Incidence of sib mating, test for inbreeding depression and potential application for reducing the impact of escaped cultivated cod on wild Atlantic cod (*Gadus morhua*).

by

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ABSTRACT

Fish often escape from aquaculture operations raising concerns of interactions with wild individuals. Due to selective breeding and high fecundity among Atlantic cod, few breeding individuals are used in culture, potentially leading to inbreeding among the offspring within the cages. If inbreeding among Atlantic cod reduces offspring survival, this could limit the risk of outbreeding depression from cod escaping as eggs or larvae, maturing and mating with wild cod. To determine if cod inbreed and what effects inbreeding has on offspring, spawned embryos from tanks of sister−brother−unrelated male trios underwent microsatellite analysis to determine parentage. Inbreeding occurred with no significant bias toward one type of male. Percent hatch, deformities, larval size, and mortality were monitored in artificially fertilized inbred and unrelated crosses. Only percent hatch differed significantly, being higher in non-inbred offspring ($P = 0.024$). One generation of inbreeding is not sufficient to determine if inbreeding would effectively reduce farmed cod offspring survival.

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Co-authorship Statement

The author of this thesis planned, designed and carried out the data collection in the lab and in the field, processed and analyzed all the data and wrote the subsequent thesis. Dr. Craig F. Purchase, Dr. Edward A. Trippel, and Dr. Ian A. Fleming provided substantial contributions to experimental design and the evolution of data interpretation, as well as providing editorial reviews.

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1. BACKGROUND INFORMATION

The current high rates of fishing around the world puts most wild stocks at the risk of collapse (Brander 2007). Aquaculture is increasing production and is anticipated to nearly match the levels of wild-caught fish by 2030 (Brander 2007). The decrease in global fisheries production has prompted the expansion of aquaculture beyond salmonids in eastern Canada, including gadoids, primarily haddock *Melanogrammus aeglefinus* and Atlantic cod *Gadus morhua*. Norway holds the most developed Atlantic cod industry and other efforts are underway along the east coast of the USA, Iceland, and until fairly recently, eastern Canada (Rosenlund & Skretting 2006). Atlantic cod production is low relative to the production of salmonids in Canada and Norway. Roughly 1 million metric tons of farmed salmon are produced annually in Norway, compared to a production capacity of 300,000 tons of farmed Atlantic cod, although these values may vary annually (Rosenlund & Skretting 2006, Husa et al. 2014). In Canada the Atlantic salmon *Salmo salar* production is around 151,000 tons (FAO 2013), and while there were 10 non-salmon aquaculture sites in 2003, including Atlantic cod, rainbow trout *Oncorhynchus mykiss*, Arctic char *Salvelinus alpinus*, haddock, and Atlantic halibut *Hippoglossus hippoglossus*, as of 2012 there are no marine sites growing cod (Chang et al. 2014).

As with any industry, there are environmental concerns. The biology of new and developing aquaculture species may be very different than salmon, and thus problems already identified in salmon may or may not be the same in others. Some of these problems include disease transmission (Heggberget et al. 1993), pollution from animal waste and excess feed (Ackefors & Enell 1994, Kelly et al. 1996), and the use of large quantities of wild fish as feed (Naylor et al. 2000).

Another common problem with aquaculture is the escape of caged fish, and also the escape of embryos from spawning events in the cages. Unlike salmon, cod spawn in the sea cages, releasing millions of eggs every spawning season (Bekkevold et al. 2002). Fertilized eggs easily drift through the cage nets into the external environment (Goldburg & Naylor 2005, Jørstad et al. 2008). Genetic markers have enabled the discovery that eggs and yolk sac larvae can disperse at least 8 km from net pens due to tidal currents and hydrographical conditions (Jørstad et al. 2008). If they survive to maturity, they could mate with wild cod and potentially introduce farmed cod genes into the wild cod gene pool (Utter 1998, Jørstad et al. 2008), and unfortunately, interbreeding among different groups of the same species does not always improve fitness (Lynch 1991).

Outbreeding depression occurs when genetic differences of interbreeding populations produce offspring with reduced fitness as a result of incompatible genes, often in the form of lost local adaptation (Dobzhansky 1950, Waser & Price 1989, Edmands 1999). Severe outbreeding depression can take place in crosses between organisms of the same species, but whose populations have been physically distant. The type of outbreeding depression most often seen in the F1 generation results from additive effects of hybridization (Waser and Price 1994, Gharrett et al. 1999). Where additive effects have altered the phenotypes of hybrid offspring, the offspring may be ill-equipped to survive in the environment of either parent (Gharrett et al. 1999, Tymchuk et al. 2007). When populations of wild fish are genetically differentiated from farmed fish (Ruzzante et al. 2000) and adapted to local conditions, the introgression of domesticated or non-local fish from aquaculture operations could interfere with co-adapted gene complexes and also result in outbreeding depression in the F2 and beyond (Bekkevold et al. 2006).

Cultivated fish species may be grown outside of their natural range (e.g., Atlantic salmon in Chile), or within their range (e.g., Atlantic salmon in Norway). At a finer scale, farmed individuals maybe raised within the natural species' range but the specific population under culture may not be from the local area. Thus, the captive fish may be genetically adapted to a set of unique environmental conditions different from those of the cage site (Read & Fernandes 2003), where they are fed, medicated for diseases, and protected from predators (Read & Fernandes 2003, Bekkevold et al. 2006, Hutchings & Fraser 2008). They may be more vulnerable to changes in the environment, including but not limited to changes in water temperature, predation, disease, and food availability (Fleming et al. 2000, McGinnity et al. 2003, Weir et al. 2005, Hutchings & Fraser 2008, Jensen et al. 2010). Outbreeding depression has been seen in plants (Waser & Price 1989, 1994), salmon *Oncorhynchus gorbuscha* (Gharrett et al. 1999), the Arabian oryx *Oryx leucoryx* (Marshall & Spalton 2000) and *Drosophila* (Aspi 2000) as early as the F1 generation. The current study evaluates the merits of one possible solution- forced inbreeding - to combat the occurrence of outbreeding depression when non-local farmed cod escape, as adults or embryos, and interbreed with the wild cod.

Inbreeding is the mating of closely related individuals. Numerous studies have indicated that it typically results in inbreeding depression, a change in phenotypes that results in reduced fitness across any number of given traits (Kincaid 1983, Lynch 1989). This is particularly troubling for very small populations, potentially lessening survival due to the loss of genetic variability caused by a greater frequency of homozygosity (Bentsen & Olesen 2002, Keller & Waller 2002). Increased homozygosity can result in

reduced fitness by unmasking deleterious recessive alleles (Charlesworth & Charlesworth 1990). The effects of inbreeding depression may be seen at some life stages and not others, and may vary greatly over the life of an organism (Carr & Dudash 2003, Grueber et al. 2010).

Within Canada, Atlantic cod, a marine groundfish in the North Atlantic, has cultural, historical, and economic significance for the people of Newfoundland, as well as numerous other cultures, as it formed the backbone of the province and was the driving force of the economy for hundreds of years (Hutchings & Myers 1995, Schrank 2005). Unfortunately, overfishing of Northwest Atlantic cod stocks caused them to crash. A series of commercial fishing moratoriums were put in place in 1992 (Taggart et al. 1994), though no substantial recovery has been documented to date.

In the wild, cod form spawning aggregations for a period of roughly two months in the winter. They are broadcast spawners and provide no parental care for their offspring (Hutchings et al. 1999). The females are repeat spawners, and produce an average of eight batches over the course of the spawning season, releasing millions of eggs over a few months (Bekkevold et al. 2002). While a female may spawn millions of eggs in a single spawning season, only a few will ever reach maturity (Svåsand et al. 2000). Between one to three months post-hatch the larvae develop into pelagic juveniles, just under two centimeters in length (Campana & Hurley 1989). There they feed in the water column until reaching roughly three to four centimeters, after which they begin to settle on the ocean bottom (Lough & Potter 1993). Predation, food availability, and habitat type at settlement may all affect larval survival (Tupper & Boutilier 1995, Vallin et al. 1999), while environmental conditions like salinity and oxygen levels, along with mortality as a

result of commercial fishing, may affect recruitment to wild stocks (Vallin et al. 1999).

While no one has been able to observe this in nature, females exhibit mate choice in captivity (Brawn 1961a, Engen & Folstad 1999, Hutchings et al. 1999). Often this decision is based on some kind of physical or auditory display by the male, be it fin size, colour, or some courtship dance or other performance (Bremner et al. 2002, Suk & Choe 2002). The courting behaviour by the males involves sound production using their drumming muscles, size displays by fully erecting their median fins, and circling the female before mating (Hutchings et al. 1999, Nordeide & Folstad 2000). Larger, more aggressive, dominant males are more likely to have reproductive success (Taggart et al. 1994, Rakitin et al. 2001, Bekkevold et al. 2002). Aggressive male cod (*Gadus callaris* L.) can form a dominance hierarchy and increase aggression in the spawning months, alternating between aggression toward male opponents and courtship behaviour toward females during the short time before a spawning event, and reducing aggressive behaviour after the spawning season (Brawn 1961b). The other male(s) may become submissive and therefore have lower reproductive success (Feindel et al. 2010). The chosen male ventrally mounts the female, matching her speed and grasping her with his pelvic fins; they align genital pores and release their gametes (Brawn 1961a). Other males may eject sperm near the mating couple in an attempt to fertilize some eggs - these males are referred to as satellite or ‘sneaker’ males (Nordeide & Folstad 2000, Rudolfsen et al. 2005).

While it is recognized that females exhibit mate choice (Hutchings et al. 1999, Rakitin et al. 1999, Rakitin et al. 2001), it is not known if they are capable of recognizing kin, or if Atlantic cod would avoid mating with related individuals. This may be important

as the broodstock selection process, and very high fecundity, means there may be thousands of related fish within a single net pen. Stocking of juveniles in sea cages in New Brunswick used to be performed in such a way that 95% of the cod were likely to have been produced by two females and the remaining 5% from opportunistic spawners, while only around 5-10 males would have sired the offspring; the potential for inbreeding was high (G. Nardi, GreatBay Aquaculture, pers. comm. 2011). Based on our current knowledge of cod spawning behaviour, related cod could end up inbreeding in the cages.

The offspring easily escape the cages as embryos and may pose a problem to the wild cod populations if they survive to maturity and interbreed (Bekkevold et al. 2006, Jørstad et al. 2008, Jensen et al. 2010). Possibly as a result of outbreeding depression, farmed-wild hybrid offspring have been shown to exhibit lower survival rates than their purely wild counterparts (McGinnity et al. 2003, Meager et al. 2010), adding to the struggle for the wild Atlantic cod populations to recover.

The objectives of the thesis were to determine if inbreeding occurs among farmed Atlantic cod, and to determine the effects of inbreeding on percent hatch, the occurrence of deformities at the embryonic and larval stages, larval size and time to starvation. Effects of inbreeding on overall survival were limited to the time to starvation after hatch as the necessary resources to rear the fish to maturity were not available.

