymer & Simberloff, 1996; Todesco et al., 2016), and is predicted to continue increasing following species introductions to foreign environments (Muhlfeld et al., 2014). The consequences of anthropogenic hybridization are often destructive, and can result in genetic swamping, outbreeding depression, and in most extreme cases, extinction (Fitzpatrick & Shaffer, 2007). This category of hybridization is a direct way for foreign species to invade native genomes, which has recently been highlighted as a significant problem forecasted to increase (Muhlfeld et al., 2017). When reproductive barriers breakdown and hybridization occurs, these events present the opportunity to address questions regarding mechanisms that maintain isolation, and how interbreeding may affect the developmental characteristics and/or fitness of offspring.

Developmental characteristics are strongly influenced by both maternal and paternal effects, often presenting complex evolutionary dynamics for species (Qvarnstrom & Price, 2001). For example, in Atlantic salmon (*Salmo salar*), timing of fertilization influences the timing of hatch and emergence, and strongly affects offspring survival and size at a common point in time (Einum & Fleming, 2000). This extent of variation influences the potential for selection, thus driving evolution. Consequently, early life is when most mortality occurs during the life-history of many species with high fecundities (Bogner et al., 2016), and what offspring experience during this time can have life-lasting effects on their reproduction and overall fitness (Clarke et al., 2016; Marshall et al., 2017). These effects, whether they are severe or benign can influence changes in population dynamics. Therefore, developmental characteristics of offspring that mediate the evolutionary trajectory of populations and species are important to understand. This is particularly interesting in the context of hybrids, considering selection is acting upon offspring whose DNA represents a merger of two species that have evolved to meet different adaptive requirements. The salmonid fishes (family: Salmonidae) are excellent model species to address this phenomenon.

Salmonids are remarkable, beautiful, and important animals that have significant value in cultural, recreational, and commercial fisheries (Quinn, 2018). In addition, they are widely cultivated in aquaculture practices. Conservation and management practices try eagerly to preserve the naturally evolved genetic structure of wild trout and salmon in their native ranges, although the forecasted future of an increasing human population and changing climate will undoubtedly cause more destruction for these fishes and their habitats (Waples & Hendry, 2008). Salmonids have been moved outside of their native ranges and introduced to foreign environments, primarily for angling, aquaculture, and commercial harvest opportunities (Jonsson & Jonsson, 2011; Quinn, 2018). Whether the ecological consequences were/are known or not, these fish have caused an array of ecological, economical, and cultural consequences (Macchi & Vigliano, 2014). Meanwhile, many populations are considered threatened in their natural ranges, where anthropogenic alterations to their habitats have had substantial effects. That being said, natural disturbances such as glaciation, volcanic activity, and landslides have been followed by gradual recovery of many stocks (Waples et al., 2008), evidence of the natural resiliency of these fishes.

Widespread introductions of salmonids have resulted in hybridization (Krueger & May, 1991), competition with native species (Macchi et al., 1999), and modifications of community structure (Martinez-Sanz et al., 2010). For example, in the Pacific Northwest of the United States, brook trout (*Salvelinus fontinalis*) were introduced and hybridize with native bull trout (*Salvelinus confluentus*), effecting their status as a threatened species (Dehaan et al., 2010). Similar effects of interbreeding have been well documented in the Rocky Mountain region of the

United States, involving introduced rainbow trout *(Oncorhynchus mykiss*) and native cutthroat trout (*Oncorhynchus clarkii*) (Muhlfeld et al., 2009). Hybridization of these two species threatens all remaining cutthroat sub-species in western North America, two of which are now extinct, and five that are listed as threatened under the Endangered Species Act (Kovach et al., 2015).

Both the rainbow trout and brown trout (*Salmo trutta*) make the top 100 list of the world's worst invasive alien species, according to the Invasive Species Specialist Group (Lowe et al., 2000). While rainbow trout are the world's most widely introduced invasive fish (Muhlfeld et al., 2014), the brown trout is arguably a similar "poster-child" of salmonid species that has toured and invaded the world by way of human transit and dispersal, while still owning the respect and love by anglers everywhere. One of the most well-known impacts brown trout have had outside of their native range is the displacement of native fish of the family *Galaxiidae* in the southern hemisphere. The latter are now only found in locations unoccupied or inaccessible to brown trout (Townsend, 1996). In New Zealand, rainbow trout and brown trout were introduced during the late 1880s and are now the top aquatic predators in the region (Wissinger et al., 2006). Brown trout are currently the most pervasive and abundant fish in New Zealand and have been responsible for the widespread decline of New Zealand's native fish (Jones & Closs, 2017).

Among the slough of ecological effects brown trout have caused following human-mediated introductions (Budy et al., 2013), one of the least recognized problems is their ability to hybridize with wild brook trout and Atlantic salmon (Quilodrăn et al., 2014). Recently, the introduction of foreign and invasive fish species has been highlighted as a major factor affecting the conservation of Atlantic salmon in North America (Gibson, 2017), a species whose range has declined significantly (Waples et al., 2008), and whose status is threatened by several other factors. North America is unique, for it is the only place in the world where hybridization of indigenous Atlantic salmon and non-native brown trout is directly influenced anthropogenically, via species introduction. Interbreeding between these two species occurs in their native range of sympatry - Europe (Álvarez & Garcia-Vazquez, 2011), and although there are reach-scale differences regarding the frequency of hybrid fish and the direction of hybridization (Matthews et al., 2000; McGowan & Davidson, 1992b), there are no distinct patterns/differences between North America and Europe (see Chapter 3).

Atlantic salmon and brown trout exhibit a high degree of natural variation directly influenced by the environments in which they inhabit (Jonsson & Jonsson, 2011). In addition to being phenotypically plastic, genetic diversity is produced and expressed among, and within populations. A great deal of information is known about these fishes, including: heritability, phenotypic plasticity of life history traits, and how selection is influenced by sources of variation in reproductive success (Hendry & Stearns, 2004). However, little is known about hybridization of these two species in North America. Brown trout were first introduced to North America (Newfoundland) in 1883 and have since invaded many watersheds on the island (Westley & Fleming, 2011). Interestingly, information regarding hybrid development in Europe where both species coevolved exists, presenting the opportunity to examine potential differences between continents, and how reproductive barriers may have weakened in North America due to long isolation.

Sexual selection plays a major role in the reproductive success of salmonids (De Gaudemar, 1998). Secondary sexual characteristics of males increase their opportunity for mating with females. The hooked jaw, enlarged adipose fin, vibrant coloration, and male-male fighting are all evolutionary traits that aid in courtship (Järvi, 1990). This type of selection is demonstrated across a wide range of taxa, and is well-known in birds, for the males are generally more “vibrant” and “attractive” than the females. Rather simply, if one is “more handsome” than the competition, that should result in greater access to a/more females. Interestingly, sexual selection occurs beyond courtship and gamete release in salmon and trout. Recent work has shown that ovarian fluid, a viscous, protein rich fluid that coats and is released with eggs can facilitate cryptic female choice for specific males (Lehnert et al., 2017). This is fascinating, considering this strong selective force occurs outside of the female’s body while in running water. Yeates et al. (2013) discovered that when sperm of Atlantic salmon and brown trout are in competition, the female’s ovarian fluid has conspecific sperm preference, biasing fertilization. This is a good demonstration of post-copulatory pre-zygotic reproductive isolation, a powerful evolutionary mechanism which can help maintain species boundaries. However, these mechanisms might breakdown and may be context dependent. For example, long isolation from brown trout could have eliminated the need or reduced the effectiveness of conspecific sperm preference for Atlantic salmon in North America.

Recently, evidence has emerged that sperm contribute more than the paternal genome, and development and fitness of subsequent offspring can be heavily influenced by variation in sperm phenotype (Evans et al., 2019). Sperm longevity has been shown to directly affect survival in zebra fish (*Danio rerio*) offspring (Alavioon et al., 2017), and size and timing of hatching of Atlantic salmon (Immler et al., 2014). In guppies (*Poecilia*), a live-bearing fish, prolonged sperm storage has been shown to impair the quality of sperm in offspring (Gasparini et al., 2017), and poor male diet has been linked with smaller body size of offspring at birth. All these examples have direct fitness implications that can result in strong selection for certain genotypes and/or phenotypes. Thus, demonstrating that the environment in which sperm is exposed to plays a large role in the evolutionary trajectory of populations. It is obvious that males contribute a lot more to offspring then what has been traditionally thought. Combining the strong selective pressures of ovarian fluid, the well-documented maternal effects associated with eggs, and more recently, the paternal affects induced by ejaculate traits, creates an enormous amount of potential variation associated with gametes.

This thesis capitalizes on the opportunity to explore post-mating consequences of hybridization where brown trout were introduced and have invaded the native range of Atlantic salmon, and whether mechanisms reducing hybrid fertilization have subsequent effects on the development of offspring. For Chapter 2, we explored the question: does sperm exposure to heterospecific ovarian fluid effect development differently than sperm exposure to conspecific ovarian fluid? For Chapter 3: what effect does hybridization have on offspring within a female? These questions are directly linked and are important for our current understanding of gamete evolution, reproductive isolation, hybridization, brown trout invasion, and Atlantic salmon conservation.

Co-authorship Statement

Data collection and writing was done by author of this thesis. Experimental planning and design was done by Dr. Craig Purchase and the author. Dr. Craig Purchase, Dr. Ian Fleming, and Dr. Peter Westley provided substantial contributions to experimental design, data interpretation, and editorial reviews for all chapters. Heather Penney provided substantial guidance and contributions to data processing and analyses.

Publication and submission status:

Chapter 2: Will not be submitted for publication, as further work is needed to make strong conclusions. It may eventually get combined with work conducted outside of this thesis to create a manuscript.

Chapter 3: (Poulos, Purchase and Penney) will be prepared for submission to a peer-reviewed journal. Chapters 2 and 3 used data that were derived from the same experiment, although the tested hypotheses and supporting background is different. The Associate Dean of the School of Graduate Studies was consulted on how to proceed with incorporating this peculiarity into the thesis. She advised to proceed in producing a manuscript style thesis, but to add this note indicating duplicate methods. Therefore, most of the methodology (aside from the data analyses) is the same for both chapters, but Chapter 2 will not be submitted for publication.

Following almost identical results among treatments for Chapter 2, it was encouraged and decided by the author, supervisor, and committee to use heterospecific ovarian fluid exposed offspring as replicates for the Chapter 3 analyses.

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Chapter 2. Does conspecific and heterospecific ovarian fluid induce ejaculate-mediated paternal effects for hatch success and timing of two closely related fish species?

Abstract

Recent evidence has shown that variation in sperm phenotype can have fitness implications on subsequent offspring. These findings challenge the longstanding view of sperm as solely a transport mechanism for paternal genes. Given the length of time salmon sperm swim before contact with eggs influences offspring development, and because of the profound effects their ovarian fluid has on sperm swimming performance, we explored offspring development as a function of sperm exposure to conspecific versus heterospecific ovarian fluid in Atlantic salmon, brown trout, and their bidirectional hybrids. We raised pure and hybrid half-sibling families from egg to yolk-sac absorption with ovarian fluid swapped intra and interspecifically among females prior to fertilization. We found no significant differences in hatch success or timing for either exposure treatment, possibly due to the methods employed or suggesting that there is no effect.

