Reproductive isolating mechanisms influencing the direction of Banded Killifish (*Fundulus diaphanus*) x Common Killifish (*F. heteroclitus*) hybridization in Porter's Lake, Nova Scotia

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A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia,
in Partial Fulfilment of the Requirements for
the Degree of Master of Science in Applied Science

August 2021, Halifax Nova Scotia, Canada

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ABSTRACT

Hybridization events provide opportunities to examine the mechanisms influencing interspecific reproductive isolation. In Porter's Lake, Nova Scotia, hybridization between two killifish species (*Fundulus heteroclitus* and *Fundulus diaphanus*) predominantly occurs with *F. diaphanus* mothers and *F. heteroclitus* fathers. To test if pre-zygotic isolating barriers contributes to this cross-direction bias, breeding behaviour was studied in the lab. *Fundulus heteroclitus* females preferred conspecific males while *F. diaphanus* females showed no preference. Additionally, all possible pairwise crosses were made *in vitro* and incubated at four salinities to test reproductive barriers related to fertilization and hybrid development. *Fundulus heteroclitus* x *F. diaphanus* (female x male) hybrids had lower fertilization and longer development times than other cross types. Together, these results suggest that both pre- and post-zygotic mechanisms contribute to the absence of *F. heteroclitus* x *F. diaphanus* hybrids in the wild, and that additional, un-measured reproductive isolating mechanisms are also likely to be quite important.

Date: August 24, 2021

ACKNOWLEDGEMENTS

First, I must acknowledge that this project would not have been possible without the immeasurable support and guidance I received from my supervisors Dr. Dalziel and Dr. Weir. The friendly and learning rich environment they provided made my time in the fish lab an absolute joy despite any frustrations or struggles. I gained and developed a great deal of skills from them for which I am tremendously grateful. I must also thank Caila Lebans for all of her help taking care of the fish and coordinating with everyone in the lab to ensure that the animals were being looked after. Also thank you to Emily Allen as well for stepping in during Caila's absence. I would also like to extend my gratitude to the members of my examining committee, Dr. Cameron, Dr. Frasier, and Dr Gilg for their time and valued feedback.

Without the support of past and present members of the SMU fish lab my entire experience throughout this project would surely have been far less enjoyable. A special thanks to Haley Drapeau and Chloe Champion for their help and good humour during field work, lab work, and fish care. Thank you to Lauren Jonah for passing along her fish identification wisdom and measuring skills. Thank you to my fellow graduate students Rachel Corney, Anna Bernhardsson, and Tori Cluney for the special support that can only be given by other struggling students. And thank you to all the other members of the fish lab that listened to my presentations and provided me with constructive feedback.

Next, I must thank all my friends and family who listened to me go on about fish constantly. I am fortunate to have a great support system that encouraged me throughout this process. A special thanks to Maddie Clarke for helping me make a lovely map of my field sites (Figure 1, it's beautiful, check it out) and helping me navigate through R.

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1. INTRODUCTION

1.1 Reproductive barriers among species

The process by which new species are formed is a key area of study in evolutionary biology (reviewed by Schluter, 2001; Butlin et al., 2012; Ravinet et al., 2017). While there is debate about how to define a species, in sexually reproducing organisms a species is typically defined as a group of individuals that interbreeds, produces viable fertile offspring, and is reproductively isolated due to biological mechanisms from other groups (Coyne & Orr, 1998; Butlin et al., 2012). New species can be formed through many different reproductive isolating mechanisms occurring prior to (pre-zygotic) or after (post-zygotic) fertilization, which might act independently or in concert (Schluter, 2001; Butlin et al., 2012) as groups diverge. Speciation may result from stochastic processes, such as when two isolated populations develop reproductive incompatibilities due to genetic drift, or they can diverge because of selection (Schluter, 2001; Butlin et al., 2012). Speciation resulting from natural selection can occur when populations adapt to differing environments (‘ecological speciation’), or when populations adapt to similar selective environments via different genetic mechanisms (‘mutation-order speciation’) (Schluter, 2001; Schluter, 2009; Butlin et al., 2012; Kulmuni et al., 2020).

Exploring the mechanisms that initiate, reinforce, and maintain reproductive isolation between two groups is key to understanding how separate species are formed and persist (Butlin et al., 2012; Kulmuni et al., 2020). Pre-zygotic barriers to reproduction occur before fertilization and can be pre-copulatory, such as behavioural differences in mating strategies or discrepancies in habitat preference among populations, or can be

post-copulatory, such as gametic incompatibilities preventing fertilization (Vigueira et al., 2008; Butlin et al., 2012; Yeates et al., 2013; Sobel & Chen, 2014). Post-zygotic barriers occur after fertilization and can include embryonic death or sterile offspring (Vigueira et al., 2008; Butlin et al., 2012; Sobel & Chen, 2014), processes that, despite successful fertilization, prevent continued gene flow between distinct populations or species. Pre-zygotic factors are generally deemed more important or stronger as they operate before post-zygotic barriers have a chance to act (Butlin et al., 2012; Montanari et al., 2016; Ostevik et al., 2016); if pre-zygotic isolation is complete, in theory post-zygotic mechanisms would be inconsequential. Moreover, strong selection for pre-zygotic barriers reduces the energy and resources potentially wasted on reproduction that will not be viable (Immler et al., 2011).

The strength of reproductive barriers can also be influenced by environmental factors. Extrinsic barriers are external to the organism, such as environmental conditions, geographical distributions, or behavioural cues that influence reproductive success and the probability of interbreeding (Rice, 1987). Intrinsic barriers involve genetic components that prevent reproduction in all environmental conditions, such as gamete incompatibility (Abbott et al., 2013). It is important to understand whether post-zygotic barriers are influenced by extrinsic and/or intrinsic mechanisms because many hybrids that are viable in laboratory conditions do poorly in the wild (reviewed by Ostevik et al., 2016). For example, hybrid inviability could be a result of intrinsic processes, wherein the offspring cannot develop due to major genetic incompatibilities, or extrinsic processes, where hybrids develop only under specific environmental conditions (Lessios, 2007; Ostevik et al., 2016).

Identifying isolating mechanisms and their relative strength can be difficult because the factors that initiate the speciation process might change or be concealed by reinforcing factors or other mechanisms over time, even after complete reproductive isolation (Butlin et al., 2012; Auffan et al., 2014; Montanari et al., 2016; Kulmuni et al., 2020). Moreover, reproductive isolating mechanisms regularly operate simultaneously or in succession over the course of evolutionary time to result in complete reproductive isolation between groups (Coyne & Orr, 1998; Schluter, 2001; Ostevik et al., 2016; Barbas & Gilg, 2018; Kulmuni et al., 2020). While disentangling reproductive isolation mechanisms is complex, studying these mechanisms is critical for understanding how they influence gene flow between species and maintain, or break down, reproductive barriers (Coyne & Orr, 1998; Schluter, 2001; Bernardi, 2013).

1.2 Hybridization

Backcrossing hybrids with their parental species can transfer genetic material from one species or population to another (Wirtz, 1999), which can homogenize populations and potentially lower overall biodiversity or provide the raw material needed for future adaptive radiation (Taylor & Larson, 2019). As such, hybridization is increasingly being recognised as having meaningful evolutionary outcomes resulting in changes to biodiversity (Nolte & Tautz, 2010). Hybridization plays an important role in the evolution and diversification of many taxa of plants and was generally thought to be less influential in animals, however, this view is changing and the role of hybridization in animals is increasingly being recognised to influence evolutionary processes (Dowling & Secor, 1997; Wirtz, 1999; Schwenk et al., 2008; Nolte & Tautz, 2010; Butlin et al., 2012).

Hybridization of divergent populations or species can have many different outcomes, with the potential for any number of genetic and/or phenotypic characteristics of the parental groups (Abbott et al., 2013; Montanari et al., 2016). While in some cases hybrids may be less fit than either parental species due to the dissociation of coevolved gene complexes, the creation of new genetic combinations through hybridization can also result in individuals that are better suited to exploit new habitats (Nolte & Tautz, 2010; Neaves & Baumann, 2011; Montanari et al., 2016). Continued adaptation to a new environment could lead to reproductive isolation between hybrids and parental species and subsequently the formation of a new species over time (Coyne & Orr, 1998; Schluter, 2001; Bernardi, 2013; Montanari et al., 2016). Alternatively, hybridization can lead to the 'collapse' of distinct species as was the case for three species of Darwin's tree finches which were reduced to two species due to hybridization (Kleindorfer et al., 2014). Hybrid zones provide a unique opportunity to study the effects of incomplete reproductive isolation or the breakdown of reproductive barriers, as well as the strength of various barriers that may be acting on the system (Vigueira et al., 2008). Subsequently, understanding how reproductive barriers influence species can contribute to our overall comprehension of speciation and local adaptation (Coyne & Orr, 2004; Bernardi, 20130). Moreover, hybridization can be affected by reproductive barriers that have a stronger influence on one sex or the other of one or both species causing unidirectional hybridization between species.

Asymmetrical hybridization, that is hybridization that occurs between females of species A and males of species B but not between females of species B and males of species A, can occur due to a variety of different intrinsic, extrinsic, pre-zygotic, and

post-zygotic mechanisms (Wirtz, 1999). Variation in breeding behaviour, wherein mating displays of one species are more attractive to con- and heterospecific mates could cause bias if individuals are able to subsequently successfully mate with conspecific individuals. In studies of swordtails, *Xiphophorus* species, females of one species prefer the courtship of heterospecific males, despite being from allopatric populations, and will hybridize when given the opportunity (Wirtz, 1999). Size differences can also affect hybridization directions in various ways. Preference for larger or smaller mates, physical or mechanical incompatibilities between sexes if mating is not feasible, or forced copulations by larger species could all contribute to asymmetrical hybridization (Wirtz, 1999; Pampoulie et al., 2021). Variation in species abundance and mate availability could also cause the more discriminating sex, usually females, to be less discriminating in choosing a mate (Pampoulie et al., 2021). For example, one hypothesis for the unidirectional hybridization between fin whales and blue whales is that the small population sizes of blue whales pressures female blue whales to mate with male fin whales, as there are few available conspecific males. By contrast, fin whale populations are large enough that the same mate-choice pressure does not occur for female fin whales, and thus only one type of hybrid is produced (Pampoulie et al., 2021). Genetic or gamete incompatibilities are also a potential barrier causing unidirectional hybridization. In many cases proteins on the surface of ova or in ovarian fluid are unrecognizable by heterospecific sperm, acting as a reproductive barrier (Palumbi, 1994; Yeates et al., 2013). Occasionally gametic incompatibility is only present between males of one species and females of the other, while reciprocal gamete interaction is unimpeded leading to unidirectional hybridization (Wirtz, 1999; Lessios, 2007).

1.3 Reproductive barriers and hybridization in fishes

Hybridization in fishes is more common than in other groups of vertebrates (Scribner et al., 2001; Hernández Chávez & Turgeon, 2007). This is because many fish species have external fertilization, synchronous spawning events with overlapping territories, limited mate availability, and a higher likelihood of secondary contact than other vertebrates (Scribner et al., 2001; Hernández Chávez & Turgeon, 2007; Montanari et al., 2016). For example, many North American minnow species, will use nests belonging to another species with an overlapping breeding season. This behaviour, coupled with external fertilization, can facilitate hybridization given synchronous occurrences of mating events during which the gametes of multiple species may be present and active at the same time (Corush et al., 2020). Fishes might also experience changes in habitat, due to anthropogenic activities (e.g., dams) or natural events, (e.g., decrease or increase in range overlap), which could lead to reproductive isolation and then secondary contact between allopatric populations (Scribner et al., 2001). Although hybridization between highly divergent groups of fishes is infrequent, it can occur in some groups (Montanari et al., 2016). For example, intergeneric hybrids of flatfishes have been reported in species that through DNA-DNA hybridization methods have been shown to be divergent in > 25% of markers (Verneau et al., 1994; Montanari et al., 2016).

Additionally, hybridization in fishes is often unidirectional (Wirtz, 1999). Contributing factors to the unidirectional bias in hybridization include the use of alternative reproductive tactics by one sex of the species (Wirtz, 1999). For example, males that sneak fertilizations by releasing sperm near mating fish can hybridize with females of another species and cause a bias in cross direction if females of their own

species do not mate with heterospecific males that do not sneak fertilizations (Wirtz, 1999). Alternative reproductive tactics can also cause hybridization direction bias due to differing gamete performance. In unidirectionally hybridizing sunfish species, interspecific sperm competition between males employing different mating strategies is thought to play an important role in this hybridization bias (Immler et al., 2011). Sperm counts of territorial sunfish males are lower than sneaker sunfish males who possess larger, more energetic sperm that are better equipped to fertilize ova (Burness et al., 2004; Immler et al., 2011). Female preference for specific males based upon size, colouration, or enthusiastic displays can also cause bias in directions of hybridization (Wirtz, 1999). Temporal constraints on gamete viability or mating opportunities could result in females mating less discriminately than males who generally do not experience the same level of temporal pressure (Wirtz, 1999). For example, female Three-spine Sticklebacks are in a position of "use it or lose it" as their eggs age, so they become much less discriminating among males as they approach the limit of their spawning time (Bakker & Milinski, 1991), which at an extreme could result in hybridization with other ecotypes (Wirtz, 1999).

A rare outcome of hybridization in fishes is the production of clonally reproducing hybrids (Neaves & Baumann, 2011; Avise, 2015). While clonal or asexual reproduction is common in many microbes, plants, and invertebrates, it is infrequent in vertebrates and in almost all cases of clonally reproducing vertebrates the source of these lineages has been hybridization of non-sister species (Neaves & Baumann, 2011; Avise, 2015; Janko et al., 2018). In these cases the asexual hybrid offspring are instantly reproductively isolated from their parental lineages, thus preventing gene flow, and maintaining reproductive

isolation, among parental species (Janko et al., 2018). Of the approximate 100 recorded cases of asexual vertebrates, natural lineages have been documented in five orders of fishes (Neaves & Baumann, 2011; Dalziel et al., 2020); one of which, Cyprinodontiformes, includes the killifish species used in the present study.

1.4 Study System: Banded and Common killifish in Porter's Lake, NS

Killifish are small topminnows of the family *Fundulidae* that occur throughout North America in freshwater, brackish water, and marine ecosystems (Wiley, 1986). These fish are commonly used in biological studies due to their abundance, hardiness, and high stress tolerance (Griffith, 1974; Burnett et al., 2007). Moreover, the reproduction and development of some fishes in this family has been well documented (Newman, 1907; Breder & Rosen, 1966; Fritz & Garside, 1974a; Hernández Chávez & Turgeon, 2007; McKenzie et al., 2017; Penney et al., 2019) and many studies have examined hybridization events among species and subspecies of killifish (Chen & Ruddle, 1970; Fritz & Garside, 1974a; Duvernell et al., 2007; McKenzie et al., 2017; Barbas & Gilg, 2018). Many species in this family hybridize naturally, are relatively easily kept in captivity and will breed in laboratory conditions (Atz, 1986; Fritz & Garside, 1974a; Griffith, 1974; Burnett et al., 2007); thus, they are a good model to examine the factors influencing reproductive isolation and hybridization in fishes.

In Atlantic Canada two species of *Fundulus* can hybridize where their ranges overlap. They are the Common Killifish (*F. heteroclitus*) and the Banded Killifish (*F. diaphanus*). These non-sister taxa began diverging 15-25 million years ago (Ghedotti & Davis, 2017). Common Killifish mainly inhabit estuaries and salt marshes but have been found in freshwater, while Banded Killifish prefer freshwater and live in streams and

lakes (Dawley, 1992; Fritz & Garside, 1974b). These divergent preferences can act as a reproductive barrier as they may prevent overlap of species ranges and any subsequent interaction between species that could lead to hybridization. Regardless of their salinity preferences, both species can survive in an extreme range of salinities (*F. diaphanus* ~0-70 parts per thousand; *F. heteroclitus* ~0-120 ppt; Griffith, 1974; Fritz & Garside, 1975; Jonah, 2019; Whitehead, 2010), allowing for species range overlap. In fact, *F. diaphanus* and *F. heteroclitus* can be found living in sympatry and hybridizing in many sites throughout the Maritimes in brackish waters of lakes, estuaries, and tidally influenced regions (Fritz and Garside 1974a; Hernández Chávez & Turgeon, 2007).

The spawning season of these fishes along the Atlantic coast spans from spring through early summer (Richardson, 1939; Breder & Rosen, 1966; Taylor et al., 1979). *Fundulus heteroclitus* spawn in marsh grasses and among algae at high tide (Newman, 1907; Breder & Rosen, 1966; Taylor et al., 1979). The fish have a reproductive cycle that is synchronous with the tides and lunar cycles (Taylor et al., 1979; Hsiao et al., 1994), though the strength of this relationship varies among populations and is weaker in more Northern populations (Petersen et al., 2010; McKenzie et al., 2017). *Fundulus heteroclitus* males develop bright blue, green, and yellow breeding colours with a distinctive black spot on their dorsal fin (Newman, 1907; Breder & Rosen, 1966) and may establish and defend a territory (Newman, 1907; Breder & Rosen, 1966); however, they display less aggressive or defensive behaviour than *F. diaphanus* (Richardson, 1939; Fournier & Magnin, 1975; Petersen et al., 2010; personal observations). Male dominance in *F. heteroclitus* is determined more by colouration rather than size, as smaller, more brightly coloured males can fend off males twice their size that are more dull in colour to

control a spawning area (Newman, 1907). It is unclear, however, if this bright colouration also serves to attract females. Female *F. heteroclitus* may indicate their desire to spawn by quickly turning sideways and flashing their bellies towards males. Male courtship involves the male pursuing a female usually slightly below her while occasionally nudging her with his head (Newman, 1907). Spawning occurs when a male holds the female with his dorsal and anal fins while pressing the female against a hard surface (Newman, 1907; Breder & Rosen, 1966). Both assume a curved body shape (Newman, 1907; Breder & Rosen, 1966) and the female vibrates for a short period (approximately two seconds) and then gametes are released into the water. The eggs will then fall away from the adults and settle in the algae or on the substrate (Newman, 1907). At higher population densities spawning can be promiscuous with several males competing to fertilize a single female's eggs, by releasing their sperm near the female as she releases her eggs (Petersen et al., 2010). During these promiscuous mating events there is still little to no male aggression occurring (Petersen et al., 2010)

Many aspects of the breeding behaviours of *F. diaphanus* are similar to *F. heteroclitus*, likely facilitating hybridization; however, there are still key differences in spawning locations and mating behaviours. Different spawning behaviours can act as reproductive barriers if cues are not interpreted properly or missed but can also facilitate hybridization if these differences are more appealing to heterospecific individuals. It should be noted that there is much less information available about the breeding behaviours of *F. diaphanus* compared to *F. heteroclitus*. *Fundulus diaphanus* males develop bright blue and green nuptial colourations along the midline of their bodies with dark bands along their sides (Richardson, 1939; Fournier & Magnin, 1975; DFO, 2011),

creating clear vertical stripes on the fish. These colours are often not as bright and do not cover as much surface area on the body as the breeding colouration of *F. heteroclitus* males. *Fundulus diaphanus* males also develop a brilliant blue/white iridescence on their anal fin (personal observations of Porter's Lake *Fundulus*). Males of *F. diaphanus* have been described as territorial and will frequently fight with other males or intruders by biting and chasing them to establish dominance (Richardson, 1939; Fournier & Magnin, 1975). Spawning and courting behaviours are similar to *F. heteroclitus* (Newman, 1907; Richardson, 1939). However, before the *F. diaphanus* male corners the *F. diaphanus* female against a surface to mate, she will release a single egg which remains attached to her by a thin filament while being followed by the male (Richardson, 1939; Breder & Rosen, 1966). This appears to be a trigger for the male to court the female towards some weeds or another surface (Richardson, 1939). Assuming a similar position to that of the *F. heteroclitus*, both *F. diaphanus* fish will quiver and release gametes (Richardson, 1939). The clutch will remain attached to the female by clear filaments until they are brushed off on surrounding vegetation or eventually detach from the female (Richardson, 1939; DFO, 2011).

The outcomes of hybridization events between *F. diaphanus* and *F. heteroclitus* appear to vary, as genetic studies of these progeny suggest that hybrids sometimes mature into sexually reproducing hybrids and other times develop into all-female asexual clonal lineages (Dawley, 1992; Hernández Chávez & Turgeon, 2007; Merette et al. 2009). Asexual reproduction in these fishes is thought to occur via gynogenesis where sperm is still required to activate the eggs, but genetic material from the male gamete is not incorporated (Dawley, 1992; Neaves & Baumann, 2011; Avise, 2015). Indeed,

backcrossing *Fundulus* hybrids with either parental species resulted in progeny that were genetically identical to the F1 females with no incorporation of any genetic material from the males in Porter's Lake fish (Dawley, 1992). Clonal reproduction in vertebrates is a relatively rare phenomenon (Neaves & Baumann, 2011; Avise, 2015), thus the study of these animals when asexual clones occur is of particular interest to understanding how meiotic abnormalities can lead to reproductive isolation.