2. INTRODUCTION

The aquaculture industry is growing as the global production and demand for fish continues to increase (Naylor et al. 2000, Brander 2007). Fish aquaculture in temperate environments is dominated by salmonids, but other taxa are now being farmed at increasing rates, including the development of cod. As mentioned above, there are a number of potential problems associated with aquaculture. One of these concerns is the escape of farmed fish and their possible negative interactions with wild fish (Thorstad et al. 2008). In the case of Atlantic salmon, occurrences of escapes have been reported in a number of countries, in small or very large numbers (Naylor et al. 2005). Farmed Atlantic salmon currently outnumber wild salmon, and escapees represent up to 50% of fish in spawning populations in Norwegian rivers (Skaala et al. 2006). Much less is known for similar issues with other cultured species, including Atlantic cod. Cod also happen to have a much higher rate of escape than salmon, because among other things, they actively bite at the cage netting (Moe et al. 2009, Zimmermann et al. 2012). For example, from 2001-2009 Norwegian Atlantic salmon escaped at a rate of 0.19% (3.93 million salmon), while cod at 1.02% (1.05 million cod) (Jensen et al. 2010). However, cod pose an additional problem.

Unlike farmed salmon, which will not spawn in sea cages, Atlantic cod do (Bekkevold et al. 2002, Jørstad et al. 2008). This means that embryos with cultivated cod genes are drifting out of sea cages into the environment, escaping through spawning (Jørstad et al. 2008, Jensen et al. 2010). As selection of cultivated species is directed away from the naturally occurring traits of wild fish, and these cultivated organisms interbreed with wild fish, it can result in alleles that are normally deleterious in the wild becoming

increasingly frequent (Lynch & O'Hely 2001 Glover et al. 2012, 2013). Cod offspring spawned in sea cages may survive until first feeding as larvae, and under the proper conditions, they may reach adulthood (Uglem et al. 2012). On top of this, farmed cod larvae have been found as far as 8km away from the sea cage (Jørstad et al. 2008). However, long term effects of escaped farmed cod have not been studied. If escaped farmed embryos or fish do survive, they may interact with wild cod with possible negative implications, including competition for food and space (Gross 1998, Atlantic salmon), mating competition (Uglem et al. 2008, Skjæraasen et al. 2010, Atlantic cod), and outbreeding depression (Tymchuk et al. 2007, rainbow trout). As the fish undergo selection leading to domestication, they could introduce a set of adaptations that differ sufficiently from the local adaptations of wild cod, as has been seen in Atlantic salmon (Hindar et al. 1991).

The potential for outbreeding depression as a result of farmed-wild cod interbreeding is of particular importance, as farmed cod may differ considerably from their wild counterparts. Whether through domestication or location of origin, captive fish may be adapted to a set of unique environmental conditions different from those of the cage site (Read & Fernandes 2003), where they are fed, medicated for diseases, and protected from predators (Read & Fernandes 2003, Bekkevold et al. 2006, Hutchings & Fraser 2008). Captive chum salmon *Oncorhynchus keta* introduced into rivers have been found to have a rate of return to their 'foreign' river that was one tenth that of their wild counterparts, which Altukhov and Salmenkova (1987) declared a sign of maladapted captive fish. Another example of domestication differentiating cultivated and wild fish is in levels of the pituitary growth hormone (PGH) in Atlantic salmon, where under artificial

conditions, farmed fish had significantly higher PGH levels resulting in body weights of almost three times that of the wild fish (Fleming et al. 2002). If escaped captive-bred fish interbreed with wild ones, local adaptations of the wild fish may be disrupted through introgression, reducing their fitness (Read & Fernandes 2003, Bekkevold et al. 2006, Araki et al. 2009).

While most of the research is on salmon, based on current research, and depending on the presence of local adaptations in the wild cod (Nielsen et al. 2009), interbreeding between escaped farmed cod and their wild counterparts could alter the fitness of wild cod (Uglem et al. 2012). Outbreeding depression has been reported as a result of spawning between escaped and wild Atlantic salmon (McGinnity et al. 2003), and the progeny of the hybrid offspring have been shown to be less fit than their non-hybrid wild counterparts (McGinnity et al. 2003). This has been seen in populations of Northwest Atlantic salmon, where the farmed broodstock originated mainly from wild salmon in non-acidified rivers. However, the rivers that many escaped salmon have over time inhabited are acidified and contain locally adapted wild salmon (Fraser et al. 2008). When the escaped farmed salmon spawn in the wild, the hybrid offspring have been found to be maladapted and suffer decreased survival rates (Fraser et al. 2008). It is not unreasonable to assume that interbreeding between non-local cultivated and wild Atlantic cod could have similar results. However, until further research is conducted on this topic, it cannot be said with certainty. It is therefore important to minimize interactions between farmed and wild cod.

Several strategies for reducing the reproductive abilities of farmed cod have been explored. Photoperiod manipulation, which hinders farmed Atlantic salmon from

maturing, has been attempted with Atlantic cod with varying success: Trippel et al. (2011a) found that increased light exposure delayed, but did not stop, maturation, while Cowan et al. (2011) were able to suppress maturation with the assistance of shading and continuous lighting. Triploidy could potentially be used as it creates severely reduced fertility in females, and aneuploid sperm in males, which although capable of fertilizing eggs, the offspring are not likely to survive past the larval stage (Feindel et al. 2011). In this study I explored inbreeding as another possible tool to mitigate the long term effects of escaping embryos on wild cod populations. Given that the broodstock of any cultivated species are chosen for very specific traits, including fast growth, disease resistance and delayed maturity, and there may be relatively few families which have those traits, there is the likely possibility, particularly for highly fecund species like cod, that thousands of related fish (mainly half- and some full-sib fish), will be grown in a single cage (Hutchings & Fraser 2008, G. Nardi, GreatBay Aquaculture pers. comm. 2011, Trippel et al. 2009).

Inbreeding in zebrafish *Danio rerio*, even at the half sibling level, can result in reduced egg fertilization, decreased survival of larvae, and smaller sized offspring (Mrakovčič & Haley 1979). Inbreeding can also increase the occurrence of deformities in rainbow trout (Aulstad & Kittelsen 1971, Aulstad et al. 1972), and cause reduced body length, weight, and survival of wild Alaskan steelhead *Oncorhynchus mykiss* (Thrower & Hard 2009). Growth rate, body weight, and food conversion efficiency at different stages of development have also been affected by inbreeding depression (Kincaid 1976a, Kincaid 1976b, Gjerde et al. 1983). If the effects of inbreeding are severe enough, for

example if embryos do not hatch or larvae do not survive, it could potentially be used as a tool to mitigate the effects of escaping cod embryos from sea cages. Based on previous research with various fish species, it is possible that by only stocking grow out cages with related fish, the resultant inbred embryos that drift out after spawning may face decreased hatch success, higher incidence of potentially lethal deformities, and less likelihood of reaching maturity, all of which could reduce the risk of farmed offspring interacting with wild cod.

Factors that could play an important role in cod reproduction are the ability of cod to recognize related fish, and the mechanisms behind mate selection. Kin recognition and mate choice may be of particular importance when it comes to the potential for inbreeding among spawning cod in cages. Kin recognition, though not well researched in Atlantic cod, can be an important factor in mating and the potential for inbreeding. Kin recognition has also been seen to effect aggression levels in chinook salmon *Oncorhynchus tshawytscha*, with increased aggression toward unrelated fish (Henkel et al. 2011). While some species of fish, juvenile salmonids and threespine stickleback *Gasterosteus aculeatus* for example, are thought to be able to recognize related individuals (Fitzgerald & Morrissette 1992, Ward & Hart 2003), other species, such as haddock (Trippel et al. 2009), do not appear to share this ability. Kin recognition does not necessarily require familiarity with one's kin (Henkel et al. 2011), and can be influenced by a number of factors, including phenotypic matching and familial imprinting in young cichlids *Pelvicachromis taeniatus* (Hesse et al. 2012). Individuals may use visual or olfactory cues as juveniles to distinguish their kin from unfamiliar fish later in life (Gerlach & Lysiak 2006). While it is thought that kin recognition could reduce

inbreeding, the mechanism to differentiate related males from unrelated males when choosing mates is absent or very weak in some species, as was thought to be the case with female guppies *Poecilia reticulate* (Viken et al. 2006). Similar to cod, male guppies court females, who then choose a mate, and until fairly recently, there was no indication of discrimination for or against related males (Viken et al. 2006). However, it has since been uncovered that cryptic female choice allows guppies to avoid inbreeding (Gasparini & Pilastro 2011), and as a result, males have been found to produce better sperm when paired with sisters to combat her natural defences to avoid inbreeding (Fitzpatrick et al. 2014). In tilapia *Sarotherodon melanotheron*, related fish group together within shoals and there has been evidence to suggest that mating is actually biased to inbreeding within these groups (Pouyaud et al. 1999). And finally, among zebrafish it appears that kin recognition occurs, and that biases toward kin changes as the fish mature, with sexually mature females preferring the odour of unrelated males, a possible mechanism for avoiding inbreeding (Gerlach & Lysiak 2006). It is not yet known whether cod can recognize related individuals, or if this would affect their mate choice.

Female cod exhibit mate choice, possibly based on secondary sexual characteristics in the form of fin displays and grunting noises produced by the males as they circle the female, pulsing the three pairs of drumming muscles on either side of the swim bladder against the bladder to produce sound (Hutchings et al. 1999, Rakitin et al. 1999, 2001, Bekkevold et al. 2002). It is believed that females may be more inclined to choose a male with larger fins, and larger drumming muscles which would produce a louder grunt (Nordeide & Folstad 2000, Rowe & Hutchings 2004), but it is yet unknown whether relatedness of potential spawning partners would also play a role.

Previous research has been undertaken on inbreeding in various marine species (Trippel et al. 2009, Houde et al. 2011), and on deformities in cod and other fish (Yousefian & Nejati 2008, Avery et al. 2009), but this is the first study to my knowledge that addresses mating between relatives of Atlantic cod and the direct effects on first generation offspring. The objectives of this study were to investigate whether or not Atlantic cod inbreed in captivity, and to examine the effects of inbreeding on offspring characteristics. Given that it is not known whether or not Atlantic cod are capable of kin recognition, I hypothesized that female cod do not differentiate between potential mates based on relatedness, and predicted no bias toward unrelated males over related males. I also hypothesized that inbreeding results in reduced offspring survival and poor offspring quality, anticipating higher mortality, reduced size, and higher incidence of deformities compared to non-inbred offspring. Two experiments were conducted: one in-tank spawning experiment to determine if cod would inbreed in captivity, and the other an *in-vitro* fertilization experiment to force inbreeding in order to determine its effects on the offspring.

3. MATERIALS AND METHODS

3.1.1 *In vivo* spawning experiment. The study specimens were from the Atlantic Cod Genomics and Broodstock Development Project (ACGBD) (Cod Genome Project, http://codgene.ca, http://genomeatlantic.ca/; Trippel et al. 2011b), created in 2005, reared in captivity and held at the St. Andrews Biological Station (Fisheries and Oceans Canada, St. Andrews, New Brunswick, Canada). Fish used were from nine F1 generation families created from the original wild broodstock, captured in the Bay of Fundy (Northwest Atlantic Fisheries Organization, Division 4X). The study specimens were fed a mix of herring *Clupea harengus*, northern shortfin squid *Illex illecebrosus* and Aesop shrimp *Pandalus montagui* for one year prior to the start of the experiment, and a marine grower diet prior to that; and were not fed for the duration of the spawning season (Fordham & Trippel 1999). The experiment used 34 fish which had spawned successfully in previous years. All fish were four years of age with known family backgrounds as a result of the ACGBD program. All individuals were weighed and measured in mid-January 2010, and Fulton's condition factor, which is important in cod mating (Kjesbu et al. 1996, Trippel & Neil 2004), was calculated for each fish ($K = [W L^{-3}] \times 100$; ± 0.1). Trios consisted of a mature female, a full sibling brother, and an unrelated male. The trios were chosen by family background and the males were matched by weight, length, and condition factor to reduce morphological features that may influence female mate choice or male dominance (Table 1). For each measurement, the males in a tank differed by no more than 11% from one another, and no more than 13% from the length of the female (Rakitin et al. 2001). On 3 February 2010, 30 cod were transferred from a holding tank and placed in their pre-assigned trios in ten 3000 L circular tanks (3 m^3, 1.83 m diameter and 1.17 m deep). The

tank conditions for the duration of the experiment ranged as follows: temperature- 5.7 to 6.3 ^{o}C, salinity- 29 to 32 ppt and dissolved oxygen- 101 to 106 %.

