Pre- and post-mating selection on male capelin

by

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Abstract

All animals, as sexual eukaryotes, undergo a biphasic lifecycle characterized by alternation between haploid and diploid phases, wherein selection occurs in both pre- and post-mating stages. The first research chapter investigated male capelin reproductive potential through semen assessment during the spawning season, aiming to test the hypothesis that male-biased body size dimorphism results from endurance rivalry, which is a form of pre-mating selection. Findings revealed that a majority of capelin exhibited abundant semen during the spawning season. Capelin demonstrated the ability to regenerate semen within two days and did not show a decline in gamete ability for fertilization during six days of captivity. This continuous readiness for mating by maintaining semen supports the hypothesis of male-biased sexual dimorphism attributed to endurance rivalry. The second research chapter examined the effects of post-ejaculation pre-fertilization sperm experiences (environmental conditions) on embryo development and, if adaptive, its potential underlying mechanisms. Capelin sperm and embryos are sensitive to salinity and represent a good system for investigating this phenomenon. Contrary to prior research findings in other fish species, this study found that post-ejaculation semen salinity experiences had no effect on embryo development. Further investigations are warranted to elucidate the mechanisms and factors influencing the mating readiness and reproductive resilience of male capelin, as well as to determine whether capelin sperm experiences beyond salinity exposure exert an influence on offspring development.

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Chapter 1. General introduction

Sexual reproduction overview

Reproduction is a biological process to produce offspring and occurs through asexual and sexual modes. Asexual reproduction requires a single individual and results in genetically identical offspring without fertilization. In contrast, sexual reproduction requires the involvement of two opposite and compatible gametes combining genetic material (usually two individuals), resulting in genetically unique offspring (Lombardi, 1998). Sexual reproduction is universal in eukaryotes, characterized by an alternation of haploid and diploid phases, known as the biphasic life cycle (Hughes & Otto, 1999; Mable & Otto, 1998; Purchase et al., 2021). The diploid phase undergoes meiotic division to produce the haploid gametes, which are known as sperm for males and egg for females. During fertilization, a sperm and egg fuse to form a diploid zygote (Lombardi, 1998; Sawada et al., 2014).

Following zygote formation, pre-mating selection acts on zygote-to-adult survival, gamete production, and mating (Andersson, 1994; Darwin, 1859, 1872; Purchase et al., 2021). This selection can have a sex-dependent impact on adult size, resulting in sexual size dimorphism, which is widespread in the animal kingdom (Andersson, 1994; Janicke & Fromonteil, 2021). The direction of the dimorphism, i.e., which sex is larger, varies depending on the mating system (Fairbairn, 1997; Pyron et al., 2013; Trivers, 1972). Females being larger is often associated with fecundity advantages, as larger females have higher energy reserves, allowing greater investment in egg production (Parker, 1992; Webb & Freckleton, 2007; but see Shine, 1988). Conversely, males being larger is often an advantage in mate competition and mate choice (Bisazza, 1993; Horne et al., 2020). Empirical studies across diverse animal taxa demonstrate that large males have an advantage in dominant contests and competitive

interactions for access to females (Bateman, 1948; Janicke & Fromonteil, 2021). The benefits can also include endurance rivalry, facilitating prolonged stay at spawning sites and thus improving mating probabilities (Ellis & Bercovitch, 2011; Orbach et al., 2019). Furthermore, it can also help in sperm competition, especially in external fertilization, by usually releasing large amounts of sperm (Parker, 1992).

Following gamete release, post-mating selection plays a crucial role in influencing gamete survival, fertilizing capability of gametes, and fertilization, which is the fusion of two gametes known as syngamy (Evans & Garcia-Gonzalez, 2016; Purchase et al., 2021). Within the context of fertilization, paternity of embryos is influenced through sperm competition and cryptic female choice. Sperm competition involves the struggle among sperm from different males to fertilize the same egg (Parker, 1970, 2020), whereas cryptic female choice biases the outcome of sperm competition (Eberhard, 1996; Firman et al., 2017).

Cross-phase effects

Both pre-mating and post-mating selection can have an impact within a phase (haploid or diploid) of the same individuals, known as within-phase trade-offs, and can extend to the subsequent phase, referred to as across-phase one-way bridges (Purchase et al., 2021). The latter can be termed "cross-phase effects" to broaden the scope of impact beyond the subsequent phase. Specifically, experiences during the diploid phase influence outcomes in the haploid phase, such as gamete-mediated parental effects (Badyaev & Uller, 2009). Conversely, experiences during the haploid phase also impact outcomes in the diploid phase, e.g. "sperm experiences" impacting embryo development (see reviews Crean & Immler, 2021; Purchase et al., 2021).

Parental effect: maternal vs paternal effects

Parental effects refer to the influence exerted by parents on their offspring beyond the transmission of genetic material (Badyaev & Uller, 2009; Mousseau & Fox, 1998). This effect includes direct influences such as resource provisioning, parental care, the natal environment, and indirect factors like epigenetic components passed to offspring through the gametes (Burgess & Marshall, 2014; Purchase et al., 2021). These diverse influences can significantly shape the offspring's phenotype and development. In early life stages, offspring are more vulnerable and have a higher chance of mortality, particularly in external developers due to direct environmental exposure (Crean & Immler, 2021; Evans et al., 2019). In such contexts, if parents can anticipate their offspring's environmental conditions and adjust their gametes' content and thus better equip their young to survive and thrive in these environments, then it would be an adaptive gamete-mediated parental effect (Burgess & Marshall, 2014; Jensen et al., 2014; Venney et al., 2022).

Among gamete-mediated parental effects, the maternal effect is often more pronounced than the paternal effect, particularly in the initial stages of an embryo's development, which heavily rely on the egg's content. This is largely due to the anisogamous nature of reproduction, where eggs, unlike sperm, are endowed with essential nutrients necessary for the offspring's early growth, especially until they develop the ability to feed independently (Jensen et al., 2014; Mousseau & Fox, 1998; Wolf & Wade, 2009). The substantial allocation of resources to eggs results in a more direct and significant impact on the offspring's development, especially during the critical early life history stages (Einum & Fleming, 1999). Moreover, the maternal contribution encompasses not just nutrition but also epigenetic components that are vital for the embryo's early developmental processes (Labbé et al., 2017). Additionally, hormones and other

biochemical factors within the egg play a crucial role in shaping the embryo's developmental path, thereby laying the basis for important physiological and behavioural characteristics in the offspring (Lubzens et al., 2010; Mommens et al., 2015).

Traditionally, it was thought that sperm, being small, solely functioned to transfer a packaged and inert paternal genome to the offspring. However, recent evidence points to a more complex paternal role, suggesting that sperm contribute more than just genetic material (Crean et al., 2013; Crean & Bonduriansky, 2014; Evans et al., 2017; Immler, 2018). This challenges the traditional view that paternal influence on offspring is confined to DNA transmission through sperm. Sperm, in addition to genetic material, also carry proteins, RNAs, and other molecular factors that may play crucial roles in offspring development, impacting gene regulation and developmental processes in the offspring (Immler, 2018; Johnson et al., 2011). The seminal fluid accompanying sperm is rich in hormones, signalling molecules, and nutrients, which may also influence the offspring's development (Kekäläinen et al., 2020; Simmons et al., 2022; Simmons & Lovegrove, 2019). Furthermore, changes in father conditions, such as dietary changes, hormonal profile alterations, sperm storage, modified social interactions or external abiotic conditions, can affect the content of their semen. Such changes in the composition of their semen can impact offspring development (Butzge et al., 2021; Evans et al., 2019; Ragsdale et al., 2022; Simmons et al., 2022).

Thus, both gamete-mediated maternal and paternal effects can impact offspring development, but the extent to which they affect offspring development represents a significant research gap (Bonduriansky & Crean, 2018).

Sperm post-ejaculation experiences

Sperm are released into foreign environments, either within the female reproductive tract or the external environment, in order to fertilize eggs. In the case of external fertilizers, the environment encountered by sperm is highly variable, with factors such as temperature, salinity, and pollutants influencing their function and viability (Alavi & Cosson, 2005; Purchase et al., 2010; Reinhardt et al., 2015). Not only does this post-ejaculation condition affect sperm phenotypic traits (Beirão, Lewis, et al., 2018; Purchase et al., 2010), but it also can cause changes at a molecular level (Lettieri et al., 2019; Lymbery et al., 2020; Marshall, 2015; Pitnick et al., 2020). Recent studies suggest that these changes in sperm post-release experiences (the environment that sperm experiences) can also impact offspring development (see review Crean & Immler, 2021). The scientific literature offers contrasting views on these effects; some researchers propose that sperm may adaptively prepare offspring for anticipated environmental conditions, a strategy that could confer evolutionary advantages (Graziano et al., 2023; Immler et al., 2014; Ritchie & Marshall, 2013). Others, however, raise concerns about the potential transmission of physiological stress from sperm to offspring, which could have detrimental effects on offspring development (Kekäläinen et al., 2018; Lymbery et al., 2021).

Lymbery et al. (2021) contributed valuable insights into the potential effects of sperm post-ejaculation exposure on offspring development. Their research demonstrates that the exposure of sperm of mussel *Mytilus galloprovincialis* to high temperatures, indicative of stressful conditions, yields adaptive effects on embryos, particularly when the embryos were subsequently incubated at ambient temperatures considered benign. However, embryos exhibited inferior performance when incubated at high temperatures. In contrast, Ritchie and Marshall (2015) and Graziano et al. (2023) propose a different dimension to this discussion. Their research on *Galeolaria gemineoa* and *Salmo salar*, respectively, using salinity and temperature exposure,

suggested the adaptive effects of sperm exposure occurs when the condition of the offspring aligns with the condition of sperm exposure. This perspective introduced a conditional aspect to the relationship between sperm experiences and offspring outcomes, offering a valuable counterpoint to existing theories. However, the complexity within this field is further accentuated by the work of Kekäläinen et al. (2018) on the sperm of European whitefish (*Coregonus lavaretus*), where they found that post-ejaculation thermal manipulation did not affect sperm phenotypes but did have maladaptive effects on offspring. These differing findings highlight a significant research gap, calling into question whether the observed effects are adaptive or maladaptive. If adaptive, further exploration is needed to uncover the potential mechanisms at play in shaping the developmental trajectory of offspring which are likely governed by two non-mutually exclusive mechanisms: haploid selection and epigenetics (Marshall, 2015; Purchase et al., 2021; Ritchie & Marshall, 2013).