Introduction

It has long been assumed that the main role of sperm is to deliver paternal genetic information to the ovum, although recent discoveries suggest that sperm contribute more than DNA (Evans et al., 2017, 2019; Krawets, 2005), and offspring fitness can be mediated by variation in sperm phenotype (Alavioon et al., 2017). This variation is directly influenced by the environment in which the father inhabits (Evans et al., 2019), and selection occurs both pre and post-ejaculation (Marshall, 2015). Simple characteristics of semen can dramatically affect fertilization (Loutradi et al., 2006), for example, sperm motility (movement of sperm cells), swimming speed (Yeates et al., 2003), and chemical parameters of ovarian fluid (Rosengrave et al., 2009) all contribute to fertilization success. As such, many aspects of semen are under selection. While components of sperm phenotype governing fertilization success have been reasonably explored, there is a lack of information on post-fertilization effects.

Two recent studies have demonstrated that variation in sperm swimming longevity affects the development and fitness of subsequent offspring in fishes. In zebrafish (*Danio rerio*), post-ejaculated longer-lived sperm (from within ejaculates) sired offspring with higher survival and greater adult male fitness when compared to offspring sired from short-lived sperm (Alavioon et al., 2017). Similar results have been demonstrated in Atlantic salmon, where sperm used to fertilize eggs 20 seconds after water activation sired offspring that developed faster than offspring sired from sperm that were activated and used for fertilization after 0 seconds, and 40 seconds (Immler et al., 2014). These findings suggest that even a simple delay in contact between water activated sperm and eggs, dramatically affects offspring development. Furthermore, in other fishes, poor diet in males has been shown to produce smaller offspring at birth (Evans et al., 2017), and prolonged sperm storage impaired sperm quality of descendent male offspring (Gasparini et al., 2017). These dramatic outcomes have substantial fitness implications on both the offspring and their parents.

Post-ejaculate selection on sperm phenotype is mediated by the female through cryptic female choice (Birkhead & Pizzari, 2002), and it has been well documented that female ovarian fluid in several species of external fertilizers can bias fertilization (reviewed by: Firman et al., 2017). This is clearly demonstrated in salmonids, as fertilization success can be high for both pure and hybrid crosses (Álvarez & Garcia-Vazques, 2011), but when sperm of two species are in sperm competition (*Salmo*), the females' ovarian fluid helps "choose" conspecific sperm (Yeates et al., 2013). Such strong sexual selection mechanisms create selective pressures influencing ejaculate traits (Lehnert et al., 2017).

As evidence increasingly demonstrates that sperm phenotype has fitness implications for offspring beyond fertilization (Alavioon et al., 2017; Evans et al., 2019; Gasparini et al, 2017; Immler et al., 2014; Marshall, 2015) and given the profound effects of ovarian fluid on sperm fertilization capacity (Lehnert et al., 2017; Rosengrave et al., 2008; Yeates et al., 2013), we expect cryptic-female choice (through ovarian fluid influences on conspecific sperm preferences) to affect offspring development independent of fertilization success. This prediction is important to help further understand sexual selection at the gametic level, and how variation in sperm affects ontogenetic development; providing an excellent model for addressing how reproductive barriers are evolving between species (Schwenk et al., 2008), and how post-copulatory sexual selection generates adaptations and drives evolution (Birkhead & Pizzari, 2002).

Here, we investigated ontogenetic development of offspring as a function of sperm exposure to conspecific versus heterosepecific ovarian fluid. We had two questions of interest: does sperm exposure to ovarian fluid affect (1) hatch success (proportion of eggs hatched), and

(2) the timing to $\geq$50% hatch (mid-hatch). We hypothesized that there are no differences in hatch success (combination of fertilization success and embryo survival) between conspecific and heterospecific exposure, as other work has shown (Yeates et al., 2013). Hatch timing varies among species but can also be mediated within species by the traits of the maternal and paternal parent. We thus hypothesized a difference, but of unknown direction. We used Atlantic salmon, brown trout, and their bi-directional hybrids for our model species, because their sperm (pure trout and pure salmon) performance has a significant range of natural variation (Gage et al., 2004; Vladic et al., 2010), and because of the intense selection induced by cryptic female choice via ovarian fluid (Yeates et al., 2013).

Methods

Gamete collection

Atlantic salmon (AS) were taken from a fish ladder on the Exploits River in Grand Falls, Newfoundland, Canada by the Environmental Resource Management Association on September 8, 2016. Fish were held in large outdoor tanks with a flow-through river water system until ready to spawn. On November 4, 2016, salmon were anesthetized with clove oil before semen and eggs were stripped by drying the urogenital pore and applying gentle pressure to the fish's abdomen. Eggs were collected in glass jars and semen in plastic bags. Gametes were packed in a cooler with ice and were brought to St. John's within 12 hours. On the same day of AS spawning, brown trout gametes were collected and stored in a similar manner around the same time, from a small stream that flows from Newfound Pond to Windsor Lake in St. John's, Newfoundland (refer to: Westley & Fleming (2011) for details on brown trout introduction and strain). Fish were dip netted and then held in a live-well for a few minutes before they were stripped for gametes.

Breeding design and fertilization

The experiment utilized a full factorial breeding design with gametes from 2 female salmon, 2 female trout, 3 male salmon and 3 male trout. To test our hypotheses, we had an ovarian fluid treatment with two levels: (1) sperm exposure to conspecific ovarian fluid (OF), and (2) sperm exposure to heterospecific OF. We swapped OF between individuals of the same species, and between species (Table 2-1 is an example of ¼ of the design, that from Salmon female 1). For example, offspring derived from Female Salmon 1 eggs had OF exposure from Female Salmon 2. By using OF from a foreign individual of the same species, we isolated heterospecific effects from simply being non-self. For fertilizations that used eggs of one species and OF from the other, a combination of equal volumes of OF were used from two females. For example, siblings derived from salmon eggs and trout OF had OF from Female Trout 1 and Female Trout 2 (Table 2-1). Whether OF exposure was the conspecific or heterosepcific treatment was dependent on the sperm species (Table 2-1). Each female produced 6 families (100 eggs per family), with 25 eggs for both treatments and in both temperatures (Table 2-1).

OF was separated from eggs using a plastic strainer lined with fine mesh nylon and collected in a 200mL glass beaker. To ensure residual OF was removed, the eggs and strainer were thoroughly rinsed using a 0.9% salt solution, which does not activate salmonid eggs, that was made from river water (Rennie’s River; next to campus) and aquarium salt (Instant Ocean). Eggs were gently poured from the strainer to a 1000mL glass beaker. Eggs and OF from each female were stored in separate beakers, labelled, covered with Petri dishes, and held in a dark refrigerator at 4.4°C for less than one hour until fertilization. This procedure was repeated for each individual female.

We calculated the average volume of OF to eggs in Atlantic salmon (~25%), and brown trout (~16%) using 7 salmon and 9 trout that were sampled on the same day, time, and location. These gametes were not used for this experiment. We were limited by amount of OF we obtained from one experimental salmon and two trout, so a volume of 12.8% OF to eggs was used to ensure the most standard OF possible per sub-family fertilization.

All female salmon #1 eggs were fertilized first, then female trout #1, then female salmon #2, then female trout #2 taking about 35 minutes per female. Each female's eggs were removed from the refrigerator, along with all the OF and semen. Semen was carefully transferred from Zip-Lock Bags to labeled and chilled 10mL beakers using a plastic 3mL pipet. 6.5mL of eggs (~ 60 eggs and no OF) were measured with a 10mL vial, and gently poured into a 200mL beaker. This was repeated twelve times (one female = 6 half-sibling families x 2 OF treatments), and beakers were placed on an aluminum tray with crushed ice covering the bottom. 9.5mL of OF was added to each beaker of eggs using a syringe for conspecific treatments, and two syringes with 4.75mL of OF was added to each beaker for heterospecific treatments (Table 2-1). Each females' OF had a unique syringe to avoid cross contamination.

Using one syringe per male, 10µL of semen was added to each beaker (already containing eggs and treatment OF), then 2mL of river water (Rennie's River; next to campus) was added using a syringe directly after. The syringe was used to briefly mix the eggs, semen, and water. Semen was exposed to a concentration of 83% OF to water ratio (9.5/11.5ml). After one tray of beakers (one female) was fertilized (OF added, semen added, 2mL water added and mixed), extra river water was poured to fill each beaker to 200mL, ~2 minutes after fertilization. The tray was returned to the refrigerator after the eggs were submerged in water. The other trays (eggs of three females) were fertilized following the same protocol. All samples were stored in a refrigerator at 4.4°C overnight to water harden.

Incubation

Five hours post-fertilization only two eggs had turned white (non-viable). Two samples of 25 viable embryos were removed from each beaker and each placed in a 5.8 cm tall x 5.8 cm inner diameter Polyvinyl Chloride (PVC) tube with a fine mesh bottom, in a plastic tray with refrigerated river water. Forty-eight PVC tubes (4 females x 6 half-sib families x 2 OF) were loaded into each incubator. Twelve PVC tubes were put in each tray (4 trays per incubator). Each tray represented embryos from one female; from top to bottom in each incubator: female salmon 1, female trout 1, female salmon 2, and female trout 2.

Two four-tray vertical incubators were setup in a refrigerated room that maintained a constant air temperature of 5°C. To determine if phenotypic responses to ovarian fluid were environmentally context dependent, treatments were exposed to two temperature regimes. These were not meant to mimic exact thermal profiles of a given population, but to represent different conditions as often experienced in early winter (Rooke et al., in review). Incubator A (temperature A) and incubator B (temperature B) followed fluctuating temperature profiles, with temperature A accumulating thermal units slower than temperature B (Figure 2-1). Two electric water chillers and two 300-watt submersible electric aquarium heaters were used to adjust the water temperature of each incubator. Pumps circulated tap water at a rate of 16L/minute through a three-stage filtration system and then through a UV light filter. Prior to hatching, water pH, ammonia, and nitrate levels were measured and recorded once a week, then every other day following first hatch (January 6, 2017) to check water quality. Approximately 35 liters of dechlorinated and chilled tap water were exchanged from each incubator every other day following the first hatch.

Embryos were initially checked twice a week then daily following first hatch. White (non-viable) eggs were removed to maintain water quality, and for data collection regarding mortality metrics. The number hatched, the number not hatched, and mortality as an embryo or alevin was recorded per PVC tube at each checking. Dead alevins were removed. PVC tubes were left in the incubator until ≤ 10 live alevins remained (sampling procedure for a different experiment).

Statistical approach

For each analysis, significance was set at α = 0.05, and assumptions of parametric statistics were checked by examining model residuals. We used a generalized mixed-effects model with binomial regression (hatch or not hatch) to analyze hatch success (Linear Mixed Effect Model, “lme4” package in R version 3.2.2). We used a linear mixed-effects model to analyze hatch time at ≥ 50% (mid-hatch). Each full model included: OF exposure (conspecific and heterospecific), sperm species (salmon and trout), egg species (salmon and trout) and temperature (A and B), and all possible interactions as fixed effects; and mother ID and father ID as random effects. For each response variable we started with the full model (Model 1). Higher order interaction terms were sequentially removed if insignificant. If interaction terms were significant the model was broken into sub-components by temperature.

Model 1:

DV ~ exposure*sperm*egg*temperature + sperm*egg*temperature + exposure*sperm*egg + exposure*egg*temperature + exposure*egg + exposure*sperm + sperm*temperature +

sperm*egg + egg*temperature + exposure*temperature + exposure + sperm + eggs + temperature + (mother-random) + (father-random) + error

Results

Hatch success

The 4-way and all 3-way interactions were non-significant and removed from the model. The OF exposure treatment had no effect on hatchability (χ^2_1 = 0.037, p = 0.951); mean percentage of eggs hatched was identical overall (conspecific = 90%; heterospecific = 90%). A high proportion of eggs hatched for each cross-type, at both temperatures for both OFs (Figure 2). None of the explanatory variables significantly affected hatch success (Table 2-2), but hatchability of the two salmon's eggs was generally higher than those of the two trout (Figure 2-2).