Seven fish died during the experiment. Of the four tanks that had mortalities, egg batches were only collected from one of them (trio one produced one batch before the female died). The three females that died were 'egg bound,' where the female fails to release eggs but continues to ovulate (Árnason & Björnsson 2011). Some of the dead fish were replaced as there were suitable fish still in the holding tank. Replacements had to fit the family requirements (related or unrelated as needed), and had to closely match the other fish in the trio in length, weight, and condition factor (Rakitin et al. 2001). All three fish in trio eight were replaced on 9 February 2010, and the non-brother in trio six was replaced on 18 February 2010.

Each tank was equipped with a surface egg-collector that was checked daily for fertilized eggs, as well as a collector over the outflow pipe for dead eggs (Thorsen et al. 2003). Eggs from the surface egg-collector were examined under a dissecting scope for fertilization; a minimum of 20 embryos from each batch were removed and preserved in 95% ethanol for genetic analysis. Additional embryos were preserved whenever possible. The embryos were almost all collected within 24 h of spawning. However, a few of the batches of embryos were only a few hours old at the time of collection. These embryos were incubated for no longer than two days to minimize differential losses due to different sires that could skew the parentage analysis (Saillant et al. 2001, Trippel et al. 2005). Average daily water temperatures during the experiment ranged from 5.7 to 6.3 ^{o}C.

On 29 March 2010 the experiment was terminated and the fish were euthanized. The barbel was removed from each adult cod and preserved in 95% ethanol for genetic analysis.

3.1.2 DNA extraction. Tissue from the preserved barbels was used to genotype the adults and embryos were used whole and intact. DNA was extracted from adult tissue and embryos following the Wizard SV 96 Genomic DNA Purification System protocol for animal tissues (Promega A2370), with a slight alteration. Instead of adding 250 µl of nuclease-free water to each well of the binding plate and incubating for two minutes, repeating for a total of 500 µl, I added 75 µl of nuclease-free water and incubated for 10-15 minutes, repeating for a total of 150 µl. DNA from the adult tissue was diluted to 25 ng μL^{-1} based on Nanodrop readings (Nanodrop ND-1000 Spectrophotometer, Thermo Scientific).

3.1.3 Multiplex design and DNA amplification. A pre-existing protocol (Wesmajervi et al. 2006) was used as a guide, with slight alterations. Four microsatellites were used (Gmo8, Gmo19, Gmo35, and Gmo37) instead of the five in Wesmajervi et al. (2006); Tch11 did not work in any of our attempts, and so was removed from the multiplex. To increase the number of successfully genotyped embryos, I used Q PCR Master Mix (5 µL), Q Solution (1 µL), 3.6 µL of DNA template, and 0.4 µL of the primer Master Mix (concentration of each primer in mix: 0.6 µM Gmo8 forward and reverse, 0.5 µM Gmo19 forward and reverse, 0.5 µM Gmo35 forward and reverse, and 0.4 µM Gmo37 forward

and reverse) for an amplification reaction volume of 10 μL. The polymerase chain reaction was carried out in a BioRad C1000 thermo cycler, and the thermal cycling parameters were as follows: denaturation at 95 ^{O}C for 15 min, 35 amplification cycles at 94 ^{O}C for 30 s, 57 ^{O}C for 90 s, and 72 ^{O}C for 90 s, then after the 35 cycles, 72 ^{O}C for 10 min, and down to 10 ^{O}C to finish.

3.1.4 DNA analysis. Adult tissue PCR products were diluted 1-20 in nuclease-free water. Due to the low yields from DNA extractions of the small number of cells found in 1-3 day embryos, embryonic PCR products were analyzed without dilution. For a 10 μL reaction mix, I added 4 μL of the PCR product, 0.2 μL of GeneScan-500-LIZ size standard, and 5.8 μL of HiDiFormamide. The samples were heated for 3 min at 95 ^{O}C and immediately placed on ice to cool before being placed in either a 3130 or 3730 Genetic Analyser (ABI 3130 and 3730 DNA Analyzer, Applied Biosystems). Data from both the 3130 and the 3730 were compared to ensure that results did not differ between machines. Peak Scanner Software (Version 1.0, Applied Biosystems, 2006) was used to read the allele sizes, and parentage was assigned by matching allele sizes between adults and embryos within a trio.

3.1.5 Statistical analysis. A log-likelihood goodness of fit *G*-test was performed to determine if there was a significant difference in the proportion of eggs sired by each male within a batch for each trio; all tests had one degree of freedom (McDonald 2009). Statistical analyses were executed in Minitab (version 15.1.1.0, Minitab Inc., 2007).

3.2.1 *In vitro* artificial fertilization. Gametes were stripped from five-year-old captive Atlantic cod from the Atlantic Cod Genomics and Broodstock Development Project, provided by the Huntsman Marine Science Centre (St. Andrews, New Brunswick, Canada). Ten families were represented by the 14 females used, with three families containing multiple females (two females in families 8 and 10, three females in family 9 in the Figures). Aquacalm (0.2 g L^{-1}; Syndel International, Vancouver, BC, Canada) was used to sedate fish before they were anaesthetized with MS-222 (76 mg L^{-1}, Aqualife TMS, Syndel Laboratories) (Butts et al. 2009). Once anaesthetized, the area surrounding the genital pore was wiped dry and pressure was applied to the abdomen to expel the eggs into a dry 1 L plastic beaker. Semen was collected from the males in a similar manner, using a dry 40 mL glass beaker, and making sure that no blood or urine entered the semen sample. Gametes were stored for no longer than one hour before use, and were kept at 6 ^{o}C immediately after collection. Semen was qualitatively examined for motility before used in artificial fertilization.

Artificial fertilization and rearing were carried out in a refrigerated room at 6 ^{o}C. All equipment was rinsed with UV sterilized salt water, dried, and chilled. Egg batches from each of 14 females were divided in half and separately fertilized with the semen of a full sibling brother and an unrelated male (Figure 1). Eggs were placed in a large glass beaker which was at least double the volume of eggs, and approximately 10 mL of seawater was added. Semen was then pipetted directly onto the eggs at a ratio of 1 mL of semen for every 100 mL of eggs, stirred with a glass rod, and left for three minutes, after

which the water level in the beaker was brought up to double the volume of eggs. After 10 minutes, excess sperm was removed from the embryos by rinsing them through a fine mesh net with 1 L of seawater. The embryos were then placed in a 1.5 L 'stock' beaker topped up with seawater. The embryos were left in the stock beaker to incubate overnight. Eggs from fourteen females were artificially fertilized between 23 February and 31 March 2010. Among the 14 females, ten families were represented, as some females were sisters. Each family was considered an independent replicate.

3.2.2 Embryo deformities For seven of the females I was able to photograph fertilized embryos at the two to four cell stages after overnight incubation and examined them for visual malformations in early cell cleavage patterns. For the remaining seven females the embryos had already progressed past the four cell stage before they could be photographed. A small random sample of embryos was removed from the stock beaker. Samples consisted of approximately 20 embryos from each female, and these embryos were photographed for visual analysis of asymmetrical development. Photographs of the inbred and unrelated embryos were analyzed for the presence of malformations in random order to reduce potential bias, and the average number of deformities per embryo was determined, based on pre-existing criteria (Shields et al. 1997, Avery et al. 2009). A blastodisc was considered normal if it had four distinct, symmetrical cells of equal size (Figure A1). The following embryo deformities were identified based on illustrations and images by Avery et al. (2009) and Shields et al. (1997): asymmetrical cells, uneven number of cells, incomplete intercell adhesion, poorly defined cell margins, vacuolar inclusions between cells, overlapping cells, extra cell bodies, only three cells (triple),

jumbled cells of varying size and position, offset/intermediate- where the four cells do not all meet at one central point, pie- when the four cells resemble a pie in shape, and marginal, where the cells are equal in size, but with three cells forming an approximate circle and the fourth on the outside of the circle.

3.2.3 Embryo rearing and percent hatch. After overnight incubation, fertilized embryos, which float at the surface of the beaker, were divided into a maximum of four 250 mL beakers of seawater with 250-300 embryos in each. The exact number of embryos in each beaker was counted; some had fewer embryos due to lower fertilization success (Figure 1). Where fertilization success was low, the embryos were divided into fewer than four beakers but these beakers had an embryo density similar to that of crosses with higher fertilization success. The temperature was maintained at 6 ^{o}C and a 50 % water change was performed daily for each beaker to maintain water quality. However, water changes were not conducted during gastrulation, as it is a time of high embryo mortality rates (Geffen et al. 2006). Dead embryos, those that were negatively buoyant and opaque in appearance, were removed and enumerated daily. The same was done with the hatched larvae, enabling estimation of percent hatch.

3.2.4 Larval survival and quality. On the day of peak hatch, which was considered Day 1 and determined as the largest single-day hatching event for each beaker, the larvae were randomly divided in half and placed in two 50 mL beakers of sea water. One of these beakers was used to monitor the time to starvation. Daily dead were recorded and

removed until all larvae had died. Fifty percent water changes were done every other day. Data from individual beakers were pooled for each male in every cross from peak hatch until 100% mortality was reached. The mean percent daily survival was calculated from the average of all inbred and unrelated crosses.

The other group was used to determine larval quality. On the day of peak hatch, five larvae were randomly selected from this group and photographed along the lateral view while under a dissecting scope so that they could be measured and examined for deformities. Larval measurements were taken at hatch and at five days post hatch (dph) using Image-Pro Plus (Version 5.1.0.20, Media Cybernetics Inc., 2004) and included total body length, myotome height, eye diameter, yolk area (not including yolk sac), and jaw length at day five only, once fully developed (Figure A2, Rideout et al. 2004).

3.2.5 Larval deformities. From the photographs taken at hatch, the presence and type of four possible deformities were recorded for each larva. Previous work on larval deformities mainly examined spinal deformities in larvae past the yolk sac stage (Aulstad & Kittelsen 1971, Gjerde et al. 1983), although a few studies identified various other deformities in the jaw, finfolds, and yolk sac (Hart & Purser 1995, Kennedy et al. 2000). Any characteristic that deviated from 'normal' (straight body, full yolk sac at hatch) was considered a deformity and included: bent tail end, bend in midsection, curved body and malformed yolk sac, where the yolk appeared shrivelled or misshaped at hatch (Figure A3). The number of deformed larvae, the average number of deformities per deformed larva, and the occurrence of each type of deformity was recorded for each cross. Larvae were euthanized after photographing. Where only one embryo hatched in a beaker, that

larva was used to estimate time to starvation; no photographs were taken as handling may have affected survival. That cross was eliminated from all of the larval size measurement and deformities analysis.