Porter's Lake, Nova Scotia, Canada, is the best-studied *F. diaphanus* and *F. heteroclitus* hybrid zone where asexual hybrids are present (Dawley, 1992; Hernández Chávez & Turgeon, 2007). Porter's Lake is connected to the Atlantic Ocean at its south end. The tides cause water to flow between the lake and the ocean at its southern end (4.643764, -63.314155; Fritz & Garside, 1974a) creating a salinity gradient across the lake. This gradient ranges from approximately 16 ppt, where the lake connects to the ocean, to 0 ppt at the northern end (Mérette et al., 2009, Jonah, 2019, personal observations, 2020). Within Porter's Lake both *Fundulus* species can be found inhabiting water that corresponds to their preferred salinities, *F. diaphanus* preferring fresh to brackish water and *F. heteroclitus* preferring brackish to marine water. Their ranges overlap in brackish water at approximately 5-10 ppt (Mérette et al., 2009, Jonah, 2019, personal observations, 2020). Exploration of the hybrids at Porter's Lake has uncovered that the majority of clonal hybrid progeny have an *F. diaphanus* mitochondrial genome (~ 100% in 2004-2007 and 96% as of 2017-2018), indicating that the maternal species has been *F. diaphanus* for most hybridization events (Dawley, 1992; Hernández Chávez & Turgeon, 2007; Dalziel et al., 2020). Genetic analysis of these hybrids also revealed the

presence of multiple clonal lineages suggesting many independent hybridization events (Hernández Chávez & Turgeon, 2007; Mérette et al., 2009; Dalziel et al., 2020).

The reason F1 hybrids of *F. heteroclitus* females and *F. diaphanus* males are so rarely found is unknown. Potential causes are differences in mating behaviours and preferences, genetic incompatibilities, and/or abiotic environmental conditions. For example, females may prefer larger and/or more colourful males, and because *F. heteroclitus* males are usually larger and more colourful than *F. diaphanus* males they may be at an advantage. It is also possible that the larger body size that *F. heteroclitus* males possess might be better equipped to defend a territory and fight other males to secure mates if they use this strategy (Breder & Rosen, 1966; Newman, 1907). These species diverged over 15 MYA, such that genetic incompatibilities resulting from the accumulation of genetic divergence may also cause a bias in fertilization success or hybrid survival. Variation in mitochondrial DNA (mtDNA), which is maternally inherited, can affect offspring fitness (Consuegra et al., 2015), thus crosses may only be viable in one direction because of mito-nuclear incompatibilities between *F. heteroclitus* mtDNA and *F. diaphanus* nuclear loci; however, such incompatibilities are predicted to most strongly occur in the F2 generation when two *F. diaphanus* nuclear alleles from a given gene might interact with the mitochondrial genome of *F. heteroclitus* . Among the hybrids in Porter's Lake, distinct mitochondrial haplotypes are present, and as such certain intermediate genetic combinations may not be viable (Hernández Chávez & Turgeon, 2007; Dalziel et al., 2020).

Additionally, variation in abiotic conditions can affect processes such as fertilization and development, which may cause variation in survival of hybrids with

either a *F. diaphanus* or *F. heteroclitus* mother (Able & Palmer, 1988; Penney et al., 2019). For example, studies of Northern and Southern subspecies of *F. heteroclitus* indicate that fish living in higher salinity environments (20-30 ppt) experienced little to no fertilization success at low salinities (5 ppt) whereas fish inhabiting lower environmental salinities (freshwater-5 ppt) had successful fertilization at low salinities (5 ppt) (Able & Palmer, 1988). The salinity gradient of Porter's Lake (0-16 ppt) makes this abiotic factor of particular interest when examining the interbreeding of fishes with divergent salinity preferences. The resulting offspring could experience fitness consequences due to their new genetic composition which may be unsuited to cope with environmental salinity. *Fundulus* hybrids have been found in areas of Porter's Lake with salinity ranging from 0-14 ppt (Mérette et al., 2009; Jonah 2019), though they appeared to be most abundant between ~8-15 ppt (Mérette et al., 2009; Jonah, 2019; personal observations, 2019-2020). Reciprocal crosses have been produced in laboratory conditions (20°C, 10 ± 2‰ salinity; Fritz & Garside, 1974a); however, natural conditions may be unsuitable for *F. heteroclitus* female and *F. diaphanus* male crosses. External fertilization means that gametes must be suited to cope with external environmental conditions. These abiotic conditions can impact gamete performance (Crean & Immler, 2021) which could affect hybridization if gamete performance is improved or hindered in one species or the other. Due to the different preferences and environmental tolerances of *F. diaphanus* and *F. heteroclitus,* each species' gametes are likely to perform better in conditions corresponding to these preferences.

1.5 Research objectives

The objective of this research is to determine which reproductive barriers lead to the bias in cross direction resulting in *F. diaphanus* mothers and *F. heteroclitus* fathers for ~96% of wild F1 hybrids in Porter's Lake. To explore the potential for pre-mating pre-zygotic reproductive barriers leading to this bias in cross direction, and the lack of *F. heteroclitus* x *F. diaphanus* (females x male) F1 hybrids, mating behaviour experiments were set up to examine variation in female preference and male aggression. Post-mating mechanisms were investigated by exploring the intrinsic pre-zygotic barrier of fertilization success and post-zygotic reproductive barriers of hatching success, embryonic mortality, and development time among cross types of *F. diaphanus* and *F. heteroclitus* (*F. heteroclitus* ♀ x *F. heteroclitus* ♂, *F. heteroclitus* ♀ x *F. diaphanus* ♂, *F. diaphanus* ♀ x *F. heteroclitus* ♂, *F. diaphanus* ♀ x *F. diaphanus* ♂). In addition to examining intrinsic effects of cross type, crosses were incubated at a range of environmentally relevant salinities (0, 5, 10, 15 ppt) to examine the extrinsic effect of this environmental parameter on post-mating reproductive isolation.

It is likely that a combination of pre- and post-zygotic and intrinsic and extrinsic factors affect the hybridization of *Fundulus* in Porter's Lake. Viability of laboratory *F. heteroclitus* female x *F. diaphanus* male F1 crosses indicates that there are no intrinsic genetic incompatibility for this cross type (Fritz & Garside, 1974a) and that pre-zygotic behavioural barriers or post-zygotic environmental conditions might be the cause of or have stronger influence on the bias in cross direction observed in wild populations.

2. METHODS

2.1 Experimental animals:

All procedures were conducted in accordance with protocols approved by the Saint Mary's University Animal Care Committee (Laboratory Animal Use Protocol 19-02A2, 20-06) and fish were collected under a Department of Fisheries and Oceans Maritime Region Scientific Collection Permit (licence #343930) issued to Drs. Weir and Dalziel.

2.1.1 *Field collection*

Fish were collected from Porter's Lake, Nova Scotia (44.742867, -63.297117), from June to August 2020 using minnow traps and a seine net. Collection occurred at four sites along the southern half of the lake (Figure 1) that have been previously used by Jonah (2019) and Mérette (2009). Most fish used for these experiments were collected from two sites (identified as 2 and 3 in Figure 1), as both species could usually be found at these sites. Fish were collected from the other sites when one or both species were scarce or mostly immature at sites 2 and 3. Due to the salinity gradient in the lake and the divergent preferences exhibited by both species, *F. heteroclitus* could usually be found at a higher salinity site (site 4 in Figure 1; average salinity ~19 ppt) and *F. diaphanus* at a lower salinity site (site 1 in Figure 1; average salinity ~6 ppt) when needed. The greatest fishing success was achieved during the afternoons on warm sunny days when the water temperature at the shore was ~ 20-25 °C. Fishes used for experiments were selected form regions of parental species range overlap as this is where hybridization is likely occurring. As such, fishes from these sympatric locations would provide the best insight into the bias in hybridization occurring in Porter's Lake

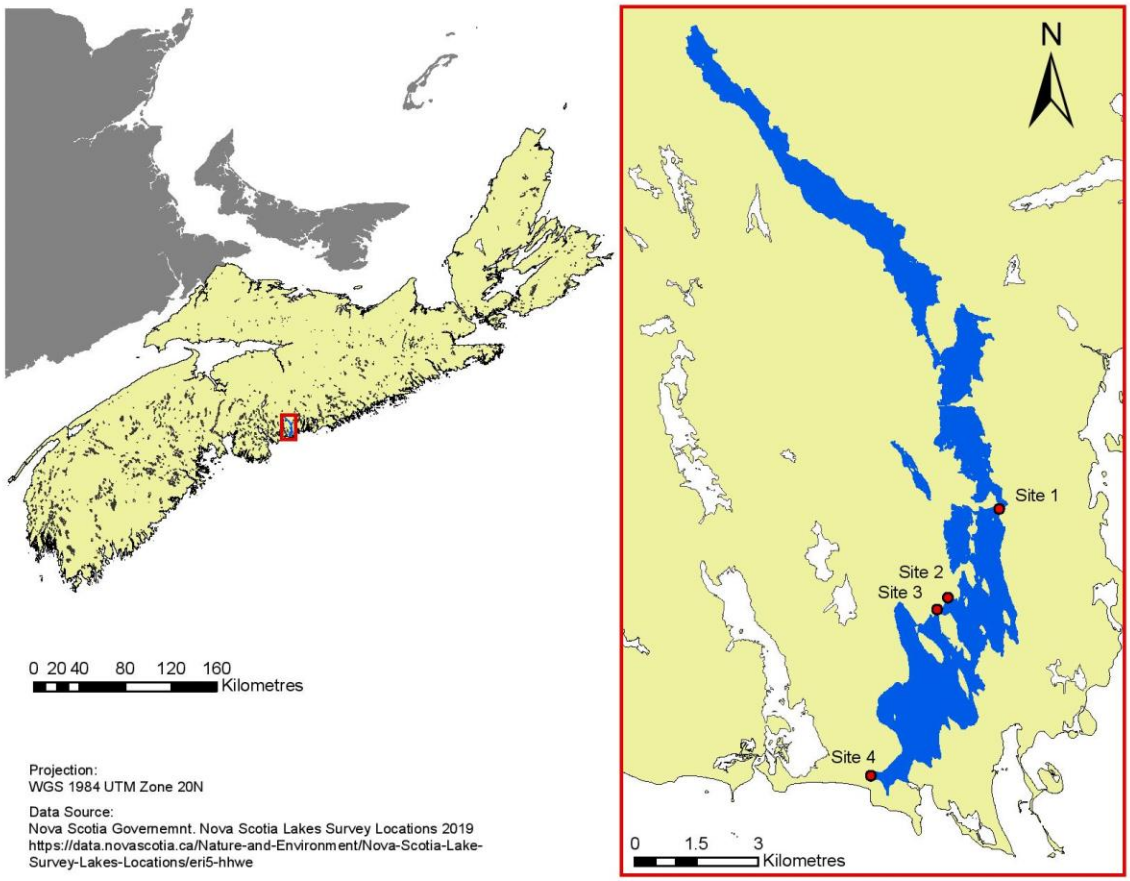

Figure 1. Map of Porter's Lake, Nova Scotia, indicating fish collection locations. Image was created using ArcMap 10.5. (Site 1 44.703427, -63.289436; Site 2 44.684037, -63.302109; Site 3 44.681442, -63.305411; Site 4 44.645063, -63.325435

2.1.2 Species and sex identification

Species were identified upon collection in the field. The two pure species are distinguishable based on body shape, colour, and size (reviewed by Gilhen, 1974; Figure 2; Figure 3); however, differentiation of hybrids from *F. heteroclitus* females is challenging. A series of quantitative measurements was used to differentiate among *F. heteroclitus, F. diaphanus,* and their F1 hybrids. The linear distance from the front of the dorsal fin to the start of the caudal fin, from the back of the anal fin to the start of the caudal fin, and the length from the dorsal end of the caudal peduncle to the ventral end were measured using digital calipers to the nearest hundredth of a cm (Figure 4). Using these measurements with the calculations outlined by Mérette (2009), the fish were assigned a probability of being each of the species or a hybrid. This method identifies species and hybrids with ~90% accuracy (Mérette, 2009; Tirbhowan, 2019). Sex was determined by colouration, as males of both species develop bright nuptial colouration (described in section 1.4), which allows males and females to be differentiated. As all F1 hybrids are expected to be female (Mérette et al., 2009) the species of any individual males could also be confidently identified due to differences in nuptial colouration. This colouration also indicated that males were sexually mature and in breeding conditions. Mature fish that could be confidently assigned to a particular species were either used to produce experimental crosses in the field or transported to the SMU Aquarium facilities for mate-choice experiments.

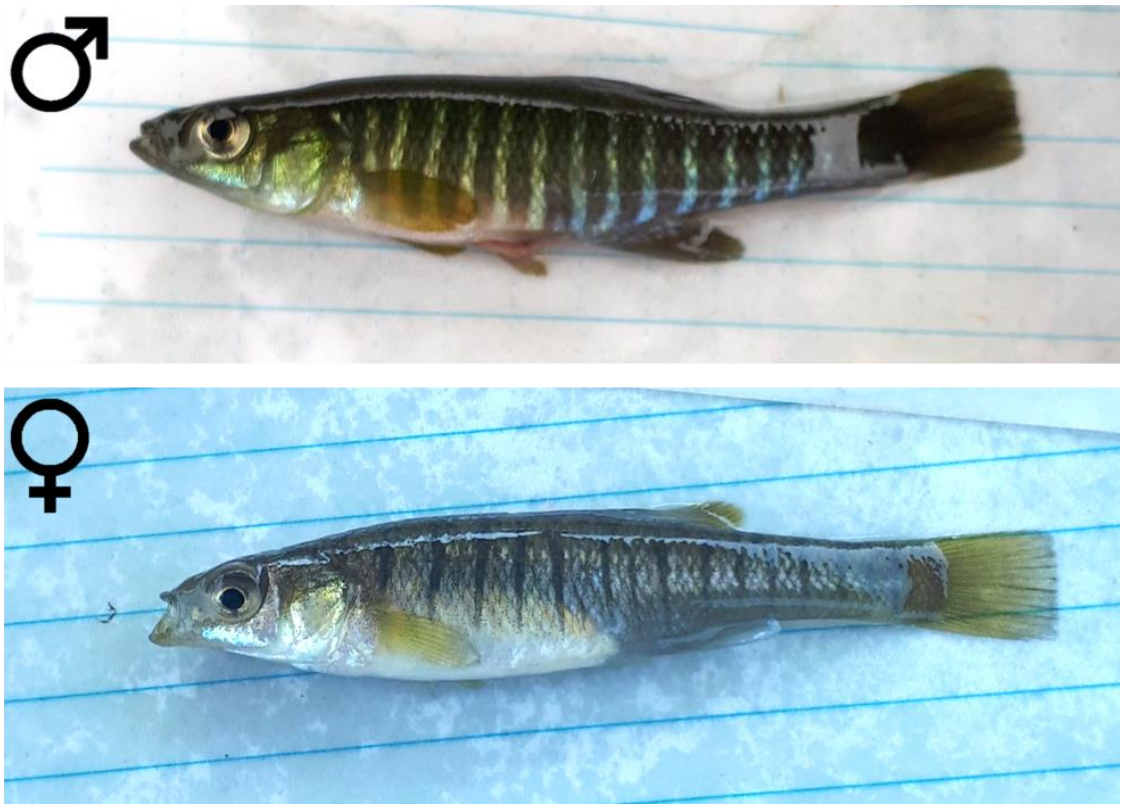

Figure 2. Male and female *F. diaphanus* in breeding condition from Porter's Lake 2020 used for *in vitro* experimental crosses.

Figure 3. Male and female *F. heteroclitus* in breeding condition from Porter's Lake 2020 used for *in vitro* experimental crosses.

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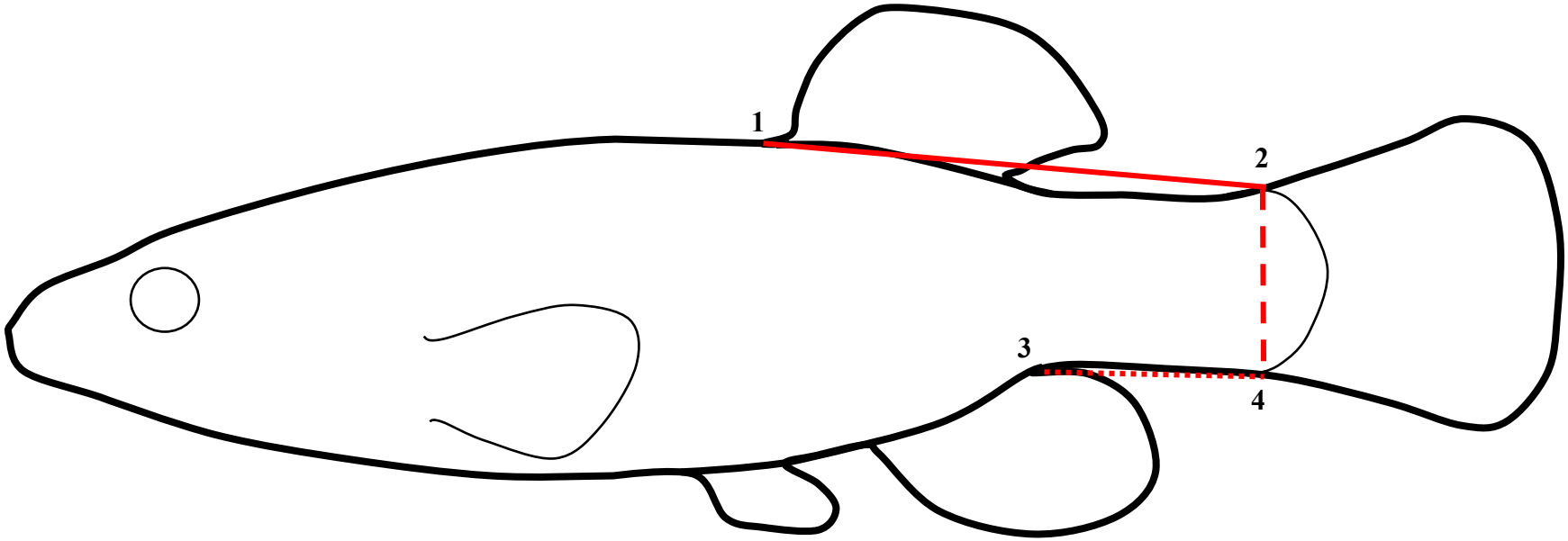

Figure 4. Diagram of measurements used to determine the species of *Fundulus* (*diaphanus* or *heteroclitus*) or their hybrids using the linear distance from: the front of the dorsal fin to the start of the caudal fin (1-2, solid line), from the back of the anal fin to the start of the caudal (3-4, dotted line), and the length from the dorsal end of the caudal peduncle to the ventral end (2-4, dashed line) following the methods of Mérette (2009).

2.1.3 *Laboratory housing*

Fish were transported back to the Saint Mary's University Aquarium facilities and housed in 15-20 gallon tanks with filters, gravel, and artificial plants. To ensure that animals remained in breeding condition, salinity, temperature, and photoperiod were set to mimic natural conditions of the hybrid zone in Porter's Lake during the breeding season (~10 ppt, ~21-23°C, 14:10 hours light:dark; Table 1). The fish were sorted into tanks separated by species and sex to prevent breeding prior to experiments. Due to their aggressive behaviour, *F. diaphanus* males were held at lower population densities and with substantial environmental enrichment to provide areas of refuge and minimize fighting among individuals. Fish were fed daily to satiation a diet comprising of beef heart flake food (Aquatic Animals Accessories Premium Beefheart Flakes), frozen *Mysis* shrimp, and bloodworms. Water quality was monitored on a weekly basis using Nutrafin and API test kits to ensure that NH_{4+}/NH_3, NO_3, NO_2 and pH levels remained within an acceptable range. Water temperature, salinity, and dissolved oxygen were measured using a YSI pro2030. Water in all tanks was changed as needed, or at least 20% weekly, to maintain water quality.

Table 1. Temperature and salinity measurements at sampling locations 1-4 of Porter's Lake (Figure 1), throughout June and July 2020 using YSI pro2030.

Site	Coordinates	Species Collected	Temperature (°C)				Salinity (ppt)			
			Min	Max	Average	Standard Deviation	Min	Max	Average	Standard Deviation
Site 1	44.703427 °N, -63.289436 °W	*F. diaphanus*	20.8	30.1	25.83	3.37	3.8	9.02	6.07	1.83
Site 2	44.684037 °N, -63.302109 °W	*F. diaphanus* & *F. heteroclitus*	20.4	28.8	24.6	2.5	8.2	15.98	10.278	2.52
Site 3	44.681442 °N, -63.305411 °W	*F. diaphanus* & *F. heteroclitus*	19.9	25	22.02	1.17	9	16.7	11.91	2.8
Site 4	44.645063 °N, -63.325435 °W	*F. heteroclitus*	19.6	21.5	19.8	0.95	16.3	21.2	19.25	2.76

2.2 Experimental design: Mating behaviour

To examine mating behaviour as a potential pre-zygotic barrier to hybridization, breeding trials were set up following the methods of McGhee et al. (2007) with some modifications. The experimental design involved three phases per trial using one female and two males (one heterospecific and one conspecific male). Four 15-gallon tanks (61 x 30.5 x 30.5 cm) were each subdivided into 3 sections; the central section (30.5 x 30.5 x 30.5 cm) was twice as large as those on either end (15.25 x 30.5 x 30.5 cm; Figure 5). The tanks were held at 23 ± 1°C at a salinity of 10 ± 0.7 ppt with a light: dark cycle of 14:10 and both end sections contained a yarn mop to mimic vegetation. Fish were placed in the experimental tanks the day before a trial. A female with a distended belly (indicative of gravidity) was placed in the large central section of the tank. One *F. heteroclitus* male and one *F. diaphanus* male were size-matched and placed in the end compartments. Opaque barriers, consisting of laminated paper, prevented fish from seeing each other during acclimation, but did not prevent waterflow between the separated compartments before the experiment began (Figure 5A). Trials were conducted in the mornings and a dark cover was placed over the tanks during the trials to minimize light as *F. heteroclitus* are known to have a higher gonadosomatic index at night (Taylor et al., 1979; Barbas & Gilg, 2018). Trials consisted of three phases outlined below (Figure 5).