Hatching timing

The 4-way interaction and the four 3-way interactions were not-significant and removed from the model. The average accumulated thermal units at which $\geq$50% of the eggs hatched did not differ between OFs (χ^2_1 = 2.362, p = 0.124, Figure 3). Sibling embryos in temperature B took more ATUs to hatch (overall mean = 471) than those in temperature A (overall mean = 404; Table 2-3, Figure 2-3). Hybrid embryos hatched earlier than pure crosses (egg*sperm, Table 2-3, Figure 2-3). For effect sizes of hatching timing between pure and hybrid embryos, refer to Chapter 3 hatching timing results.

Discussion

Results from this experiment suggest that sperm exposure to ovarian fluid type has no effect on the hatch success, or the timing to mid-hatch for Atlantic salmon, brown trout, or their bi-directional hybrids. Therefore, our hypothesis regarding hatch success was supported and our hypothesis for hatch timing was not. Hatch success was generally high as other work has shown (Yeates et al., 2013), and the same proportion for conspecific and heterospecifc ovarian fluid (OF) exposure. Timing was significantly affected by temperature treatment, supporting that the rate in which thermal units accumulate affects the amount needed for eggs to hatch. Timing was also significantly affected by the maternal species, and the egg and sperm interaction, suggesting that Atlantic salmon, brown trout, and their hybrids hatch at different times.

We were curious if ovarian fluid would have an effect on the development of two closely related species, given quality varies among females and can affect individual ejaculates differently (Urbach et al., 2005). We predicted a difference between conspecific and heterospecific exposure, considering OF can enhance sperm velocity, motility, and straightness of swimming (Alonzo et al., 2016; Lehnert et al., 2017), but in which direction was unknown. These nuances are important to understand considering the environment in which sperm are exposed can determine the fitness of offspring (Evans et al., 2017), and highlights the major role sperm play on the evolutionary trajectory of populations.

Within salmonids, earlier emergence and larger sizes at hatching have been shown to provide a competitive advantage in the wild (Skoglund et al., 2012). This may result in selection against those who do not hatch within an optimum time, making this metric an important nuance of offspring survival. The factors influencing timing of emergence (not the same as hatching but correlated) have been clearly documented as a maternal effect (Einum & Fleming, 2000). More recently, the timing of hatching has been shown to be mediated by variation in sperm phenotype (Immler et al., 2014). Our findings suggest that hatch success and timing to mid-hatch of Atlantic salmon, brown trout, and their bi-direction hybrids is not affected by sperm exposure to conspecific vs heterospecific ovarian fluid. This is particularly important to address in North America, where brown trout are invasive and can interbreed with closely related Atlantic salmon (Jonsson & Jonsson, 2011). In the context of hybridization, sperm is being exposed to heterospecific ovarian fluid in nature.

Our lack of result could be due to the exact methods used. Immler et al. (2014) found effects on offspring if sperm swam for 20s in water before contact with eggs. We did not activate sperm and make them swim in the OFs before contact with eggs, nor did we have a water only control. We thus do not know if OF in general has an effect on offspring (both conspecific and heterospecific OF could vary from water in the same way), or if OF source affects offspring if sperm swim in it for a longer time prior to fertilization. However, this experiment is a preliminary step towards understanding if exposure affects offspring development, gamete evolution, and the role of paternal traits in offspring development. However, this experiment should be further explored using water as a control and repeating the delay contact conditions performed by Immler et al. (2014).

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Tables

Table 2—1 Full factorial breeding design sample – all half-sibling families derived from one female (Female Salmon 1) in Temperature A. Half-sib families were replicated for each female for Temperature B. Each row represents a half-sibling family consisting of 25 eggs. The "Cross Code" in the far-right column represents: Female ID/OF ID x Male ID. MS represents Male Salmon, MT = Male Trout, FS = Female Salmon, FT = Female Trout.

Temperature	Egg Spp	Female ID	Sperm Spp	Male ID	Crosstype	Exposure	OF Spp	OF ID	Cross Code
A	Salmon	FS1	Salmon	MS1	Pure	Con	Salmon	FS2	FS1/FS2xMS1
A	Salmon	FS1	Salmon	MS2	Pure	Con	Salmon	FS2	FS1/FS2xMS2
A	Salmon	FS1	Salmon	MS3	Pure	Con	Salmon	FS2	FS1/FS2xMS3
A	Salmon	FS1	Trout	TM1	Hybrid	Hetero	Salmon	FS2	FS1/FS2xTM1
A	Salmon	FS1	Trout	TM2	Hybrid	Hetero	Salmon	FS2	FS1/FS2xTM2
A	Salmon	FS1	Trout	TM3	Hybrid	Hetero	Salmon	FS2	FS1/FS2xTM3
A	Salmon	FS1	Salmon	MS1	Pure	Hetero	Trout	FT1+2	FS1/FT1+2xMS1
A	Salmon	FS1	Salmon	MS2	Pure	Hetero	Trout	FT1+2	FS1/FT1+2xMS2
A	Salmon	FS1	Salmon	MS3	Pure	Hetero	Trout	FT1+2	FS1/FT1+2xMS3
A	Salmon	FS1	Trout	TM1	Hybrid	Con	Trout	FT1+2	FS1/FT1+2xTM1
A	Salmon	FS1	Trout	TM2	Hybrid	Con	Trout	FT1+2	FS1/FT1+2xTM2
A	Salmon	FS1	Trout	TM3	Hybrid	Con	Trout	FT1+2	FS1/FT1+2xTM3

Table 2—2 Analysis of deviance output table for hatch success. P-value associated with the explanatory variable (far left column) shows that none of the variables or interactions had any effect on hatch success.

Variable	Chisquare	df	P
Temperature	0.584	1	0.445
Sperm Spp	0.079	1	0.778
Exposure	0.037	1	0.951
Egg Spp	3.085	1	0.079
Temperature * Sperm Spp	0.394	1	0.530
Temperature * Exposure	0.060	1	0.807
Exposure * Egg Spp	0.004	1	0.948
Sperm Spp * Exposure	0.536	1	0.464
Sperm Spp * Egg Spp	0.027	1	0.870
Temperature * Egg Spp	0.219	1	0.640

Table 2—3 Analysis of deviance output table for timing of 50% hatch. P-value associated with the explanatory variable (far left column) showing temperature, egg, and the egg and sperm interaction had significant effects on hatch timing.

Variable	Chisquare	df	P
Temperature	356.551	1	<0.0001
Egg Spp	28.473	1	<0.0001
Sperm Spp	0.574	1	0.448
Exposure	2.362	1	0.124
Temperature * Egg Spp	10.239	1	0.001
Temperature * Sperm Spp	2.872	1	0.090
Egg Spp * Sperm Spp	15.548	1	<0.0001
Temperature * Exposure	2.632	1	0.105
Egg Spp * Exposure	1.007	1	0.316
Sperm Spp * Exposure	1.468	1	0.226

Figures

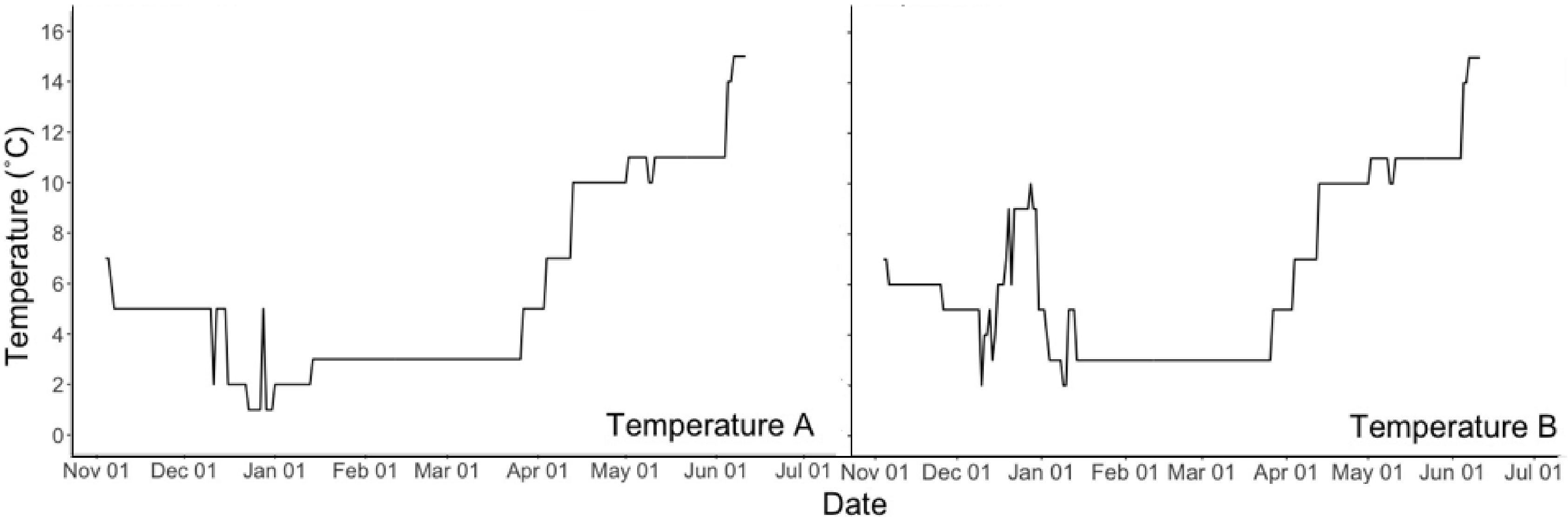

Figure 2—1 Profiles of temperature A (mean = 5.67°C; CV = 63.84) and temperature B (mean = 6.29°C; CV = 54.21) during time of incubation (November 5, 2016 – June 13, 2017).