3.2.6 Statistical analysis. Data for females that were in the same family were averaged for statistical analyses, as were data from the beakers for each male in a cross. Sample sizes were as follows: N = 10 independent comparison groups (20 half-sib families) for embryo deformities and hatch data, and N = 9 for larval deformities, time to starvation, and size measurements. A paired t-test was used to compare differences between inbred and non-inbred offspring hatch data and the occurrence of embryo deformities (arcsine transformed proportions, Sokal & Rohlf 1995). The same test was used on untransformed means to determine if there was a difference in the mean number of deformities per deformed embryo. A two-way ANOVA was used to compare the prevalence of deformities among larvae using arcsine transformed proportions with relatedness (inbred vs. non-inbred) and cross as variables, and untransformed means were used with the same general linear model to determine if there was a difference in the mean number of deformities per deformed larva. A two-way ANOVA was used to analyse time to starvation between inbred and non-inbred crosses, where the crosses themselves were pooled, so the variables were relatedness and days. Body length, myotome height, eye diameter, and yolk area were analysed using a three-way ANOVA where relatedness, cross, and day were variables, to account for measurements taken at hatch and at five dph. An interaction term (*relatedness · day*) was included, but found not to be significant. The residuals were checked for normality and homogeneity to meet the assumptions for

ANOVA. Jaw length, measured only at five dph, was analysed using a paired t-test. A Bonferroni correction was applied to the size measurement data to account for multiple tests of size, with the resulting $\alpha = 0.01$. Statistical analyses were performed using Minitab (version 15.1.1.0, Minitab Inc., 2007).

4. RESULTS

4.1 *In vivo* competitive spawning experiment. Only five of the ten trios spawned. These trios produced 1to 10 batches, with an average of 3.8 batches (Table 2). Of the 19 batches from all spawning trios, single paternity was observed in five batches, all of which were fertilized by the unrelated male in each trio. Multiple paternity occurred in the rest of the batches (n = 14), some of which were dominated by the brother. The brother did not have sole paternity of any batches. The number of eggs fertilized by each male within a batch for all trios differed significantly, with the exception of two batches in trio two, where each male fertilized approximately half the batch. For the majority of the batches, one male dominated the fertilizations (Table 2). Of the five trios that spawned, the unrelated male had the greatest fertilization success in trios one, two, and three, while the related male was dominant in trios four and five (Table 2). Inbreeding occurred in at least one batch for each trio.

4.2 *In vitro* fertilization experiment. The percent occurrence of deformities did not differ between embryos of inbred and non-inbred crosses (Table 3, Figure 2), or in the average number of deformities per deformed embryo. The two most prevalent embryo deformities were unequal cell size and asymmetry; approximately 20% of the embryos, inbred and unrelated crosses combined, had these two deformities (Table 4). Incomplete intercell adhesion was the third most common, with 19% of all embryos examined showing this deformity, and poorly defined cell margins were seen in 14.9% of the embryos. The remaining deformities occurred in fewer than 7% of all the embryos examined. Similar results were found for the percent of larvae with deformities (Figure 3)

and the average number of deformities on each deformed larva (Table 3). The most common larval deformity was a bent tail, affecting 66.9% of all the sampled larvae combined, while bent midsections were found on 15.4% of the larvae. Curved spines and malformed yolk sacs were equally prevalent, affecting 8.8% of the total number of larvae sampled.

None of the differences in the occurrence of embryo or larval deformities were significant. The unrelated offspring of both females in family nine had a higher incidence of embryo deformities, the opposite of which was seen in family ten (Figure 2). When it came to larval deformities, there was no consistency in the prevalence of deformities in the offspring of females from family eight or nine (Figure 3); for example, two out of three inbred offspring from family nine's females had the most deformities compared to their non-inbred counterparts, while the third female's inbred offspring had a lower incidence of larval deformities.

Percent hatch among offspring of unrelated crosses was significantly than their inbred counterparts (42.2% ± 6.73%, 33.6% ± 6.16% respectively; mean ± standard error) (Table 3, Figure 4). In five of the inbred crosses and four of the non-inbred crosses only one embryo hatched from a single beaker (these larvae were only used to measure time to starvation, not larval size). With an α of 0.05, the only significantly different size measurement was myotome height, where offspring of unrelated crosses had a significantly higher myotome height then their inbred counterparts ($F_{(1,28)} = 7.72$, $P = 0.01$, Table 5). However, myotome height was not significant after Bonferroni correction ($\alpha = 0.01$). Similarly, there was no difference in total body length ($F_{(1,28)} = 2.61$, $P =$

0.118), eye diameter ($F_{(1,28)} = 2.13$, $P = 0.155$), yolk area ($F_{(1,28)} = 0.12$, $P = 0.736$), and jaw length ($T_{(1,18)} = 1.87$, $P = 0.095$) (Figure 5, Tables A1, A2, A3, A4). Time to starvation did not differ significantly between inbred and non-inbred crosses (Table 3, Figure 6).

Table 1. Fork length (cm), body weight (kg), and Fulton's condition factor (*K*), of each Atlantic cod *Gadus morhua* used in the *in vivo* competitive spawning experiment, as well as replacement fish used due to death of an originally assigned cod (Rep- replacement, F- female, B- brother, NB- non-brother). Family designations illustrate the relatedness of each fish, where the same family number indicates fish that are full siblings. The females in trios 6-10 did not spawn (egg bound or died and had no suitable replacement).

Trio	Fish	Family	Length (cm)	Weight (kg)	*K*
1	F	1	64.1	4.31	1.64
	B	1	64.0	3.54	1.35
	NB	4	65.0	3.84	1.40
2	F	2	61.5	4.30	1.85
	B	2	53.0	3.50	2.35
	NB	8	53.6	3.42	2.22
3	F	3	63.7	4.71	1.82
	B	3	61.1	4.22	1.85
	NB	9	59.3	4.38	2.10
4	F	4	67.9	5.33	1.70
	B	4	67.5	5.08	1.65
	NB	8	65.1	4.26	1.54
5	F	5	62.2	4.23	1.76
	B	5	60.5	3.76	1.70
	NB	9	59.9	3.16	1.47
6	F	6	57.9	3.02	1.55
	B	6	56.7	2.21	1.21
	NB	5	54.7	2.23	1.36
	Rep NB	4	62.6	3.21	1.31
7	F	7	70.9	5.15	1.44
	B	7	69.7	4.79	1.41
	NB	6	68.8	4.23	1.30
8	F	8	64.3	3.51	1.32
	B	8	62.5	3.59	1.47
	NB	9	62.5	4.46	1.83
	Rep F	4	73.2	5.24	1.34
	Rep B	4	62.6	3.21	1.31
	Rep NB	8	61.3	2.89	1.26

Continued...

9	F	4	65.0	4.90	1.78
	B	4	64.0	3.93	1.50
	NB	1	59.9	3.29	1.53
10	F	5	61.2	3.90	1.70
	B	5	60.0	3.36	1.56
	NB	9	60.6	3.38	1.52

Table 2. Number of batches of eggs produced by each trio of Atlantic cod *Gadus morhua* that spawned and the number of genotyped embryos sired by each male per batch, and pooled for the trio (percent of total eggs for batch/trio shown in parentheses). A total of 19 batches were produced. The log-likelihood goodness of fit G-test was used to determine whether there was a significant difference in the proportion of embryos sired by each male within a batch (degrees of freedom = 1 for all batches).

Trio	Batch	Brother	Unrelated	G value, P value
1	Feb 27	2 (4.3)	44 (95.7)	47.32, <0.001
2	Pooled	66 (17.6)	310 (82.4)	
2	Feb 15	1 (5.9)	16 (94.1)	15.96, <0.001
2	Feb 16	0	36 (100)	49.91, <0.001
2	Feb 17	1 (7.1)	13 (92.9)	12.2, <0.001
2	Feb 19	0	24 (100)	33.27, <0.001
2	Feb 23	3 (7.3)	38 (92.7)	35.37, <0.001
2	Feb 25	1 (3.1)	28 (96.6)	31.50, <0.001
2	Mar 2	2 (4.2)	46 (95.8)	49.91, <0.001
2	Mar 9	12 (18.5)	53 (81.5)	27.97, <0.001
2	Mar 11	25 (52.1)	23 (47.9)	0.08, 0.773
2	Mar 15	21 (38.9)	33 (61.1)	2.69, 0.101
3	Pooled	1 (0.98)	101 (99.02)	
3	Feb 11	0	21 (100%)	29.11, <0.001
3	Feb 12	0	39 (100%)	54.07, <0.001
3	Feb 20	1 (2.4)	41 (97.6)	48.77, <0.001
4	Pooled	27 (87.1)	4 (12.9)	
4	Feb 7	18 (85.7)	3 (14.3)	11.89, <0.001
4	Feb 28	9 (90)	1 (1)	7.36, <0.01
5	Pooled	70 (72.2)	27 (27.8)	
5	Feb 10	0	15 (100)	20.79, <0.001
5	Feb 11	36 (90)	4 (10)	29.45, <0.001
5	Feb 17	34 (81)	8 (19)	17.32, <0.001

Table 3. Full statistics for deformity, hatch, and starvation data of Atlantic cod *Gadus morhua* collected from the in vitro fertilization experiment: mean (± standard error; standard deviation for time to starvation) for related and unrelated crosses, the T or F statistic, and P value ($\alpha < 0.05$).

Variable	Mean, Inbred	Mean, Unrelated	Statistic	P Value
Percent occurrence of embryo deformities	77.1 (±10.46)	71.9 (±14.92)	$T_{(1,8)} = 0.95$	P = 0.395
Average number of malformations per deformed embryo	3.1 (±0.99)	2.6 (±0.90)	$T_{(1,8)} = 1.41$	P = 0.23
Percent of larvae with deformities	30.6 (±6.91)	34.9 (±7.67)	$F_{(1,28)} = 0.33$	P = 0.57
Average number of deformities on each deformed larva	0.5 (±0.14)	0.4 (±0.09)	$F_{(1,28)} = 2.84$	P = 0.104
Percent hatch	33.7 (±7.20)	42.2 (±7.39)	$F_{(1,9)} = 7.3$	P = 0.024
Time to starvation (days)	15.4 (±1.97)	16.8 (±1.95)	$F_{(1,553)} = 1.31$	P = 0.254

Table 4. Percent of each type of Atlantic cod *Gadus morhua* embryo and larval deformity found in the inbred and unrelated crosses for both inbred and unrelated crosses combined. There was no significant difference between inbred and unrelated offspring.

Deformity	Inbred	Unrelated
Embryos		
Incomplete intercell adhesion	18.2	19.9
Vacuolar inclusions between cells	7.7	4.3
Cell margins poorly defined	15.8	14.0
Unequal cell size	20.6	20.4
Asymmetry	20.6	22.0
Jumbled	3.4	2.2
Overlapping	1.0	0.0
Extra cell bodies	2.9	2.7
Offset/intermediate	3.8	7.0
Triple	3.4	3.8
Pie	1.0	1.6
Marginal	1.9	2.2
Larvae		
Bent tail	59.2	75.4
Bent midsection	22.5	7.7
Curved spine	9.9	7.7
Malformed yolk sac	8.5	9.2

Table 5. Three-way ANOVA table for the response variable of myotome height of Atlantic cod *Gadus morhua* larvae as influenced by the variables of relatedness (Trt), family, and day (one and five dph), showing interaction terms. The factors day and relatedness are present to control for variation.