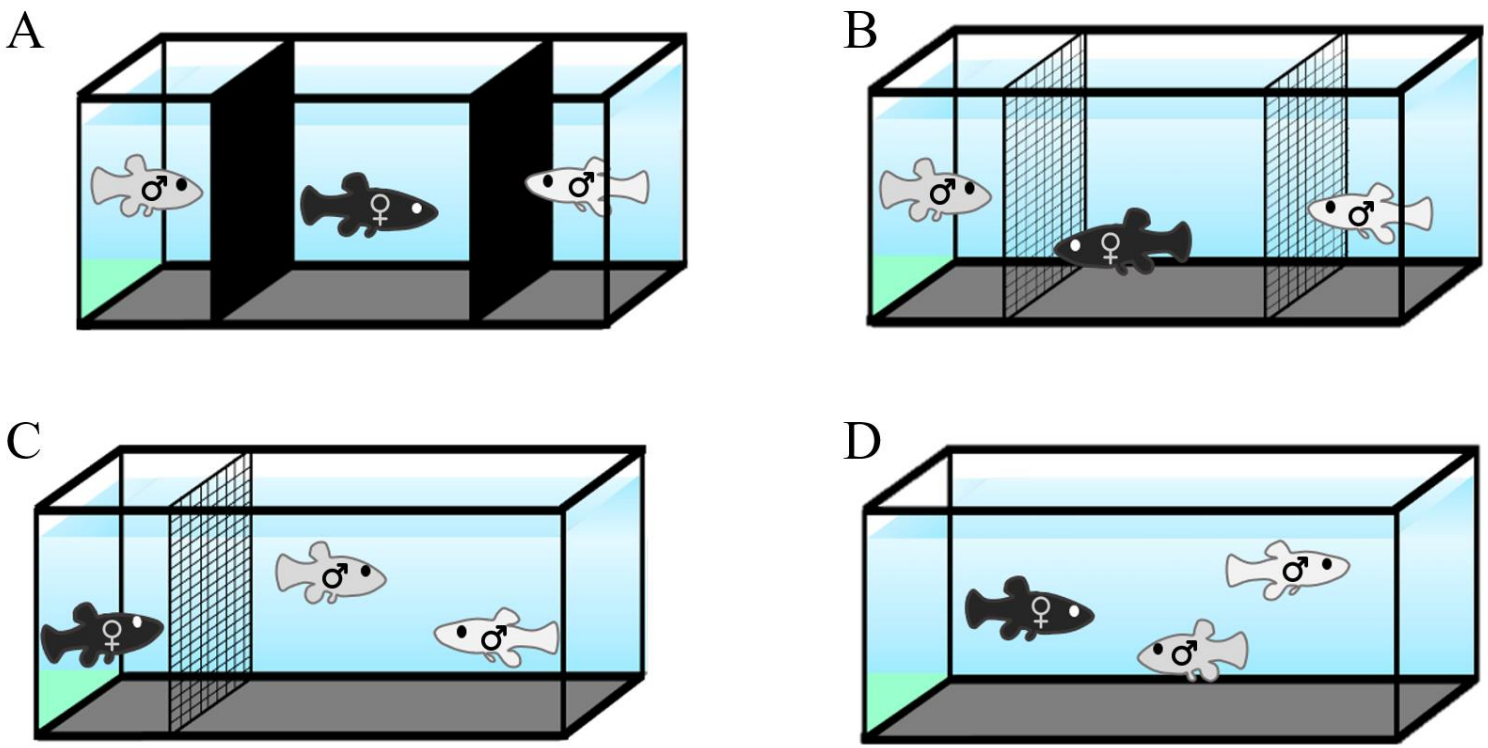

Figure 5. Depiction of mating behaviour trial process. The top left-hand panel (A) indicates the initial placements of the fish, female (black, middle) and males (greys, ends) during acclimation, with opaque barriers separating the fish. Phase 1 (B), the trial began with the opaque barriers being removed, leaving mesh barriers to separate the individuals and allowing the fish to see each other. Phase 2 (C), the female was moved to one of the flanking sections while the males were allowed to interact. And phase 3 (D), all 3 individuals were free to interact with each other.

Phase one of a trial consisted of removing the opaque barriers (Figure 5B). This allowed the transmission of any visual cues but prevented physical contact between the fish. The female was observed for 30 minutes to determine how much time she spent within 7 cm of either male; the proportion of time spent with each male was used as a measure of female preference. Phase two (Figure 5C) involved interaction between the males without access to the female. During this phase, the female was isolated in one of the side sections while the second mesh barrier was removed so that the males were able to physically interact in the larger section. The number of aggressive behaviours between males were recorded for 30 minutes. The behaviours recorded were based on descriptions by Newman (1907) and Breder & Rosen (1966), as well as personal observations in the housing tanks and during preliminary trials. These included attacks, chasing, and aggressive displays. Attacks involved biting and shoving; in cases when both fish were fighting back and forth each male was scored for an attack. Chasing was characterized by one male deliberately following the other while the latter attempted to retreat. Aggressive displays between the two species differed in that *F. heteroclitus* would flare their fins and curve their bodies towards their opponent while *F. diaphanus* would curve their bodies back and forth making a wiggling motion. It should be noted that when males attacked each other, often the recipient of the attack would defend themselves, and this was also scored as aggressive behaviour during data collection. *Fundulus diaphanus* males, being the more aggressive species, were usually the instigators during these interactions. The third and final phase of a trial (Figure 5D) involved removal of the last mesh barrier to allow all three fish to interact. During this phase, aggressive behaviours were recorded for a 60 min period. If mating occurred anytime during this phase, it was also noted. Each phase of the trial was recorded with an Enviro R jvc (GZ-R460D) video camera. A total

of 20 trials were conducted, with 10 per focal female species. Fish were normally only used once, with the exception of two *F. diaphanus* males which were reused for 2 trials due to lack of individuals.

2.2.1 *Fish tagging*

Fish used for breeding experiments were tagged for individual identification after experimental use to ensure that the procedure and any subsequent secondary effects did not interfere with their behaviour during the experiment. The animals were anesthetized in MS-222 (Tricaine mesylate) to minimize pain and discomfort during the procedure. Once a fish was anesthetized, characterized as a loss of equilibrium, it was re-measured (standard length and species identification measurements; Figure 4), weighed, and tagged. Fish were tagged by a subcutaneous injection of Visual Implant Elastomer (NorthWest Marine Technology) of different colours in specific locations on each individual fish. A fin clip (~2mm^2) was also taken at this time from their caudal fin for later genetic analysis to confirm species identification from morphological measurements.

2.2.2 *Genetic analysis*

Genetic analysis was used to confirm the species of females, and in particular to differentiate *F. heteroclitus* females from F1 hybrids. DNA was extracted from fin clips using Omega Bio-Tek EZNA Tissue DNA Kits following the manufacturer's instructions. Following the procedures outlined in Tirbhowan (2019), polymerase chain reactions (PCR) were performed to amplify a portion (~ 660 bp) of the D loop region of the mitochondrial genome (mtDNA). Restriction enzyme digestions with HphI were then used to determine the maternal species of individual fishes by identifying a restriction site unique to *F. diaphanus* (Hernández Chávez & Turgeon, 2007; Tirbhowan, 2019). This

unique restriction site produced three bands (166, 211, & 215 bp) in *F. diaphanus* mtDNA when products were run on 3% agarose gel stained with ethidium bromide to be viewed, while the *F. heteroclitus* amplification product was just cut once so only produced two bands (211 & 381 bp). A combination of visual characteristics, measurements (Figure 4), and genetic identification methods were used to identify females of either species and hybrids. If the mitochondrial D-loop and species identification measurements did not match, microsatellite regions of the nuclear DNA containing diagnostic species-specific alleles were also amplify as described in Tirbhowan (2019). If individuals possessed an *F. diaphanus* and *F. heteroclitus* allele at these loci, they were marked as hybrids to be excluded from data analysis.

2.3 Experimental design: *In vitro* crosses

Crosses of both parental species and each hybrid type, described as the species of the female x the species of the male, were made both in the field and in the lab using the same methods. Species were identified by morphological measurements (Figure 4) and males and females were selected based on brightness of male nuptial colouration and evidence of developed eggs (distended stomachs) in females. Applying gentle pressure to the abdomen released eggs (Atz, 1986; Dawley, 1992), which were placed in a petri dish. Milt was obtained from *F. heteroclitus* males by applying pressure to the abdomen and collected with a capillary tube. Milt was unobtainable from *F. diaphanus* males in this manner, so they were euthanized with clove oil in the field and testes were obtained via dissection. The milt or testes, which were cut up, were mixed gently with the eggs for 1 min and then clutches were split into 1 mL of each of the different salinity treatments (0, 5, 10, 15 ppt) for a minimum of 30 minutes. Eggs were checked for fertilization by

looking for the fertilization envelope under a dissection microscope. The fertilization envelope, which develops when eggs are fertilized, is typically clearly visible approximately 2 hours after fertilization (Armstrong & Child, 1965). If fertilization was uncertain, eggs were checked the next day for the fertilization envelope. The clutches were then rinsed off with water of the corresponding salinity treatment and placed in tanks of each of the experimental salinity treatments. Groups of eggs were kept in separate labeled petri dishes within the same experimental tanks. The petri dishes had holes cut in the tops that were covered with a mesh screen. The dishes were sealed with parafilm and rubber bands and weighted down with rocks, nuts, and washers. For four crosses (2 *F. diaphanus* x *F. diaphanus* and 2 *F. diaphanus* x *F. heteroclitus* crosses) eggs were pooled from 2 *F. diaphanus* females to obtain a larger clutch size to split among the experimental treatments and 3 *F. diaphanus* males fathered 2 clutches each (2 *F. diaphanus* x *F. diaphanus* and 4 *F. heteroclitus* x *F. diaphanus* crosses) due to lack of other males.

Experimental tanks at four salinities (0 ppt, 5 ppt, 10 ppt, 15 ppt) held the developing eggs at room temperature (averaging ~21°C) and contained air lines to aerate the water, filters, and a rearing solution of methylene blue to help prevent fungal infection. Tank temperature and salinity were monitored daily using a Hanna HI 98192 EC/TDS/NaCl/Resistivity probe and water changes were performed weekly or as needed to maintain the appropriate salinity. Embryos were checked daily for deaths, infections, and hatching. Dead and diseased eggs were recorded and removed. On the 5^{th} and 10^{th} days the development stages were recorded and compared to those described in Armstrong and Child (1965) and Penney et al. (2019). The eggs were transferred to

separate Tupperware containers of their respective salinity treatments at room temperature on the 10th day to accurately track hatching among clutches and salinity treatments. The water in the container was also monitored and adjusted as needed to maintain salinity. Once hatched, the fish were transferred to separate containers at 10 ppt salinity based on cross type, with different families of each cross-type pooled to rear fish for future experiments. A total of 55 crosses were made (14 *F. diaphanus* x *F. diaphanus*;13 *F. diaphanus* x *F. heteroclitus*; 14 *F. heteroclitus* x *F. diaphanus*; 14 *F. heteroclitus* x *F. heteroclitus*).

2.4 Statistical analysis

R version 4.0.4 (R Core Team, 2021) was used for statistical analyses and plots were generated using the package 'ggplot2' (Wickham, 2016). Generalized linear models (GLMs), generalized linear mixed models (GLMMs), from the 'lme4' package (Bates et al., 2015), and analysis of deviance tables were used to analyze data and determine significant factors. *Post hoc* Tukey multiple comparisons tests were used to check for significant differences among estimated marginal means with the 'emmeans' package (Lenth, 2021). The fit of models was checked by plotting residuals on quantile-quantile plots.

2.4.1 Behavioural experiments

GLMMs with normal error distributions were used to test the fixed effect of the species of the males and the species of the females with individual females as a random effect on female preference for males. The response variable, the time females spent near each male over the course of the 30 min phase 1 of the trials, was calculated as a proportion of the total time of trials to use in analyses.

Male aggression analysis was performed on the totaled counts of all male aggressive behaviours (displays, attacks and fights, and chases) during phases 2 and 3 of the mating trials. Phases 2 (30 mins) and 3 (60 mins) were analysed both separately and together with the complete total counts male aggressive behaviours towards males and females. Counts of behaviours from phase 2 of the trials were analysed using GLMMs with negative binomial error distributions. Male and female species were used as fixed effects and trial numbers were used as random effects. Male-male aggression and male-female aggression during phase 3 of the trials as well as total male-male aggression over the course of all phase of trials were analysed using GLMMs with Poisson error distributions with the same fixed and random effects.

2.4.2 In vitro crosses

The effect of cross type (*F. diaphanus* x *F. diaphanus, F. diaphanus* x *F. heteroclitus, F. heteroclitus* x *F. diaphanus, F. heteroclitus* x *F. heteroclitus*) and incubation salinity (0, 5, 10, 15ppt) on the fertilization success, embryonic mortality, and hatching success were tested using GLMs with binomial error distributions. Models were weighed by the number of eggs split among salinity treatments (fertilization success) and the number of fertilized individuals per group (survival and hatching) to account for differences in clutch sizes using the 'weights =' function from the 'stats' package (R Core Team, 2021).

Time to hatching was analyzed using negative binomial GLMs. Analysis was conducted on the average hatch time in days of each group of eggs from each clutch split in each of the salinity treatments. Salinity, cross type, and temperature were used as fixed effects and models were weighted by the number of hatched individuals per egg group. The average room temperature over the course of development from fertilization to

hatching was used as a covariate for these analyses. Analyses were also performed on development time using the residuals of hatching time and temperature to correct for temperature and analysed using salinity and cross type as fixed effects. Residuals were normally distributed and as such were analysed using GLMs with normal error distributions.

2.5 Quantification of isolating barriers

The strength of reproductive isolation of barriers was quantified for females of both species following the methods used in Barbas & Gilg (2018) which were based on methods by Sobel & Chen (2014) and Ramsey et al. (2003). The model developed by Sobel & Chen (2014) calculates reproductive isolation (RI) assuming a linear relationship between the probability of gene flow between species, calculated as the proportion of heterospecific (H) reproductive success over the sum of heterospecific and conspecific (C) success, and reproductive isolation. The results of these calculations range from -1 to 1 indicating success of heterospecific pairings or conspecific pairings respectively.

$$RI = 1 - 2\left(\frac{H}{H + C}\right)$$

Pre-zygotic mate preference and fertilization success as well as post-zygotic hatching success were all quantified using this method. However, due to the successive nature of reproduction the relative contribution (RC) to RI of each barrier is affected by the specific life stages during which each barrier can act (Ramsey et al., 2003; Barbas & Gilg, 2018). That is to say, each subsequent barrier can only prevent gene flow that has not previously been inhibited by an earlier barrier. Calculating the absolute contribution (AC) of each barrier accounts for the sequential structure of reproductive events.

Estimates of AC and total RI were calculated using the methods outlined by Ramsey et al. (2003).

$$AC_n = RI_n\left(1 - \sum_{i=1}^{n-1} AC_i\right)$$

$$RI_{Tot} = \sum AC$$

Mate preference experiments were all conducted at 10 ppt however these measures are not expected to vary across salinities as both species of fish can tolerate all experimental salinities tested in the present study. As such RI for mate preference was used to calculate AC and total RI at each salinity. The RC of each barrier was then estimated as the proportion of the AC to total RI as described in Ramsey et al. (2003).

$$RC = \frac{AC}{RI_{Tot}}$$

3 RESULTS

3.1 Mating behaviours

3.1.1 Female preference

The species of both males and females, as well as the interaction of these factors, had a significant effect on female preference (Table 2). On average, both species of females spent more time with conspecific males rather than heterospecific males (Figure 6). While *F. heteroclitus* females spent significantly more time with conspecific males, the proportion of time *F. diaphanus* females spent with conspecific males was not significantly different than the time spent with heterospecific males (Figure 6). The time *F. heteroclitus* females spent with conspecific males is also significantly higher than the time *F. diaphanus* females spent with conspecific and heterospecific males (Figure 6).

Table 2. Summary of analyses of deviances of pre-zygotic mating behaviour GLMs with male and female species as factors used in analyses.

	Female Preference			Male-male Aggression (Phase 2)		
Factor	df	X^2	p-value	df	X^2	p-value
Male species	1	6.80	0.0091*	1	25.08	< 0.0001*
Female species	1	13.73	0.0002*	1	0.06	0.81
Male species x Female species	1	20.78	< 0.0001*	1	0.0018	0.97

	Male-male Aggression (Phase 3)			Male Aggression (Total)		
Factor	df	X^2	p-value	df	X^2	p-value
Male species	1	33.53	< 0.0001*	1	91.29	< 0.0001*
Female species	1	16.03	< 0.0001*	1	6.63	0.0100*
Male species x Female species	1	3.41	0.06	1	2.80	0.09

	Male-Female Aggression (Phase 3)		
Factor	df	X^2	p-value
Male species	1	23.68	< 0.0001*
Female species	1	3.04	0.08
Male species x Female species	1	0.14	0.14

* significant factors

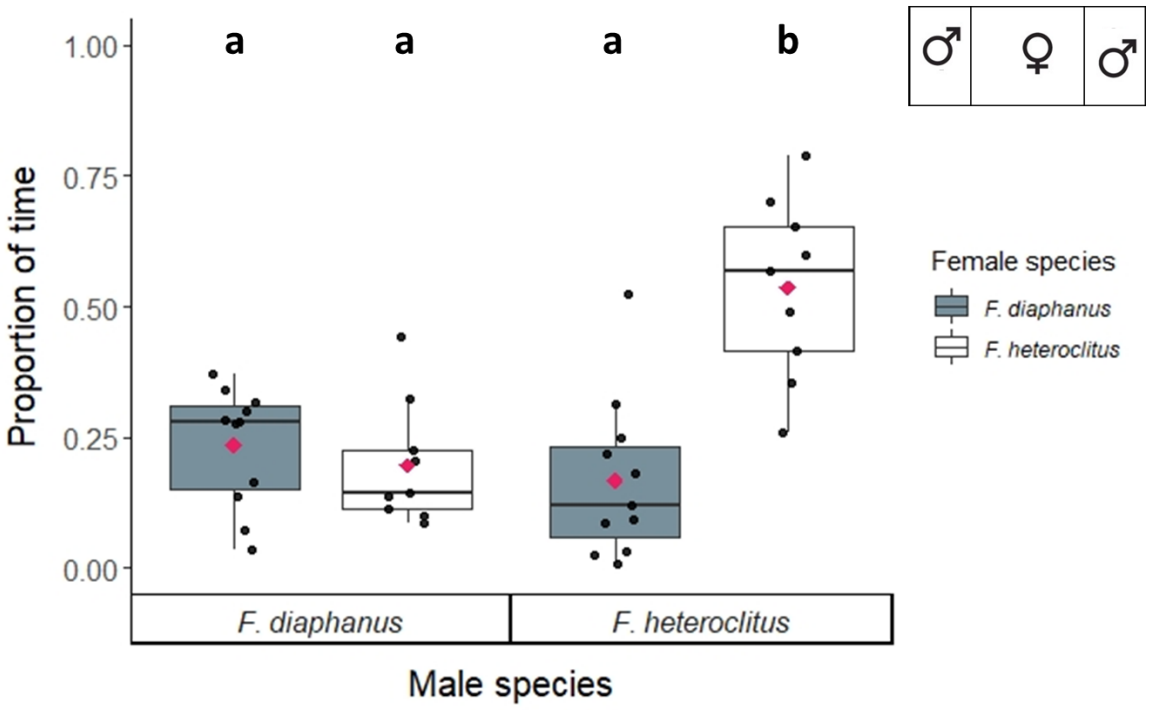

Figure 6. Proportion of time females (*F. diaphanus* or *F. heteroclitus*) spent within 7 cm of the flanking tank sections containing either *F. diaphanus* or *F. heteroclitus* males during phase 1 of the mating behaviour trials (n = 20; 10 females of each species). Pink diamonds represent estimated marginal mean time each species of female spent with each species of male. Significant differences ($p < 0.05$) among groups, based on *Post hoc* Tukey multiple comparisons tests, are represented by different letters at the top of the plot. The panel in the top right corner depicts the position of each fish in tanks during the phase 1 trials.

3.1.2 Male aggression

During the second phase of the behavioural trials when males could interact with each other but not with the female, the only variable that significantly affected male aggressive behaviours (displays, attacks and fights, and chases) was the species of the male (Table 2). The mean aggression displayed by *F. diaphanus* males during phase 2 of the mating trials tended to be higher than that of *F. heteroclitus* males; however, this difference was not significant (Figure 7).

During phase 3 of trials, when males had access to the female, four conspecific *F. heteroclitus* mating events occurred. No other mating events were observed. Significant predictors of male aggression during phase 3 of the trials included the species of the males and species of the females (Table 2). Mean male-male aggression was higher for males of both species in trials with *F. diaphanus* focal females, however, they were not significantly different from trials with *F. heteroclitus* females (Figure 8). There were also no significant differences in male-male aggression when males were in the presence of conspecific or heterospecific females. *Fundulus diaphanus* males displayed significantly more aggressive behaviours towards the other males in the presence of *F. diaphanus* females than *F. heteroclitus* males in the presence of *F. heteroclitus* females (Figure 8). Male aggression towards females during phase 3 of experimental trails was only significantly affected by the species of the male (Table 2), and *F. diaphanus* males displayed significantly more aggressive behaviour towards females than *F. heteroclitus* males (Figure 9).

Total male-male aggression during phases 2 and 3 of mating trials and male-female aggression during phase 3 was significantly affected by the species of both males

and females but not their interaction (Table 2). *Post-hoc* comparisons revealed that both *F. diaphanus* and *F. heteroclitus* males were not significantly more aggressive in the presence of one species of female or the other. Additionally, neither species of male was more aggressive in trials with focal *F. diaphanus* females or *F. heteroclitus* females. In the presence of heterospecific females, there were nor significant differences in male aggression between the species. *Fundulus diaphanus* males displayed significantly more aggressive behaviours in the presence of *F. diaphanus* females than *F. heteroclitus* males in the presence of *F. heteroclitus* females (Figure 10).