Epigenetics

Epigenetics is the study of alterations in gene expression beyond changes in the DNA sequence (Donkin & Barrès, 2018). Sperm are equipped with a repertoire of epigenetic elements, including DNA methylation, non-coding RNAs, and histone modifications, each capable of inducing changes in gene expression (Immler, 2018; Jenkins & Carrell, 2012; Johnson et al., 2011; Labbé et al., 2017). Following ejaculation, these components can be modified by external conditions (Pitnick et al., 2020) and may become integral to the process of epigenetic regulation, a mechanism that subtly shapes gene expression without altering the DNA sequence. As one example, Lymbery et al. (2021) exposed the sperm of mussels to heat and revealed that while

there were no phenotypic changes in motility, the sperm exposed to higher stressful conditions had lower hsp90 gene mRNA levels.

For epigenetic alterations in sperm to exert an impact, they must transfer to the embryo and also influence the development of the resulting offspring (Fitz-James & Cavalli, 2022). This forms the basis of the anticipatory hypothesis, proposing that offspring are primed for conditions anticipated by the sperm, thereby enhancing their performance in such environments (Burgess & Marshall, 2014; Lymbery et al., 2021; Marshall, 2015; Ritchie & Marshall, 2013). It is essential to note, however, that the effectiveness of these modifications hinges on the alignment of environmental conditions encountered by the offspring with those experienced by the sperm. In scenarios where conditions deviate, the modified sperm may not confer advantageous traits to the offspring (Lymbery et al., 2021).

Haploid selection

Haploid selection is defined as the phenomenon where the phenotype under selection is determined by alleles located on a haploid or effectively haploid genome (Joseph & Kirkpatrick, 2004). While haploid gametic selection is widely acknowledged in the plant kingdom and finds practical applications in agriculture, its relevance in the animal kingdom is notably limited (Immler, 2019). This limitation in animals, diplontic taxa, is primarily attributed to the absence of mitosis in the haploid phase, which leads to the traditional perception of inert DNA in this phase.

In the animal kingdom, the haploid selection concept in eggs, the female gametes, is markedly constrained compared to sperm, the male gametes. This discrepancy arises because a substantial majority of mature eggs or ovules produced by a female are highly likely to be

fertilized (Haldane, 1924 as cited by Immler, 2019). Conversely, male gametes, or sperm, face competition among sperm within an ejaculate (Sutter & Immler, 2020). In a single ejaculation, numerous sperm are released, but the limited availability of eggs constrains the chances of successful fertilization to only a minute fraction of the sperm (Alavioon et al., 2017, 2019; Immler et al., 2014).

Sperm are the product of spermatogenesis, a process in which diploid cells undergo meiosis to produce haploid cells. Each sperm in an ejaculate is recognized for its uniqueness, and it is traditionally assumed that the sperm phenotype, including swimming ability, is predominantly determined by the diploid genome, with no influence from the haploid genome (Borowsky et al., 2018; Braun et al., 1989). However, recent findings challenge this assumption, suggesting a role for the haploid genome in shaping sperm phenotypes (Alavioon et al., 2017; Borowsky et al., 2018; Immler, 2019). However, the mechanisms involved in this process require further investigation. A study in mice revealed that sperm with lower DNA fragmentation reached the fallopian tube (Hourcade et al., 2010), and in boars, chromatin-unstable sperm had a reduced likelihood of reaching oocytes in vivo (Ardón et al., 2008), highlighting a link between sperm genotype and phenotype. Research on a fish species, the Mexican cavefish *Astyanax*, demonstrates a direct correlation between sperm haplotypes and phenotypic variance (Borowsky et al., 2018). Likewise, studies on the zebrafish (*Danio rerio*), further substantiate this connection, highlighting the influence of sperm haplotypes not only on phenotypic diversity but also on the fitness of the resulting offspring (Alavioon et al., 2017). Together, these suggest that sperm phenotype may be partly determined by the sperm's haploid DNA.

Genes expressed in a haploid state would undergo direct exposure to selection, possibly exerting influence over sperm phenotypes such as morphology, motility, and viability

(Immler, 2019; Immler et al., 2014). This, in turn, would shape the competition dynamics among sperm within an ejaculate. Sperm carrying "high-quality" genes may manifest phenotypically superior traits, including larger size and enhanced motility, offering a potential selective advantage in fertilization outcomes (Alavioon et al., 2019). The competitive landscape post-ejaculation, influenced by external conditions, determines significantly which sperm phenotype achieves success in fertilization (Holt & Look, 2004). The developmental trajectory of offspring is directly influenced by which specific sperm successfully fertilizes the egg out of all the sperm in an ejaculate (Alavioon et al., 2017, 2019; Borowsky et al., 2018; Immler, 2019). The epigenetic component of fertilizing sperm can possibly play an additional pivotal role in shaping the subsequent development of the offspring.

Study species: capelin

Capelin (*Mallotus villosus*) is a small, cold-water marine pelagic schooling species that occurs in the North Pacific and North Atlantic. It is a forage fish belonging to the Osmeridae family, which has an important ecological role in transferring energy from zooplanktons to vertebrates such as other larger fishes, marine mammals, and seabirds (Buren et al., 2014; Carscadden & Vilhjálmsson, 2002). In Iceland and Norway, they primarily spawn in offshore demersal sites, but in Newfoundland, most capelin spawn on beaches (DFO, 1991). Due to this annual beach spawning event, it is an iconic species in Newfoundland. It favours pebbles sized 0.5 cm to 2.5 cm for spawning and shows a preference for water temperatures of 5-8 °C. Spawning is predominately during the night or on cloudy days (DFO, 2019; Penton et al., 2012; Templeman, 1948).

Capelin males and females are almost morphologically identical before sexual maturity (DFO, 1991). The typical total length at maturity falls within the range of 13 to 20 cm. External features include an elongated and slender body characterized by a prominent central dorsal fin and a smaller adipose fin, just preceding the caudal fin. The coloration encompasses from olive to green above the lateral line, transitioning to a silver hue below (Sleggs, 1933; Templeman, 1948). Sexual dimorphism becomes evident approximately four to five weeks before spawning, with males undergoing discernible morphological changes. Sexually mature capelin males are larger than females and undergo changes in morphological features, including enlarged fins and the development of spawning ridges (DFO, 1991; Jangaard, 1974; Orbach et al., 2019; Templeman, 1948).

During the spawning season, capelin males strategically position themselves near the beach awaiting for females (DFO, 1991; Nakashima, 1987; Ressel, 2019). As females are ready for spawning, they move onto the beach to join the males. Both sexes move onto the beach, engage in mating, and are carried back by the next wave. A single female often spawns with two males at the same time (DFO, 1991; Orbach et al., 2020). The enlarged fins and spawning ridges of males may facilitate the retention of females during mating (Orbach et al., 2019). It is thought that males participate in multiple mating sessions within a spawning season (DFO, 1991). Post-spawning mortality is high among capelin; however, some females can survive to spawn in a subsequent year whereas males are semelparous (Flynn et al., 2001).

Capelin exhibit great inter-annual recruitment variability, with significant implications for both the ecosystem and commercial fisheries (Carscadden & Vilhjálmsson, 2002). The Newfoundland capelin stock experienced a significant decline in 1990-1991 and has remained at low abundance for three decades (DFO, 2019; Murphy et al., 2018). Post-collapse,

capelin matured and spawned at ages 2-3, 1-2 years earlier than the pre-collapse period (DFO, 1991; Murphy et al., 2018). This temporal shift in maturation results in a spawning population that is both younger and smaller in size. Furthermore, the timing of peak beach spawning has been persistently delayed by about 3 weeks since 1991 in contrast to the pre-collapse spawning period. This delayed spawning and the potential environmental mismatch affecting offspring development, along with the production of weaker year classes, may have inhibited capelin stock recovery (Cushing, 1990; Murphy et al., 2021; Purchase, 2018).

The complexity of capelin reproductive biology is further complicated by the sensitivity of their sperm and embryos to salinity, which results in suboptimal performance at higher salinity (Beirão et al., 2018, Purchase, 2018 and references therein). Additionally, capelin release pre-activated sperm, the only known external fertilizing vertebrates to release sperm that is already active. This phenomenon is hypothesized to serve as an adaptation to the sensitivity of their sperm to salinity levels, thereby optimizing fertilization success in marine environments (Beirão et al., 2018).

Thesis organization

Adult capelin males are larger compared to females (Jangaard, 1974). It is proposed that this male-biased sexual size dimorphism aids in endurance rivalry due to no evident interaction-dependent male competition and no correlation between female mate choice and male size (Orbach et al., 2019). This implies that larger male bodies, with higher energy reserves, facilitate extended stays at mating sites, enhancing mating opportunities. But they also need a continual supply of semen for multiple matings. However, given their unusually small testes (GSI ~1%; Orbach et al., 2020; Ressel et al., 2020), compared to most fishes (Tsikliras et al.,

2010; Stockley et al., 1997), they do not start spawning with abundant semen. And their unique sperm (the only known externally fertilizing vertebrate to release pre-activated sperm; Beirão et al., 2018), may inhibit their ability to regenerate semen quickly and may also be particularly sensitive to sperm aging.

Chapter 2 of this thesis focused on gamete quality dynamics with specific objectives of assessing semen quality variation among males, investigating semen regenerative capacity, and exploring the impact of delayed spawning on gamete quality in capelin. Through this examination, the study aims to shed light on gamete quality dynamics in the context of the endurance rivalry hypothesis.

Chapter 3 of this thesis investigates the effect of sperm experience on embryo development. Given the inconsistent findings from several studies, there is a need to determine whether there is an adaptive effect of sperm experience on embryo development (Graziano et al., 2023; Kekäläinen et al., 2018; Lymbery et al., 2021; Ritchie & Marshall, 2013). The second research chapter's objective extends beyond mere confirmation, centring on elucidating the underlying adaptive mechanisms. Specifically, the chapter aims to discern which potential mechanism - haploid selection or epigenetics - exert a more significant impact on shaping the developmental trajectory of capelin offspring.

Beach-spawning capelin stands out as an ideal model organism for investigating the potential impact of sperm experiences on offspring development. Despite their marine habitat, both capelin sperm and embryos exhibit sensitivity to high salinity (Beirão et al., 2018; Purchase, 2018). The successful development of embryos in ocean salinity conditions suggests the potential transmission of adaptive traits, wherein sperm post-ejaculation experiences may impart effects conducive to enhanced embryo survival in such conditions. The importance of

manipulating the salinity conditions of sperm and embryos in controlled experiments becomes evident in this context. Therefore, in Chapter 3, I exposed capelin sperm to benign and stressful salinity conditions, followed by embryo incubation at matched and mismatched salinity conditions from the initial sperm exposure. This methodical technique allows for determining whether sperm experiences have an impact on offspring development.