Source	DF	Seq SS	Adj SS	MS	F	P
Day	1	0.001	0.001	0.001	14.69	<0.0001
Trt	1	0.001	0.001	0.001	7.72	0.010
Family	9	0.021	0.021	0.002	34.19	<0.0001
Error	28	0.002	0.002	0.000		
Total	39	0.025				

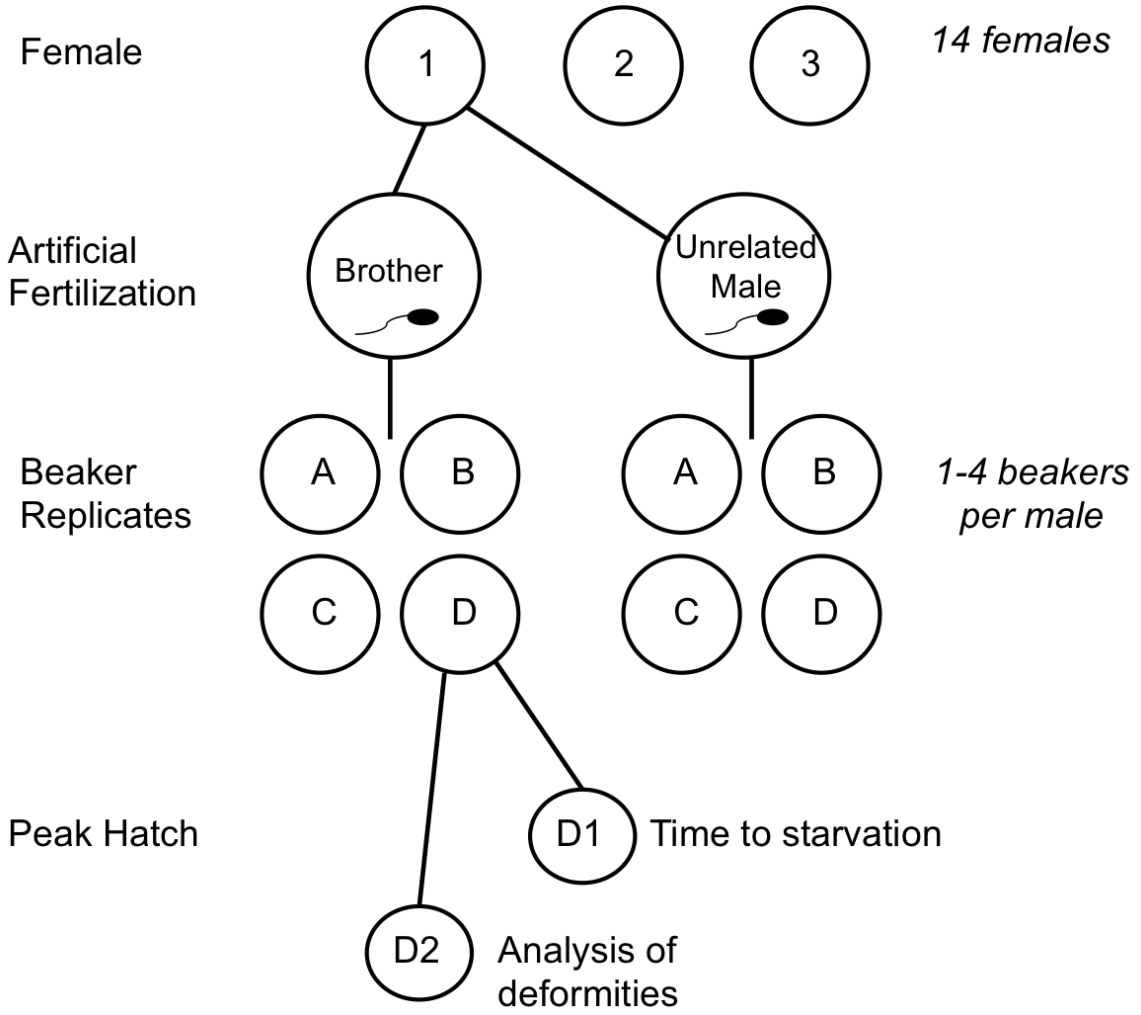

Figure 1. Illustration of artificial fertilization process. Eggs from each female Atlantic cod *Gadus morhua* were crossed with sperm from a full-sibling brother and an unrelated male and incubated overnight. Embryos from each cross were divided equally into 250ml beakers until hatch. At peak hatch the larvae from each beaker replicate were removed and subdivided into 50ml beakers for measurement of deformities and time to starvation.

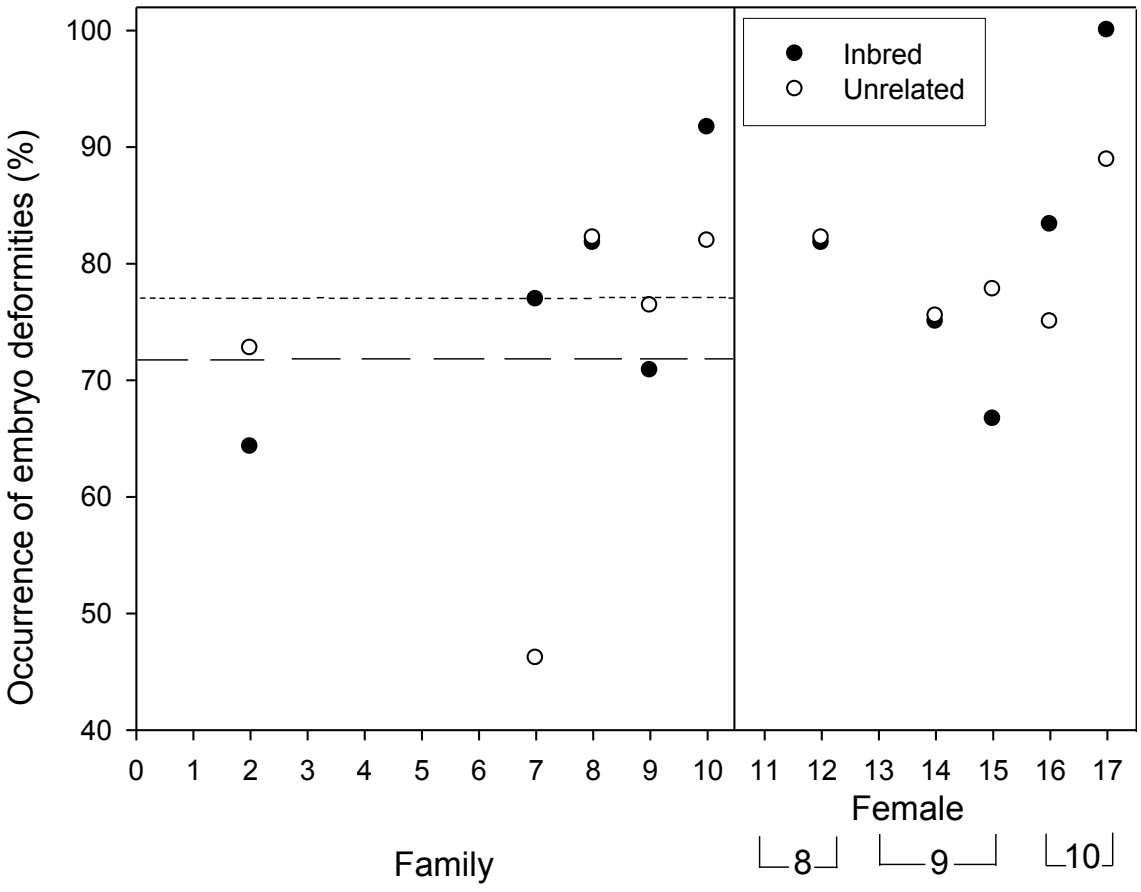

Figure 2. Occurrence of embryo deformities (percent) in the seven crosses of inbred (dark circle) and unrelated (open circle) Atlantic cod *Gadus morhua* that were photographed at the four cell stage. Families 1, 3-5, 6, and one female in family eight could not be photographed at the four cell stage. The dashed line indicates the mean for unrelated offspring and dotted line indicates the mean for inbred offspring. Families with more than one female are separated by individual female in the right panel, with the family indicated by the number bracket, and the females within that family directly along the x axis. The occurrence of embryo deformities was a percentage determined by one sample from each

female before the embryos had been separated into multiple beakers, therefore standard error could not be calculated.

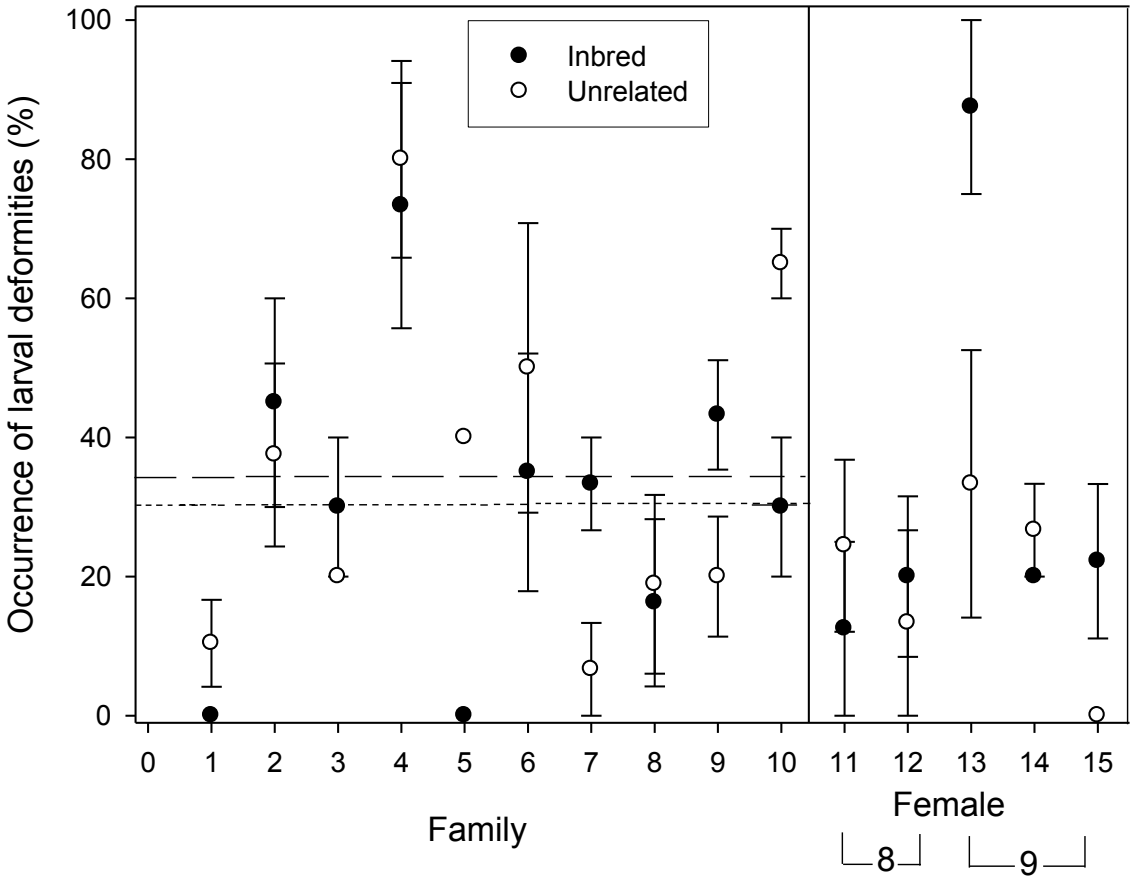

Figure 3. Mean occurrence of larval deformities (percent, with standard error) of offspring from inbred (black circle) and unrelated (open circle) crosses of Atlantic cod *Gadus morhua* at hatch. The dashed line indicates the mean for unrelated offspring and dotted line indicates the mean for inbred offspring. Families with more than one female are separated in the right panel, with the family indicated by the number bracket, and the females within that family directly along the x axis. Samples were taken from each of the replicate beakers for each female, allowing the standard error to be calculated.