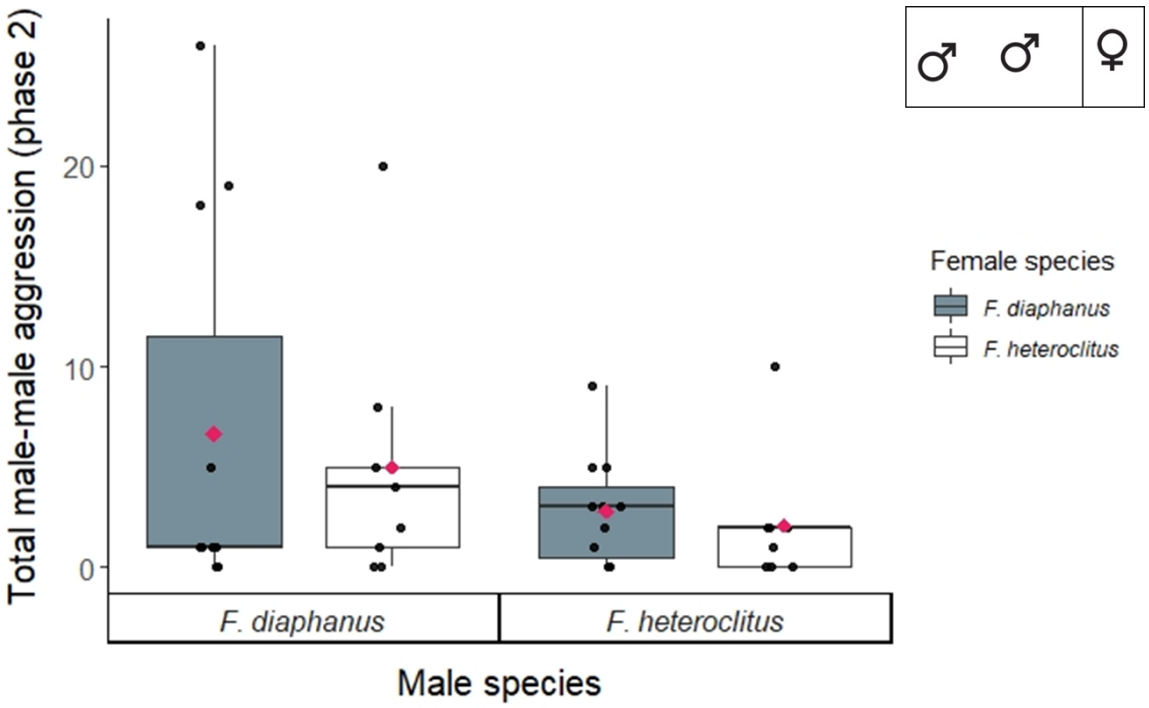

Figure 7. Counts of all male-male aggression during the second phase of the mating trials when the female remained isolated from males (n = 40; 20 males of each species). Pink diamonds represent mean aggression of males. The panel in the top right corner depicts the position of each fish during phase 2 of the trials.

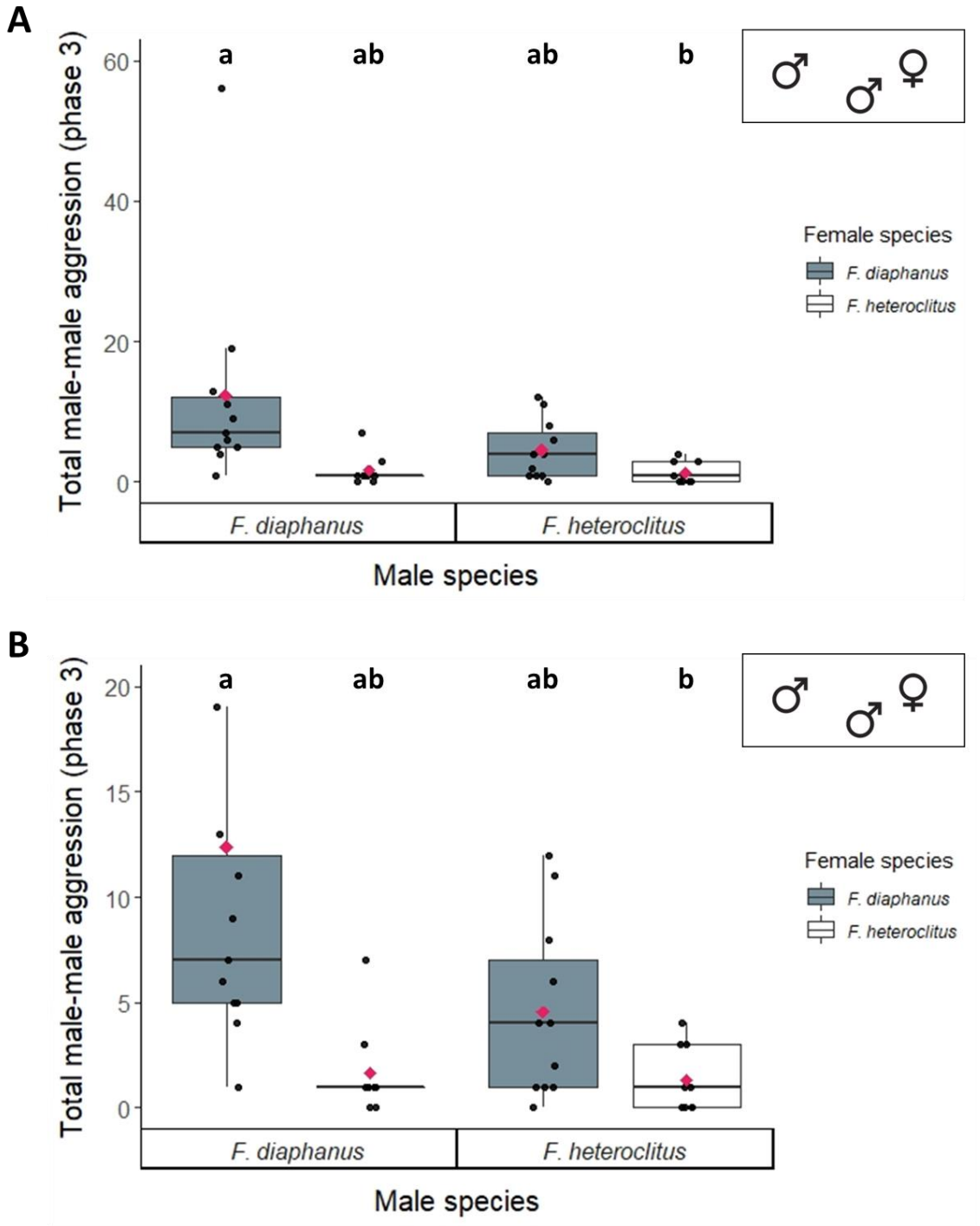

Figure 8. Total counts of all male-male aggression during the third phases of the mating trials (n = 40; 20 males of each species). Panels A and B represent the same data however panel B has a limited y axis excluding data beyond 20 aggression counts (1 data point). Significant differences ($p < 0.05$) among groups, based on *Post hoc* Tukey multiple comparisons tests, are represented by different letters at the top of the plot. Pink diamonds represent mean aggression of males. The panel in the top right corner depicts the position of each fish during phase 3 of the trials.

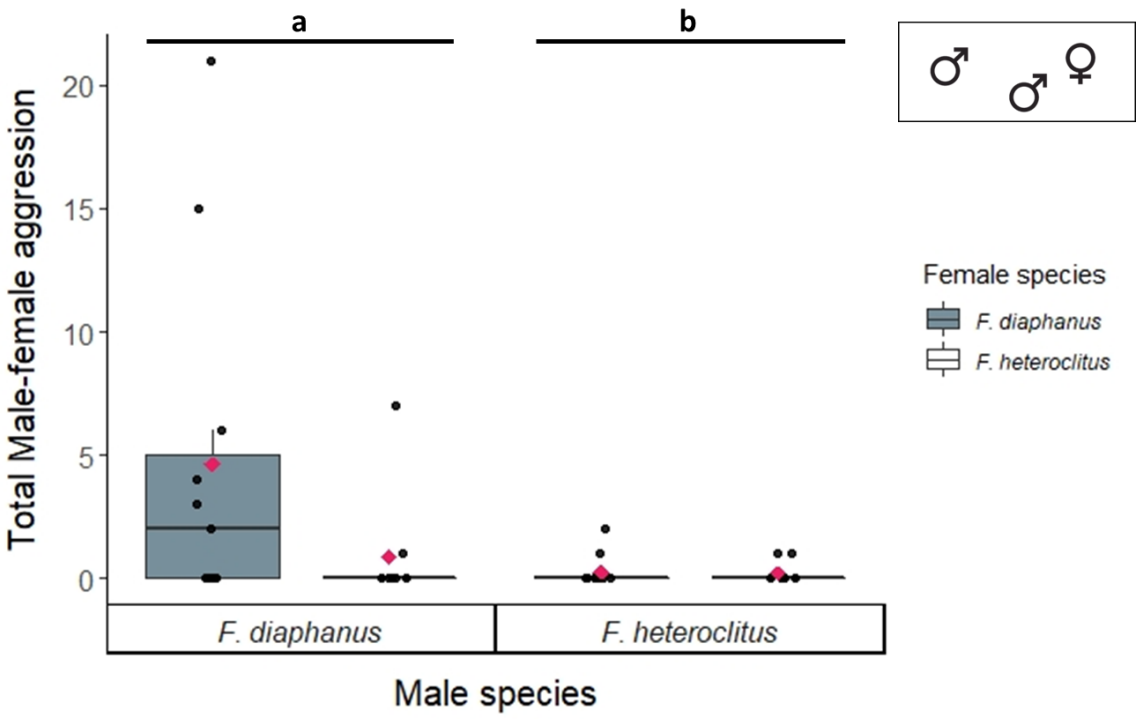

Figure 9. Counts of all male aggression towards females during the third phase of the mating trials when the males and female could interact (n = 40; 20 males of each species). Significant differences ($p < 0.05$) among the species of male, based on *Post hoc* Tukey multiple comparisons tests, are represented by different letters at the top of the plot. Pink diamonds represent mean aggression of males. The panel in the top right corner depicts the position of each fish during phase 3 of the trials.

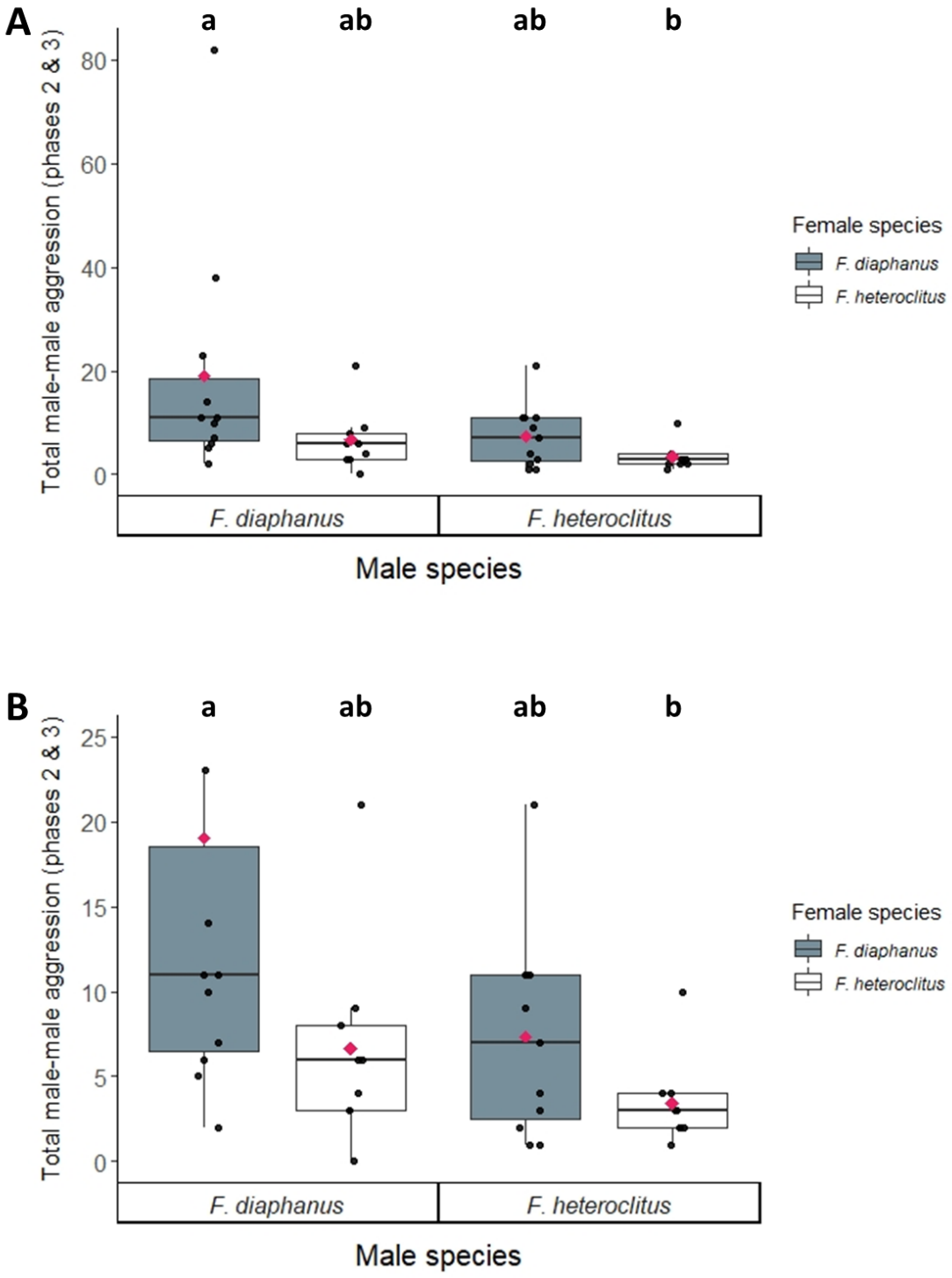

Figure 10. Total counts of male aggression towards other males during the second and third phases of the mating trials and towards females during the third phase (n = 40; 20 males of each species). Panels A and B represent the same data, but panel B has a limited y axis excluding data beyond 25 aggression counts (2 data points). Significant differences ($p < 0.05$) among groups, based on *Post hoc* Tukey multiple comparisons tests, are

represented by different letters at the top of the plot. Pink diamonds represent mean aggression of males.

3.2 *In vitro* crosses

3.2.1 Fertilization success & hatching success

A total of 1050 eggs out of 1318 from all cross types were successfully fertilized (~80%) and while *F. heteroclitus* prefer higher salinities and *F. diaphanus* prefer lower salinities, eggs were fertilized at all test salinities (0, 5, 10, 15 ppt). The method of sperm collection (capillary tube or dissection) and application to the eggs (milt or testes) was tested as a factor, but the collection method did not affect fertilization success (data not shown). Both cross type and salinity treatment had a significant effect on fertilization success; however, the interaction between these factors was not significant (Table 3). Fertilization success was highest for pure *F. heteroclitus* crosses (~89%), followed by *F. diaphanus* x *F. heteroclitus* (female x male) hybrids (~87%) and pure *F. diaphanus* (~82%). *Fundulus heteroclitus* female x *F. diaphanus* male hybrids had the lowest success (~68%), which was significantly lower than all other cross types (Figure 11). Although salinity treatment had a significant effect on fertilization success, there were no significant differences in *post-hoc* comparisons of salinity treatments. Across all species, mean fertilization success was lowest in the 0 ppt treatment (~76%) and the highest in both the 5 ppt and 15 ppt treatments (~85%) while the 10 ppt treatment was in the middle (~80%).

Of the 1050 eggs fertilized from all cross types and across all salinities, 22 of the embryos were lost or damaged during transport and handling. Of the remaining 1028 fertilized embryos, 432 individuals hatched (~42%). Hatching success was significantly affected by cross type and salinity treatment as well as the interaction between these variables (Table 3). This interaction was driven by a difference in hatching success

between pure *F. heteroclitus* crosses at 15 ppt, which had significantly higher success than pure *F. diaphanus* crosses at 10 ppt (Figure 12). No other significant differences were observed in hatching success among species or salinities. Hatching success was lowest at 0 ppt for all cross types except pure *F. diaphanus* (Figure 12), which had higher hatching success at lower salinities (0 & 5 ppt). Pure *F. heteroclitus* had increasing hatching success as salinity increased from 0 to 15 ppt (Figure 12). Hybrid *F. heteroclitus* x *F. diaphanus* crosses had higher hatching success at intermediate salinities (5 & 10 ppt) and *F. diaphanus* x *F. heteroclitus* crosses had the most hatching success at 10 ppt.

Table 3. Summary of analyses of deviances of fertilization and hatching success GLMs. Factors are fixed effects of cross type (Type; *F. diaphanus* x *F. diaphanus, F. diaphanus* x *F. heteroclitus*, *F. heteroclitus* x *F. diaphanus*, and *F. heteroclitus* x *F. heteroclitus*) and experimental salinity treatment (Salinity; 0, 5, 10, 15 ppt) used in analyses.

	Fertilization Success			Hatching Success		
Factor	df	X^2	p-value	df	X^2	p-value
Cross Type	3	70.91	< 0.0001 *	3	61.21	< 0.0001 *
Salinity	3	10.32	0.0161 *	3	8.85	0.0313 *
Cross Type x Salinity	9	8.32	0.50	9	21.52	0.0105 *

* significant factors

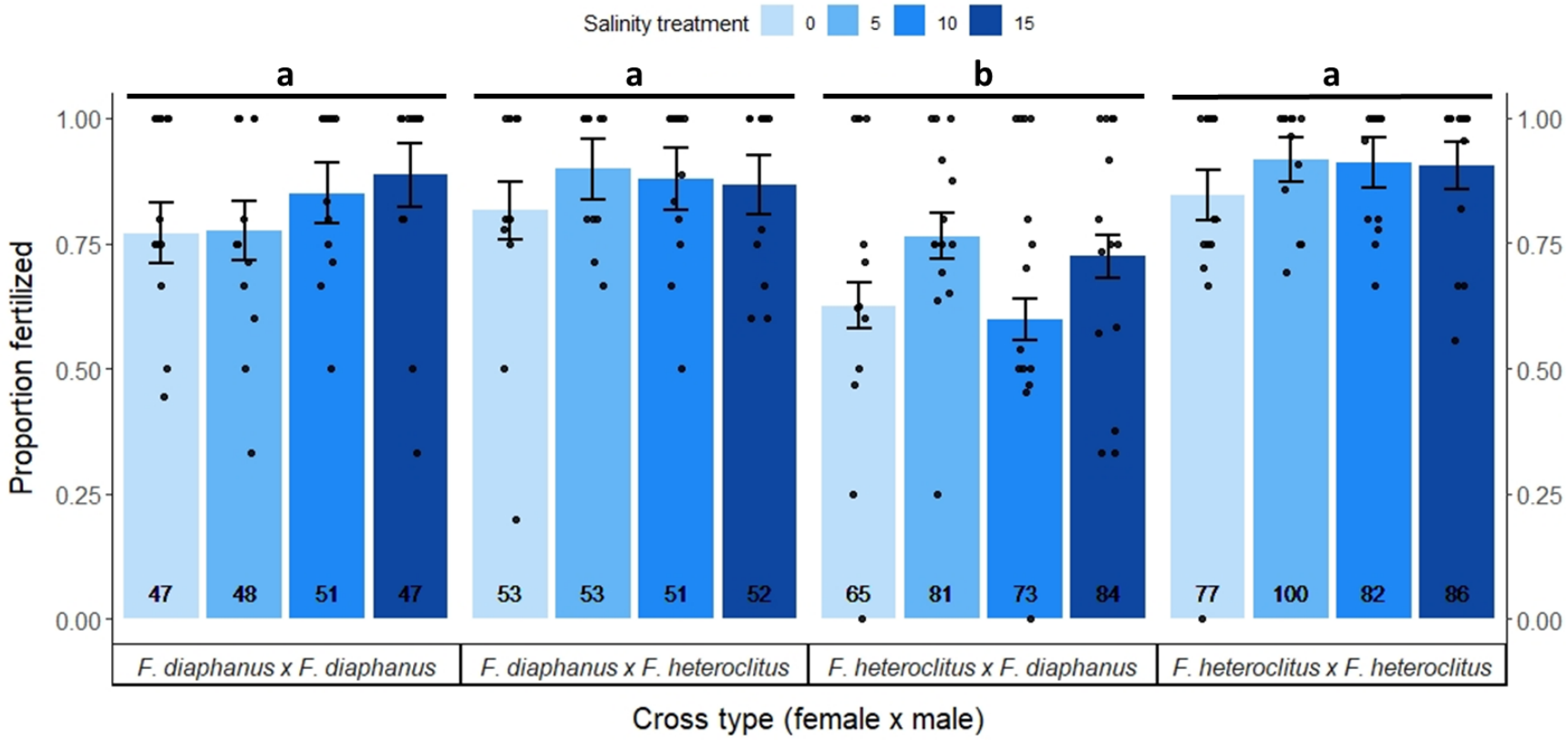

Figure 11. Proportions of fertilized eggs across cross types and salinity treatments. Bars represent estimated marginal means of fertilized eggs, data points represent the proportion of fertilized eggs from each clutch at each salinity treatment and error bars represent ± one standard error. The total number of fertilized individuals from all crosses (pooled) can be seen at the bottom of the bars. Significant differences ($p < 0.05$) among cross types, based on *Post hoc* Tukey multiple comparisons tests, are represented by different letters at the top of the plot.