Co-authorship statement

Ranjan Wagle played a crucial role in designing, planning, data collection, processing, analysis, and subsequent chapter writing. Dr. Craig Purchase conceived the studies, substantially contributed to experimental design, fieldwork, statistical analysis, and assisted in editing and reviewing all thesis chapters. Connor Hanley made significant contributions to fieldwork, editing, and review in Chapter 2, deserving inclusion as a co-author. Chapters 2 and 3 are presented as draft manuscripts, using the pronoun "we," while Chapters 1 and 4, were written specifically for this thesis, use the pronoun "I."

Publication and submission status

Chapter 2: Wagle R., Hanley C.P. and Purchase C.F. Endurance rivalry in capelin: insights from among-male semen properties, semen regenerative capacity and impacts of delayed spawning.

Chapter 3: Wagle R. and Purchase C.F. Can sperm experience prior to fertilization improve offspring performance beyond paternity in an external fertilizing fish?

*The second and third chapters are intended for publication and have been prepared for submission to a peer-reviewed journal.

297 *Appendices are included for both chapters in the thesis, but are not planned to be included with
298 the chapters when they are published.

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Chapter 2. Endurance rivalry in capelin: insights from among-male semen properties, semen regenerative capacity and impacts of delayed spawning

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Abstract

Male-biased adult sexual size dimorphism is often the result of intra-sexual selection, driven by male-male competition. Capelin (*Mallotus villosus*) exhibit male-biased sexual size dimorphism but lack contests/fighting, and female mate choice, if present, is unrelated to male size. Consequently, it is hypothesized that adult sexual size dimorphism in capelin is due to a mating system that favours larger males with greater energy reserves, enabling them to compete for prolonged access to mating opportunities through endurance rivalry. To enable this, males need a continual supply of semen through the spawning season. However, they have unusually small testes and are predicted to deplete stored semen rapidly, and their unique sperm physiology may constrain the ability to regenerate it. We found that the majority of capelin sampled on the beach had adequate semen, but instances of no semen were observed toward the end of the spawning season. Capelin held in laboratory tanks could regenerate semen within two days. Capelin held in captivity for 6 days did not exhibit a decline in gamete ability to achieve fertilization success. These results provide support for the endurance rivalry hypothesis.

Introduction

Sexual size dimorphism (SSD) refers to adult body size difference between males and females within a species. It results from males and females having different selection pressures for size and is widespread in the animal kingdom (Andersson, 1994; Darwin, 1872; Horne et al., 2020; Rennie et al., 2008). Female-biased SSD (females being larger than males) is commonly attributed to natural selection, favouring large females in egg production capacity (but see Shine, 1988). In contrast, male-biased SSD is often the result of intra-sex sexual selection driven by male-male competition (Cox et al., 2003; Fairbairn, 1997; Pyron et al., 2013; Trivers, 1972). The majority of fish species have female-biased SSD (Bisazza, 1993; Webb & Freckleton, 2007), but there are exceptions that can provide insights into selection dynamics and mating systems (Bisazza, 1993; Brockmann, 2001; Orbach et al., 2019; Parker, 1992).

Capelin (*Mallotus villosus*) is one such exception species (Orbach et al., 2019; Templeman, 1948). In addition to being larger, adult males also exhibit secondary sexual characteristics having enlarged fins and unique spawning ridges (DFO, 1991; Jangaard, 1974; Orbach et al., 2019). These secondary sexual characteristics have recently been hypothesized to facilitate physical contact with females while spawning, rather than serving as a means of mate choice (Orbach et al., 2019). Despite the male-biased SSD in capelin, there is no evidence of contests/fighting for mating opportunities, and if females do exhibit some sort of mate choice, it is not based on male size (Orbach et al., 2020). It is likely that large males benefit in some way, as achieving a larger size usually requires a longer period of growth or increased foraging risk (Rennie et al., 2008); thus, there is a trade-off between the benefits of size and the survival probability costs associated with delayed maturation (Roff, 1986).

Orbach et al. (2019) recently proposed that the capelin male-biased SSD is due to a mating system based on endurance rivalry, which is interaction-independent male-male competition (Andersson, 1994). Capelin migrate inshore to spawn, and once spawning begins at a beach, males remain very close by as spawning repeatedly starts and stops with changing environmental conditions and the arrival of new females (DFO, 1991; Jeffers, 1931). By staying at the site for longer periods, males would increase their chances of mating, which may be extended by larger bodies having more energy reserves. However, simply remaining at the spawning site for a prolonged period is insufficient to secure more matings, as males also require enough semen for multiple matings. This could be achieved by having a large amount of semen at the onset of the spawning season or by producing new semen for each mating. However, capelin have unusually small testes with a gonadosomatic index of around 1% (Beirão et al., 2015; Orbach et al., 2020; Ressel et al., 2020), and thus, the first scenario does not occur. If the mating system is based on endurance rivalry, we, therefore, predict (1) that once spawning begins at a site, if sampled next to spawning beaches, some males will not contain any semen (used up).

In addition to having extremely small testes, capelin also possess unique sperm, being the only known external fertilizing vertebrate to release pre-activated sperm (Beirão et al., 2018a). Despite unique sperm physiology, we predict (2) that capelin can quickly regenerate semen (the ability to replenish semen stock) as they spawn. The unique sperm physiology may make the sperm particularly sensitive to delayed spawning due to the semen aging (over maturation) effect (Gasparini et al., 2014; Reinhardt, 2007; Reinhardt & Turnell, 2019), which we addressed as a third objective.

Methodology

Among male variation in semen quality – spawning beach surveys

Spawning male capelin were captured during June and July 2023, using a dip net on three beaches of the Avalon Peninsula, Newfoundland, Canada: Middle Cove Beach (47° 39' 2.75" N, 52° 41' 45.24" W) on 29th June; 2nd, 4th, 6th,10th, 12th and 14th July, Bellevue Beach (47° 38' 9.28" N, 53° 46' 51.09" W) on 7th, 8th, 9th July, and Holyrood Beach (47° 23' 21.8" N, 53° 07' 34.5" W) on 21st July. Fish from these beaches are thought to be from the same population (Dodson et al., 1991; Penton et al., 2014).

At capture, the total length of haphazardly selected males was measured, and semen was assessed by gently pressing the abdomen until no further release of semen occurred. Semen quality was visually scored directly upon release on the fish's body in the field according to the categories: none (no semen), poor (thick pelleted semen), fair (watery/dilute semen – relatively low sperm concentration) and excellent (whiter/denser semen – relatively high sperm concentration). Poor quality semen may not have the ability to fertilize eggs or be released during ejaculation. For fair and excellent semen quality, the quantity was scored as relatively low, medium or high. This categorization of semen quality and quantity was based on years of observations.

To confirm the effectiveness of the semen quality categorization, two males, each from fair and excellent categories, were selected. Using the same amount of semen, excellent quality males had 6.75 times higher fertilization success than those deemed of fair quality's with the same females (Appendix 2-1).

Semen regenerative capacity – lab experiment with wild fish

Semen regenerative capacity (the ability to replenish semen stock) was assessed in a blocked design with four block comprised of capelin captured at Middle Cove on June 29, July 4, and July 10, and Holyrood Beach on July 21. Fish were transported in aerated coolers to the Ocean Sciences Centre (OSC), Memorial University of Newfoundland, St. John's, NL, Canada. Within each block, all males (~ 80) were measured for total length and stripped to remove all their semen, which was then scored for quality and quantity using the categories previously described. They were then kept in a 1,000-liter flow-through seawater tank maintained at 4 to 8 °C. Capelin generally do not feed while spawning, and thus no food was provided in the tanks. No fish died while being held in the tank.

On alternate days over 6 days (duration of captivity), 10-20 males were selected randomly and stripped again to score semen quality and quantity following the same assessment categories and then killed (each fish semen was assessed twice, including the day at capture, i.e., day 0). One block (June 29 Middle Cove) deviated from this alternate-day pattern, as assessments were conducted on the second, third and fourth days.

Impact of delayed spawning on the quality of gametes – lab experiment with wild fish

General design: A scenario was established by separating male and female fish, placing them in tanks and holding them for six days in order to assess the impact of delayed spawning on gamete quality in block design. Fertilization was carried out every other day in each block from the day the fish were captured (on days 0, 2, 4 and 6). Gamete quality was assessed by their fertilizing ability. A partial pooling approach was used, wherein random subsets of

capelin were sampled to minimize the influence of individual variation on the interpretation of the effect of delayed spawning. Each fish was used only once.

Capture and Hold Technique: The three blocks contained male and female capelin captured at Middle Cove Beach on July 4 and 10, and Holyrood Beach on July 21. They were transferred to OSC as described above, but males and females were kept separately in tanks with ~ 50 fish each. Blocks were divided into two groups: each one comprised a male and a female tank (2 tanks for males and 2 tanks for females for each block). Fertilization was performed between males and females within each group (e.g., Block 1, Group A, Tanks F1, M1; Block 1, Group B, Tanks F2, M2).

Fertilization: To carry out fertilization on each fertilization day within each block, four females from each group were randomly selected and eggs were collected by gently pressing their abdomen (Purchase, 2018). The eggs from each female were placed into separate flexible teflon trays. A standardized process was employed whereby eggs from four females of the same group were mixed in fixed proportions of 3 grams each, creating 12 grams of two mixed egg batches (one per group). This approach was taken so that every female was represented equally, ensuring a more representative approach to the female population within these batches from each female group. To evaluate each male's semen quality without the confounding influence of sperm competition, semen from four males from each male group was used separately for fertilization, avoiding any mixing. This resulted in four separate fertilization sets from a tank group (in total eight fertilization sets from two groups) per block, wherein each

706 tank group, 20 µL semen from each of the four different males was used and mixed with each 0.4
707 gm of eggs (about 2000 eggs) from the mixed eggs batch (residual eggs were discarded).
708
709 **Fertilization assessment:** Capelin eggs are sticky upon contact with water, so to
710 make observations of individual eggs possible for fertilization assessment, a solution of 600
711 mg/L tannic acid mixed in 30 psu water was used to remove the stickiness of the eggs of each
712 fertilization set. This was then poured off and rinsed thrice with 30 psu water (Purchase, 2018).
713 Eggs were incubated in the dark at 4° C using plant growth incubators. After 18 hours,
714 approximately 300 eggs per fertilization set were examined under a microscope to score the
715 fertilized eggs (8- and 16-cell differentiation stage) and unfertilized eggs (no cell division)
716 (Beirão et al., 2018b).
717 To check the repeatability of our fertilization assessment, the fertilization rates of a
718 randomly selected fertilization set from each block were assessed twice using different sets of
719 eggs (Appendix 2-2). There was no mortality among the males and females from any capture
720 date while they were held in the tank.
721
722 **Statistical analysis**
723 Graphs depicting the patterns of semen quality, quantity and regeneration capacity
724 during the spawning period were plotted to assess variations in semen quality and quantity. Data
725 were adjusted by converting to proportionate scores for consistent comparison across semen
726 quality, quantity, and capture dates and locations. Box plots were used to depict the relationship
727 between capelin total length, semen quality and quantity, and semen regenerating capacity.