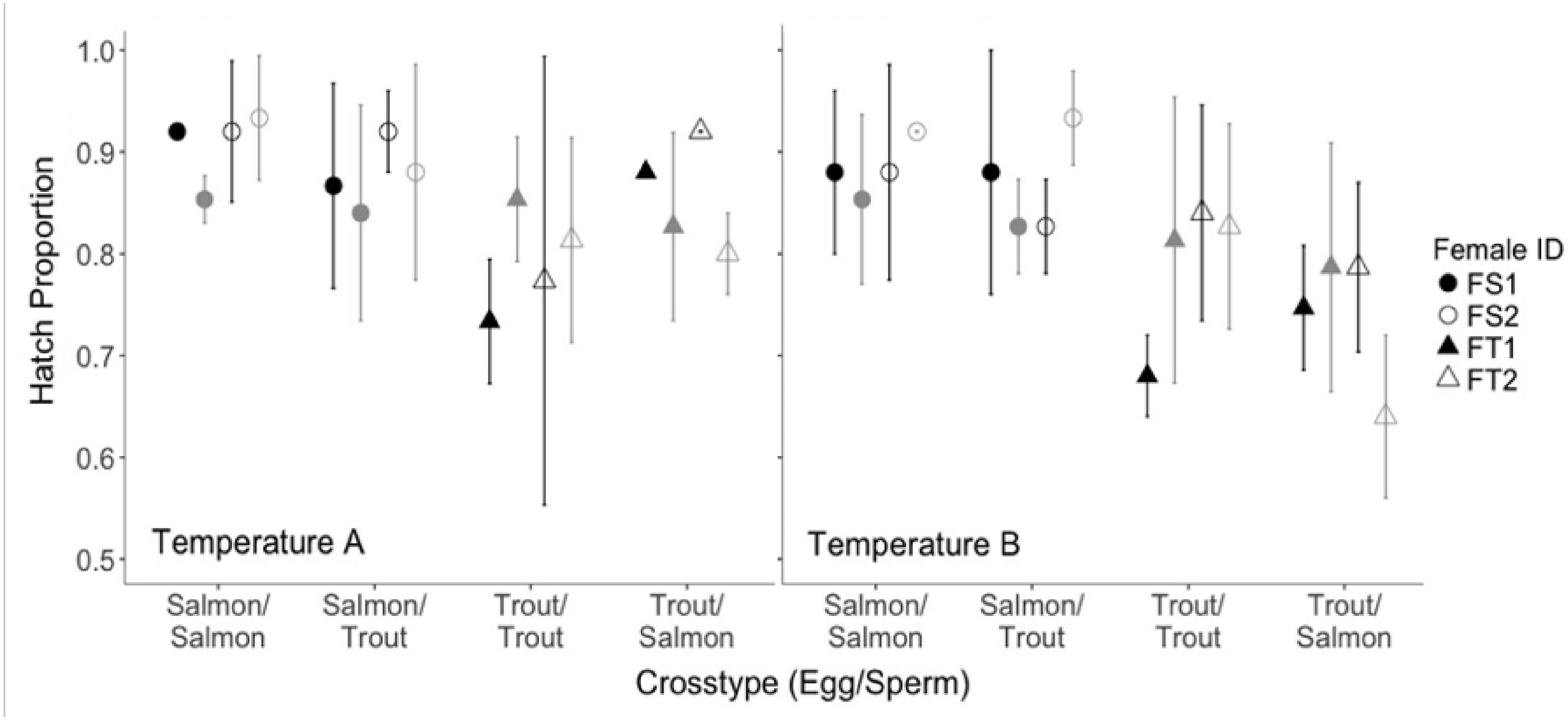

Figure 2—2 Average hatch proportion (hatch success) in temperature A (left) and temperature B (right) for each cross-type (egg/sperm; Table 1), as a function of conspecific ovarian fluid exposure treatment (black symbol), and heterospecific ovarian fluid exposure treatment (grey symbol). Circles represent female salmon, triangles represent female trout. Solid points represent female 1, open points represent female 2. Error bars represent standard deviation of 3 fathers. No error equals identical hatch.

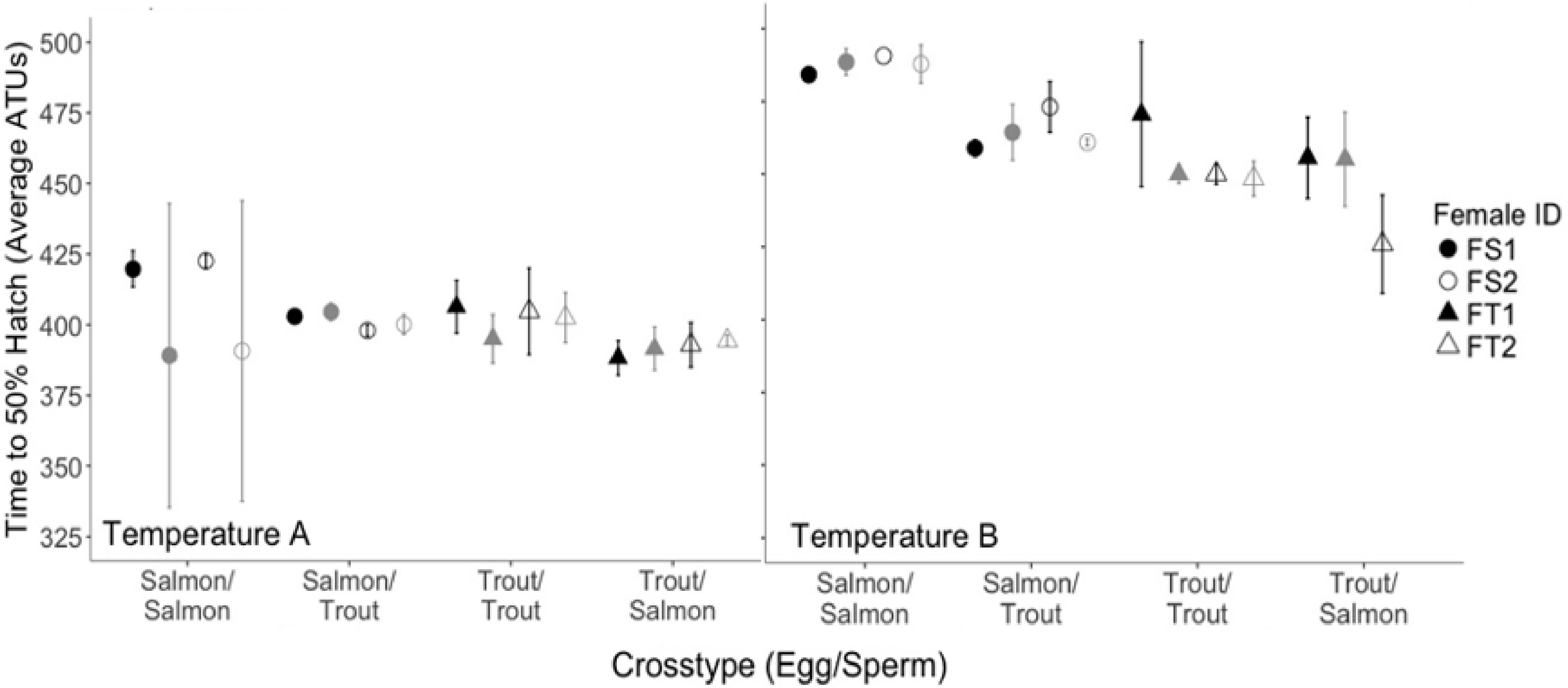

Figure 2—3 Average time (accumulated thermal units) to 50% hatch (mid-hatch) as a function of conspecific ovarian fluid exposure treatment (black symbol), and heterospecific ovarian fluid exposure treatment (grey symbol). Circles represent female salmon, triangles represent female trout. Solid points represent female 1, open points represent female 2. Error bars represent standard deviation of 3 fathers. No error equals identical hatch timing.

Chapter 3. Developmental characteristics of an invasive and indigenous hybrid freshwater fish (*Salmo trutta* x *Salmo salar*)

Abstract

Both biotic and abiotic reproductive isolation creates and maintains the integrity of species. Anthropogenic activities can degrade barriers to reproduction, promoting hybridization. Atlantic salmon and brown trout (*Salmo*) hybrids are found in Europe where both species are native and coevolved, and in North America where brown trout were introduced. We review the existing knowledge of F_1 hybrid frequency, the direction in nature (if reported), and the results of laboratory experiments observing ontogenetic development. The literature regarding the ontogenetic development of hybrids in North America is limited and contradictory to what has been found in field studies, for all hybrids found in nature are offspring of female trout, while this cross-type seems to suffer high rates of post-hatch mortality in laboratory experiments. Many of the experiments failed to control for individual variation in gamete quality, which may distort results. We thus experimentally examined the developmental characteristics of pure Atlantic salmon, pure brown trout, and bi-directional hybrid offspring within individual females. We predicted hybridization would cause a high rate of post-hatch mortality, shortly after hatching for offspring derived from trout mothers, as previous work has shown. Hybridization had no effect on hatch success, but in both species caused a given female’s eggs to hatch slightly earlier, and for salmon only, caused high rates of mortality post-hatch compared to their pure conspecifics. Our findings suggest that hybridization causes earlier mortality for salmon mothers, the opposite of what others have found.

Introduction

The biological species concept is widely used to define what a species is, but this textbook definition is challenged when two species hybridize. Botanists have long viewed natural hybridization as an important evolutionary process that promotes novel adaptations and produces new species (Mallet, 2007). By contrast, zoologists have focused primarily on the mechanisms that prevent interbreeding, likely because animals hybridize less frequently than plants (Rhymer & Simberloff, 1996). Conservation biologists, who seek to use science to maintain and restore natural resources are mainly concerned with anthropogenic hybridization, and the subsequent loss of biodiversity, genetic swamping, outbreeding depression, and biological invasion (Fitzpatrick & Shaffer, 2007; Todesco et al., 2016). The rate of anthropogenic hybridization is increasing, following human-mediated introductions of non-native taxa, habitat alteration, and climate change (Hegarty, 2012; Kovach et al., 2016; Muhlfeld et al., 2014). Although the consequences of hybridization vary widely among taxa (Abbott et al., 2013), it has been emphasized that interbreeding between invasive and native species is a significant problem that is predicted to increase (Muhlfeld et al., 2009; Muhlfeld et al., 2017).

Introducing species to foreign environments where closely related species naturally exist commonly results in interbreeding (Verhoeven et al., 2010). This phenomenon has been observed in fishes more than any other vertebrate group (reviewed by Scribner et al., 2001), and salmonids provide several examples. Salmonids have been stocked and/or introduced around the globe for angling, farmed for food, and prized for their mere beauty and high degree of natural variation. Most research has concluded that hybridization events among salmonids are problematic, and a threat to native diversity. For instance, in the Rocky Mountain region of the United States, invasive hybridization of introduced rainbow trout (*Oncorhynchus mykiss*) and native cutthroat trout (*Oncorhynchus clarkii*) is directly related to widespread stocking that ceased decades ago (Muhlfeld et al., 2017). In the same geographic region, dispersal of hybrid individuals with high proportions of rainbow trout genes are found to be the primary factor causing further interspecific hybridization with cutthroat trout (Kovach et al., 2015; Muhlfeld et al., 2014). Moreover, on Vancouver Island, British Columbia, frequency of hybridization of this species pair is significantly correlated to the level of anthropogenic disturbance, including logging, urban development, and stocking of rainbow trout (Heath et al., 2010). In the Pacific Northwest of the United States, hybrids of introduced brook trout (*Salvelinus fontinalis*) and native/endangered bull trout (*Salvelinus confluentus*) have been documented. They interbreed bidirectionally, which is to say that both species can be the mother or father in crosses, and F_1 hybrid offspring are fertile and reproduce (DeHaan et al., 2010).

The genus *Salmo* provides an interesting case of hybridization, with the suggestion that natural hybridization of Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) was described in the 17th century (Makhrov, 2008). It has been well documented using molecular markers (Solem et al., 2014) that the two species hybridize in Europe where they coevolved (Álvarez & Garcia-Vazquez, 2011; Hartley,1996 Jansson & Ost, 1997), and in North America (McGowan & Davidson, 1992b) where brown trout were introduced in 1883 (Westley & Fleming, 2013). Whereas hybrids have been well studied in their native range of sympatry, they have received less attention in North America where brown trout are invasive. Given Atlantic salmon diverged between regions about 600,000 years ago (Cauwelier et al. 2012), and because salmon and trout in North America have only been in contact for 136 years (maximum), the opportunity exists to determine if, and potentially how reproductive barriers have changed due to the isolation between species.

The frequency of hybrids is an important metric that can reflect the strength of reproductive isolating mechanisms between species (Li & Maki, 2015). Although salmonids have a wide range of chromosome numbers and are variable among regions (Hatley, 1987), North American and European Atlantic salmon have (n=54-60; Brenna-Hansen et al., 2012; Galbreath & Thorgaard, 1994), and brown trout (introduced to NA) have the same in both regions (n=76-84; Hartley, 1987). Any continental differences in hybrid frequency are therefore unlikely due to post-zygotic developmental incompatibilities. Due to long isolation between North American Atlantic salmon and brown trout, it seems predictable (as others have suggested: Hubbs, 1955; Verspoor, 1988) that hybrids would be more common in North America considering frequency can increase following species introductions (Quilodán et al., 2014), and because mechanisms (behavioral/courtship rituals) that evolved in sympatric populations (Europe) to prevent/reduce interbreeding may breakdown over time. Following a review by Jordan et al. (2007) and Makhrov (2008), and additional work published since (Table 3-1), we conclude there is no distinct pattern of hybrid frequency between continents. Frequencies are largely reflective of disturbance effects, as reports of sites with high proportions of hybrids are caused by habitat alteration, fish parasites, stocking, and escaped farmed fish (Solem et al., 2014). In addition, survival and persistence are context dependent, and how well offspring perform is determined by numerous environmental (Casas-Mulet et al., 2014) and biological factors (Houde et al., 2015). Hybrid frequencies in the wild are likely to vary in time and space, therefore cannot always be assumed to represent a given area.