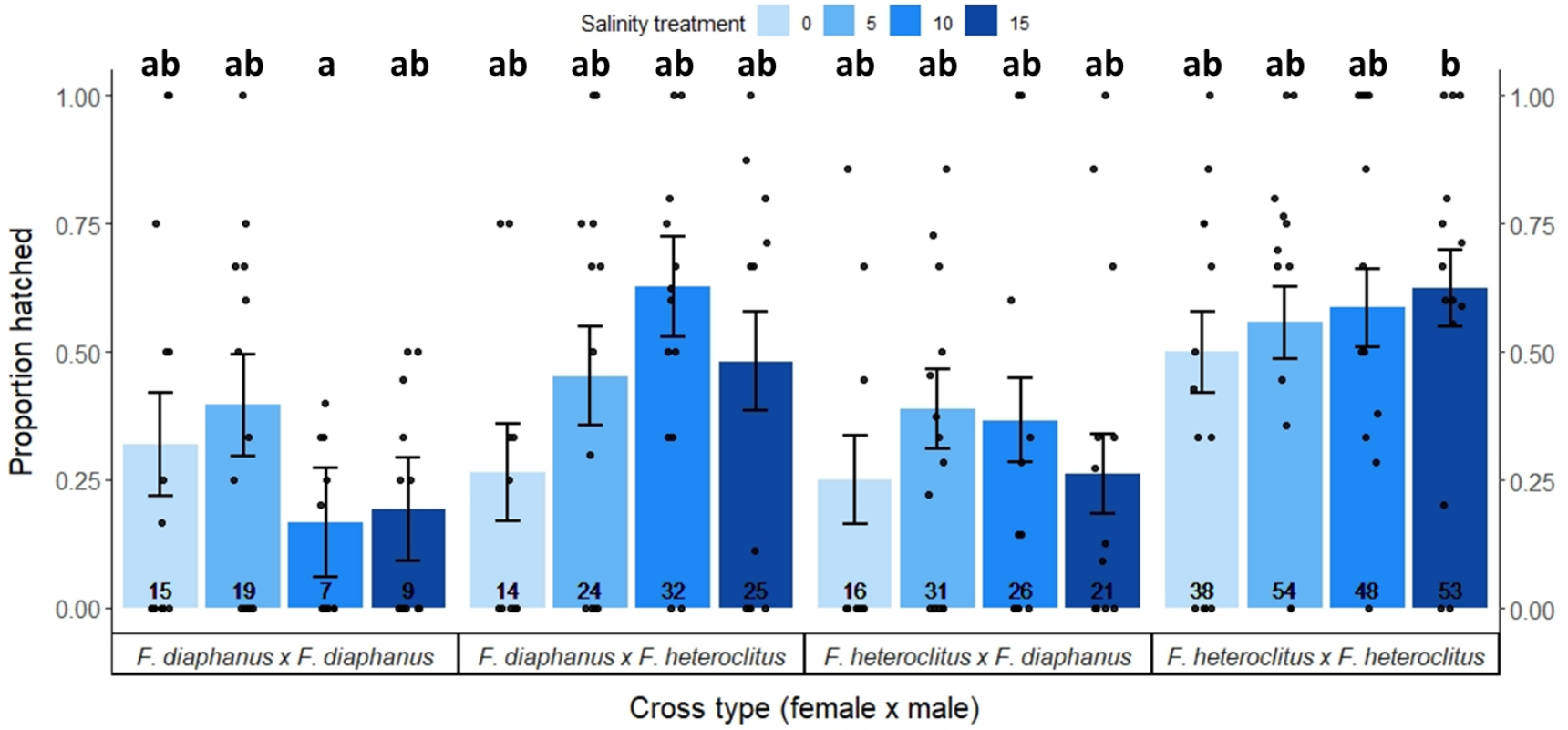

Figure 12. Proportions of hatched individuals across cross types and salinity treatments. Bars represent estimated marginal means of hatched fish, data points represent the proportion of hatched individuals from clutches at each salinity treatment and error bars represent standard error. The number of hatched fish from all crosses (pooled) are the numbers at the bottom of the bars. Significant differences ($p < 0.05$) among groups, based on *Post hoc* Tukey multiple comparisons tests, are represented by different letters at the top of the plot.

3.2.2 Mortality

Embryonic death from fertilization to the fifth day of development was significantly affected by cross type, salinity treatment, and the interaction of these factors (Table 4). *F. heteroclitus* crosses at 5 ppt, 10 ppt, and 15 ppt had significantly lower embryonic mortality than other cross types within the first 5 days of development (Figure 13A). Pure *F. heteroclitus* crosses had the highest mortality at 0 ppt (~41%), but this value was not significantly different from any other cross types at any of the salinity treatments.

From the fifth day of development to the tenth fish mortality was only significantly affected by cross type (Table 4). Pure *F. heteroclitus* crosses had the highest mortality during this time (~8%) which was significantly higher than pure *F. diaphanus* crosses (~2%) and *F. heteroclitus* x *F. diaphanus* crosses (~3%), while *F. diaphanus* x *F. heteroclitus* cross mortality (~4%) was not significantly different from pure *F. heteroclitus* (Figure 13B).

From the tenth day of development and beyond mortality was again influenced only by cross type (Table 4). Pure *F. heteroclitus* crosses had the highest mortality during this time (~11%) which was significantly higher than *F. diaphanus* x *F. heteroclitus* hybrids which had the lowest mortality (~3%; Figure 13C). Pure *F. diaphanus* crosses had the next lowest mortality (~5%) and *F. heteroclitus* x *F. diaphanus* hybrids had the second highest mortality (~6%); these were not significantly different from other cross types.

Table 4 Summary of analyses of deviance of embryo mortality GLMs at days 5, 10, and beyond 10 of development. Factors are fixed effects of cross type (*F. diaphanus* x *F. diaphanus*, *F. diaphanus* x *F. heteroclitus*, *F. heteroclitus* x *F. diaphanus*, and *F. heteroclitus* x *F. heteroclitus*) and experimental salinity treatment (Salinity; 0, 5, 10, 15 ppt) used in analyses.

	Day 5 Mortality			Day 10 Mortality			Day 10+ Mortality		
Factor	df	X	p	df	X	p	df	X	p
Cross Type	3	125.702	< 0.0001*	3	18.98	0.0002*	3	14.65	0.0021*
Salinity	3	26.096	< 0.0001*	3	7.44	0.06	3	0.0046	0.95
Cross Type x Salinity	9	17.33	0.044*	9	12.13	0.21	9	5.09	0.17

* significant factors

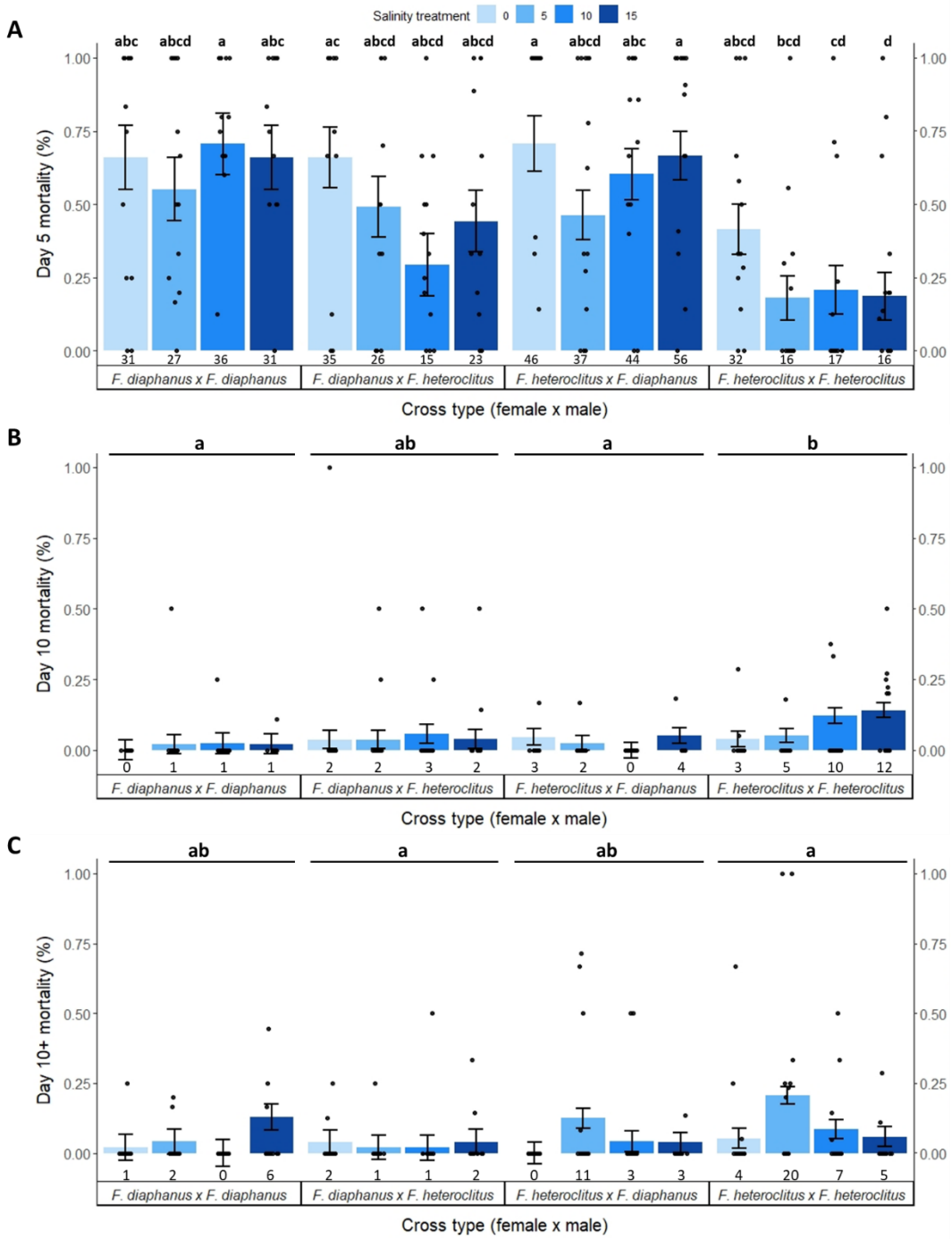

Figure 13. Embryonic mortality at days 5 (A), 10 (B), and beyond 10 (C) across cross types and salinity treatments. Bars represent estimated marginal means of dead embryos, data points represent the mean deaths per clutches at each salinity treatment and error bars represent standard error. The number of individual deaths from all crosses (pooled) are represented by the numbers under the bars. Significant differences ($p < 0.05$) among groups, based on *Post hoc* Tukey multiple comparisons tests, are represented by the different letters at the top of the plot.

3.2.3 Development time

Development time varied from 11 to 26 days, averaging ~14.5 days across all cross types at all salinities. Cross type and temperature, but not incubation salinity, were significant predictors of time to hatch (Table 5). Pure *heteroclit*us crosses had a significantly shorter development time than *F. heteroclitus* x *F. diaphanus* hybrid crosses (Figure 14A). On average *F. heteroclitus* x *F. diaphanus* hybrids also had a longer development time than pure *F. diaphanus* crosses and *F. diaphanus* x *F. heteroclitus* hybrid crosses, but not significantly so.

As temperature was a significant factor in analysis of development time, the residuals of the linear regression of temperature versus development time were used to correct for temperature. In doing so, the effect of cross type remained significant (Table 5). To further explore the effect of temperature by a different method, temperature was used as a covariate, and found pure *F. heteroclitus* crosses had a significantly faster development time than *F. heteroclitus* x *F. diaphanus* crosses (Figure 14B). Hybrid *F. heteroclitus* x *F. diaphanus* crosses also had significantly slower development time than reciprocal *F. diaphanus* x *F. heteroclitus* crosses (Figure 14B).

Table 5. Summary of analyses of deviances of development time GLMs. in days and as residuals. Factors are fixed effects of cross types (Type), experimental salinity treatments (Salinity), and temperature for non temperature corrected data.

	Development Time (Days)			Development Time (Residuals)		
Factor	Df	X^2	p-value	df	X^2	p-value
Type	3	22.86	<0.0001*	3	12.37	0.0062*
Salinity	3	4.05	0.26	3	2.29	0.51
Temp	1	133.14	<0.0001*	-	-	-
Type x Salinity	9	6.75	0.66	9	13.45	0.14
Type x Temp	3	0.55	0.91	-	-	-
Salinity x Temp	3	1.78	0.62	-	-	-
Type x Salinity x Temp	9	3.11	0.96	-	-	-

* significant factors

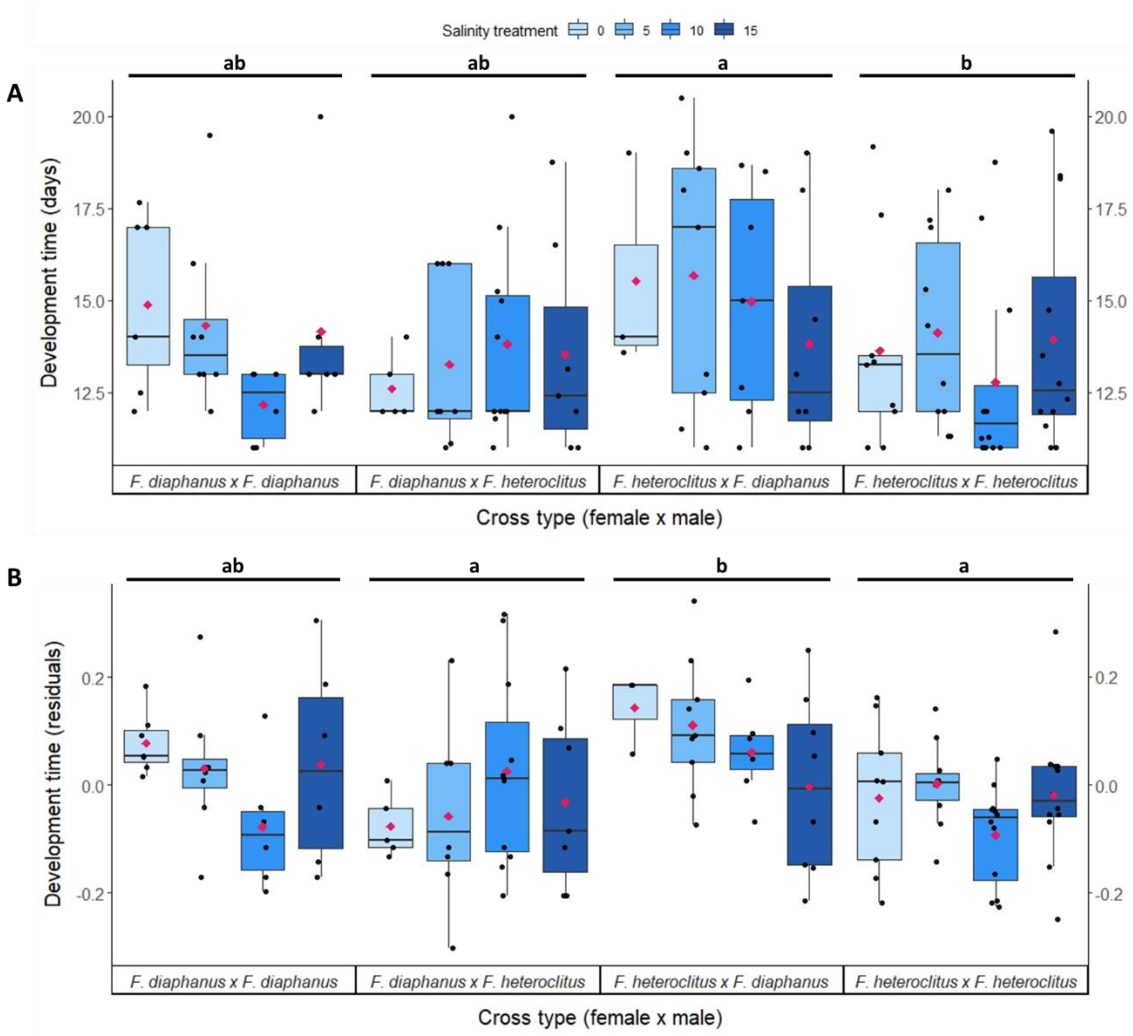

Figure 14. Development time of each cross type at different salinity treatments. Data points indicate mean hatching time per clutch at each salinity. Pink diamonds represent grand means at each salinity. Significant differences among cross types based on post-hoc analysis are represented by different letters at the top of the plots. Panel A represents development time in days and panel B represents the residuals of temperature corrected data.

3.3 Quantification of isolating barriers

As mate preference was only tested at 10 ppt, subsequent absolute contribution (AC) and relative contribution (RC) calculations for fertilization and hatching at each salinity treatment were conducted using the mate preference values at 10 ppt. Reproductive isolation (RI) was consistently stronger in *F. heteroclitus* females compared to *F. diaphanus* females. None of the isolating barriers measured for *F. heteroclitus* females favoured hybridization while some fertilization and hatching success measures indicated greater success for *F. diaphanus* females when mating with F. heteroclitus males than conspecifics.

Reproductive Isolation (RI) calculations showed that mate choice was the strongest measured barrier for females of both species. Total isolation, which typically varies from 0 (no isolation) to 1 (complete isolation) was moderately high for *F. heteroclitus* females at all salinities (~0.60-0.75), while *F. diaphanus* had a relatively low score at 0 ppt (~0.22) and had negative scores at 5, 10, and 15 ppt (Table 6). These negative values, which indicate that the barriers tested do not cause any isolation, were because fertilization and hating success of *F. diaphanus* eggs were often higher in hybrid crosses than conspecific crosses. Moreover, the relative contributions of hatching success for *F. diaphanus* resulted in values greater than 1 (1 indicating complete isolation) due to the negative total isolation scores. The AC and RC scores for *F. heteroclitus* females maintain mate preference as the strongest barrier and indicate that hatching success at each salinity was a stronger barrier than fertilization success.

Table 6. Strengths and contributions (relative and absolute) of reproductive isolating barriers between *F. diaphanus* and *F. heteroclitus*. As mate preference was only tested at 10 ppt only one value was obtained per species which subsequently were used to calculate AC for fertilization and hatching at each experimental salinity. RC values were also based on previous calculations using mate preference at 10 ppt.

Reproductive Isolation								
Barrier	*F. diaphanus* ♀				*F. heteroclitus* ♀			
Pre-zygotic								
Mate preference	0.167				0.463			
Salinity	**0**	**5**	**10**	**15**	**0**	**5**	**10**	**15**
Fertilization Success	-0.028	-0.074	-0.017	0.011	0.169	0.091	0.191	0.111
Post-zygotic								
Hatching Success	0.094	-0.067	-0.580	-0.430	0.333	0.230	0.407	0.179

Absolute Contribution								
Barrier	*F. diaphanus* ♀				*F. heteroclitus* ♀			
Pre-zygotic								
Mate preference	0.167	0.167	0.167	0.167	0.463	0.463	0.463	0.463
Salinity	**0**	**5**	**10**	**15**	**0**	**5**	**10**	**15**
Fertilization Success	-0.024	-0.062	-0.014	0.010	0.091	0.049	0.103	0.060
Post-zygotic								
Hatching Success	0.081	-0.519	-0.4916	-0.354	0.149	0.112	0.177	0.086

Total Reproductive Isolation	0.224	-0.414	-0.339	-0.178	0.702	0.624	0.743	0.608

	Relative Contribution							
Barrier	*F. diaphanus* ♀				*F. heteroclitus* ♀			
Pre-zygotic								
Mate preference	0.745	-0.403	-0.492	-0.939	0.659	0.742	0.623	0.761
Salinity	**0**	**5**	**10**	**15**	**0**	**5**	**10**	**15**
Fertilization Success	-0.105	0.149	0.042	-0.054	0.129	0.078	0.139	0.098
Post-zygotic								
Hatching Success	0.360	1.254	1.451	1.993	0.212	0.180	0.238	0.141

4 DISCUSSION

Genetic analyses of *F. diaphanus* and *F. heteroclitus* F1 hybrids have revealed that the majority of hybrids (~96%-100%, depending on sampling year) are the product of *F. diaphanus* females mating with *F. heteroclitus* males (Dawley, 1992; Hernández Chávez & Turgeon, 2007; Dalziel et al., 2020); however, the cause of this bias in hybridization direction had not previously been explored. The results of the present study suggest that both pre- and post-zygotic reproductive isolating mechanisms could be leading to the bias in hybridization events of *Fundulus* species in Porter's Lake and the prevalence of *F. diaphanus* female x *F. heteroclitus* male F1 hybrids.

From a pre-zygotic perspective, females exhibited different preferences, with *F. heteroclitus* preferring conspecific males while *F. diaphanus* showed no preference for conspecific males. Also, male *F. diaphanus* were typically more aggressive than *F. heteroclitus* males toward their competitors and potential mates possibly driving them away. In terms of pre-zygotic fertilization success and post-zygotic development and hatching, *F. heteroclitus* ♀ x *F. diaphanus* ♂ hybrids had a significantly lower fertilization success than all cross types and a significantly longer development time than reciprocal F1 hybrid and pure *F. heteroclitus* crosses. However, hatching success did not significantly vary between hybrids and parental crosses. Overall, the metrics tested in this study suggest that pre-zygotic barriers (i.e., mate choice and fertilization success) are the primary drivers of unidirectional hybridization in these fishes

4.1 Mating behaviour

4.1.1 Female preference

If mate preference contributes to the unidirectional hybridization observed in wild populations, we might expect to see a preference for males of one species over the other. While it was observed that *F. heteroclitus* females appeared to prefer conspecific males, *F. diaphanus* females did not show a clear preference based on the metric used in this work (Figure 6). Previous research involving *F. heteroclitus* hybridization events with another *Fundulus* species, *F. grandis*, found that the strength of pre-zygotic reproductive isolation in the form of conspecific mate choice was stronger in *F. heteroclitus* females (Barbas & Gilg, 2018). As is the case here, *F. heteroclitus* females seem to exhibit strong preference for males of their own species while *F. diaphanus* females do not appear to have a preference, which may make them more likely to hybridize.