To analyze the data on the impact of delayed spawning on the quality of gametes, we used a generalized linear mixed model (GLMM) with a binomial error distribution ('lme4' package; Bates et al., 2015) using the 'cbind' function to combine fertilized and unfertilized eggs as the response variable. The model included fixed-effect categorical variables of holding duration and random effect of captured date (block), tank group and fertilization set. However, to account for overdispersion, we switched to a GLMM with a beta-binomial error distribution ('glmmTMB' package; Brooks et al., 2017; Lymbery et al., 2021). Statistical analyses were performed in R v. 4.3.0 (R Core Team, 2023), and graphs were made using the 'ggplot2' package (Wickham, 2016).

Results

Among male variation in semen quality

Across 11 sampled dates (June 29 to July 21), semen quality showed notable variation among capelin. The majority of sampled capelin (53%) had excellent quality semen, ranging from a minimum of 34% on July 14 to a maximum of 90% on July 4 (Figure 2-1). Fair quality semen, on average over 11 sample dates, was found in 31% of the fish, reached its highest proportion at 56% on July 2 and its lowest at 8% on July 10. Only 9% of sampled capelin had poor-quality semen. About 7% of sampled capelin had no semen, which was not observed until July 9. Around 16% of the sampled males had poor quality semen or no semen and is believed to lack the ability to fertilize any eggs (Figure 2-1).

The proportions of excellent and fair-quality semen in capelin fluctuated without a clear temporal trend, while instances of capelin with no semen increased slightly over the

spawning period. However, interpreting a temporal trend is challenging due to the small sample size and confounding factors of time and space.

Capelin with excellent semen quality were often accompanied by high quantity of semen (Figure 2-2). The majority of capelin (76%) had a high quantity of semen within the excellent quality semen, while 54% of capelin exhibited a medium quantity of fair quality semen. Very few capelin with low quantity semen were present across both quality categories (3% in excellent while 9% in fair quality semen).

There was no relationship between semen quality and male size (Figure 2-3). Body size was not related to the quantity of fair quality semen, but larger males had a higher quantity of excellent-quality semen compared to smaller males (Figure 2-4).

Semen regenerative capacity

Across all four capture dates (blocks), the majority of males had semen of excellent (53%) and fair (21%) quality at the time of capture (day 0) (Figure 2-5). After complete stripping on day 0, many capelin were able to regenerate excellent and fair quality semen again after two days (was not assessed on day 1) in all blocks (36% excellent and 41% fair), and the proportion increased with additional days in three out of the four blocks (Figure 2-5). Although the fish were not tracked individually, it is likely that fish with poor quality semen upon capture also had the poor quality on subsequent days. Regarding temporal trends, the capelin captured on June 29 could regenerate semen quicker than on July 4, which was quicker than on July 10. However, capelin sampled on July 10 initially had lower semen quality than other blocks. Those captured on July 21 had a reduced ability to regenerate semen over days.

In terms of quantity of excellent and fair quality semen, across all four blocks, on the capture day (day 0), most capelin had high and medium quantities of semen (fair quality: 39% high and 48% medium quantities: excellent quality: 68% high and 29% medium quantities) (Figures 2-6 & 2-7). Of those capelin who were able to regenerate excellent quality semen on subsequent days, the capelin captured on June 29 and July 4 exhibited a higher quantitative regeneration of semen compared to those captured on July 10 and July 21 (Figure 2-6). The regenerative capacity of fair quality semen in terms of quantity also followed a similar trend, albeit with a relatively lower regenerative quantity than excellent quality semen (Figure 2-7).

There was no relation between male size and the quality of regenerated semen (Figure 2-8). Body size was not associated with the quantity of fair-quality regenerated semen as well as quantity of excellent quality regenerated semen (Figure 2-9).

Impact of delayed spawning on the quality of gametes

The delay in artificial capelin spawning over 6 days from capture showed no discernible influence on fertilization success (chi-square = 3.28, d.f. = 3, p = 0.349) (Figure 2-10).

Discussion

Beach-spawning capelin males strategically position themselves near the beach, awaiting females to initiate spawning (DFO, 1991; Jeffers, 1931). Hence, males must be continuously prepared for spawning, and "compete" to extend their presence at spawning sites, a reproductive strategy known as endurance rivalry (Andersson, 1994; Orbach et al., 2019). Our data on semen quality assessment in the field revealed that a majority, but not all, of capelin had

795 adequate semen throughout the spawning season (not the same fish sampled). Furthermore,
796 excellent semen quality was often accompanied by relatively high quantities. We found no
797 relation between fish total length and semen quality, which is consistent with Orbach's (2020)
798 findings that sperm swimming characteristics did not correlate with body length; however, the
799 quantity of excellent quality semen was related to body size. As predicted, we found some
800 capelin with no semen, but only towards the end of the sampling period. Previous work at the
801 same study sites has, at times, observed many males that contain no semen (Purchase,
802 unpublished). These results indicate that capelin deplete their semen stores, but despite unique
803 sperm and abnormally small testes, through rapid semen regeneration, they maintain their ability
804 to seize continual mating opportunities.
805 Capelin, despite possessing unusually small testes (Ressel et al., 2020), exhibited a
806 remarkable capacity for semen regeneration, with the majority of captive fish regenerating semen
807 within two days (we did not assess them after one day). Individuals who did not completely
808 replace their semen after 2 days (different individuals were sampled each day), generally
809 continued to improve production after more time (sampling days 4 and 6). Research on species
810 such as brown trout (*Salmo trutta caspius*), turbot (*Scophthalmus maximus*), and rainbow trout
811 (*Salmo gairdneri*) also demonstrated their semen regeneration capability, with sampling intervals
812 set at biweekly for brown trout, weekly for turbot and also weekly for rainbow trout (did not
813 investigate shorter intervals); however, upon repeated sampling of the same fish at each
814 successive interval, these species experienced a gradual decline in semen quality
815 (Büyükhatipoglu & Holtz, 1984; Hajirezaee et al., 2009; Suquet et al., 1992). Our data on higher
816 semen regenerative capacity on successive sampling may be due to our distinctive sampling
817 approach, where each fish was sampled only once for semen assessment throughout the entire

duration of captivity, meaning fish not sampled on day 2 had extra days for semen production. We found no relation between capelin body size and the quality and quantity of regenerated semen. However, we speculate that body size may influence how often semen regenerates, warranting further investigation in future research. Nevertheless, this rapid semen regeneration in capelin within two days improves mating opportunities and aligns with the concept of endurance rivalry, maximizing mating success.

Extended delays in spawning can lead to gamete over-maturation (gamete aging), affecting gamete quality and subsequent fertilization success and embryo development across species (Azin Mohagheghi Samarin & Lahnsteiner, 2015; Bobe & Labbé, 2010; Gasparini et al., 2014; Hay, 1986; Reinhardt & Turnell, 2019). Each species has a specific timeframe for optimal reproductive output post-gamete maturation. For instance, rainbow trout (*Oncorhynchus mykiss*) and other salmonids experienced decreased egg quality if females retained eggs for a week post-ovulation, while in Atlantic halibut (*Hippoglossus hippoglossus*), egg quality diminishes 4 to 6 hours post-ovulation, and in tilapia (*Oreochromis niloticus*) and goldfish (*Carassius auratus*), less than 2 hours (Bromage et al., 1994 and references therein). Hay (1986) observed no effect on fertilization when spawning was delayed for up to two weeks in Pacific herring (*Clupea herengus pallasii*); however, longer delays negatively affected embryo development but not fertilization success. Turbot males exhibited reduced fertilizing capacity towards the end of the spermiation period (Suquet et al., 1998), while guppies (*Poecilia reticulata*), when males retained sperm for 12 days, displayed decreased sperm velocity (Gasparini et al., 2014; Gasparini et al., 2017). Our study found that delayed spawning (gamete aging) of up to six days in capelin had no negative effect on the gamete's (sperm and eggs) ability for fertilization. However, although fertilization success remained unaffected, there may be a detrimental effect on embryo

development (Purchase, unpublished; Hay, 1986), which warrants further investigation. Nevertheless, within the context of the endurance rivalry concept, this implies that capelin semen fertilization capacity remained unaffected by delayed spawning (sperm aging) for up to six days, highlighting males' ability to wait for mating opportunities without compromising reproductive fitness.

Acknowledgements

Assistance in collecting capelin and conducting experiments was provided by Kaitlyn Gladney and Nick Murphy. Funding was provided via Memorial University and grants to Craig Purchase from the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, and the Research and Development Corporation of Newfoundland and Labrador. All procedures followed the Canadian Council on Animal Care guidelines for the use of research animals (Memorial University protocols 18-02-CP, 22-02-CP) and Fisheries and Oceans Canada permit NL-6666-22.