Atlantic salmon and brown trout are among the best-studied fish in the world (Klemesten et al., 2003), yet the existing knowledge of their hybridization is rather ambiguous (Quilodrăn et al., 2014). In southern Europe, most hybrids found in nature have salmon mothers and trout fathers (Álvarez & Garcia-Vazquez, 2011; Matthews et al., 2000), while reciprocal cross-types are found in other European locations (Hartley, 1996; Hurrel & Price, 1991; Paaver et al., 2001; Solem et al., 2014). However, in limited North American sampling the opposite appears true where brown trout seem to always be the maternal species (Gephard et al., 2000; McGowan & Davidson, 1992b; Verspoor, 1988). It is not fully understood why hybridization is directional, but ecological and behavioral factors seem relevant (Solem et al., 2014). Alternative reproductive tactics such as sexually mature sneaking parr, habitat alteration, stocking, escaped farmed fish, and scarcity of spawners have been frequently suggested as explanatory variables (Makrov et al., 2008; Solem et al., 2014). Laboratory experiments in both continents have demonstrated directional mortality post hatching for hybrids derived from brown trout mothers (reviewed in Table 3-2), suggesting post-zygotic reproductive isolation occurs for this cross-type (Álvarez & Garcia-Vazquez, 2011; McGowan & Davidson, 1992a; Oke et al., 2013); possibly explained by genetic and/or developmental incompatibilities (Álvarez & Garcia-Vazquez, 2011). However, field studies and laboratory experiments are contradictory, considering this cross-type has been well documented in the wild (Table 3-1).

For Atlantic salmon and brown trout hybrids to exist in nature, several processes must align and function between species. First, reproduction of parent species has to overlap spatially and temporally allowing the gametes of two species in reproductive state to physically contact. Second, pre-mating behavior/rituals must be similar, and genitalia and gametes have to mechanically function between species. Third, post-copulatory pre-zygotic sexual selection mechanisms such as sperm competition and cryptic female choice have to be absent or weak enough to allow fertilization of heterospecific gametes. Finally, offspring must be viable through ontogenetic development long enough to be observable. Considering the frequency of hybrids is reflective of those who survived ontogenetic development, and because hybrid survival seems to be directional, why this cross-type persists in nature is up for debate. Given 1) both published experiments in North America that have explored hybrid viability (McGowan & Davidson 1992a; Oke et al., 2013) did not control for maternal variation (see Table 3-2), an extremely important nuance due to the high degree of variation among individuals; 2) because McGowan & Davidson (1992a) had suspected mortality caused by physical damage and gamete quality; and 3) considering the extreme asymmetric survival of hybrids reported by Alvarez & Garcia-Vasquez (2011) is suspect (Table 2, Solem et al., 2014), there is a need to further explore this topic.

Controlling for comparisons within a female to avoid confounding egg quality, we hypothesized hybridization would have a larger effect on offspring derived from brown trout than salmon females (Álvarez & Garcia-Vazquez, 2011; McGowan & Davidson, 1992a; Oke et al., 2013). We also hypothesized hatch success to be high for each cross-type, and because brown trout eggs hatch earlier than Atlantic salmon eggs (Jonsson & Jonsson, 2011; Makhrov, 2008), hatch timing would be earliest for pure trout, followed by trout hybrids, salmon hybrids, and lastly pure salmon. We measured (1) hatch success (proportion of eggs fertilized and survived to hatch), (2) timing when ≥50% of the embryos hatched (mid-hatch), and (3) timing when ≥25% of alevins died (post-hatch mortality) and evaluated whether hybridization patterns were environmentally context dependent with regard to incubation temperature.

Methods

Gamete collection

Native Atlantic salmon (AS) were taken from a fish ladder on the Exploits River (contains no brown trout) in Grand Falls, Newfoundland, Canada on September 8, 2016. Fish were held in large outdoor tanks with a flow-through river water system until ready to spawn. On November 4, 2016, salmon were anesthetized with clove oil before semen and eggs were stripped by drying the urogenital pore and applying gentle pressure to the fish's abdomen. Eggs were collected in glass jars and semen in plastic bags. Gametes were carefully packed in a cooler with ice and were brought to Memorial University in St. John's within 12 hours. On the same day of AS spawning, brown trout gametes were collected and stored in a similar manner around the same time, from a small stream that flows from Newfound Pond to Windsor Lake in St. John's, Newfoundland (refer to: Westley & Fleming (2011) for details on brown trout introduction and strain). Fish were dip netted and then held in a live-well for a few minutes before they were stripped for gametes.

Breeding design and fertilization

The experiment utilized a full factorial breeding design with gametes from 2 female salmon, 2 female trout, 3 male salmon and 3 male trout, creating twenty-four half-sibling families. There was full replication of each half-sibling family that differed in ovarian fluid treatment from Chapter 2. The results showed little variation among ovarian fluid treatments, therefore we used them for replicates for each half-sib family in this study (Female Salmon 1 example: Table 3-3).

Fertilization took place between 1:30 a.m. and 3 a.m. on November 5, 2016. All female salmon #1 eggs were fertilized first, then female trout #1, then female salmon #2, then female trout #2. Each female's eggs were removed from the refrigerator, along with semen from the 6 males. Semen was carefully transferred from Zip-Lock Bags to labeled 10mL beakers using a small pipet. 6.5mL of eggs (~ 60 eggs) were measured with a 10mL vial and gently poured into a 200mL beaker. This was repeated twelve times (one female = 6 half-sibling families x 2 replicates), and beakers were placed on an aluminum tray with crushed ice covering the bottom. 10µL of semen was then added to each beaker using a syringe, followed by 2mL of river water. The syringe was used to briefly mix the eggs, semen, and water. After one tray of twelve beakers had been fertilized, watered, and mixed, river water was poured into each beaker to submerge all the eggs. The tray was then returned to the refrigerator. The other three trays (females) were fertilized following the same protocol. All samples (48 beakers) were stored in a refrigerator at 4.4°C overnight to water harden the eggs.

Incubation

Five hours post-fertilization only two eggs had turned white (non-viable). Each brood was split, with two samples of 25 embryos removed from each beaker and each placed in a 5.8 cm tall x 5.8 cm inner diameter Polyvinyl Chloride (PVC) tube with a fine mesh bottom, in a plastic tray with refrigerated river water. Forty-eight PVC tubes, 12 per tray, were loaded into each incubator. Each tray represented embryos from one female, from top to bottom: female salmon 1, female trout 1, female salmon 2, and female trout 2.

Two, four-tray Marisource vertical incubators were used in a recirculating system at a rate of 16L/minute, and through a UV light filter. Electric water chillers and 300-watt submersible electric aquarium heaters were used to adjust the water temperature of each incubator. Incubators were setup in a refrigerated room at a constant temperature of 5°C. Incubator A and incubator B followed fluctuating temperature profiles, with incubator A accumulating thermal units slower (mean = 5.67°C; CV = 63.84) than incubator B (mean = 6.29°C; CV = 54.21; Figure 1). Prior to hatching, water pH, ammonia, and nitrate levels were measured and recorded once a week, then every other day following first hatch (January 6, 2017) to ensure water quality was okay. Approximately 35 liters of degassed tap water were exchanged from each incubator every other day following the first hatch.

Embryos were initially checked twice a week then daily following first hatch. White (non-viable) eggs and dead embryos and alevins were removed to maintain water quality, and for data collection regarding mortality metrics. The daily number hatched, the number not hatched, and mortality as an embryo or alevin was recorded per PVC tube. PVC tubes were left in the incubator until ≤ 10 live alevins remained (sampling methodology was employed for another experiment).

Statistical approach

For each analysis, significance was set at α = 0.05, and assumptions of parametric statistics were checked by examining model residuals. We used a generalized mixed-effects model (Model 1) with binomial regression (hatch or not hatch) to analyze hatch success (Linear Mixed Effect Model, "lme4" package in R version 3.2.2). A linear mixed-effects model was used to analyze timing to ≥ 50% hatch (accumulated thermal units at which 50% of the total hatched per tube hatched), and post-hatch mortality (time at which ≥ 25% of the hatched alevins died). Due to our sampling protocol to accommodate another experiment, we were limited to analyzing mortality at ≥ 25%. The timing of mortality was quantified as ATUs from fertilization (all individuals fertilized at the same time), for hatching over different days and specific individuals could not be subsequently tracked.

Each full model included: sperm species (salmon and trout), egg species (salmon and trout) and temperature (A and B), and all possible interactions as fixed effects; and mother ID and father ID as random effects. For each response variable we started with the full model. The three-way interaction term was insignificant for each metric. It was removed from the model, and the models ran in a simpler form.

Model 1:

DV ~ sperm*egg*temperature + egg*temperature + sperm*egg + sperm*temperature + sperm + egg + temperature + (mother-random) + (father-random) + error

Results

Hatch success

Although hatch success yielded variable results from different parents (Figure 3-2), it was high overall, as predicted by previous studies. Controlling for individual variation in egg quality among females, there were no significant differences among cross-types (sperm and egg interaction: $\chi^2_1 = 0.027$; $p = 0.87$), or between temperatures ($\chi^2_1 = 0.584$; $p = 0.44$). Hatch success was highest for salmon eggs (pure mean = 89%; hybrid mean = 87%), followed by hybrid trout eggs (mean = 80%), and lastly pure trout (mean = 79%) (Figure 3-2).

Hatching timing

Controlling for egg quality among females by using within female comparisons, hybridization caused timing to 50% hatch to occur earlier (Figure 3-3). There were differences in hatch timing among cross-types (egg and sperm interaction: $\chi^2_1 = 17.51$; $p < 0.0001$). The effect was statistically significant for trout eggs at both temperatures (temperature A: $\chi^2_1 = 8.60$; $p = 0.003$; and temperature B: $\chi^2_1 = 3.98$; $p = 0.046$), and for salmon in only one temperature (temperature A: $\chi^2_1 = 0.405$; $p = 0.524$; and temperature B: $\chi^2_1 = 90.485$; $p < 0.0001$). In all cases the effect size was small (average = -2.90%, range = -0.51% to -5.07%). The hatch timing means between individual females within a species were very similar (< 2% difference). Incubation temperature had more of an effect than hybridization on the accumulated thermal units to hatch. The interaction of temperature and egg species had a significant effect on hatch timing (χ^2_1 = 10.27; p = 0.001). Temperature had a difference of 15.1% in effect size between temperature A and temperature B for salmon embryos, and 11.8% for trout embryos.

Post-hatch mortality timing

Controlling for individual variation in egg quality, cross-type (egg and sperm interaction) had a significant effect on timing since fertilization for the mortality of $\geq$ 25% of hatched embryos (χ^2_1 = 5.24; p < 0.022). It was driven by the large effect (average = -18.4%; range = -30.06% to -11.4%) within salmon females. When run separately by egg species, the sperm species of fertilization (whether hybrid or pure cross) was significant for salmon eggs (χ^2_1 = 50.36; p < 0.0001), but not trout eggs (χ^2_1 = 0.25; p = 0.614). The effect size and pattern of hybridization on salmon eggs was consistent for both females (Figure 3-4). Mortality timing was highly variable for pure trout (temperature A: CV = 25.25, mean = 798.16; temperature B: CV = 19.54, mean = 1003.08) and trout egg hybrids (temperature A: CV = 26.98, mean = 906.75; temperature B: CV = 35.63, mean = 969.33), meaning death occurred over a wide range of ATUs, compared to pure salmon that died within a tighter window of time (temperature A: CV = 1.16, mean = 1216.50; temperature B: CV = 2.49, mean = 1316.33) and salmon hybrids (temperature A: CV = 17.83, mean = 984.66; temperature B: CV = 9.50, mean = 1101.54). Timing appears not to differ between salmon hybrids and trout hybrids.