Visual displays and male colouration in the *Fundulidae* family are known to affect mating and female preference in some species, such as *Lucania goodei, Leptolucania ommata*, *Fundulus notti,* and *F. cingulatus* (e.g. Foster, 1967; Fuller & Noa, 2010), but the role of these displays in *F. heteroclitus* and *F. diaphanus* spawning is unclear. Bright nuptial colouration in *F. heteroclitus* males is involved in establishing dominance (Newman, 1907; Foster, 1967). Although not quantified in this study, *F. heteroclitus* male nuptial colouration was quite different, and typically much brighter than *F. diaphanus* (Figure 2 & 3). The differences between these visual displays might partially explain the patterns observed here, as the duller colours of *F. diaphanus* males might fail to attract *F. heteroclitus* females. In many species of fish, females will prefer larger and/or more brightly coloured males as these traits can indicate that a male is in good condition,

possesses good genes, and/or is dominant to other males (Reynolds & Gross, 1992; Ryan & Keddy-Hector, 1992; Johnstone, 1995; Godin & Dugatkin, 1996; Kraak & Bakker, 1998). Body condition may also play a role in the hybridization bias. Comparing length to weight of the fish used, at the same standard lengths *F. heteroclitus* typically weighed more than *F. diaphanus* (Figure A1). In this study, males were visually size matched for trials, when possible, to account for any advantages larger males would possess, and in most cases, this resulted in clear conditional differences between the males. *Fundulus diaphanus* appeared slimmer (lower condition factor) while *F. heteroclitus* had a "chunkier" appearance (higher condition factor) which could also be a trait *F. heteroclitus* females find more attractive, thus contributing to stronger pre-zygotic isolation in females of this species. However, it is not yet clear which traits females of either species find attractive, if these traits relate to male dominance, and if any preference or dominance awards males greater mating success. Uncovering such information would be highly beneficial to determine why such a strong bias in hybridization direction occurs in Porter's Lake.

4.1.2 Male aggression

F. diaphanus males were far more aggressive than *F. heteroclitus* males, as predicted from previous descriptions and reports of *F. diaphanus* males as aggressive and territorial (Newman, 1907; Breder & Rosen, 1966; Fournier & Magnin, 1975; DFO, 2011). Overall, *F. diaphanus* males displayed more aggressive behaviour towards other males and females during all phases of mating trials. Without access to a female (Phase 2) no significant differences in male aggression were observed; however, mean aggression by *F. diaphanus* males was higher in trials with both species of females (Figure 7).

Fundulus diaphanus males are territorial and as such they may have been attempting to establish and defend a territory. Mean aggression of both species of males during phase 2 was higher in trials with focal *F. diaphanus* females. While this could indicate that both species of males are more interested in *F. diaphanus* females, it is likely that this is simply due to the recipient of attacks, usually *F. heteroclitus*, defending themselves from the instigators, usually *F. diaphanus*. As expected due to the differences in described mating behaviours (Newman, 1907; Richardson, 1939; Breder & Rosen, 1966; Fournier & Magnin, 1975, DFO, 2011), male-male aggression differed significantly between species when they were in the presence of conspecific females and had access to them (Phase 3). *Fundulus diaphanus* males were significantly more aggressive in the presence of a *F. diaphanus* female than *F. heteroclitus* males with *F. heteroclitus* females while no other significant differences were observed between groups. Establishing dominance via aggression, as *F. diaphanus* males do, might contribute to the bias in hybridization direction observed in natural *Fundulus* populations in Porter's Lake as this aggression is also directed towards females. McGhee et al. (2007) found that dominant bluefin killifish (*Lucania goodei*, family Fundulidae) males exhibiting higher levels of male-male aggression also exhibited higher levels of male-female aggression and had greater mating success (McGhee et al., 2007). This positive association of male-male and male-female aggression appears to be consistent in *F. diaphanu*s males as they were significantly more aggressive towards females than *F. heteroclitus* males (Figure 9). However, if there is a direct cost to associating with aggressive males, specifically risk of injury due to dominant males also displaying aggression towards females (e.g. McGhee et al., 2007), *F. diaphanus* females might be more willing to mate with heterospecific *F. heteroclitus* males to avoid aggression from conspecific males if the opportunity presents itself.

Moreover, *F. heteroclitus* females might be deterred by aggressive males as *F. heteroclitus* have been described as more docile than *F. diaphanus* (Newman, 1907; Richardson, 1939; Petersen et al., 2010). Despite evidence that higher levels of aggression can increase mating success (McGhee et al., 2007) and lower aggression was observed by *F. heteroclitus* males in all interactions, conspecific *F. heteroclitus* matings were the only mating events observed during this study. *Fundulus diaphanus* do not appear to survive as well in laboratory conditions as *F. heteroclitus* and as such may not have been willing to mate (Newman, 1907, Personal observations 2019-2020).

Another consideration that could not be tested in this study, as no mating events involving *F. diaphanus* females were observed, is the release of a single egg by *F. diaphanus* females before mating. As previously discussed, mating of *F. diaphanus* involves the release of a single eggs by the female which remains attached to her by a thin filament at which point males have been observed to increase their courtship leading females to a spawning area and spawning takes place shortly thereafter (Richardson, 1939; Breder & Rosen, 1966). *Fundulus heteroclitus* females do not exhibit this trait and without this signal, a *F. diaphanus* male might not know that a *F. heteroclitus* female is ready to spawn and thus never attempt to spawn with her. Moreover, the egg below *F. diaphanus* females is not predicted to prevent *F. heteroclitus* males from mating with them, thus causing a bias in hybridization direction.

4.2 In vitro crosses

4.2.1 Fertilization success

Variations in fertilization success were predicted to follow salinity preferences of parental species, with *F. diaphanus* pure crosses being more successful at low salinities (0

& 5 ppt), *F. heteroclitus* pure crosses at higher salinities (10 & 15 ppt), and hybrids having the most success at intermediate salinities (5 & 10 ppt). Incubation salinity was a significant predictor of fertilization success; however, *post-hoc* comparisons revealed no significant differences between salinity treatments, which did not match predictions. This differs from previous studies examining fertilization in *Fundulus* species showing that salinity affects fertilization success in that fertilization success was lower in salinities that differed greatly from the salinities to which fish would normally be exposed (Rao, 1974; Bush & Weis, 1983; Palmer & Able, 1987; Able & Palmer, 1988). However, the ranges of salinities tested in these other studies was larger (0-30 + ppt) than those presented here (0 – 15 ppt), which could explain why no significant differences were observed among salinity treatments; salinity ranges chosen for this study were selected based on observations from the natural environment where the species co-occur.

Hybrid *F. heteroclitus* x *F. diaphanus* crosses had significantly lower fertilization success (~60-75%) than other cross types. This aligns with the observed bias in cross direction in wild populations. Fertilization success of pure crosses and *F. diaphanus* x *F. heteroclitus* hybrids was ~75-85%, which is similar to what has previously been reported in *F. heteroclitus* at 5, 10, and 15 ppt (Rao, 1974; Bush & Weis, 1983; Palmer & Able, 1987; McKenzie et al., 2017). Overall fertilization success for all cross types at 0 ppt was slightly lower than the other treatments, expect *F. heteroclitus* x *F. diaphanus* crosses which had slightly lower fertilization at 10 ppt, but still comparatively high when contrasted with previous studies where *F. heteroclitus* had no or very low fertilization success in freshwater (with the exception of freshwater populations; Rao, 1974; Palmer & Able, 1987; Able & Palmer, 1988). Palmer and Able (1987) did find that marine *F. heteroclitus* populations acclimated to freshwater were able to obtain some fertilization

success in freshwater while unacclimated fish had no success in freshwater. Porter's Lake is a brackish environment and the fish used in this study were mostly collected between 5 and 10 ppt, as such they may be better suited for reproduction in lower salinities. Palmer and Able (1987) observed that when placed in freshwater, *F. heteroclitus* eggs from marine populations became opaque and there were no signs of fertilization or development. This change from clear to opaque eggs was observed, but was not restricted to pure *F. heteroclitus* crosses nor to the 0 ppt salinity treatment. The range of salinity treatments tested was chosen based on the salinity gradient present in Porter's Lake, as such it would appear that environmental salinity is not causing the bias in hybridization of *Fundulus* in Porter's Lake via fertilization success.

Gametes of both parental species were able to function in all salinity treatments as evidenced by the similar, relatively high, fertilization success of both pure cross types and *F. diaphanus* x *F. heteroclitus* crosses at each incubation salinity (~75-90%). Moreover, the method of sperm collection (milt via capillary tube for *F. heteroclitus* males or testes via dissections for *F. diaphanus* males) did not significantly affect fertilization success.

The lower fertilization success for *F. heteroclitus* x *F. diaphanus* crosses may suggest some conflict between *F. heteroclitus* ova and *F. diaphanus* sperm. Incompatibilities in gamete recognition are one possible mechanism leading to this lower fertilization. Proteins on the surface of gametes play an important role in fertilization success, particularly for organisms with external fertilization (Palumbi, 2009). It is possible that *F. diaphanus* sperm cannot recognize *F. heteroclitus* ova as well as *F. diaphanus* eggs due to reduced surface protein recognition. Fertilization in teleost fishes occurs when a spermatozoon enters the egg via a small opening called the micropyle

(Coward et al., 2002). The chorion of the egg is quite thick and teleost sperm do not have an acrosome to penetrate the egg (Coward et al., 2002). As such proteins on the outer layer of the egg serve to guide the sperm to the opening; if *F. diaphanus* sperm have reduced recognition of these proteins on *F. heteroclitus* eggs fertilization success would be greatly lowered. A second potential incompatibility mechanism may arise if ovarian fluid influences relative fertilization success by con- or heterospecific sperm. In some cases, ovarian fluid is needed for sperm activation (Coward et al., 2002), and it can also serve to direct sperm and prolong sperm activity (Yeates et al., 2013; Zadmajid et al., 2019). Congeneric Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) can and do hybridize when they share spawning grounds; however, one of the mechanisms that prevents interspecific fertilization is ovarian fluid causing preferential fertilization by conspecific sperm (Yeates et al., 2013). Ovarian can provide an environment that increases the motile lifespan of con or heterospecific sperm yet promote a more linear swimming trajectory guiding sperm to the micropyle for conspecific sperm only (Yeates et al., 2013; Zadmajid et al., 2019). It is possible that *Fundulus heteroclitus* ovarian fluid might lower the chances of hybridizing with *F. diaphanus* males if the sperm are not as well stimulated by it as conspecific sperm might be; this hypothesis should be tested in future research.

4.2.2 Development and Mortality

Embryonic death over the course of development was significantly lower in pure *F. heteroclitus* crosses than all other cross types on the fifth day of development. This is not surprising as *F. heteroclitus* are typically robust, and the durability of their eggs and embryos first made them ideal model organisms (Atz, 1986). *Fundulus heteroclitus*

mortality was then significantly higher than *F. diaphanus* and *F. heteroclitus* x *F. diaphanus* crosses on the tenth day and significantly higher than *F. diaphanus* x *F. heteroclitus* hybrids beyond the tenth day. There were no differences in mortality between hybrid crosses or *F. diaphanus* crosses, again suggesting fertilized *F. heteroclitus* x *F. diaphanus* embryos are able to develop unimpeded and any reproductive barriers leading to the lack of *F. heteroclitus* x *F. diaphanus* hybrids in natural populations are occurring before or after embryonic development. Mortality was highest within the first 5 days of development in all crosses and then increased again slightly before hatching. This trend has been observed in certain euryhaline fishes including *Fundulus* (Rao, 1974).

Within normally developing *F. heteroclitus*, embryos should reach stages 27-28 (112-128 hours) on the fifth day of development and stages 34-35 (228-252 hours) by the tenth day at 20 ± 0.2 °C, at which point they are ready to hatch (Armstrong & Child, 1965). By the fifth day of development embryos that had not ceased development were between the stages of 25-28 as described by Armstrong & Child (1965). By these stages the main body is clearly visible, circulation has begun, and embryonic movement can be observed. The embryos that had stopped developing were at approximately stages 11-18, which are the blastula to gastrula stages. Significant differences in embryonic mortality were observed among cross types and salinity treatments within the first five days of development, but none of these differences were between hybrid crosses. Previous research of various euryhaline fishes, including herring, plaice, and killifish, suggests that before the closure of the blastopore, embryos may struggle with osmoregulation at sub-optimal salinities and need to divert energy away from development to cope with osmotic stress, which can lead to longer development times or embryonic death (Bunn et al.,

2000; Rao, 1974). As many of the deaths in the first 5 days occurred as eggs stopped developing near the blastula to gastrula stages and mortality was highest at 0 ppt for all crosses except pure *F. diaphanus* where 0 ppt was the second highest mortality, it is possible that this is due to osmoregulatory stress.

On the tenth day of development and beyond *F. heteroclitus* crosses had significantly higher mortality than *F. diaphanus* crosses and *F. heteroclitus* x *F. diaphanus* hybrids (day 10) and *F. diaphanus* x *F. heteroclitus* crosses (day 10+; Figure 13B & C). The similar mortality rates exhibited by both hybrid cross types again suggest that there are no restrictions on hybrid development once fertilized.

As the parental species have similar development times, any variation in development time between hybrid types could affect hybridization direction. Development time of hybrid *F. heteroclitus* x *F. diaphanus* crosses was significantly longer than *F. heteroclitus* and *F. diaphanus* x *F. heteroclitus* crosses when the temperature over the course of development was accounted for (Figure 14B). Salinity did not affect development time which would indicate that once the embryos reached a certain stage, they were able to osmoregulate efficiently at the tested salinities. Previous studies of *Fundulus* species testing wider salinity ranges (0-30+ ppt) found that development time was increased at lower and higher levels as more or less energy was needed to be diverted from development to maintain homeostasis (Brown et al., 2012; Rao, 1974). It is unclear why *F. heteroclitus* x *F. diaphanus* hybrids have longer development times, but this may be sub-optimal in natural conditions, as longer development times could also increase risk of predation. Many organisms prey on fish eggs including insects, amphibians, and other fish and eggs are unable to defend

themselves or escape predation (Purcell, 1985; Schaeffer & Margraf, 1987; Paradis et al., 1996; Bunn et al., 2000).

4.2.3 Hatching success

Hatching occurs when hatching enzymes break down the chorion. Hatching enzymes are secreted by hatching gland cells which migrate during development to one of four final configurations in the embryo which differs from species to species (Inohaya et al., 1997). Despite lower fertilization success, *F. heteroclitus* x *F. diaphanus* hybrids did not have significantly lower hatching success, which, coupled with the lack of differences in embryonic mortality between hybrid crosses, would suggest that there are no major genetic incompatibilities preventing development of these crosses once fertilized. The only significant difference in hatching success observed was pure *F. diaphanus* crosses at 10 ppt having lower success than pure *F. heteroclitus* at 15 ppt. As *F. diaphanus* exhibit a preference for lower salinities (Dawley, 1992; Fritz & Garside, 1974b), the higher success at 0 and 5 ppt and lower success at 10 and 15 ppt is not surprising. *Fundulus heteroclitus*, prefer higher salinities (Dawley, 1992; Fritz & Garside, 1974b), and had increasing success with increasing salinity from 0 to 15 ppt. Both types of hybrid crosses had similar success. Given the similarities of development between *F heteroclitus* and *F. diaphanus* (Armstrong & Child, 1965; Penney et al., 2019) and the lack of significant differences in hatching overall, the hatching mechanism is likely similar between the species and does not seem to be affected by hybridization.

4.3 Strength of reproductive isolating barriers

Reproductive isolation was much stronger in *F. heteroclitus* females than *F. diaphanus* females. Barbas & Gilg (2018) also found stronger reproductive isolation in *F.*

heteroclitus when hybridizing with *F. grandis*. These similar results from laboratory testing suggest that *F. heteroclitus* females have strong isolating mechanisms that may not be present for some other killifish species in laboratory conditions.

There are many potential factors that could lead to this strong reproductive isolation in *F. heteroclitus*. Because visual displays in some *Fundulids* are known to play a role in breeding (Newman, 1907; Foster, 1967), variations in male nuptial colouration might strongly influence con- or heterospecific mating success when female *F. heteroclitus* are given a choice between two males with different breeding colours, as is the case with *F. diaphanus*. Mate choice is one of the first steps of reproduction, as such there is potential for this pre-zygotic phenomenon to be a strong reproductive isolating barrier. Nevertheless, it is necessary to account for the strength and roles of subsequent pre-zygotic barriers and post-zygotic barriers as well when examining reproductive isolation.

The strength of RI and absolute contributions of fertilization success did not vary greatly between species, however, hatching success greatly favoured hybridisation for *F. diaphanus* females at 5 10, and 15 ppt salinity. While this might suggest that the observed bias in Porter's Lake may be due to preference for *F. diaphanus* females to hybridize, analysis of hatching success revealed no significant differences between pure *F. diaphanus* crosses and either hybrid crosses and no differences between hybrid crosses (Figure 12). This strong favour towards hybridization for *F. diaphanus* females observed here may be due to the low *F. diaphanus* survival observed in the laboratory (Newman, 1907, Personal observations 2019-2020) creating a form of pseudo hybrid vigor resulting in negative total RI scores and RC scores of greater than 1. As such, the low survivorship

of *F. diaphanus* in laboratory conditions makes it difficult to estimate the strength of various reproductive barriers in these conditions. Moreover, it should be noted that the relative strength and contributions of post zygotic mechanisms in this study may be underestimated as not all potential barriers were tested and only early life stage post-zygotic barriers were examined in laboratory conditions (Ramsey et al., 2003; Barbas & Gilg, 2018). The continued effects of environmental salinity on fry development and survival as well as other environmental conditions (e.g. oxygen content, predation, available spawning territory) also were not tested in this study. While there is some evidence that pre- (mate preference) and post-zygotic mechanisms (fertilization success and development time) are causing unidirectional hybridization of *F. diaphanus* females and *F. heteroclitus* males in Porter's lake there are likely many other mechanisms that need to be explored to explain the very strong directionality observed in the wild population.

4.4 Future directions

There is evidence that both pre- and post-zygotic reproductive isolating mechanisms contribute to the bias in hybridization direction observed in wild species of *Fundulus* in Porter's Lake wherein pre-zygotic barriers appear to be stronger. Further investigations into these mechanisms could provide further insight into the mechanisms specifically influencing hybridization events. To study additional pre-zygotic mechanisms, behavioural observations could involve an examination of female choice between two con- or two heterospecific males of varying sizes and intensity of nuptial colour. This could lend further insight into the characteristics that females prefer, and whether these differ between species and if males of one species exhibits more of these

characteristics than the other. In addition, the relationship between male aggression and dominance and female preference could then also be tested to see if traits preferred by females are those possessed by dominant males and lead to higher mating success (e.g. McGhee et al., 2007). Also, the link between higher male-female aggression and mating success discussed previously could be examined. To effectively study sexual selection and reproductive isolation both male and female behaviour must be studied together ideally using multiple metrics to obtain more accurate results (Fuller, 2001; St John & Fuller, 2019).

Quantifying fertilization success of observed mating events to compare to *in vitro* fertilization might also be beneficial in understanding pre-zygotic reproductive barriers experienced by these fishes as high sperm concentrations, as used in our *in vitro* crosses, can result in higher fertilization success between gametes with lower compatibility (Palumbi, 2009; McKenzie et al., 2017). Another potential avenue would be to examine sperm competition and fertilization success of ova given both con- and heterospecific sperm as well as comparing and switching ovarian fluid on ova to check gamete compatibility in both directions (Immler et al., 2011; Yeates et al., 2013). This would provide insight into whether or not hybridization is affected by gametic cryptic female choice choices and how sperm recognize and react to con and heterospecific ova (Yeates et al., 2013).

Perhaps the most exciting result of this study was the production of *F. heteroclitus* x *F. diaphanus* hybrids that have survived in the laboratory for a year (Summer 2020-Summer 2021). These fish appear to be healthy and some males have developed nuptial colouration. Possessing both parental species and both types of F1

hybrid crosses provides many opportunities to compare their respective physiologies and behaviours. However, we do not yet know if these fish are fertile, and if so if they reproduce clonally or sexually. If these hybrids reproduce sexually, male and female mating behaviours of hybrids could be tested as described above with other hybrids and parental species to test if F2 generations can be produced and if so, examine how F2 generations might be affected by pre- and post-mating reproductive barriers. Due to the absence of male hybrids in Porter's Lake (Dawley, 1992; Hernández Chávez & Turgeon, 2007; Merette et al. 2009), but the ability to produce these fish in the lab (Fritz & Garside, 1974a; personal observations 2020-2021), it would be interesting to see how both hybrid crosses compare to each other and males of the parental species in terms of their attractiveness to females and their ability to compete for mates. If possible, backcrossing hybrids with parental species could also provide insight into any post-mating reproductive isolating mechanisms and genetic incompatibilities the F2 generations may face as a result of new genetic combinations. These *Fundulus* species live in sympatry in many locations in the Maritimes, which provides opportunities to study and compare various populations. Despite F1 hybrids showing no major intrinsic post-zygotic barriers, it is possible that these might only be observed at the F2 generation in the form of Dobzhansky-Muller-Bateson incompatibilities (Presgraves, 2010) if these lab-produced hybrids do reproduce sexually.