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Chapter 2 figures

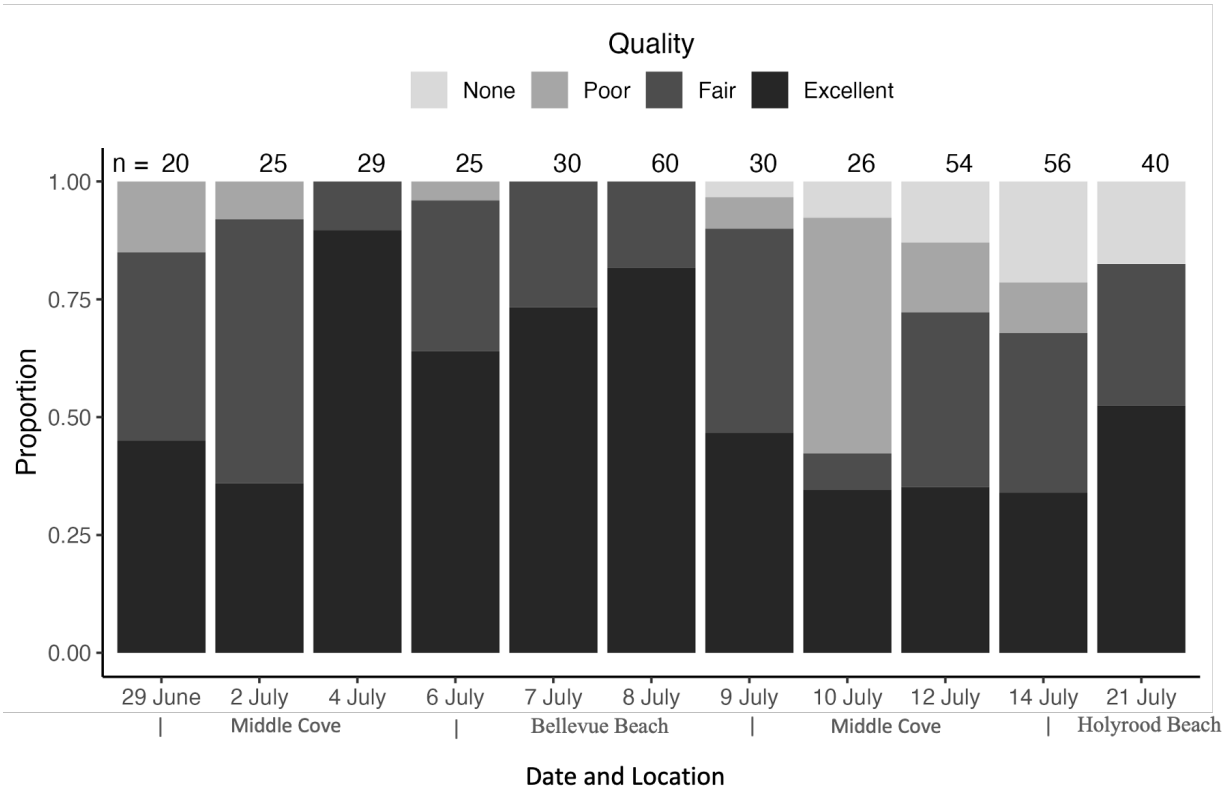
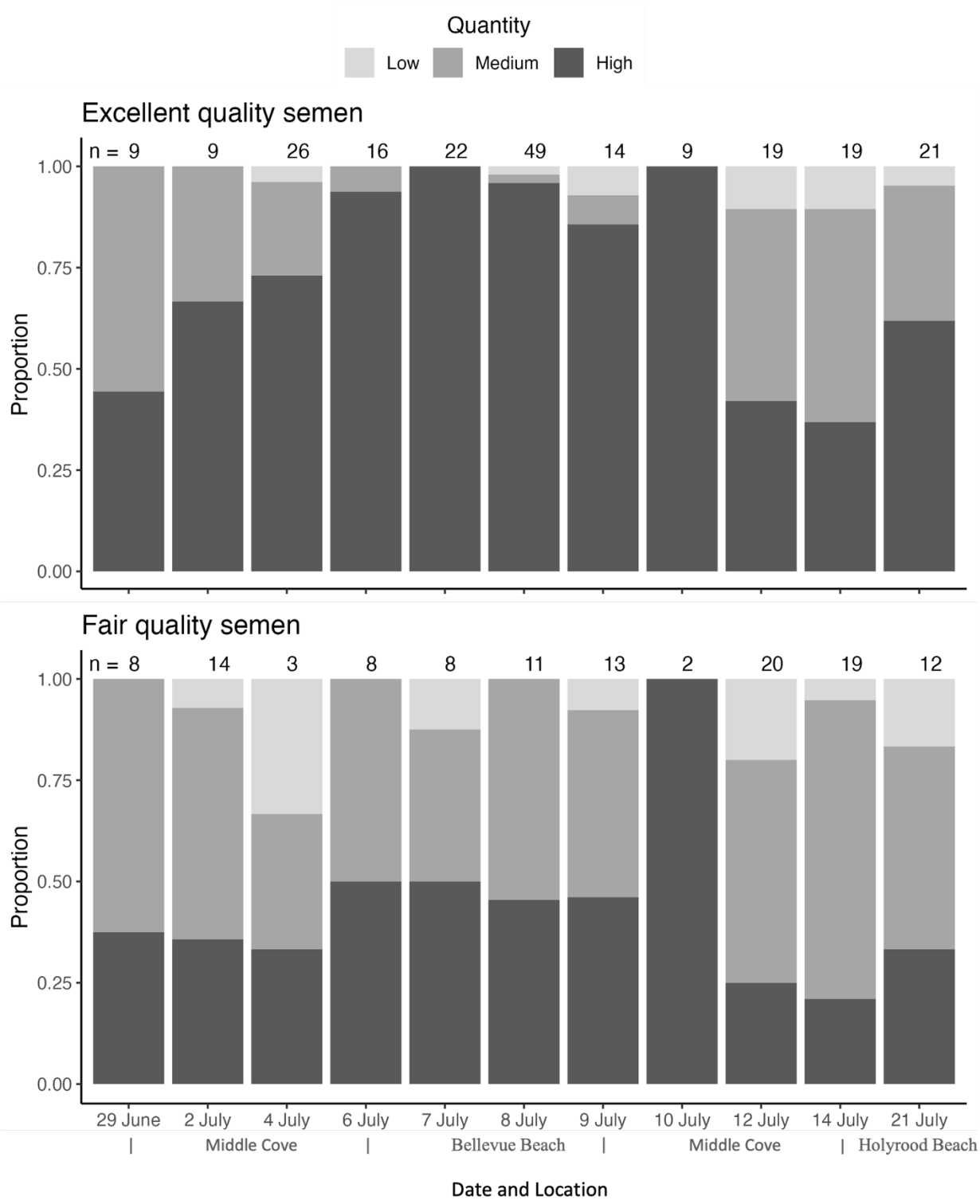
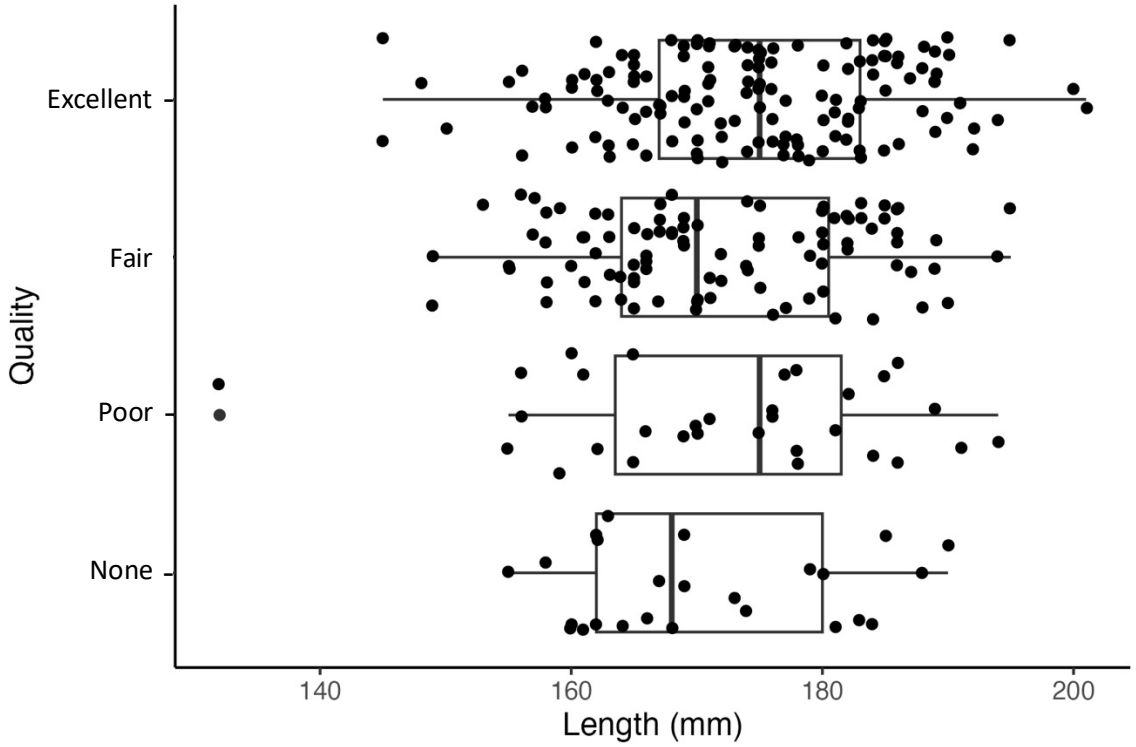


Figure 2-1. Variation in capelin semen quality scored in the field at different beaches in eastern Newfoundland during the 2023 spawning period. Semen quality was categorized into four groups, each denoted by different colours. The total number of capelin scored on specific dates is indicated above each respective bar. The y-axis represents proportionate scores, standardized through conversion to proportionate values, normalizing the data for comparison.

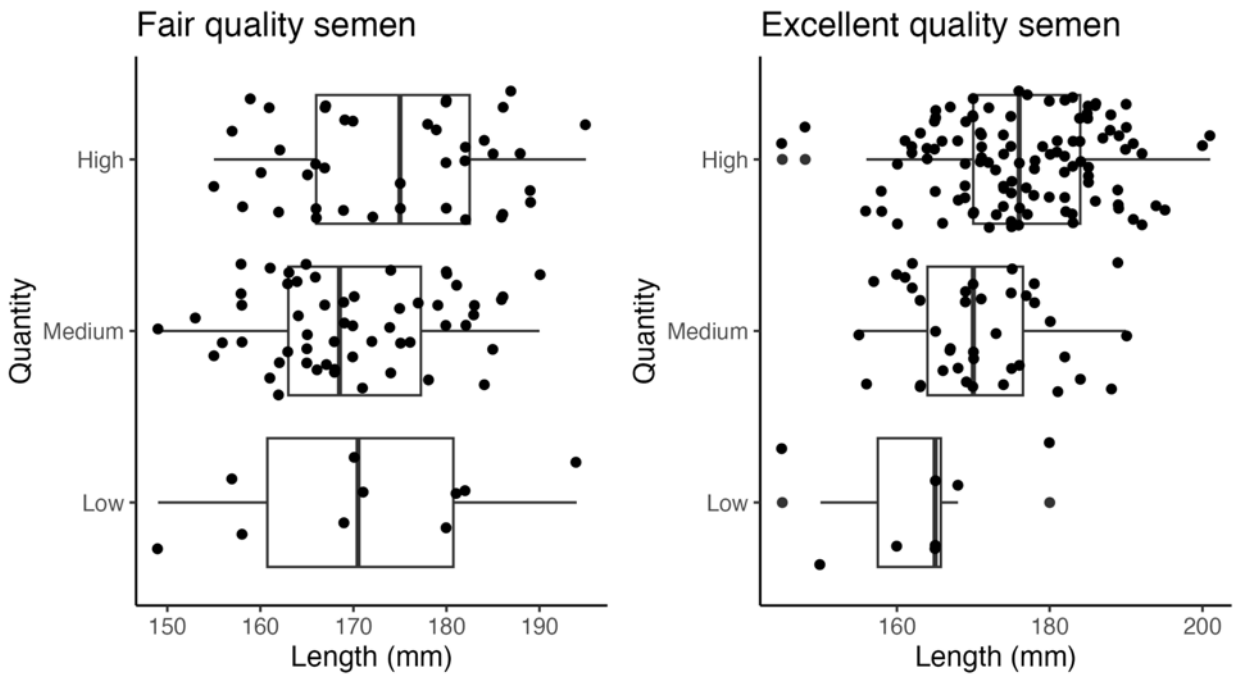


996 Figure 2-2. Variation in capelin semen quantity within the excellent and fair quality categories
997 scored in the field across different beaches in eastern Newfoundland during the 2023 spawning
998 period. Relative semen quantity was categorized into three groups, each denoted by different

colours. The total number of capelin scored within each of the two quality categories on specific dates is indicated above each bar. The y-axis represents proportionate scores, standardized through conversion to proportionate values, normalizing the data for comparison.