Discussion

The results from this experiment did not support the prediction based on previous studies (Álvarez & Garcia-Vazquez, 2011; McGowan & Davidson, 1992a) that hybridization has a greater effect on brown trout than Atlantic salmon embryo development. We found similar biological responses to hybridization for salmon and trout eggs in hatch timing, and slightly faster mortality for hybrids from salmon eggs but not trout eggs, though overall mortality between hybrid cross-types did not differ. Although our sample size was small, our experiment was done in a controlled manner, and it is thus clear that the assertion of strong bidirectional mortality of hybrids produced from trout mothers and salmon fathers (Álvarez & Garcia-Vazquez, 2011) is at least not universal. Moreover, the result of faster salmon egg mortality may help explain why only directional crosses (brown trout mothers x Atlantic salmon fathers) offspring are observed in nature (Gephard et al., 2000; McGowan & Davidson, 1992b).

The overall proportion of eggs hatched was ≥79% for each cross-type in both temperature treatments. Therefore, fertilization success was undoubtedly high for both pure and hybrid families, supporting reproductive isolation is not mediated by gamete incompatibility in this hybrid pair, and heterospecific fertilization success can be very high bi-directionally. Similar results have been reported in Europe by Álvarez & Garcia-Vazquez (2011). Barriers to fertilization success do exist at the gametic level when sperm of Atlantic salmon and brown trout are competing to fertilize eggs (Yeates et al., 2013, there was no sperm competition in our experiment). The female's ovarian fluid of both species has been shown to have cryptic female choice, mediating sperm competition towards conspecific sperm preference and biasing fertilization (Yeates et al., 2013). This type of selection potentially contributes to reproductive isolation without wasting energetic investments into lost egg production, and likely occurs where sympatric populations exist. It is not known if isolation between species has caused these mechanisms to break down over time in North America, and as reviewed, it is apparent that hybrid frequency can be very high, very low, or sometimes intermittent in both continents.

We expected hybrids to hatch intermediately to pure offspring and predicted those derived from trout eggs to hatch before salmon egg hybrids. Given timing to mid-hatch has been well documented in these species, we predicted pure trout to hatch first, and pure salmon to hatch last (Embody, 1934; Jugwirth & Winkler, 1984; Kane, 1988). We found trout egg hybrids began hatching first, followed by pure trout, salmon egg hybrids and lastly pure salmon. The difference in ATUs between pure and hybrid trout was very small in both temperatures (<10 ATUs), while pure and hybrid salmon was <5 ATUs (temperature A), and 23.42 ATUs (temperature B). Considering the water temperature was 3°C during this time, it equates to about 3 days difference between when pure trout and hybrid trout reached mid-hatch, which is likely not biologically meaningful. Moreover, hybridization for salmon and trout eggs from each female caused timing to mid-hatch (50% hatch) to happen earlier. The observed pattern was consistent for salmon eggs and trout eggs in both temperature treatments, and statistically significant for trout at both temperatures and salmon at one temperature. Emergence timing is commonly shown to affect offspring performance through establishment of territory and/or competition linked to body size, where earlier timing of these events can be beneficial (Skoglund et al., 2012). However, while our data suggests relative hatch timing is slightly context dependent varying with temperature regime, our sample size was small, and the effect size of hybridization was very small. Therefore, there is little if any biological significance regarding this metric.

Due to our sampling protocol, we were limited by the duration of time we could effectively monitor post-hatch mortality due to logistical constraints, therefore we analyzed this metric when ≥25% of hatched offspring died. It is based on ATUs since fertilization as we do not know when individual alevin hatched. Hybridization had no significant effect for trout mothers, but did for salmon mothers, and the pattern of faster hybrid mortality was consistent for both females. Although the data suggest statistical significance, whether this is biologically meaningful is questionable. We did not feed the hatchlings, and thus our metric of 25% post-hatch mortality could be interpreted as timing to starvation. At 6°C (mean in temperature A and B treatments), ATUs to 50% ready for exogenous feeding for brown trout and Atlantic salmon is predicted to be roughly 750-1000 based on Figure 2.1 and 3.1 from Solomon & Lightfoot (2008). Given that the mean ATUs to ≥25% mortality for salmon egg hybrids was 985 in temperature A and 1105 in temperature B, and 907 ATUs in temperature A, and 969 ATUs in temperature B for trout egg hybrids, we believe our findings are not exclusively a function of starving. Numerous individuals survived until 1200+ ATUs in temperature A, and 1375+ ATUs in temperature B. However, given the temperatures in the current experiment were fluctuating, comparing means to a constant temperature presents constraints. This nuance is important to address considering directional hybrid mortality in other studies seems to happen shortly after hatching when alevins have sufficient amounts of yolk remaining (Álvarez & Garcia-Vazquez, 2011; McGowan & Davidson, 1992a). Considering we monitored post-hatch mortality for a short amount of time, additional work is necessary and should monitor mortality beyond yolk-sac absorption.

While considering our findings are not consistent with the two experiments addressing hybrid viability in North America (McGowan & Davidson, 1992a; Oke et al., 2013), it is crucial to point out that variability in egg quality among mothers was not addressed in those studies. In addition, McGowan & Davidson (1992a) had issues with suspected poor gamete quality and potential physical damage to eggs in the incubator. Moreover, offspring were grouped together by cross-type shortly after hatching in that experiment; further distorting the ability to observe parental effects. Due to the difference in peak spawn timing of brown trout and Atlantic salmon, earlier experiments may have experienced high mortality of offspring derived from brown trout mothers due to the over-ripening of eggs (Makhrov, 2008). Our experiment did not have this complication, and we controlled for individual variation. Therefore, we believe our results could be accurate to what is observed in the region.

It is apparent that the existing knowledge of Atlantic salmon and brown trout hybridization is ambiguous. Quilodrán et al. (2014) suggested the consequences of hybridization of these species are demographically mediated, not genetically, while others have made suggestions regarding genetic incompatibilities (Álvarez & Garcia-Vazquez, 2011). Our findings support an environmental effect influencing differences in timing events, which has been well documented by others. In addition, different chromosome numbers between species may be responsible for genetic incompatibilities that occur beyond fertilization. Moving forward, further examination is needed to investigate these ideas. Although our sample size was small (n=10; 2 females of each species, 3 males of each species) and fails to capture variation expressed within or among populations, our experimental design was powerful, for we looked at the effects of hybridization *within* a female; thus, controlling for the strong maternal traits salmonids exhibit (Houde et al., 2011). Despite these shortcomings, we found hatch success to be similar to what has been reported from Europe (high) but found post-hatch mortality results are the opposite (selection against hybrids derived from salmon eggs; Table 3-2). Additional work is needed to understand the role of reproductive isolating mechanisms and their influence on mediating hybridization in this hybrid pair.

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Tables

Table 3—1 Accessible published literature in English regarding the observed frequency of Atlantic salmon and brown trout hybrids in nature and the direction of hybridization in accordance to geographical region, the extent of sample site(s) and the number of fishes analyzed (life stage sampled varied among studies). Percentages in "Direction of hybridization" column are within the "Mean frequency."

Reference	**Region**	**Sample site(s)**	**(n) Fishes analyzed**	**Direction (♀x♂)**	**Mean frequency (%)**
North America					
Beland et al. 1981	Nova Scotia	1 river	56	No information	1.80
Verspoor 1988	Newfoundland	10 watersheds	786	No information	0.90
McGowan & Davidson 1992b	Newfoundland	9 rivers	792	Directional (BTxAS)	4.67*
Gephard et al. 2000	United States	1 river	137	Directional (BTxAS)	0.81*
Europe					
Crozier, 1984	Ireland	1 watershed	426	No information	3.60*
Garcia de Leaniz & Verspoor 1989	Spain	4 watersheds	175	No information	2.30
Jansson et al. 1991	Sweden	1 river	332	No information	13.00*
Hurrel & Price 1991	England	6 rivers	559	Bi-directional (ASxBT) = 62.3% (BTxAS) = 37.7%	1.40
Jordan & Verspoor 1993	Britain	23 rivers	5697	No information	1.00*
Youngson et al. 1993	Scotland	16 rivers	2373	Directional (ASxBT)	1.00*
Hindar & Balstad 1994	Norway	Multiple rivers	8665	No information	0.24
Elo et al. 1995	Norway, Finland	2 rivers	2024	No information	0.15

Reference	**Region**	**Sample site(s)**	**(n) Fishes analyzed**	**Direction (♀x♂)**	**Mean frequency (%)**
Gross et al. 1996	Sweden	4 watersheds	482	No information	1.60
Hartley 1996	Britain	1 river	55	Bi-directional (BTxAS) = 90% (ASxBT) = 10%	18.18*
Jannson & Ost 1997	Sweden	1 river	2256 323 restored site	No information	1.60 41.50*
Matthews et al. 2000	Ireland	13 rivers	4135	Directional (ASxBT)	1.20*
Paaver et al. 2001	Estonia	5 rivers	821	Bi-directional	2.80*
Garcia-Vazquez et al. 2001	Spain, France	7 rivers	721	Directional (ASxBT)	2.53*
Castillo et al. 2008	Spain, France	8 rivers	1630	Bi-directional	7.81* 1.39
Castillo et al. 2010	Spain	10 rivers	1652	Directional (ASxBT)	1.35
Chelenkova et al. 2011	Bulgaria	19 watersheds	146	No information	8.90*
Adams et al. 2014	Scotland	6 rivers 4 rivers	281 48 migratory	Bidirectional (BTxAS) = 80% (ASxBT) = 20%	0.70 10.40*
Solem et al. 2014	Norway	1 river	232	Bi-directional (BTxAS) = 85% (ASxBT) = 15%	27.00*

Notes: *Means marked with (*) mentioned disturbance factors likely responsible for high frequency; Direction of hybridization for Adams et al. 2014 is the analysis for the migratory fish sample only; Most literature is unclear about the size/extent of areas sampled, but either listed number of rivers or number of catchments/watersheds - a river is a drainage feature of a watershed, and a watershed is the drainage area.*

Table 3—2 Accessible published literature in English of hybrid viability (survival) and the developmental stage analyzed by cross direction in laboratory experiments in accordance to geographical region. Constraints and/or limitations highlight the evaluation of female/male contributions, control for individual variation, and general issues encountered.