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Supplementary Material

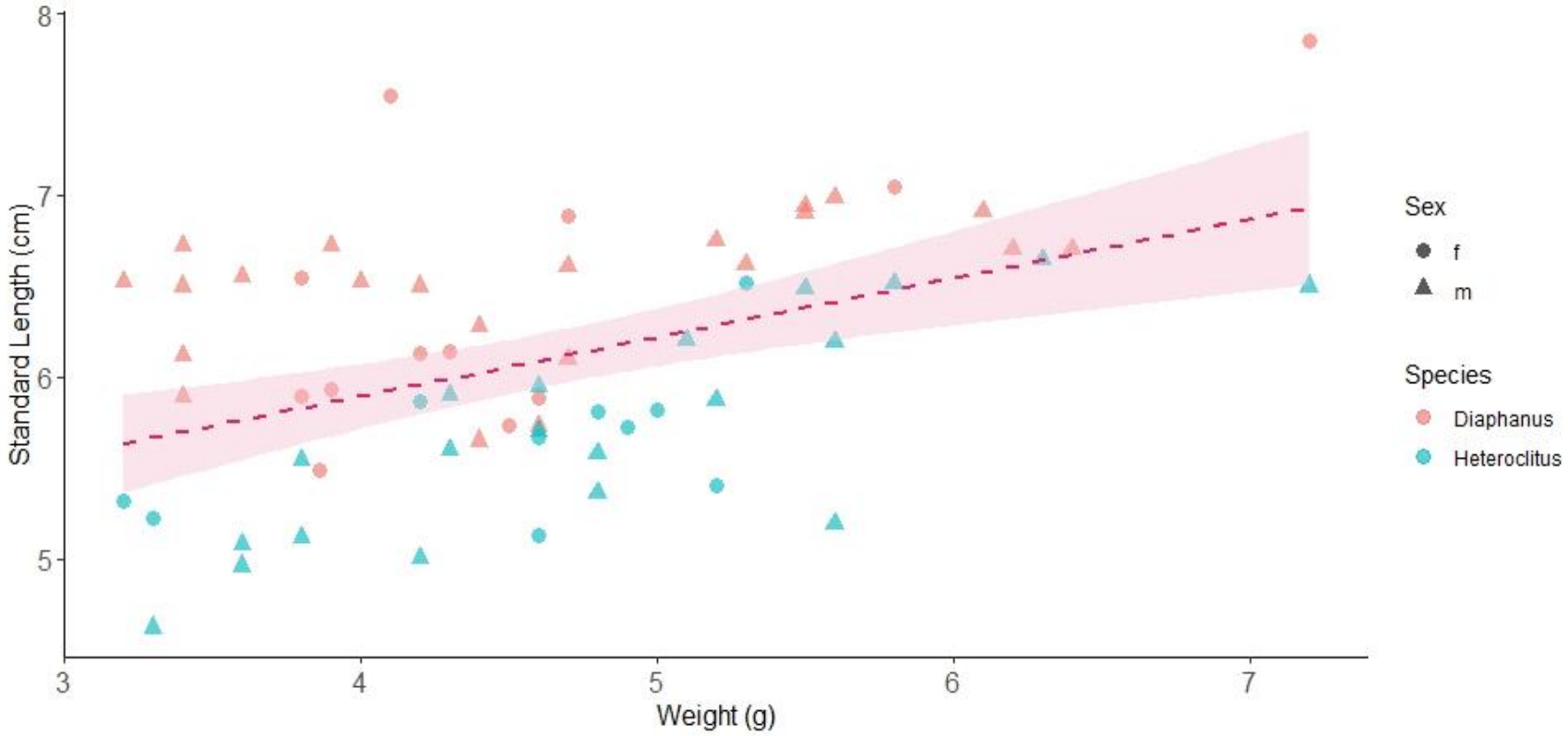

Figure A1. Standard lengths and weights of fish used in breeding trials. Data points represent individual fish, colours indicate species, and shapes indicate sex.

Table A1. Summary of in vitro crosses.

Cross Type (female x male)	Total Clutches	Salinity Treatments	Eggs per Treatment	Total Eggs	Fertilized Eggs	Total Fertilized	Hatched Embryos	Total Hatched
F. diaphanus x F. diaphanus	14	0	61	236	47	193 (184)*	15	50
		5	62		48		19	
		10	60		51		7	
		15	53		47		9	
F. diaphanus x F. heteroclitus	13	0	65	242	53	209	14	95
		5	59		53		24	
		10	58		51		32	
		15	60		52		25	
F. heteroclitus x F. diaphanus	14	0	108	452	65	303 (292)*	16	94
		5	106		81		31	
		10	122		73		26	
		15	116		84		21	
F. heteroclitus x F. heteroclitus	14	0	91	388	77	345 (343)*	38	193
		5	109		100		54	
		10	93		82		48	
		15	95		86		53	
Total	55		1318		1050 (1028)*		432	

*22 embryos were lost and/or damaged during transport and handling in some way after fertilization was confirmed

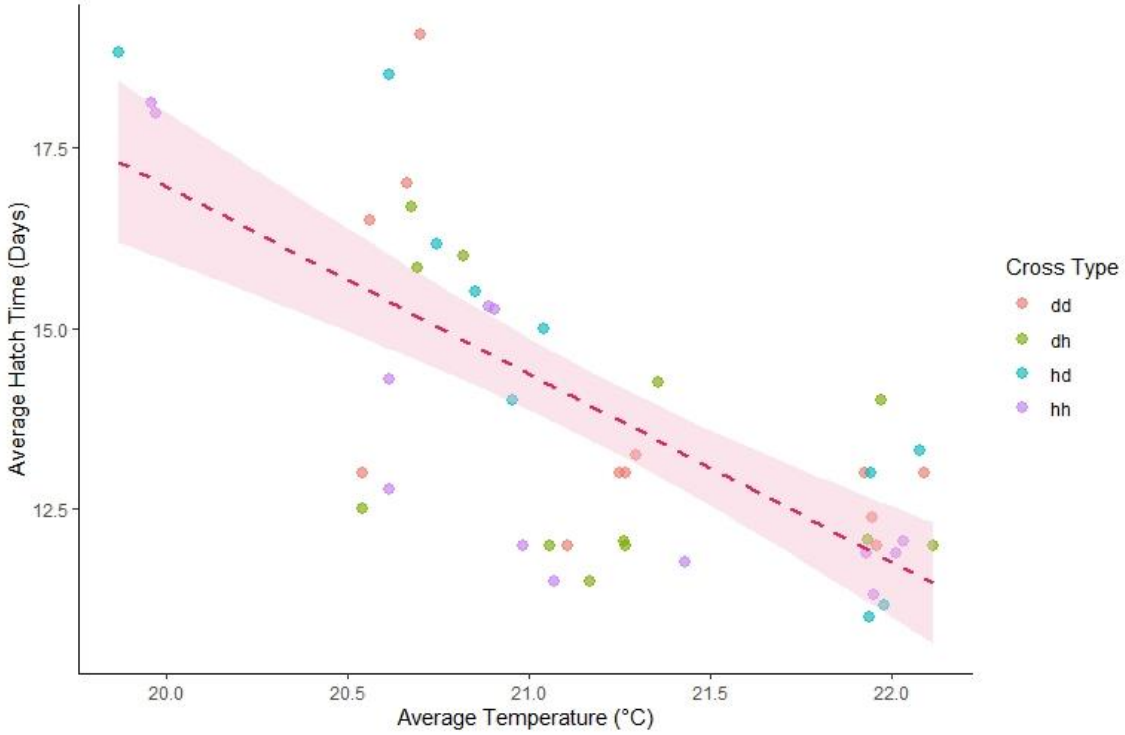

Figure A2. Average temperatures over the course of development time of crosses. Data points are the average development time in days of individual clutches.

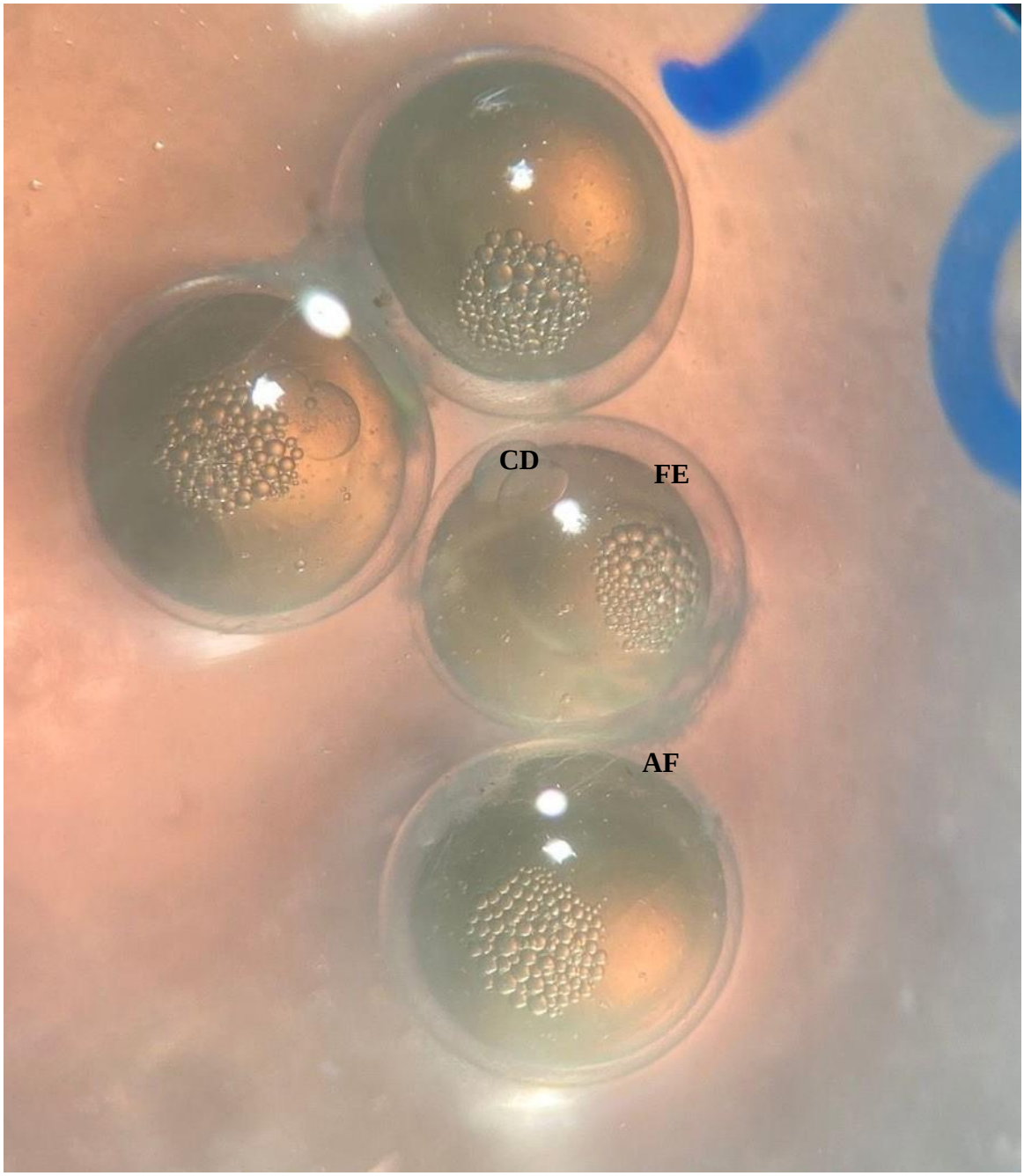

Figure A 3. Fertilized *F. diaphanus* embryos (~1-5 hours). Fertilization can be confirmed via the presence of the fertilization envelope (FE) creating a clear distinction between the yolk and the outer egg. A few attaching filaments (AF) can be seen on the outer surface of the eggs while the beginnings of cell division (CD) can be seen on the surface of the yolks.

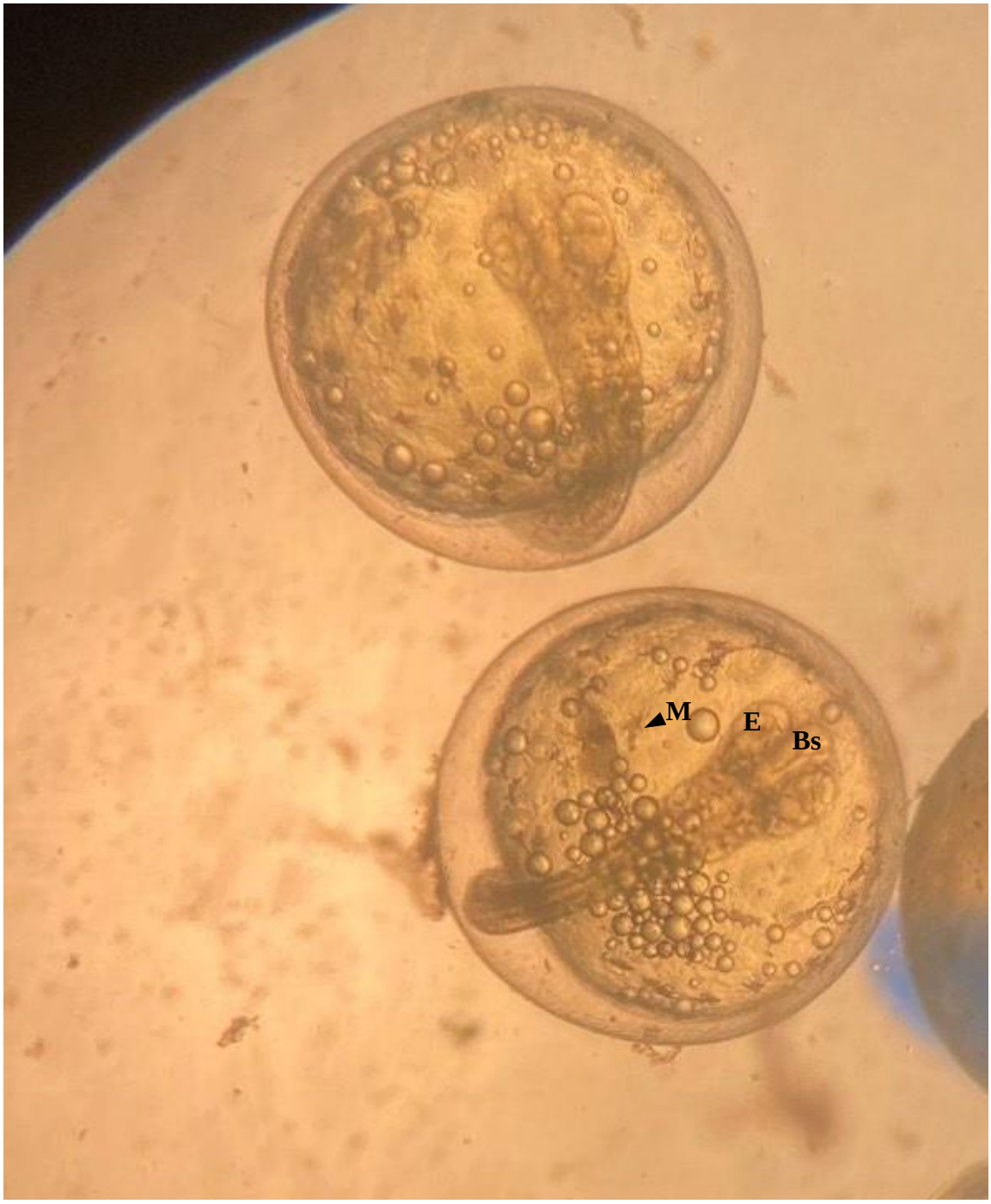

Figure A 4. *Fundulus diaphanus* embryos at approximately stage 27-28 (112-128 hours) compared to the description of *F. heteroclitus* development by Armstrong & Child (1965). At this stage the embryonic body is clearly visible wrapping around the yolk and movement can be observed. Clearly distinguishable features include the eyes (E), fore- mid- and hindbrain (Bs) on the head as well as melanophores (M) expanding on the yolk sac.

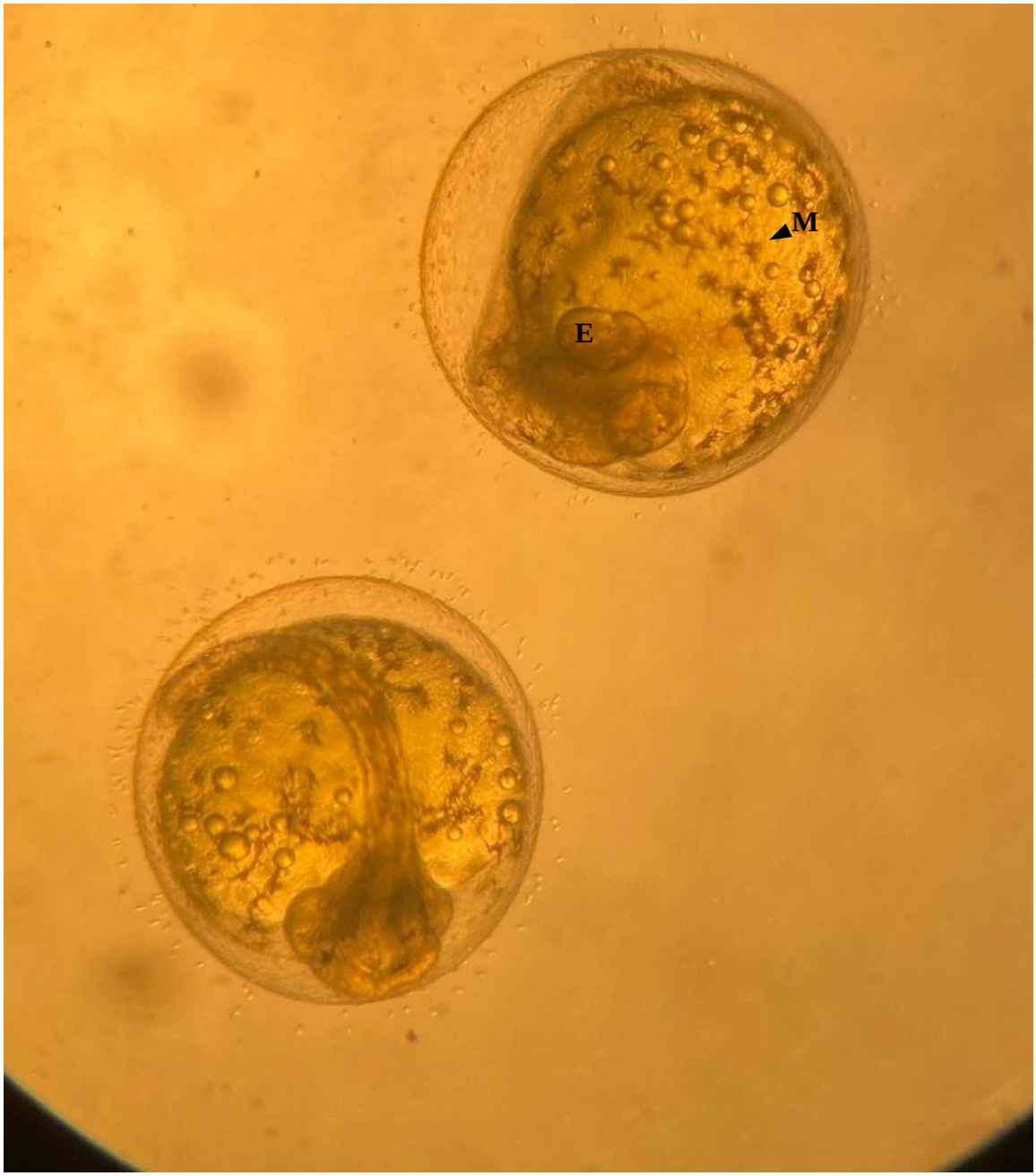

Figure A 5. Developing *F. diaphanus* x *F. heteroclitus* (female x male) hybrid embryos at approximately stages 26-27 of development as described by Armstrong & Child (1965). Similar features to those present in Figure A 4 can also be seen above.

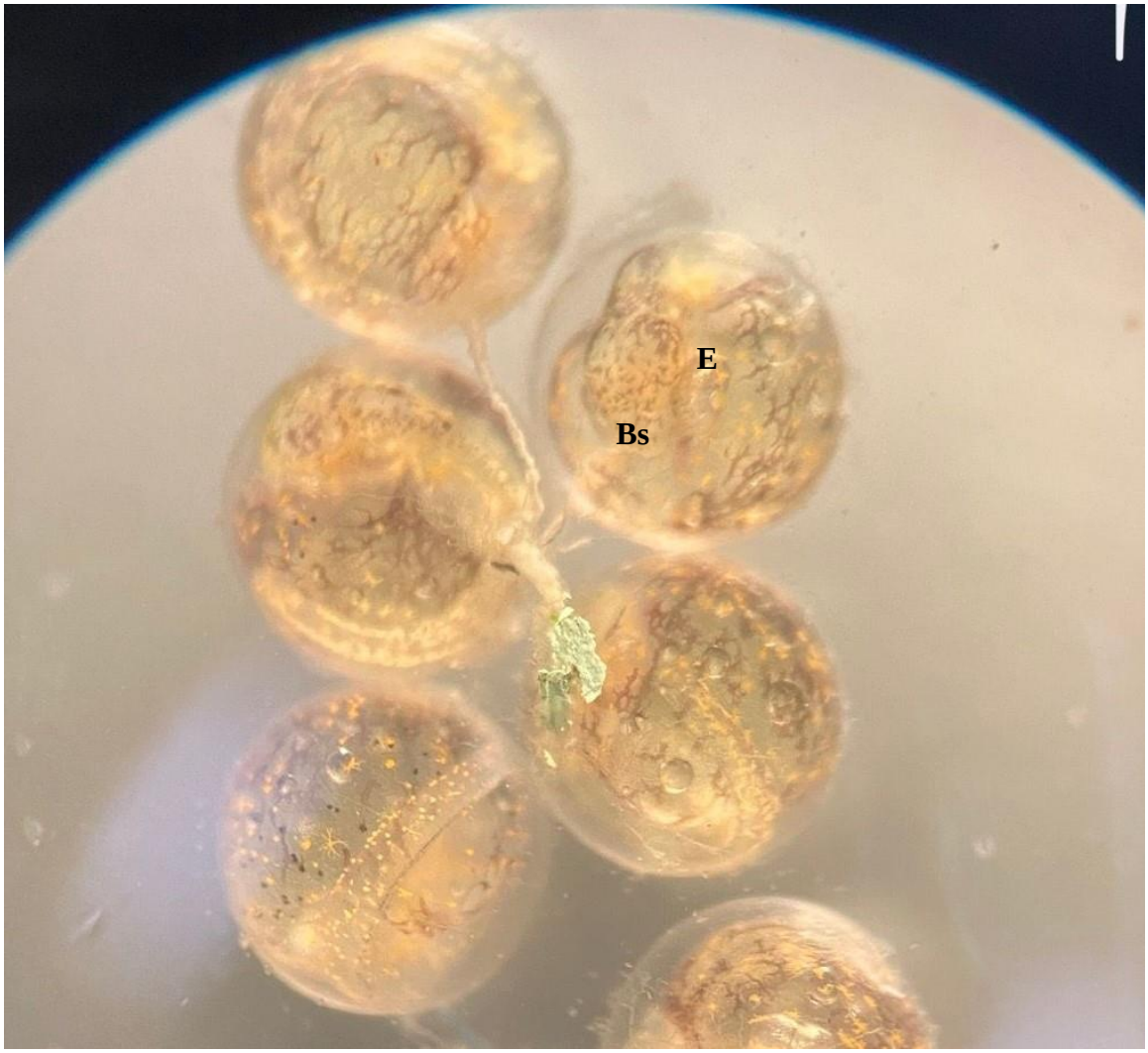

Figure A 6. Developing *F. heteroclitus* x *F. diaphanus* (female x male) hybrid embryos at approximately stages 28-30 of development as described by Armstrong & Child (1965). At these stages pigmentation develops rapidly and similar features to those present in Figure A 4 can also be seen above.