1002 Figure 2-3. Variation in capelin semen quality across different fish total lengths scored in the
1003 field across different beaches in eastern Newfoundland during the 2023 spawning period. The
1004 thick line within the box plot represents the median, the box represents the interquartile range
1005 (25th and 75th quartiles), and the whiskers extend to 1.5 times the interquartile range from the
1006 first and third quartiles, respectively.



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1008 Figure 2-4. Variation in capelin semen quantity across different fish total lengths in two semen
1009 quality categories, fair and excellent, scored in the field across different beaches in eastern
1010 Newfoundland during the 2023 spawning period. The thick line within the box plot denotes the
1011 median, the box represents the interquartile range (25th and 75th quartiles), and the whiskers
1012 extend to 1.5 times the interquartile range from the first and third quartiles, respectively.

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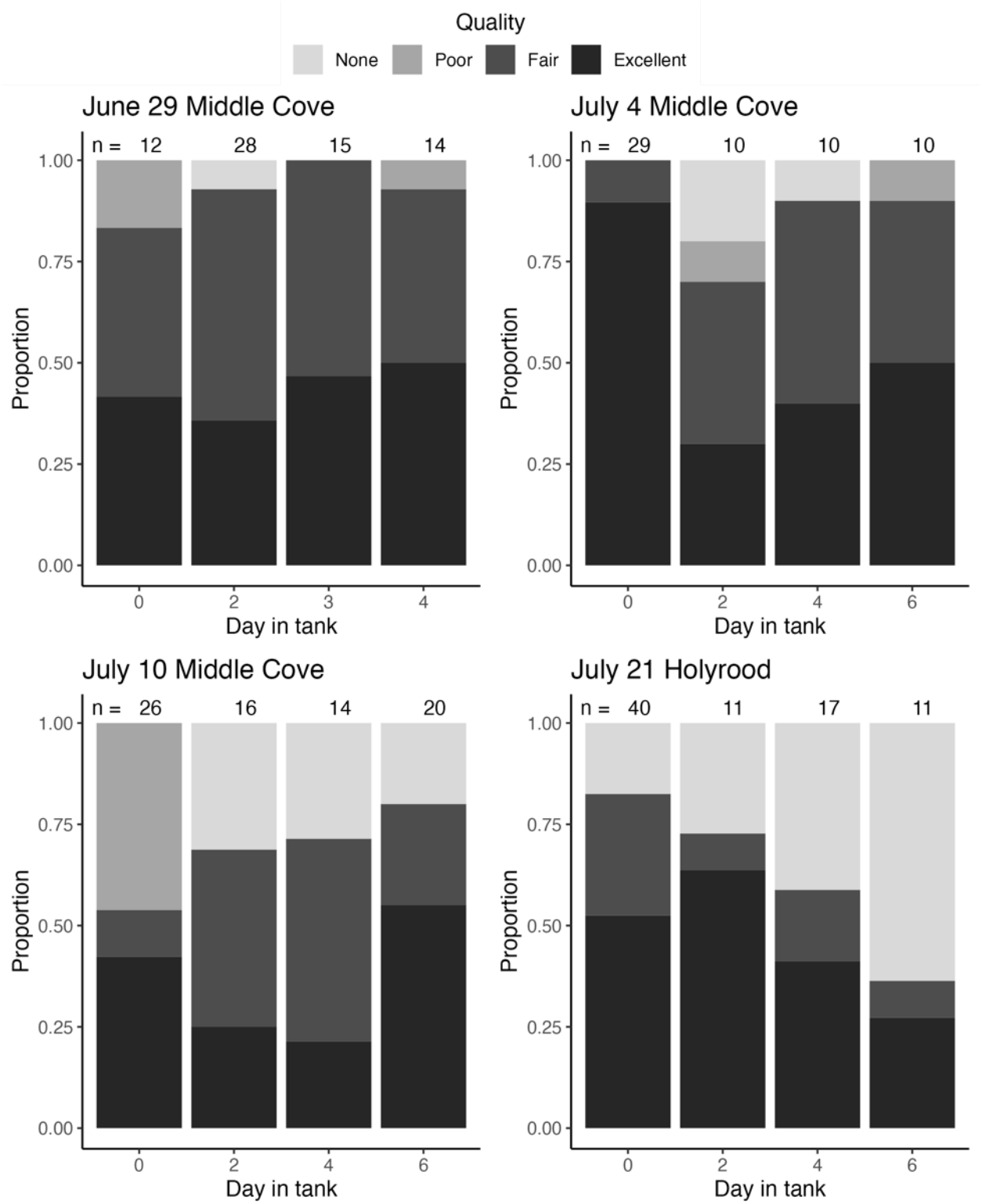
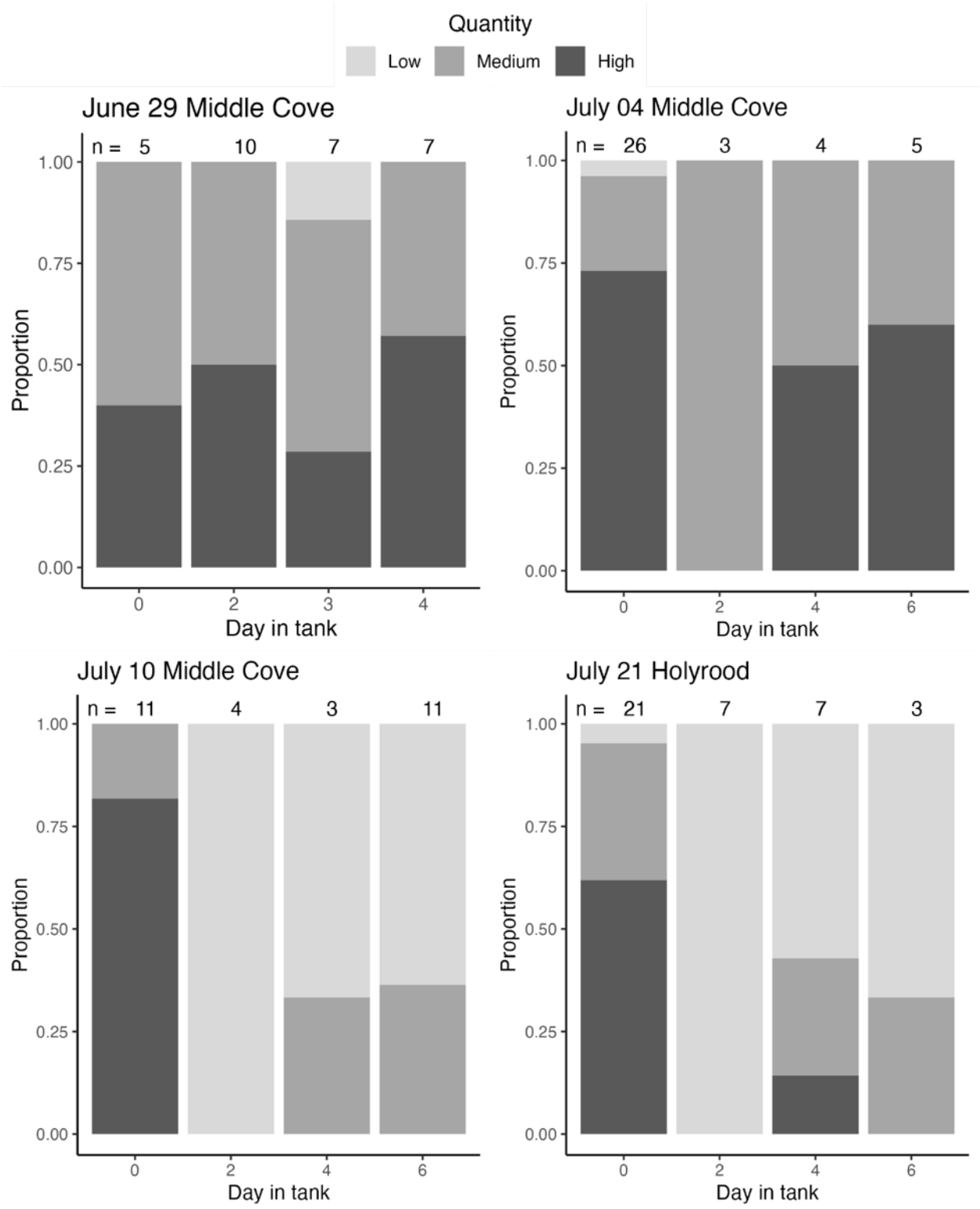


1014 Figure 2-5. Assessment of the regenerative capacity of capelin semen based on quality.

1015 Completely stripped male capelin were kept in a tank, and every alternate day over a 6-day

1016 period, a subset was scored to assess their semen regeneration ability based on four categories of