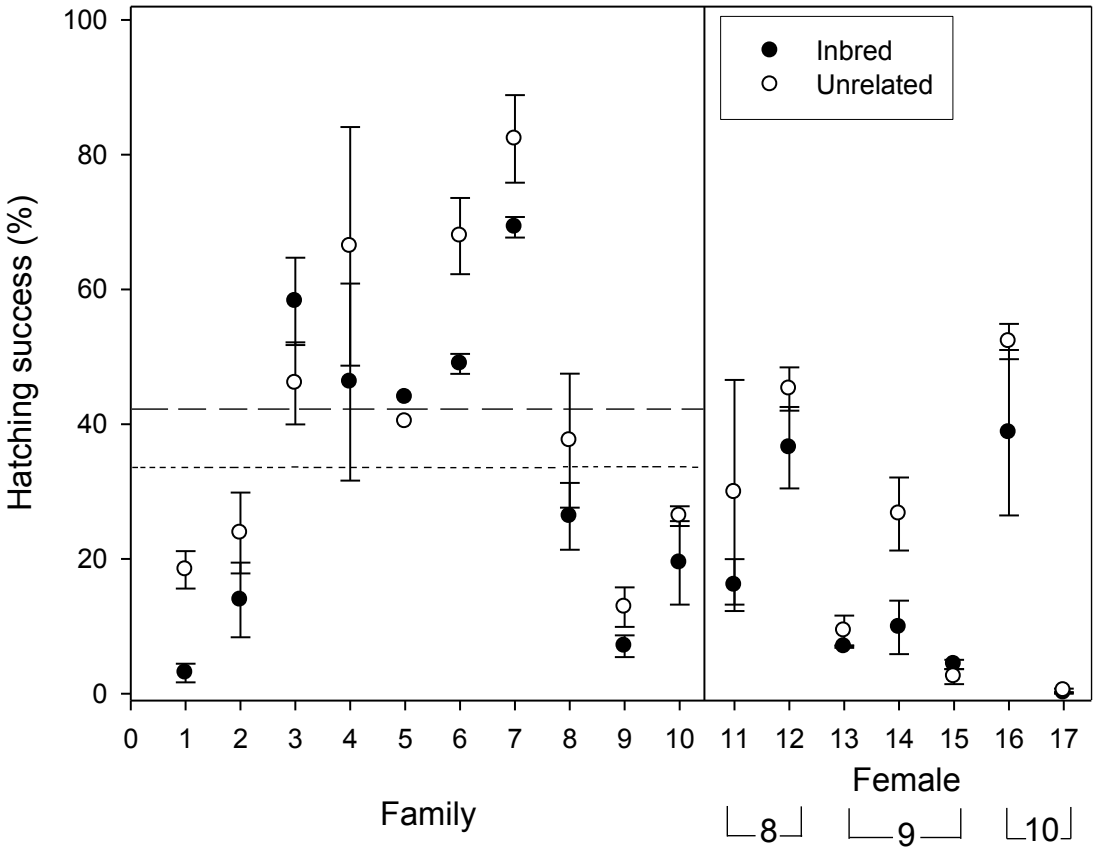

Figure 4. Mean hatching success (percent, with standard error) of offspring from inbred (black circle) and unrelated (open circle) crosses of Atlantic cod *Gadus morhua*. The dashed line indicates the mean for unrelated offspring and dotted line indicates the mean for inbred offspring. Families with more than one female are separated in the right panel, with the family indicated by the number bracket, and the females within that family directly along the x axis.

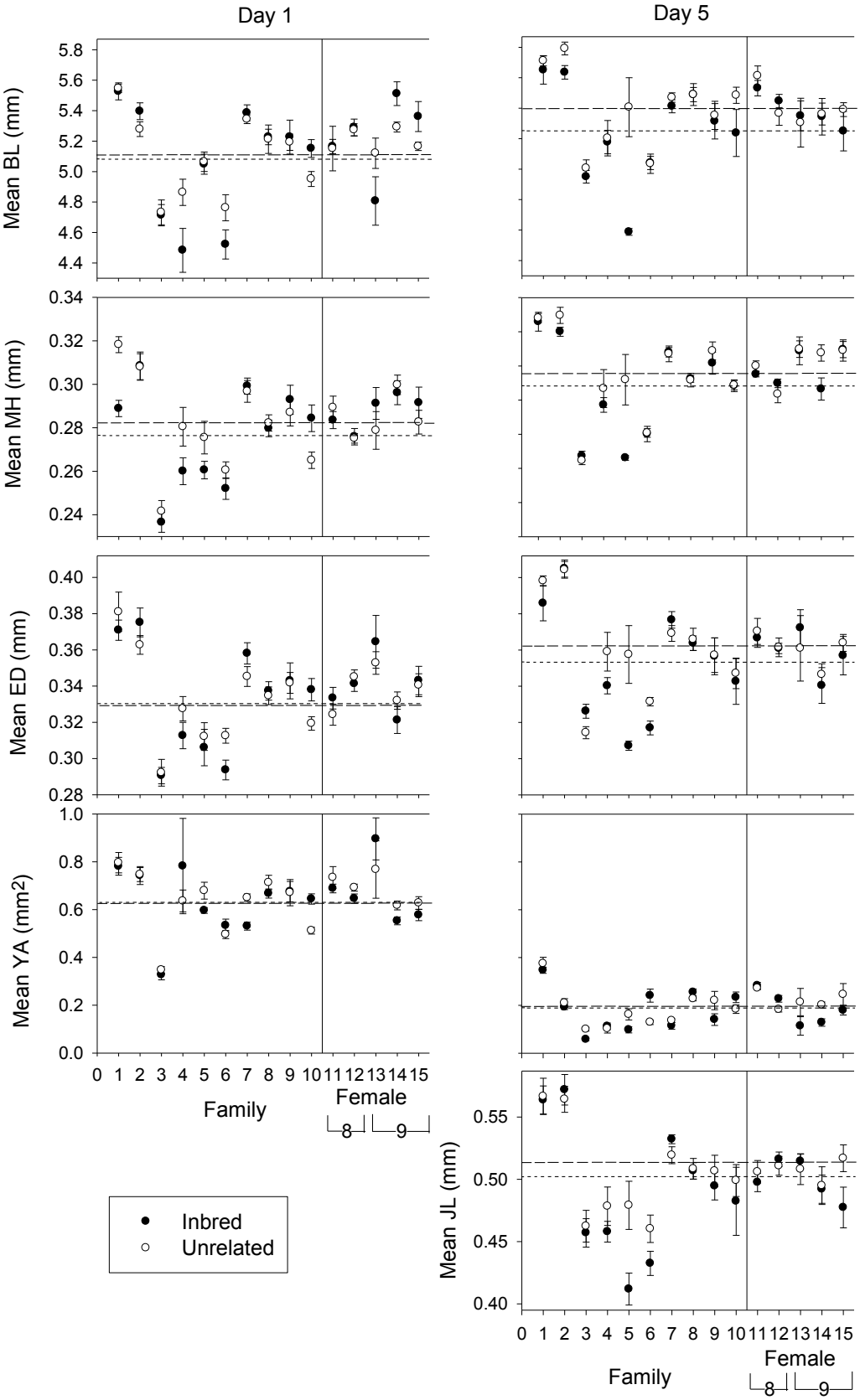
Day 1
Day 5
Mean BL (mm)
Mean MH (mm)
Mean ED (mm)
Mean YA (mm²)
Mean JL (mm)
Family
Female
8
9
Inbred
Unrelated

Figure 5. Mean body length (BL), myotome height (MH), eye diameter (ED), yolk area (YA) and jaw length (JL) (± standard error) of inbred (dark circle) and unrelated (open circle) Atlantic cod *Gadus morhua* larvae on day 1 and day 5 (jaw length at day 5 only). The dashed line indicates the mean for unrelated offspring and dotted line indicates the mean for inbred offspring. Families with more than one female are separated in the right panel, with the family indicated by the number bracket, and the females within that family directly along the x axis.

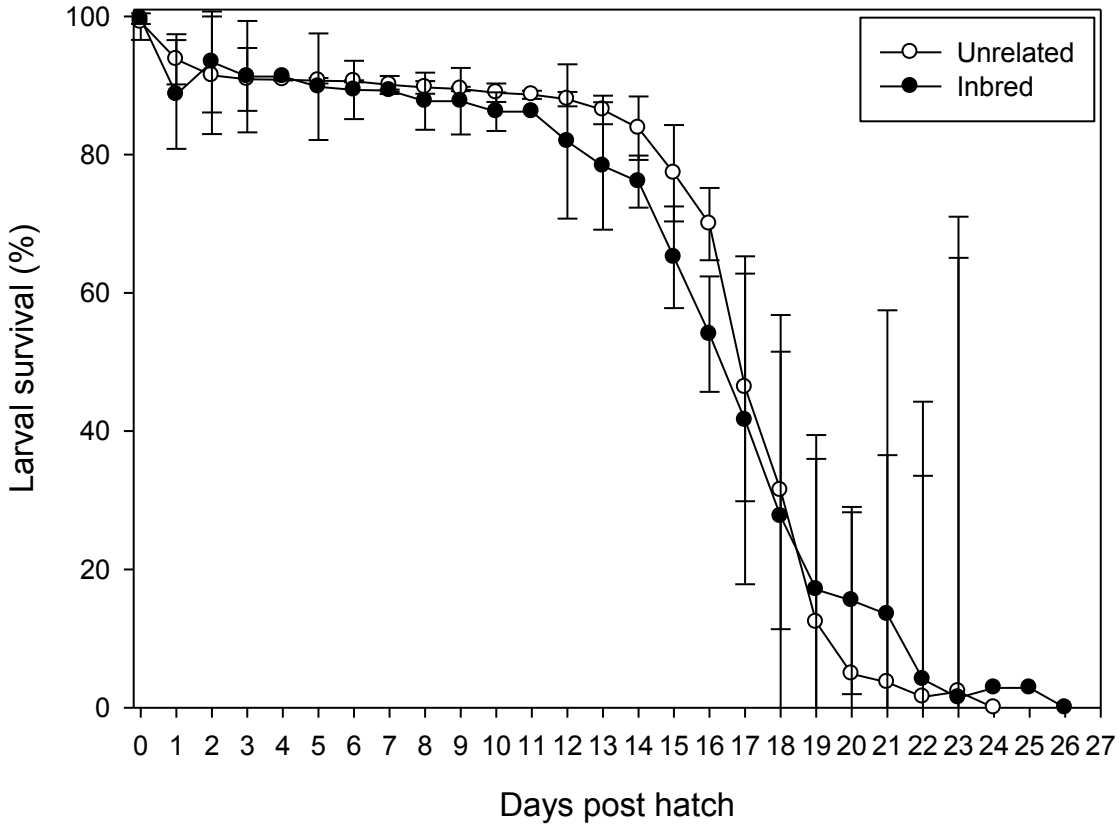

Figure 6. Mean cumulative larval survival (percent, with standard deviation) of offspring from inbred (black circle) and unrelated (open circle) crosses of Atlantic cod *Gadus morhua* after hatch.