Reference	**Region**	**Developmental stage(s) analyzed**	**Survival (♀x♂)**	**Evaluation of maternal/ paternal contribution**	**Control for individual variation**	**Other constraints/limitations**
North America (laboratory experiments)						
McGowan & Davidson 1992a	Newfoundland	·Fertilization to hatching ·Hatching to yolk-sac absorption	·Directional post-hatch mortality for (BTxAS)	Yes	No	·Cross-types were grouped together after hatching ·Suspected egg mortality caused by physical damage and poor gamete quality ·Total (n) of eggs for each family were rounded to the nearest 10; poor statistical evidence
Gray et al. 1993	United States	·Fertilization to eyed, hatching, and yolk-sac absorption	·High mortality for all offspring ·Highest mortality for (BTxAS)	Yes	No	·Very high mortality for all experimental cross-types ·Families derived from 3 single-pair fertilizations; weak design
Oke et al. 2013	Newfoundland	·ca. 100 days following start of exogenous feeding	·Directional post-hatch mortality for (BTxAS)	Yes	No	
Europe (laboratory experiments)						
Refstie & Gjedrem 1975	Norway	·Fertilization to eyed, and hatching	·High mortality for salmon egg cross-types	Yes	No	·Vague methodology ·Number of individuals used in experiment not mentioned

Reference	**Region**	**Developmental stage(s) analyzed**	**Survival (♀x♂)**	**Evaluation of maternal/ paternal contribution**	**Control for individual variation**	**Other constraints/limitations**
			·Highest mortality for pure salmon			
Blanc & Chevassus 1979	France	·Fertilization to eyed, and 15th day post hatching	·High mortality to hatching for trout egg hybrids ·Very high mortlaity15 days post hatching for trout egg hybrids	Yes	No	·Vague methodology ·No information post eyed-stage for salmon egg hybrids ·Conspecific sperm were pooled, distorting variation in fathers
Babiak et al. 2002	Poland	·Fertilization to eyed, and to mid yolk-sac absorption	·High mortality for trout egg hybrids than pure trout	Yes	No	·Conspecific eggs were pooled ·Salmon egg cross-types were not created in this experiment ·How mid yolk-sac absorption was determined not mentioned
Garcia-Vazquez et al. 2002	Spain	·Fertilization to fry stage	·Very low survival for all cross-types ·Lowest survival for pure salmon	Yes	Yes	·Survival percentages were calculated using an "estimated" number of eggs; not an actual count ·Survival estimates assumed all eggs were fertilized

Reference	**Region**	**Developmental stage(s) analyzed**	**Survival (♀x♂)**	**Evaluation of maternal/ paternal contribution**	**Control for individual variation**	**Other constraints/limitations**
Álvarez & Garcia-Vazquez 2011	Spain	·Fertilization to yolk-sac absorption	·Directional post-hatch mortality for (BTxAS)	Yes	Yes	·Eggs assumed not fertilized if they did not reach eyed stage ·Figure 2 has no error bars to evaluate if cross-direction trend was universal across individual parents ·Figure 4 lines should reach 100% given they are adjusted to show timing of what hatched ·Unclear if fish were grouped together by cross-type after hatching or not

Table 3—3 Full factorial breeding design sample – all half-sibling families derived from one female (Female Salmon 1) in Temperature A. Half-sib families were replicated for each female for Temperature B. Each row represents a half-sibling family consisting of 25 eggs.

Temperature	**Egg Spp**	**Female ID**	**Sperm Spp**	**Male ID**	**Cross-type**	**Replicate**
A	Salmon	FS1	Salmon	MS1	Pure	Replicate 1
A	Salmon	FS1	Salmon	MS2	Pure	Replicate 1
A	Salmon	FS1	Salmon	MS3	Pure	Replicate 1
A	Salmon	FS1	Trout	TM1	Hybrid	Replicate 1
A	Salmon	FS1	Trout	TM2	Hybrid	Replicate 1
A	Salmon	FS1	Trout	TM3	Hybrid	Replicate 1
A	Salmon	FS1	Salmon	MS1	Pure	Replicate 2
A	Salmon	FS1	Salmon	MS2	Pure	Replicate 2
A	Salmon	FS1	Salmon	MS3	Pure	Replicate 2
A	Salmon	FS1	Trout	TM1	Hybrid	Replicate 2
A	Salmon	FS1	Trout	TM2	Hybrid	Replicate 2
A	Salmon	FS1	Trout	TM3	Hybrid	Replicate 2

Figures

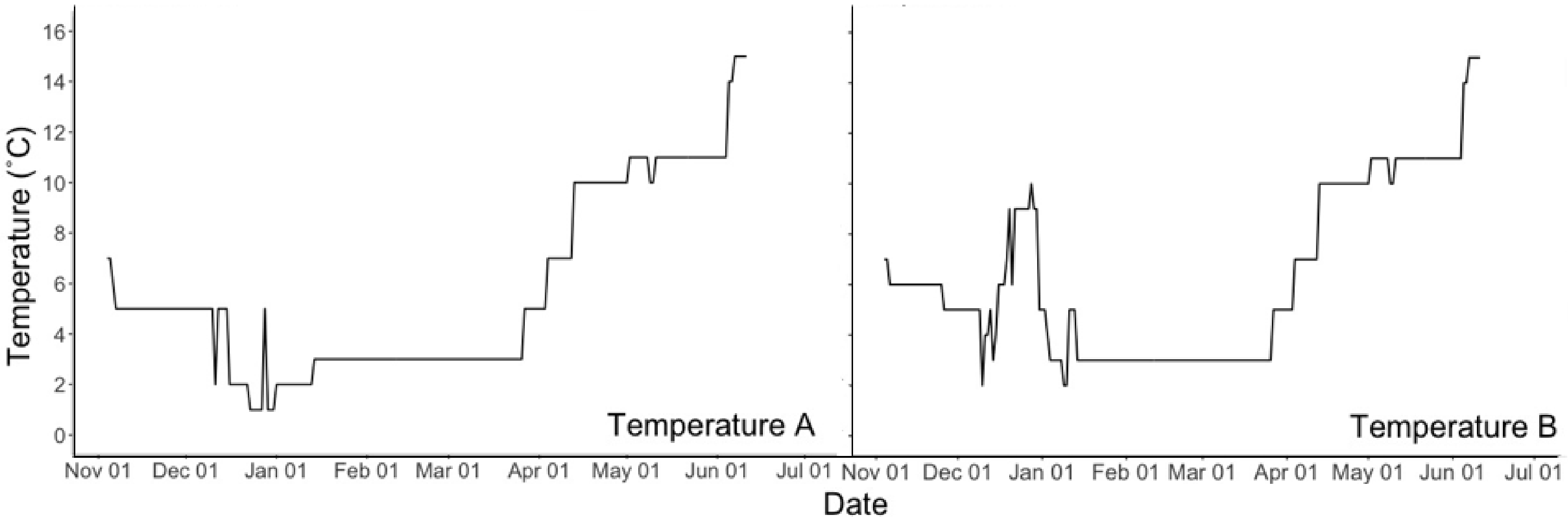

Figure 3—1 Profiles of temperature A (mean = 5.67°C; CV = 63.84) and temperature B (mean = 6.29°C; CV = 54.21) during timeof incubation (November 5, 2016 – June 13, 2017).

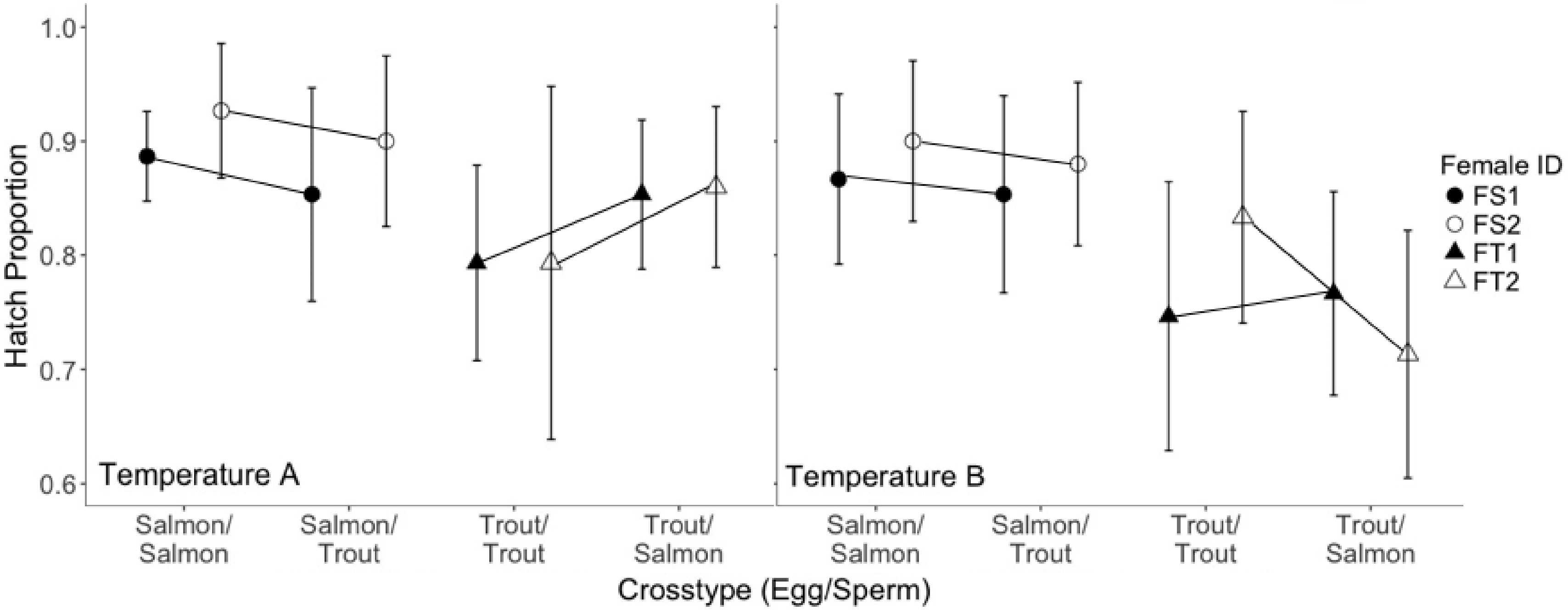

Figure 3—2 Proportion of eggs hatched in temperature A (left) and temperature B (right) for each cross-type (egg/sperm). Circles represent female salmon, triangles represent female trout. Solid points represent female 1, open points represent female 2. Error bars represent standard deviation of 3 fathers. Lines connecting points represent the effect size (percent change) of hatch proportion within an individual females' eggs for males of different species.

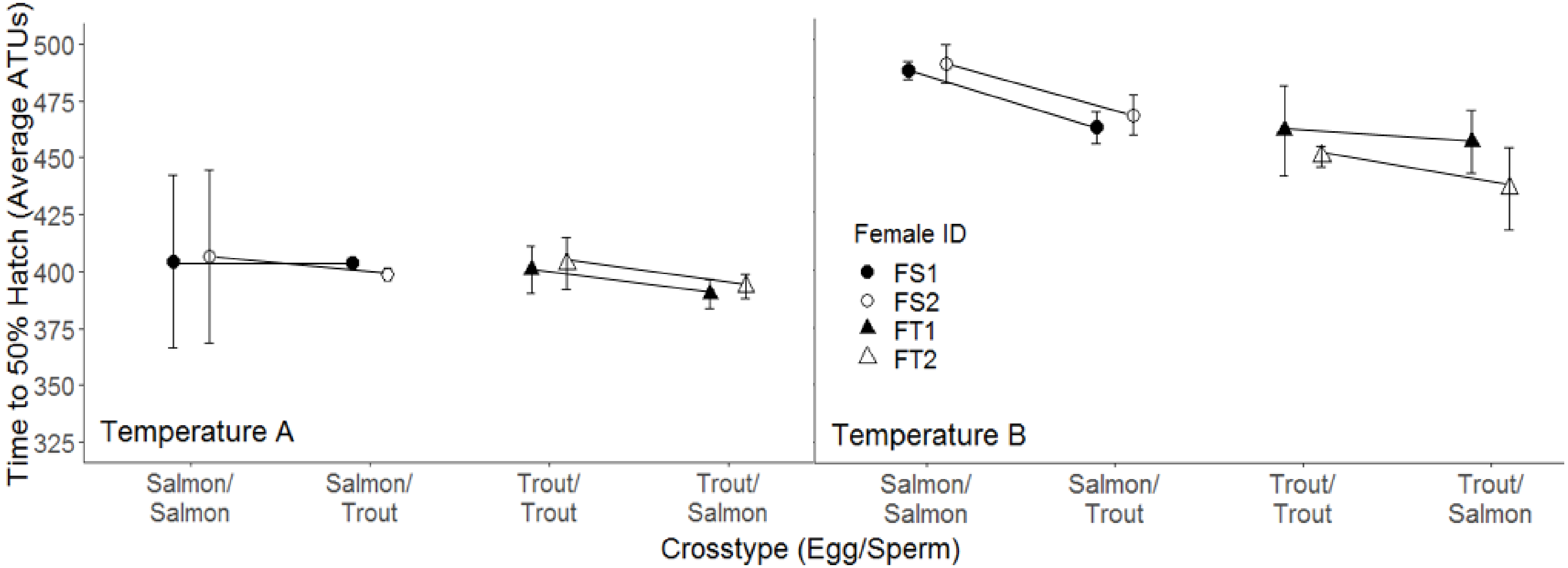

Figure 3—3 Average time (accumulated thermal units) to 50% hatch (mid-hatch) for each cross-type (egg/sperm) in temperature A (left) and temperature B (right). Circles represent female salmon, triangles represent female trout. Solid points represent female 1, open points represent female 2. Error bars represent standard deviation of 3 fathers. Lines connecting points represent the statistically significant effect size (percent change) of hybridization within an individual females' eggs.