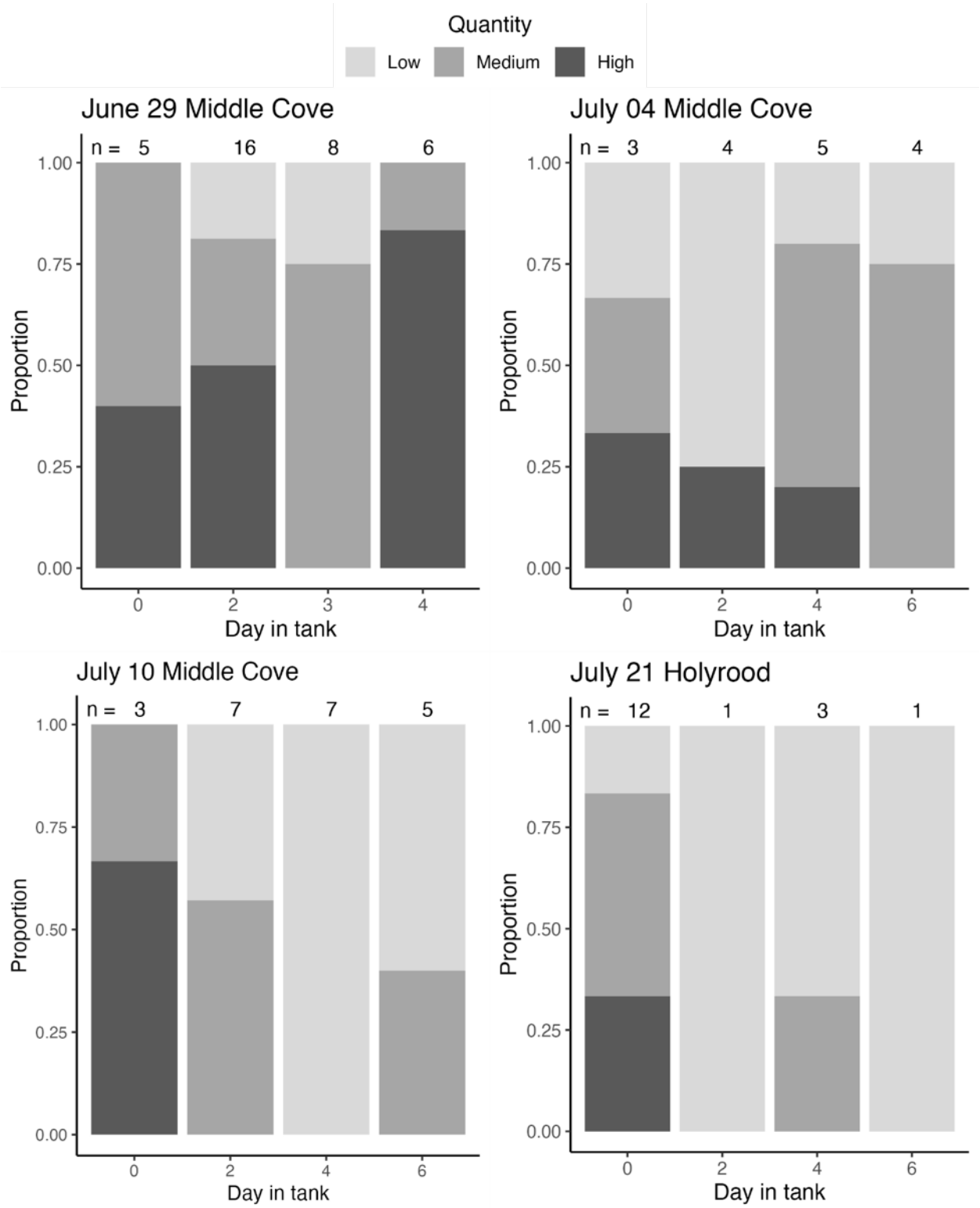
semen qualities, each distinguished by distinct colours. The total number of capelin scored on specific dates is indicated above each respective bar. The y-axis represents proportionate scores, standardized through conversion to proportionate values, normalizing the data for comparison. The 2023 capture date and location are provided at the top of each bar plot.



1022 Figure 2-6. Assessment of the regenerative capacity of excellent quality capelin semen based on

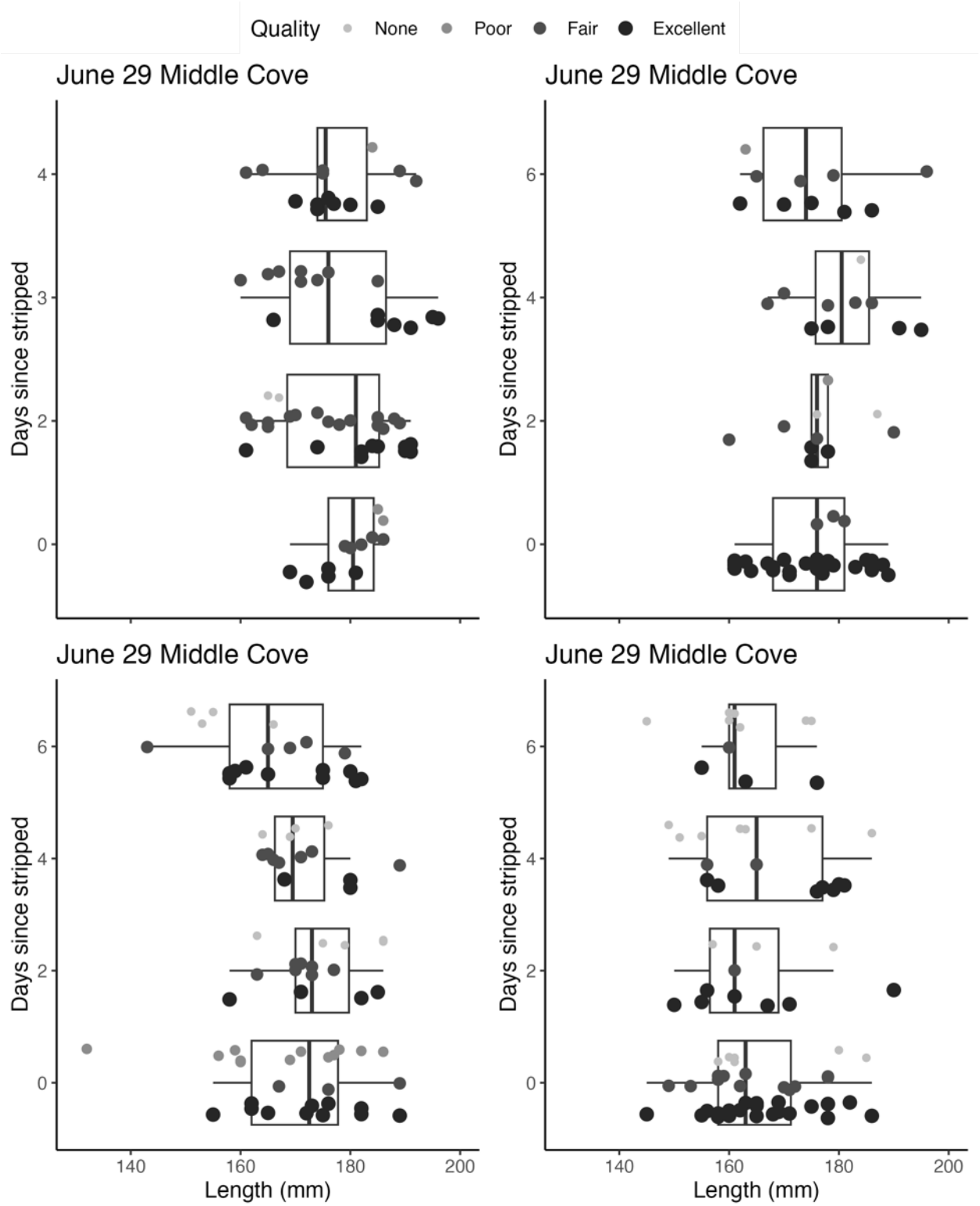
1023 quantity. Completely stripped male capelin were kept in a tank, and every alternate day over a 6-

day period, a subset of capelin was scored to assess their semen regeneration ability based on three quantity categories, each distinguished by a unique colour code. The total number of capelin scored on specific dates is indicated above each respective bar. The y-axis represents proportionate scores, standardized through conversion to proportionate values, normalizing the data for comparison. The 2023 capture date and location are provided at the top.



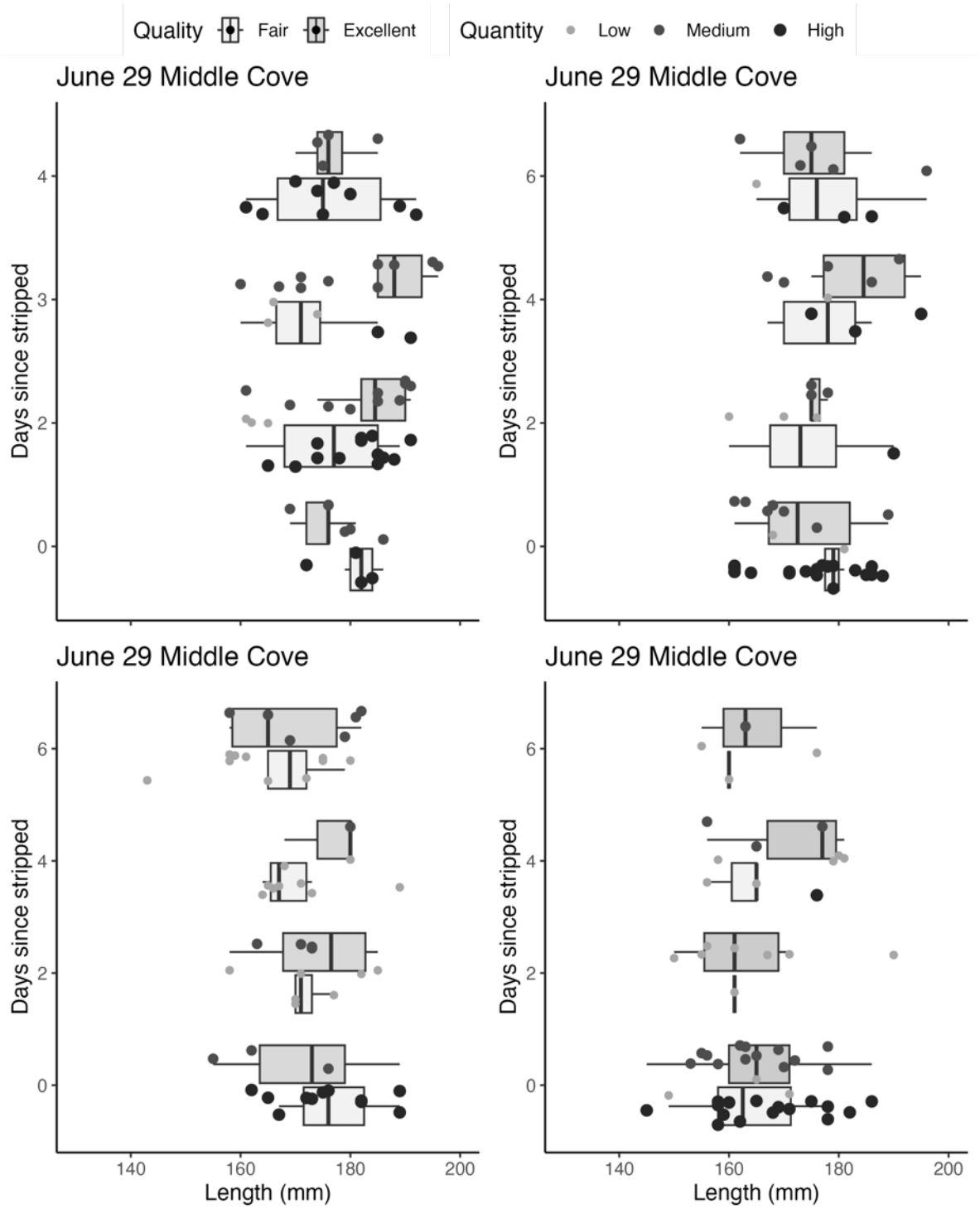
1029 Figure 2-7. Assessment of the regenerative capacity of fair quality capelin semen based on
1030 quantity. Completely stripped male capelin were kept in a tank, and every alternate day over a 6-
1031 day period, a subset of capelin was scored to assess their semen regeneration ability based on

three quantity categories, each distinguished by a unique colour code. The total number of capelin scored on specific dates is indicated above each respective bar. The y-axis represents proportionate scores, standardized through conversion to proportionate values, normalizing the data for comparison. The 2023 capture date and location are provided at the top.