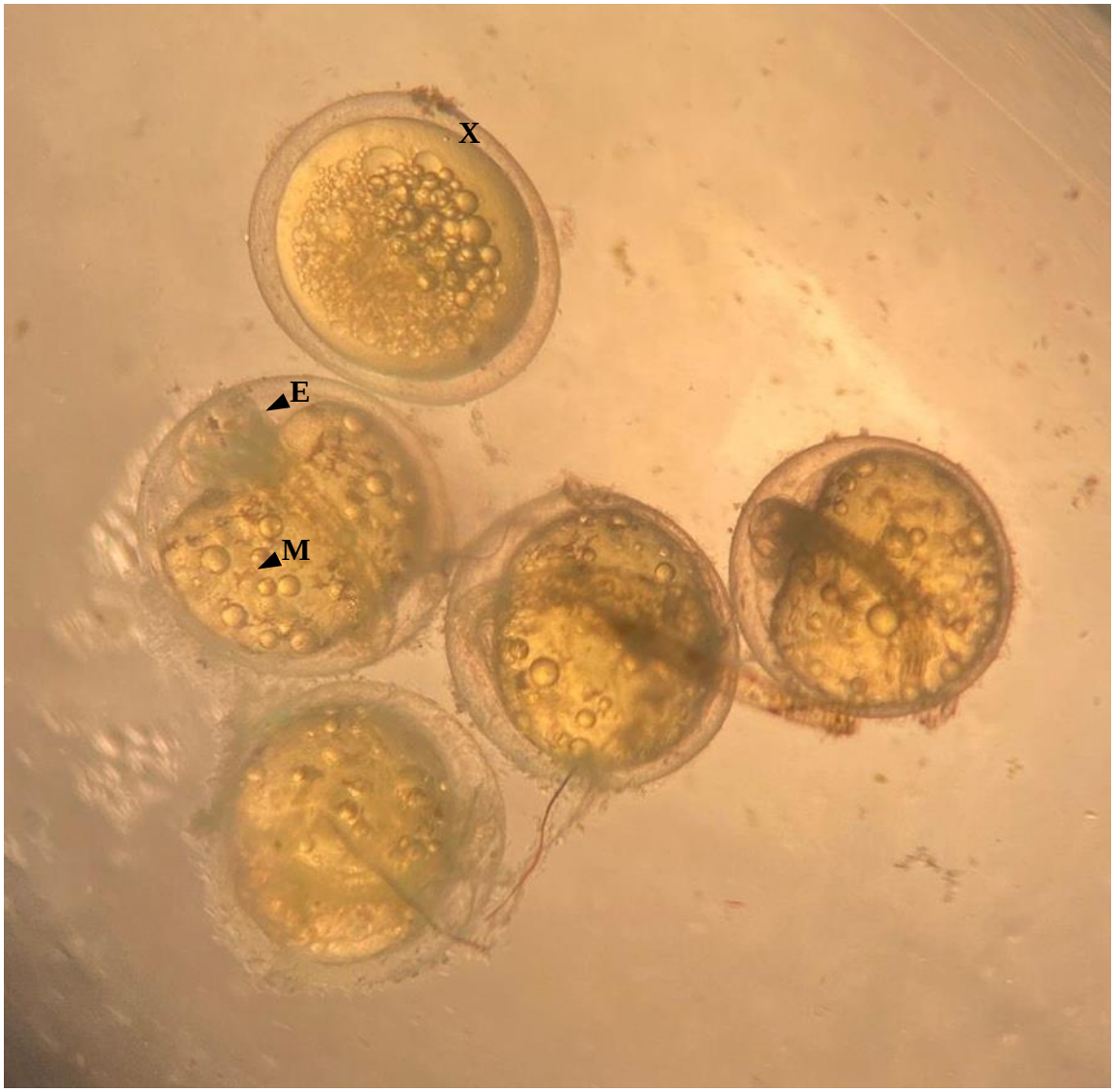

Figure A 7. Developing *F. heteroclitus* embryos at approximately stages 26-27 and one individual (at the top of the image; X) that ceased developing at approximately stages 12-15 as described by Armstrong & Child (1965). Similar features to those present in Figure A 4 can also be seen above.

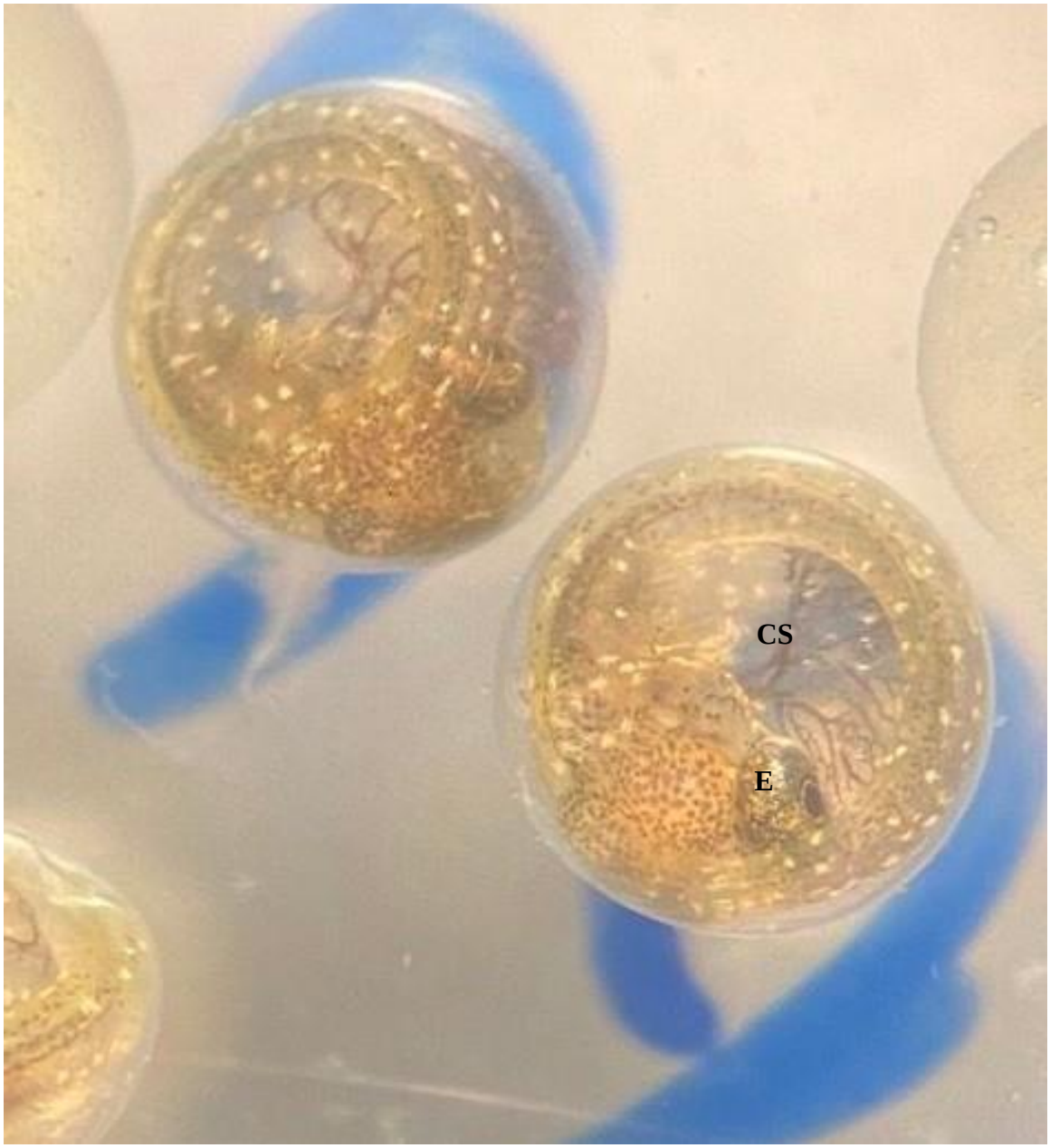

Figure A 8. *Fundulus diaphanus* embryos at approximately stage 33-34 (216-228 hours) of development as described by Armstrong & Child (1965). At this stage embryos are ready to hatch. The body has taken up most of the space in the egg, the tail can be seen wrapping around the egg back towards the head, eyes (E) are clearly visible as well as some of the circulatory system (CS)

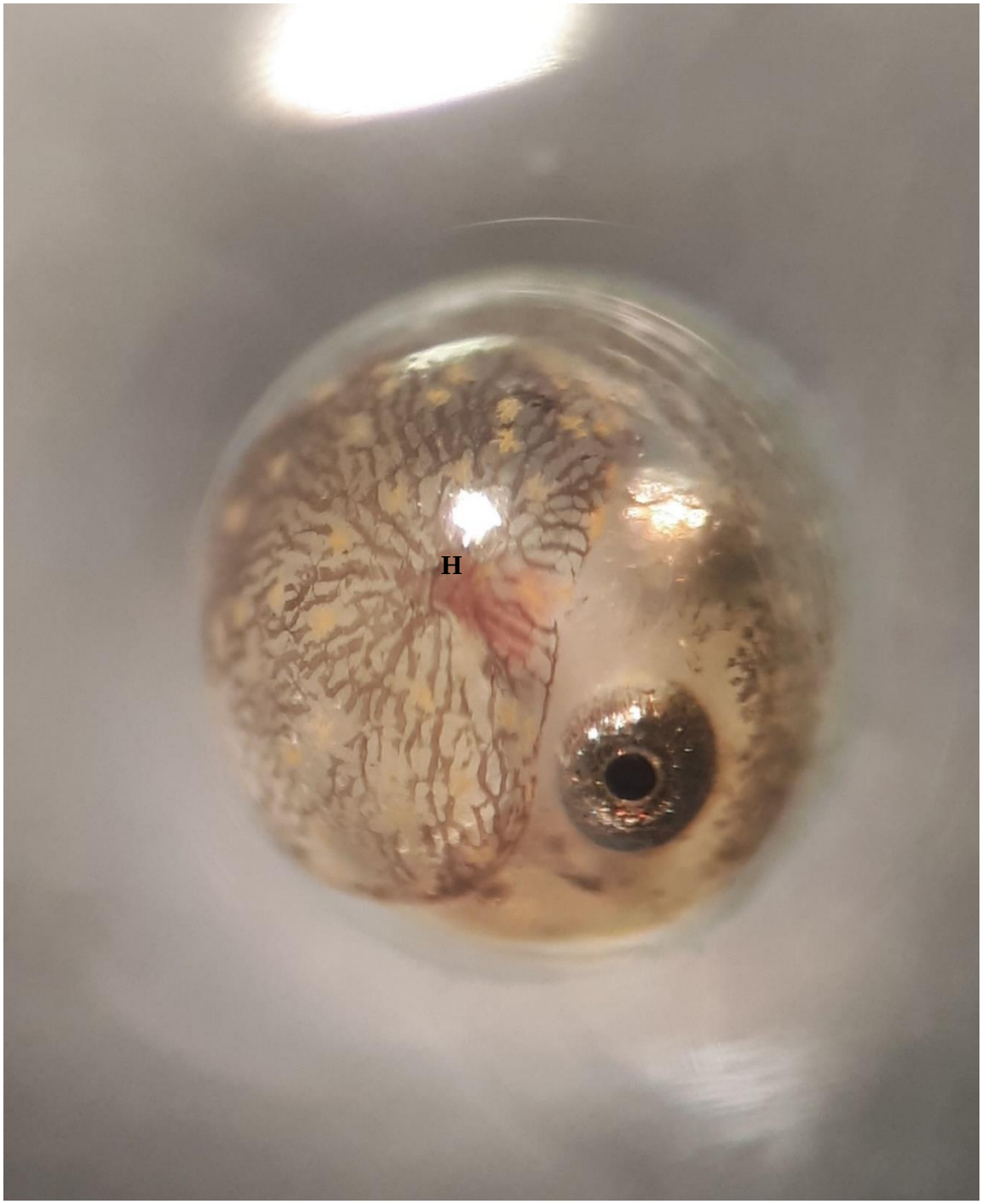

Figure A 9. Developing *F. diaphanus* x *F. heteroclitus* (female x male) hybrid embryo at approximately stage 33-34 of development as described by Armstrong & Child (1965). Similar structures as those visible I Figure A 8 and the heart (H) are visible.

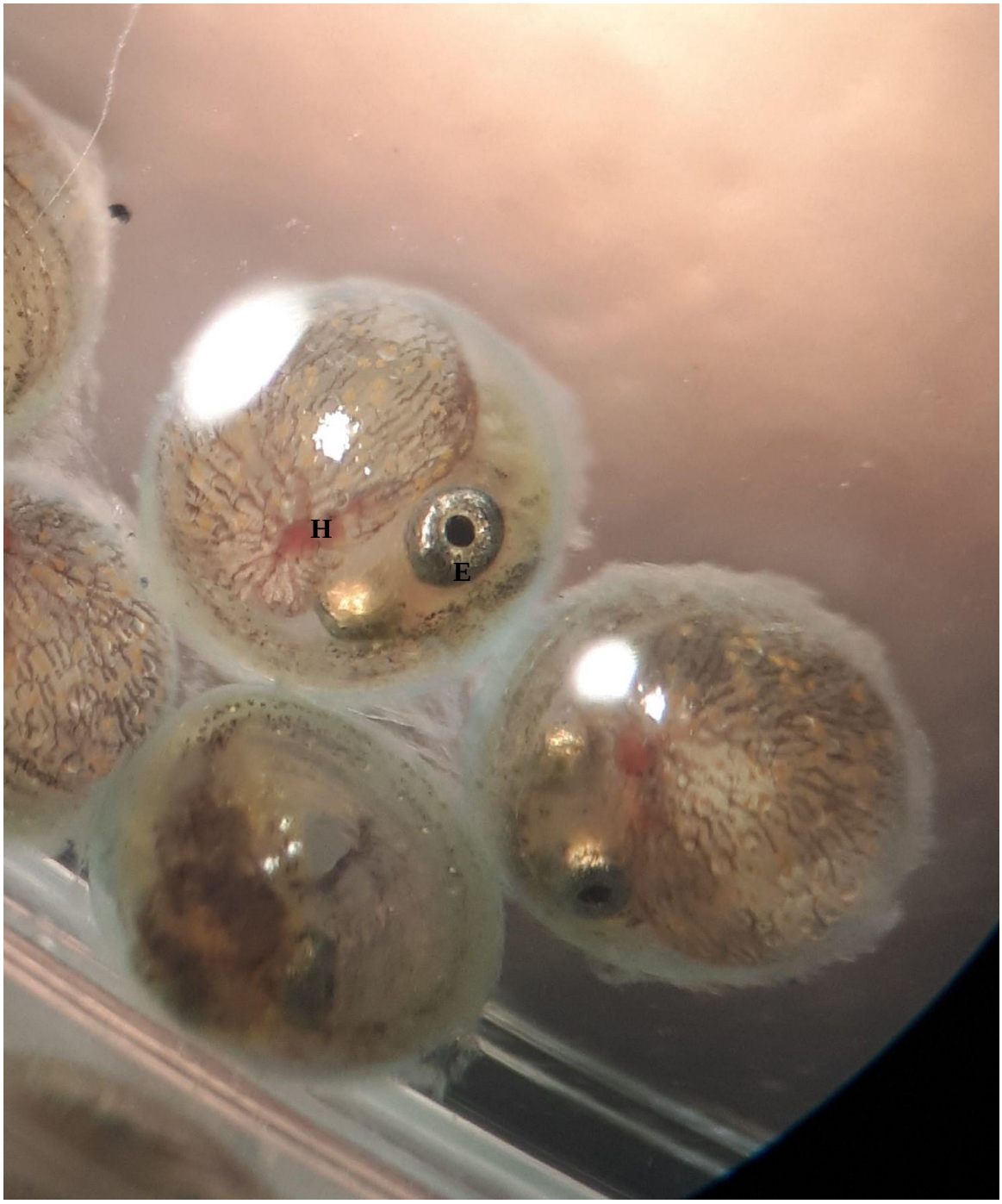

Figure A 10. Developing *F. heteroclitus* x *F. diaphanus* (female x male) hybrid embryos at approximately stages 33-34 of development as described by Armstrong & Child (1965).

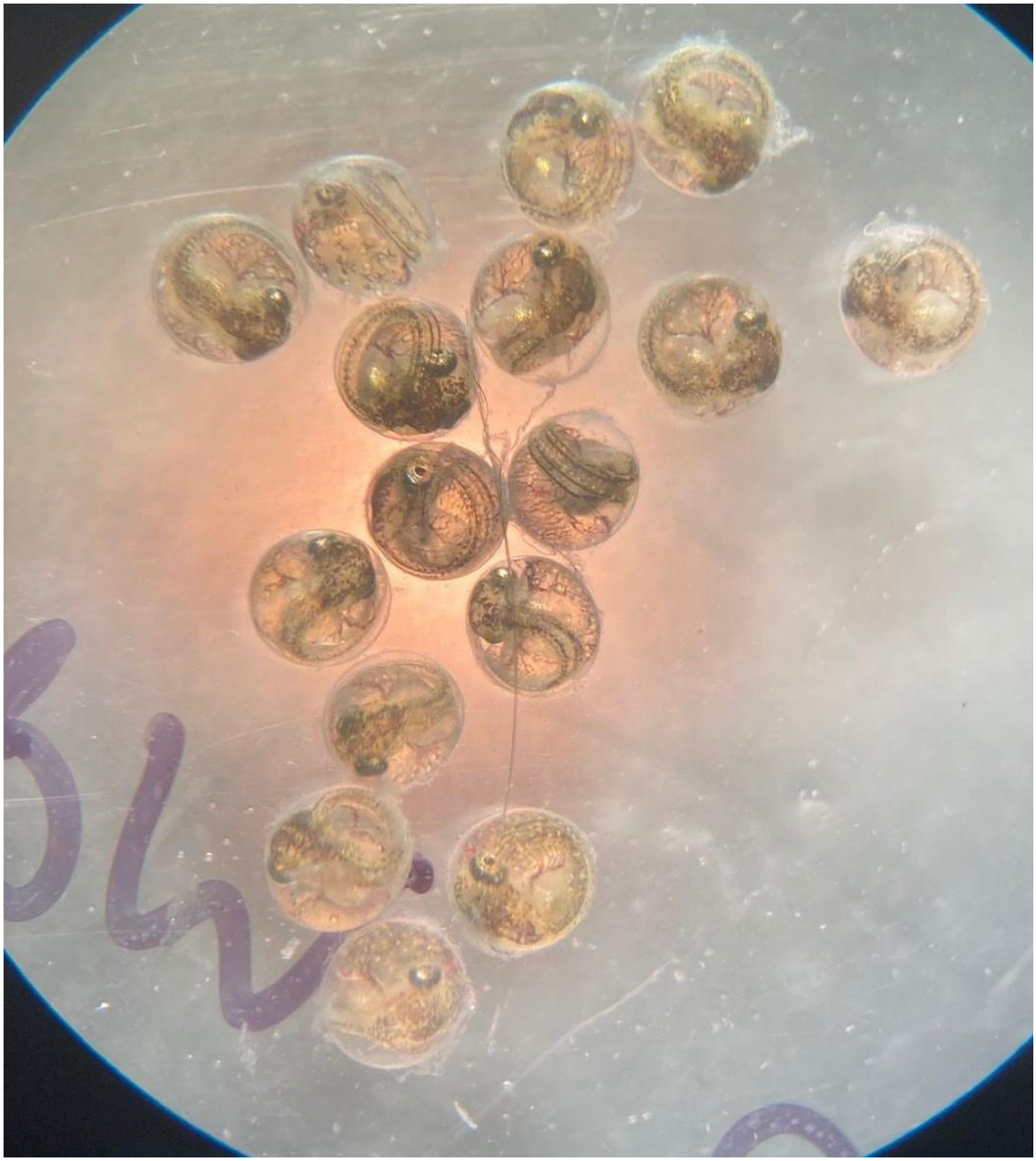

Figure A 11. Developing *F. heteroclitus* embryos at approximately stages 33-34 as described by Armstrong & Child (1965). Similar features to those present in Figures A 8-10 can also be seen above.