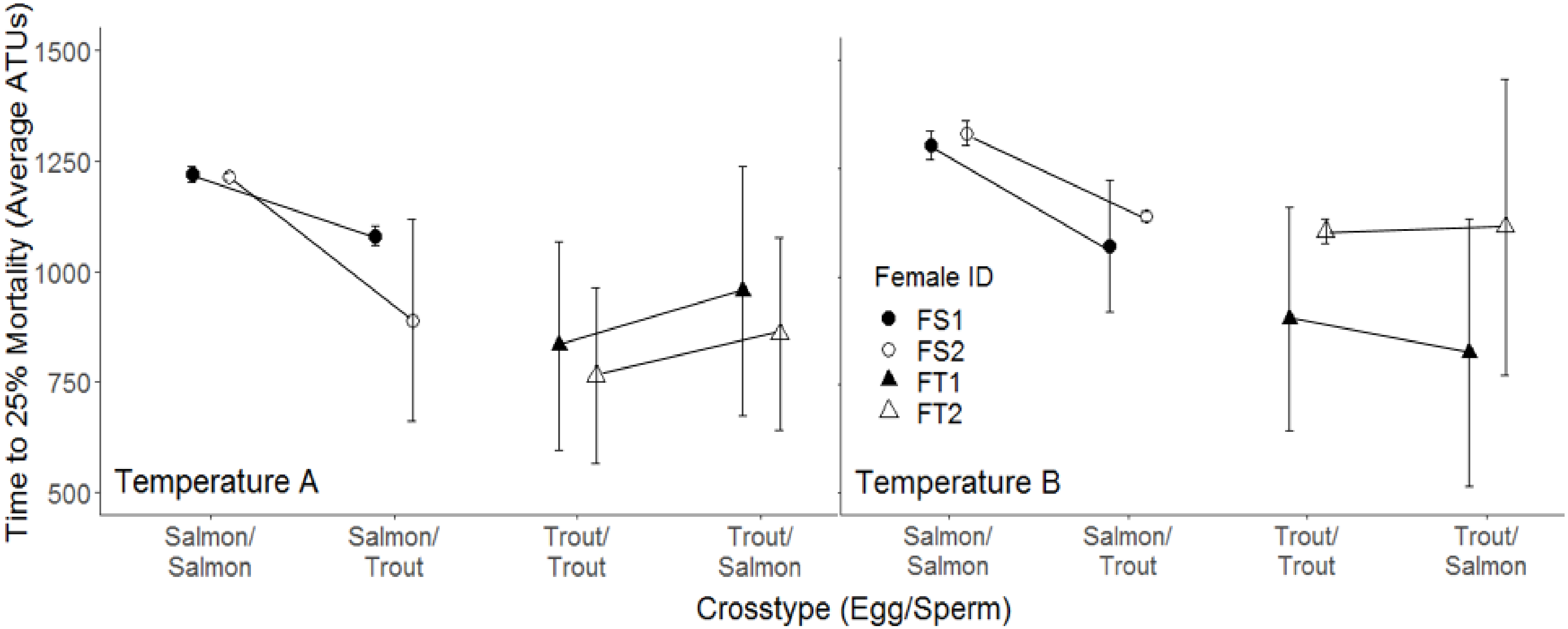

Figure 3-4. Average time (accumulated thermal units) to ≥25% post-hatch mortality for each cross-type (egg/sperm) in temperature A (left) and temperature B (right). Circles represent female salmon, triangles represent female trout. Solid points represent female 1, open points represent female 2. Error bars represent standard deviation of 3 fathers. Lines connecting points represent the effect size (percent change) of hybridization within an individual females' eggs. Hybridization had a significant effect on salmon eggs, but not trout eggs.

Chapter 4. Conclusions

This experiment investigated the developmental characteristics of Atlantic salmon and brown trout hybrids while making direct comparisons within a clutch of eggs from each female to control for maternal effects. In addition, the full-factorial breeding design we used allowed us to control for paternal effects; which have recently been shown to have a significant influence on the fitness of offspring (Alavioon et al., 2017). This is an extremely critical nuance for developmental studies, due to the strong parental traits associated with the genotype and/or phenotype in salmonids (Houde et al.,2011). Therefore, our results accurately describe the development of offspring from each parental source. It is important to highlight that other experiments that have researched this topic did not control for this (McGowan & Davidson, 1992b), and have instead made conclusions directly among cross-types (pure trout, pure salmon, and bi-directional hybrids). In addition, making direct comparisons among females of the same species to answer a "species-level" questions are difficult within the salmonids, for these animals exhibit a high degree of variation among populations, and within populations. However, our experiment used an approach where we could accurately describe development on the individual level for each parental source. Our findings suggested there is no differential effect on developmental characteristics of subsequent offspring when sperm is exposed to conspecific or heterospecific ovarian fluid, and hybridization has a significant effect on the hatch timing and mortality timing, but not hatch success of offspring.

Given more research on fishes has supported the idea that sperm phenotype effects offspring development and fitness (Alavioon et al., 2017; Evans et al., 2017; Krawets, 2005), and what sperm is exposed to matters (Immler et al., 2014), we explored how exposure to ovarian fluid could potentially influence development. We found no effect for either ovarian fluid treatment (conspecific and heterospecifc exposure), but our lack of result could be due to the methods we used. Immler et al. (2014) swam/activated sperm in water for 10 seconds before contact with the eggs. We did not activate sperm or make them swim in the ovarian fluid treatments before contact with the eggs. In addition, we did not have a water control. Therefore, we do not know if ovarian fluid in general influences offspring development, or if ovarian fluid source affects offspring if sperm swim in it for a longer time prior to fertilization. It is important to highlight that maternal effects can alter paternal effects based on the phenotypic characteristics of her mate (Evans et al., 2019). This nuance could have potentially confounded our experiment and could be why we did not see a difference in exposure treatments. However, despite the shortcomings in our methodology, this experiment is a useful preliminary step towards understanding how sperm exposure effects offspring. Moving forward, these questions of interest will be pursued by others within the research lab, for it is an important to explore the nuances of paternal traits; a topic that has received far less attention/interest than maternal traits in salmonids. In addition, the evolutionary implications of sperm phenotype induced paternal traits are unknown and present a wealth of opportunity for future research.

Hybridization had no effect on the proportion of eggs that hatched for both reciprocal crosses, suggesting that fertilization success can be very high for bi-directional Atlantic salmon x brown trout offspring. These findings parallel what has been observed in Europe (Álvarez & Garcia-Vazquez, 2011), and are a good indicator that gametes between species are completely functional, and in both directions. This demonstrates that post-copulatory prezygotic isolating mechanisms between species are likely absent when sperm from only one of the species encounters the eggs. Therefore, in the “eyes” of Atlantic salmon conservation in North America, if the two species can physically contact one another, if courtship succeeds, and if gametes are released and fertilized, the female's reproductive investment is completely wasted. This is particularly true given hybrids are typically sterile.

An interesting observation in this experiment was the effect hybridization had on hatch timing for every female. Heterospecific sperm (hybridization) caused eggs to hatch earlier in both temperatures and for each cross-type, and although the effect size was small, the pattern was consistent. These findings demonstrate the importance that sperm play in the development of offspring, for our results support the inference that sperm can influence the timing of when eggs hatch, as others have demonstrated (Immler et al., 2014). However, their experiment was interested with what sperm were exposed to (similar to our experiment for Chapter 2), but nonetheless, it is very obvious that sperm matter.

The timing to mid-hatch is a common metric used to address questions about development (Granath et al., 2004; Wang et al., 2007), because when offspring hatch has a strong effect on the survival of individuals (Arnold et al., 2004; Bradbury et al., 2004). It's been shown that those who emerge (not the same as hatching, but linked) earlier can have a competitive advantage over individuals that hatch later due to larger body size and establishment of territory (Skoglund et al., 2012). Our results therefore indicate that hybrid offspring may have a competitive advantage over their pure lineage half-sibling brothers and sisters. Considering this result poses a harmful effect on the status of North American Atlantic salmon, this topic should be explored more thoroughly. Perhaps hybrids hatch earlier and outcompete pure Atlantic salmon at a very early developmental age.

It's been documented that in fishes, offspring survival during ontogenetic development is a key indicator of population viability through time (Whitney et al., 2014). Our mortality data showed that hybridization caused earlier death within salmon females shortly after hatching, but not within trout females, suggesting that hybrids derived from salmon eggs suffer a fast rate of post-hatch mortality. This is the opposite of what others have found, for European and North American experiments both demonstrated that female brown trout and male Atlantic salmon hybrids die shortly after hatching (Álvarez & Garcia-Vazquez, 2011; McGowan & Davidson, 1992a). However, our results were analogous to what has been observed in the wild in North America, where all hybrids documented have been derived from brown trout females. Post-hatch selection for hybrids with salmon mothers in our experiment may explain why only the reciprocal cross is found in nature.

This study generated some limitations that should be considered while interpreting the results. Our sample size was small (n=10 fish) and generated a lack of diversity within species (salmon and trout each came from one stock). Therefore, what we found does not capture variation that exists within, and among populations. Obtaining gametes was difficult logistically given we wanted to collect gametes from both species within the same day for accurate results. We struggled to find unspawned female brown trout, due to individuals frequently moving in and out of the sample area during the night. Further research should include individuals from multiple stocks and from several watersheds to provide stronger evidence for species-level questions. That being said, our full-factorial breeding design was an important way to see variation within any individual.

In conclusion, Atlantic salmon and brown trout hybridization in North America provided a unique opportunity to investigate the nuances of offspring development between an indigenous and an introduced invasive species, while in most of their native range they are sympatric. The questions explored in Chapter 2 and Chapter 3 provided important insights that should be explored more thoroughly in effort to support the conservation of Atlantic salmon stocks in North America – a species whose status remains threatened by a multitude of factors. Although they are prized, loved and an important species for anglers, the wealth of impacts introduced brown trout have on indigenous fishes (and other taxa) should be enough evidence to manage them as an invasive species (Warner et al., 2015). Or perhaps, *Salmo trutta* should be widely accepted and managed as the "new" naturalized salmonid species in North America – something that is potentially already happening. An interesting consideration is the fact that anthropogenic effects tend to have permanent effects of salmonids, while natural disturbances including glaciation, volcanic activity, and landslides have been followed by gradual recovery for millions of years (Waples et al., 2008). These fishes are quite resilient if we give them a chance.

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