5. DISCUSSION

There was no collective bias in paternity from the *in vivo* experiment. However, the brother in each of the five spawning tanks sired embryos, indicating that sib mating occurred. Given that cultivated Atlantic cod inbreed and that individuals in sea cages spawn, there may be inbred farmed embryos escaping through the cage netting into the environment. Inbreeding has been studied on a number of fish species, including guppies (Viken et al. 2006, Fitzpatrick et al. 2014), haddock (Trippel et al. 2009), and rainbow trout among others (Kincaid 1983). Inbreeding actually occurred in haddock (Trippel et al. 2009) and wild populations of rainbow trout (Kincaid 1983).

There are several potential limitations to the *in vivo* experiment. Along with being only one generation removed from the wild broodstock, there could have been physical, chemical, or behavioural differences between males in a tank rather than relatedness that influenced female mate choice. However, in this experiment it is impossible to tell if the female actually chose a specific mate, as with the small size of the tanks it was possible for both males to release sperm and fertilized eggs, as occurred in all of the tanks. Paternity could have been influenced by three things: the timing of gamete release, which is behavioural and may be related to mate choice (Brawn 1961a); spatial matching of gamete release, where the female chooses to release her gametes close to her chosen mate, but the other male also releases its gametes in close proximity, as the small tanks allow (Nordeide & Folstad 2000, Rudolfsen et al. 2005); and finally, sperm quality of each male could differ substantially, a non-behavioural factor affecting the likelihood of siring eggs (Rudolfsen et al. 2008, Skjæraasen et al. 2009). Males in the study were closely matched by colour, length, weight, and condition factor; however, behavioural

dominance through aggressive interactions from one male toward the other may have played a role in male fertilization success (Brawn 1961b, Hutchings et al. 1999, Rowe et al. 2008). In addition, only half of the females spawned, reducing the sample size. Future studies may wish to increase the sample size, using fish from different origins and history, particularly a long maintained domesticated broodstock, perform the experiment in sea cages with more potential mates for the females to choose from, and collect embryos from multiple spawning pairs within each tank. In our experiment, while determining that mating between siblings occurs among cultivated cod, we did not determine whether or not Atlantic cod have kin recognition; future studies may wish to address this as well.

The percent of embryos that hatched from unrelated parents was significantly higher than those that hatched from related parents, though the difference was modest. The same has been reported in inbred wild rainbow trout, where inbreeding reduced hatch success by 53% (Kincaid 1983), and in threespine stickleback where inbred fish had lower fertilization success and decreased hatch success than their non-inbred counterparts (Frommen et al. 2008). These results suggest that inbreeding could decrease farmed offspring viability, and therefore the likelihood of escaped embryos surviving to maturity, reducing the number of farmed cod interacting with their wild counterparts.

Although the presence of deformities in the embryos and larvae was extremely common, there was no significant difference in prevalence or severity of deformities between offspring from related and unrelated parents. However, embryos were only analyzed for deformities at the four cell stage, and larvae at hatch. While there was no significant difference in the occurrence of deformities, hatching success between inbred and non-inbred crosses differed. It could be that early deformities are corrected before

hatch in inbred and unrelated offspring, while fatal deformities may form later in development in inbred offspring, affecting hatch (Vallin & Nissling 1998). Normal development of a cod embryo involves cellular cleavages which form symmetrical cells until approximately the 16 or 32 cell stage, at which point symmetry is lost (Avery et al. 2009). Unusual cell cleavage has been associated with egg mortality, reduced hatch rates, and deformities in larval cod (Pickova et al. 1997, Vallin & Nissling 1998). Cleavage deformities may include asymmetrical blastomeres, poor adhesion between cells, and blastomeres of different size and shape (Shields et al. 1997). Depending on when abnormalities occur, some can be serious and result in cell death, while less serious deformities can potentially be corrected (Vallin & Nissling 1998, Avery et al. 2009). Vallin & Nissling (1998) found that deformed embryos are capable of hatching as normal, active larvae. The ability to follow individual embryos through all stages of development would have allowed us to determine if any of the embryo deformities corrected themselves before hatch.

Larval deformities in aquaculture specimens such as rainbow trout and greenback flounder can be recognized as early as hatch, while some are not noticeable until they reach harvest size (Aulstad & Kittelsen 1971, Gjerde et al. 1983, Hart & Purser 1995, Yousefian & Nejati 2008). Abnormalities may be caused by infections and a range of environmental factors such as low oxygen levels and non-optimal temperatures, as has been found in rainbow trout and herring (Holliday & Blaxter 1960, Aulstad & Kittelsen 1971, Hart & Purser 1995). One generation of full-sibling inbreeding in rainbow trout can also cause deformities (Aulstad & Kittelsen 1971, Yousefian & Nejati 2008), while other studies have found no relation between first generation inbreeding and the

occurrence of deformities (Gjerde et al. 1983). Aulstad and Kittelsen (1971) found lethal deformities in rainbow trout in the form of body curvatures just after the resorption of the yolk sac. Curvature of the body can be the result of a deformed vertebral column or abnormal muscle configuration (Aulstad & Kittelsen 1971, Yousefian & Nejati 2008). Other deformities found in aquaculture specimens, particularly rainbow trout, include a shortened tail (Gjerde et al. 1983), jaw deformities (Hart & Purser 1995), and length at hatch, and can affect time of first feeding in herring (Holliday & Blaxter 1960) and long term growth (Yousefian & Nejati 2008). Even a fish with normal appearance may be deformed depending on the location and seriousness of the deformity (Gjerde et al. 1983).

Time to starvation and the other size measurements (body length, eye diameter, yolk area, and jaw length) did not differ significantly between the inbred and non-inbred offspring. In the half-sibling families there was very little consistency among females regarding deformities. In one family the inbred offspring had the most embryo deformities, while the non-inbred offspring had the most in another family. Among sisters from three families there was no consistency within each family for larval deformities. Although untested, this could potentially have been the result of paternity effects from each of the different sires. Paternity can have a significant impact on early life history traits in haddock, including hatching success (Rideout et al. 2004). Given these results, it would be interesting to repeat the experiment with a focus on paternity effects, as well as a greater number of families and a higher number of females within each family to determine if the same inconsistency would be found, or if there would be a noticeable family effect.

Some of the limitations to the *in vitro* experiment were that the embryos and

larvae did not face any of the potential stressors that the sea cage or wild environment may present, like changes in water temperature for example, which could influence hatch success. Due to constraints in time and resources, deformities were only measured in embryos at the 4-cell stage and larvae at hatch, size measurements were limited to hatch and day 5, and offspring survival was only measured to starvation. It is possible that had the resources been available to rear the larvae and examine deformities, size, and survival of individual offspring on a long-term basis, there may have been some observable differences between those of related and unrelated parents. However, most mortality typically occurs over the period measured.

There is the possibility that had the resources been available to rear the larvae and examine deformities, size, and survival on a long-term basis, there may have been some observable difference between offspring of related and unrelated parents. Differences in body size of inbred and non-inbred fish have been noted in Alaskan steelhead after several years at sea (Thrower & Hard 2009). One study showed that in relation to maternal effects of larval body size of cod, variation seen in juveniles was still apparent in offspring nearing two years old (Tosh et al. 2010). Another study used otolith back-calculations to determine that fast growing larvae had better chances of survival in the long term (Campana 1996). Similarly, differing body weights and lengths of sea bass *Dicentrarchus labrax* L. caused by maternal effects were still present at harvest (Dupont-Nivet et al. 2008). If the larvae were reared for a much longer time, subsequent measurements might show that any initial differences could be an indication of long-term size differences, with higher overall growth in non-inbred individuals producing a larger cod with better chances of survival and reproductive success.

As has been seen with Atlantic salmon, aquaculture could be responsible for two means of genetic pollution through introgression- the introduction of farm-selected and also potentially non-native genes into wild populations (McGinnity et al. 1997, Edmands 1999, Fraser et al. 2008). If the fish have been intensely selected for domestication, they can introduce domestic adaptations that disrupt local adaptations of wild cod (Hindar et al. 1991). In Canada, while there was cod farming the fish reached F2 generation, while in other programs it may be hard to tell just how domesticated the cod are as wild cod may be added to 'refresh' the broodstock. Even if cod are not overly domesticated, in many cases the cod in sea cages are not local to the cage site (Bekkevold et al. 2006) and may be maladapted to the environmental conditions outside of the sea cages where they are not fed or protected from predators (Fleming et al. 2000, McGinnity et al. 2003, Read & Fernandes 2003, Bekkevold et al. 2006, Hutchings & Fraser 2008). There is the risk that they will mate with wild cod and cause outbreeding depression to occur, where the hybrid offspring, regardless of inbreeding, may be less suited to survival and may be more vulnerable to changes in the environment that may include changes in water temperature, predation, disease, and food availability (Weir et al. 2005, Hutchings & Fraser 2008, Jensen et al. 2010). Along with escapes through spawning, it is important to note that a rip in the net or any other cage damage would most likely result in stocked cod escaping (Jørstad et al. 2008, Jensen et al. 2010, Zimmermann et al. 2012). Due to past fishing efforts, many wild cod populations are already struggling (Hutchings 1991, COSEWIC 2010) and interbreeding with escaped farmed cod could be detrimental to their overall survival, although more research is necessary to confirm this possibility.

Inbreeding has been shown to have a wide range of negative impacts on rainbow

trout (Cooper 1961), Atlantic salmon, carp *Cyprinus carpio* L. (Moav & Wohlfarth 1976) and brook trout *Salvelinus fontinalis*, producing offspring that are less fit for survival than their non-inbred counterparts, even after just one generation of full-sibling mating (Aulstad and Kittelsen 1971, Kincaid 1976a,b, Mrakovčič & Haley 1979, Gjerde et al. 1983). If that is the case with farmed Atlantic cod as well, should any of the escaped embryos survive to maturity as documented in Norwegian fjords (van der Meeren et al. 2012), there is the possibility that they may perform poorly compared to wild adult fish, although there is no clear evidence of this in cod yet. The results of this experiment indicate that there are no major effects of inbreeding in the earliest life stages of the F1 generation. This has also been the case in inbred Atlantic salmon (Houde et al. 2011). However, the reduced hatch rates of inbred offspring may help to mitigate some of the problem of egg escape. The full scope of inbreeding effects on the F1 generation may be better determined over a longer period of time with fed larvae.

Stocking the cages entirely with full-sibling fish to ensure inbreeding could be a means of reducing the occurrence of farmed-wild cod interactions, at least through the escape of embryos. Or better yet, if an efficient production method could be established, cages could potentially be stocked with only one gender of fish so no spawning takes place. Unrelated to inbreeding, one particular area in which stocking cages with one extended family of cod could have a very positive effect is in the ability to trace escaped cod back to specific farms, perhaps encouraging more responsible farming practices (Glover et al. 2011). There may be concerns about the genetic homogenizing potential on the receiving wild cod population if large numbers of fish from one family escaped from a cage, but as it stood in Canadian cod aquaculture while there were active cod farms, there

was already very little family variation in each cage (G. Nardi, GreatBay Aquaculture, pers. comm. 2011). As mentioned earlier, some work has already been carried out help minimize the occurrence of genetic introgression when farmed cod escape. This includes triploidy (Feindel et al. 2011) and photoperiod manipulation (Trippel et al. 2011a).

As long as cod spawn in sea cages, embryos will escape. However, if the difference in hatch success seen in the *in vitro* experiment translates to sea cage situations, this could be a start to reducing the impact of escapes through spawning. At this point in time, more research is needed on cod in general, as much of the available research is on salmonids, the life-history traits of which are very different from those of Atlantic cod. It is important to determine what, if any, are the long-term effects of inbreeding on first generation offspring, as well as further tools to reduce the negative effects of aquaculture on wild Atlantic cod populations.

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7. APPENDIX

Table A1. Mean Atlantic cod *Gadus morhua* larval trait measurements on day 1 and day 5 for offspring of related and unrelated parents (± standard error), as well as corresponding P values ($\alpha = 0.01$) and F or T statistic. Jaw length was only measured on day 5 as the jaw is not fully developed at hatch.

			Day 1		Day 5	
Trait	P Value	Statistic	Inbred	Unrelated	Inbred	Unrelated
Total body length (mm)	0.118	$F(1,28) = 2.61$	5.032 (±0.1070)	5.066 (±0.0912)	5.250 (±0.1041)	5.395 (±0.0816)
Myotome height (mm)	0.010	$F(1,28) = 7.72$	0.276 (±0.0073)	0.284 (±0.070)	0.287 (±0.0090)	0.294 (±0.0085)
Eye diameter (mm)	0.155	$F(1,28) = 2.13$	0.333 (±0.0097)	0.333 (±0.0082)	0.352 (±0.0099)	0.360 (±0.0086)
Yolk area (mm^2)	0.736	$F(1,28) = 0.12$	0.628 (±0.0440)	0.637 (±0.0408)	0.179 (±0.0284)	0.185 (±0.0257)
Jaw length (mm)	0.095	$T(1,18) = 1.87$	-	-	0.491 (±0.0169)	0.504 (±0.0119)

Table A2. Three-way ANOVA table for the response variable of body length of Atlantic cod *Gadus morhua* larvae as influenced by the variables of relatedness (Trt), family, and day (one and five dph), showing interaction terms. The factors day and relatedness are present to control for variation.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	0.747	0.747	0.747	23.95	<0.0001
Trt	1	0.081	0.081	0.081	2.61	0.118
Family	9	2.512	2.512	0.279	8.95	<0.0001
Error	28	0.873	0.873	0.031		
Total	39	4.212				

Table A3. Three-way ANOVA table for the response variable of eye diameter of Atlantic cod *Gadus morhua* larvae as influenced by the variables of relatedness (Trt), family, and day (one and five dph), showing interaction terms. The factors day and relatedness are present to control for variation.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	0.005	0.005	0.005	57.83	<0.0001
Trt	1	0.000	0.000	0.000	2.13	0.155
Family	9	0.027	0.027	0.003	32.32	<0.0001
Error	28	0.003	0.003	0.000		
Total	39	0.036				

Table A4. Three-way ANOVA table for the response variable of yolk area of Atlantic cod *Gadus morhua* larvae as influenced by the variables of relatedness (Trt), family, and day (one and five dph), showing interaction terms. The factors day and relatedness are present to control for variation.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	2.030	2.030	2.030	448.79	<0.0001
Trt	1	0.001	0.001	0.001	0.12	0.736
Family	9	0.330	0.330	0.037	8.1	<0.0001
Error	28	0.127	0.127	0.005		
Total	39	2.487				

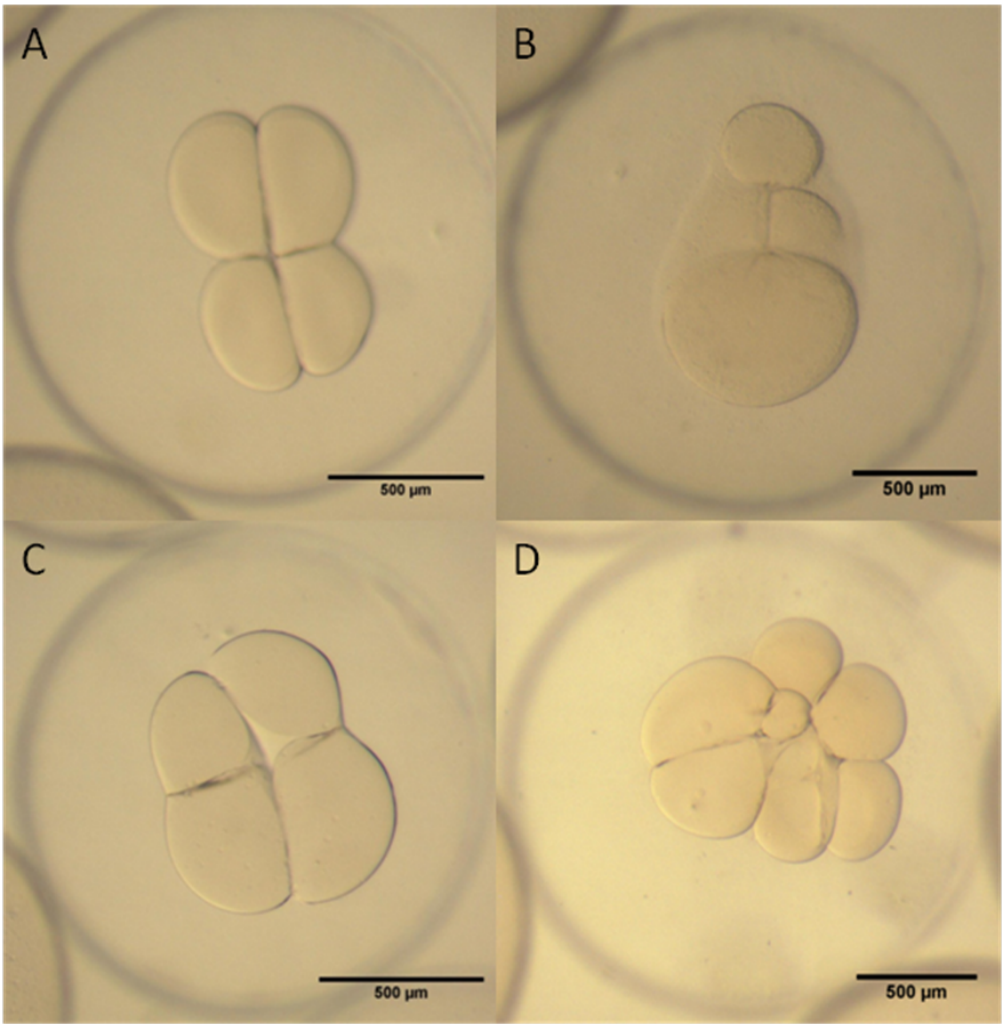

Figure A1. Photographs of Atlantic cod *Gadus morhua* embryos around the four-cell stage. Photograph A shows a normal embryo at the four cell stage and was used for comparison to determine the presence of deformities in other embryos. The embryos pictured in B-D had the following deformities: B- asymmetrical, unequal cell size, cell margins poorly defined, incomplete inter-cell adhesion, marginal, C- asymmetrical, unequal cell size, vacuolar inclusions between cells, D- asymmetrical, unequal number of cells and cell size, vacuolar inclusions between cells, incomplete inter-cell adhesion, jumbled. Scale bars represent 500 µm.

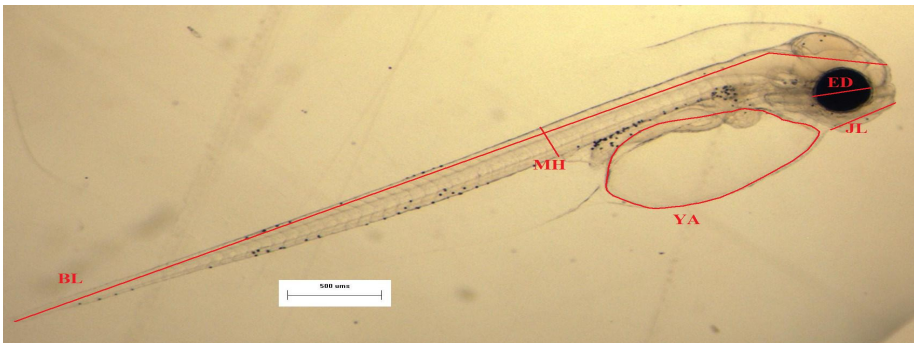

Figure A2. Illustration of measurements recorded on Atlantic cod *Gadus morhua* larvae at hatch and day 5 (jaw length at day 5 only). BL- body length, MH- myotome height, ED- eye diameter, YA- yolk area, JL- jaw length.

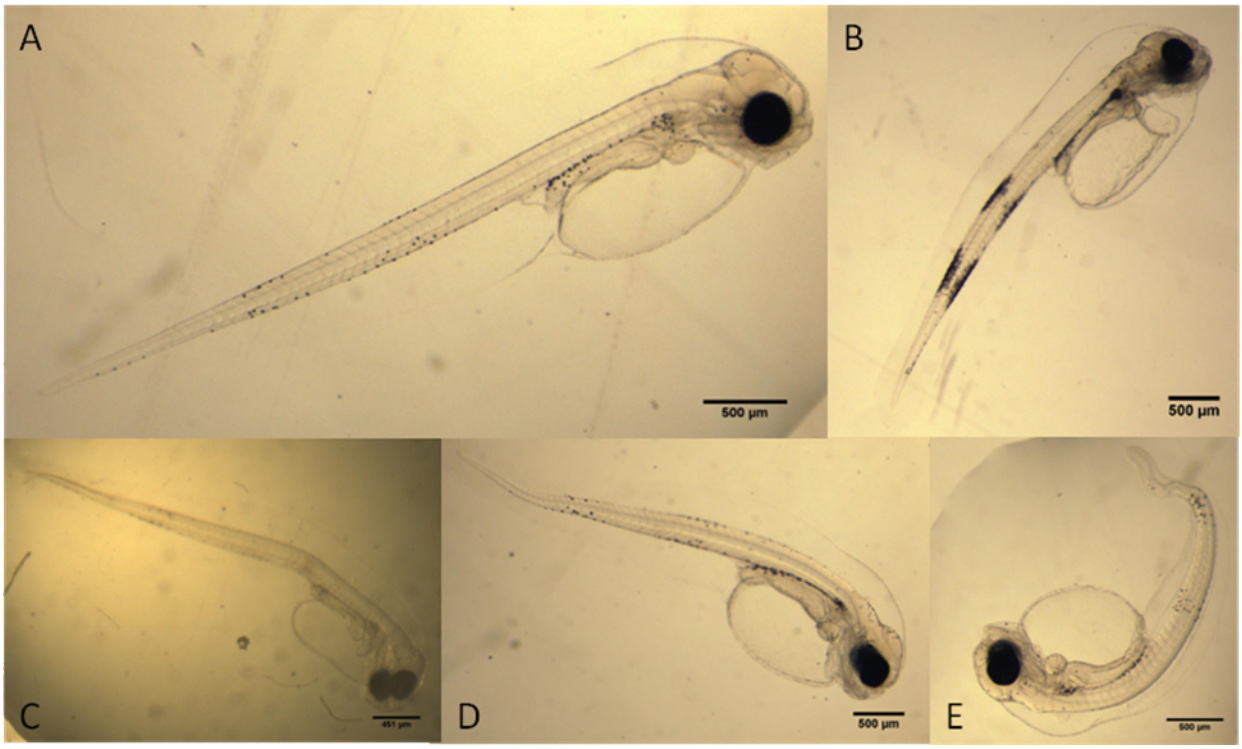

Figure A3. Photographs of Atlantic cod *Gadus morhua* larvae at hatch (Day 1). A- Normal larva used as reference for identifying deformities in other larvae, B- larva with malformed yolk, C- larva with bent midsection, D- larva with bent tail, E- larva with curved spine and severely bent tail. Scale bar in each photo represents 500 µm.