1037 Figure 2-8. Variation in capelin semen regenerative capacity across different fish total lengths
1038 based on quality. Completely stripped capelin were kept in tanks, and a subset scored every
1039 alternate day over a 6-day period to assess semen regeneration in the year 2023. Semen quality is

represented by size-differentiated colour points, with larger and darker points denoting better quality. The thick line within the box plot represents the median, the box represents the interquartile range (25th and 75th quartiles), and the whiskers extend to 1.5 times the interquartile range from the first and third quartiles, respectively.



1045 Figure 2-9 Variation in capelin semen regenerative capacity across different fish total lengths
1046 based on quantity. Completely stripped capelin were kept in tanks, and a subset scored every
1047 alternate day over a 6-day period to assess semen regeneration in the year 2023. Different box
1048 colours indicate semen quality, while semen quantity is represented by size-differentiated colour

1049 points, with larger and darker points denoting better quantity. The thick line within the box plot
1050 represents the median, the box represents the interquartile range (25th and 75th quartiles), and
1051 the whiskers extend to 1.5 times the interquartile range from the first and third quartiles,
1052 respectively.
1053

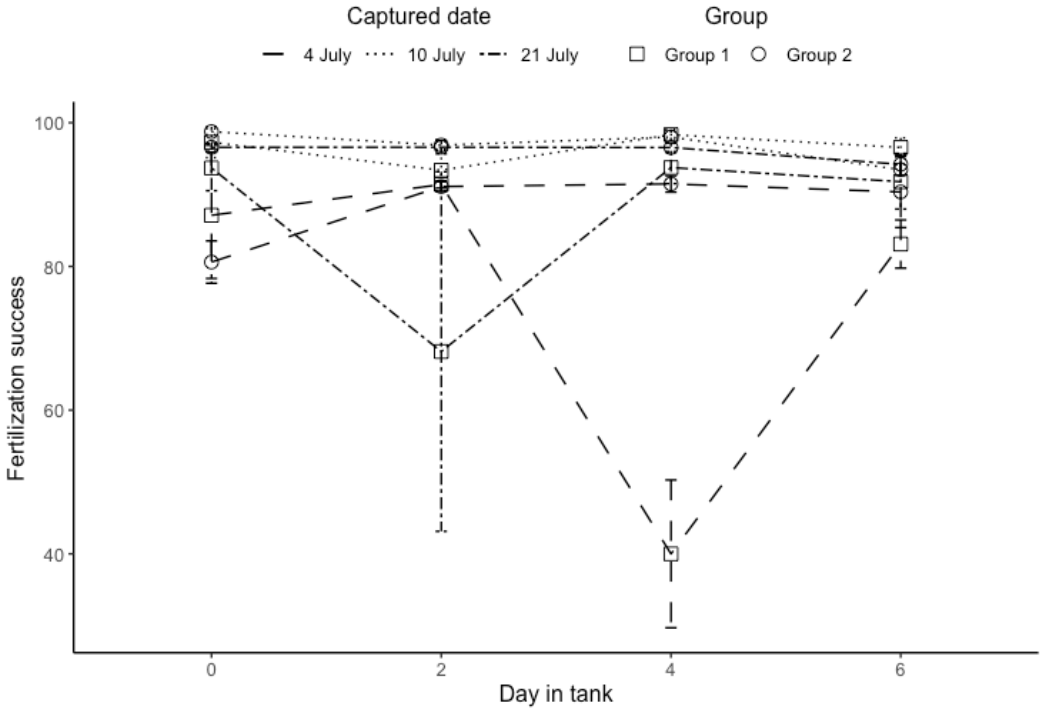
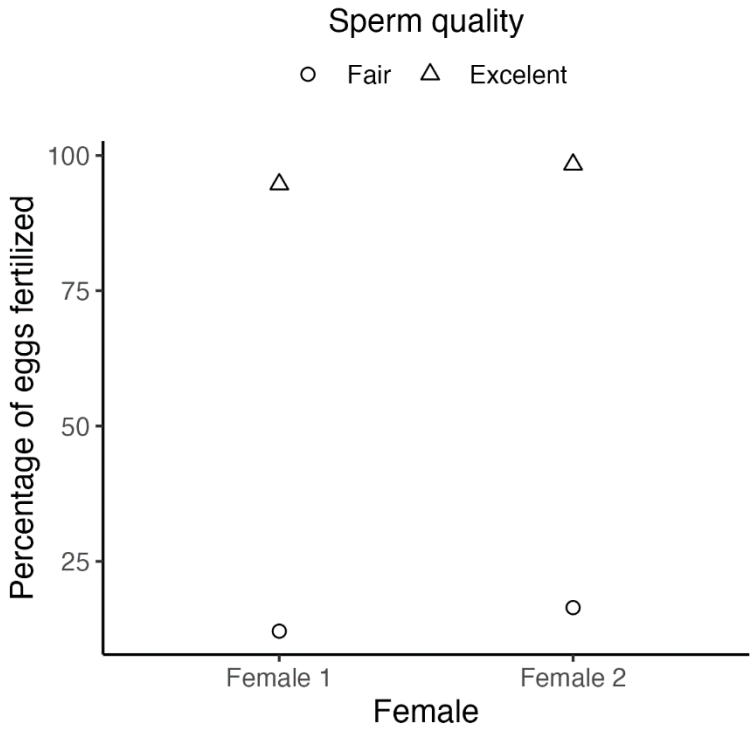


Figure 2-10. Effect of delayed spawning on capelin gametes (semen and eggs) quality, quantified by fertilization success. Data are shown as means (± s.d.), averaged within the capture date and tank group and calculated by giving equal weight to each fertilization set. The 2023 capture dates are indicated by different lines, whereas tank groups by different shapes.

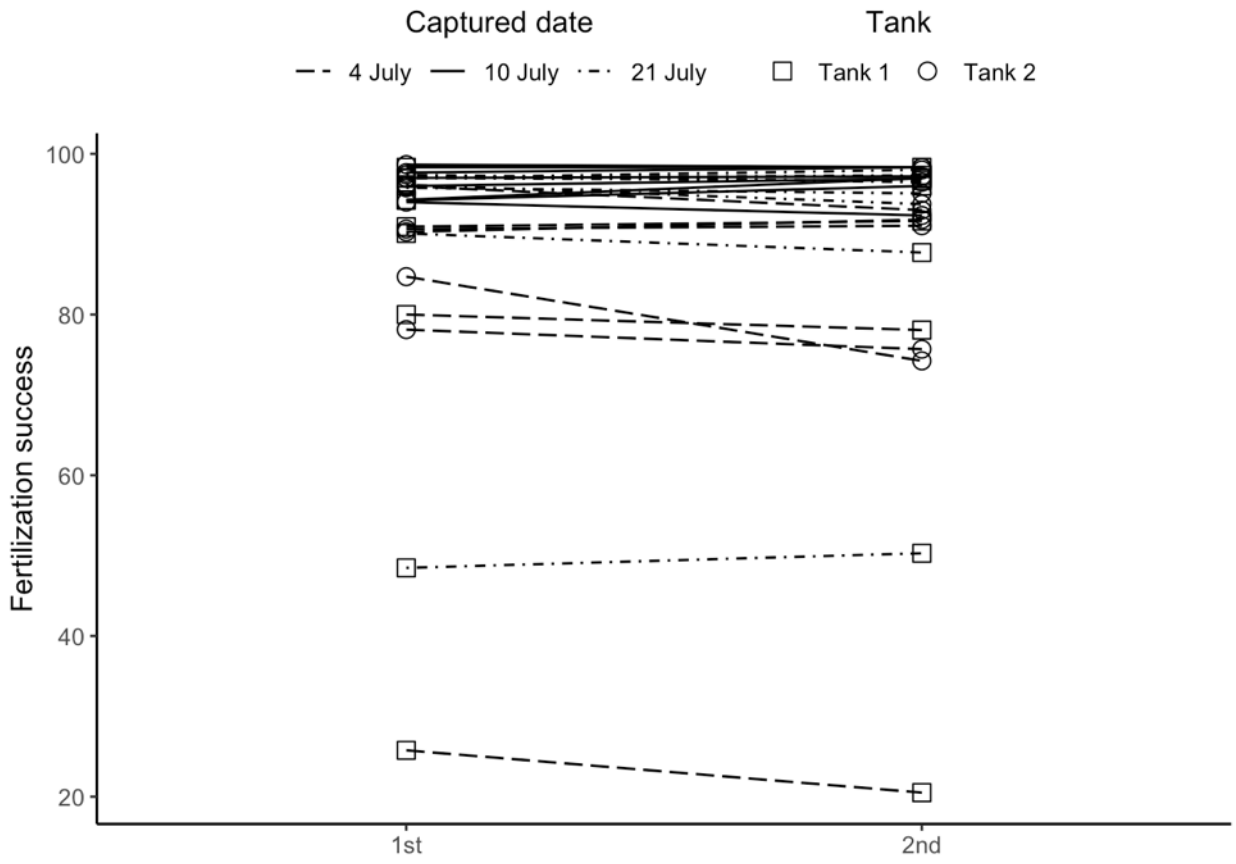
Chapter 2 appendices



Appendix 2-1. Comparing the fertilizing capacity of semen of two quality categories, fair and excellent, using two females and two males of each semen quality category. The data indicates that the categorization of sperm quality into fair and excellent is meaningful, as excellent semen quality results in 6.75 times higher fertilization rate compared to fair semen quality.

For fertilization, semen from a male of each quality category was used to fertilize the eggs from the same female, and this was repeated with another female, utilizing two females and four males in total. Dry fertilization was conducted using 20 µL of semen and 0.4gm eggs, following Purchase (2018) protocol. Eggs were incubated in the dark using a plant growth incubator at 4°C. After 18 hours, approximately 300 eggs from each cross were examined under a microscope to

1072 distinguish fertilized eggs (8- and 16-cell differentiation stage) from unfertilized eggs (no cell
1073 division) (Beirão et al., 2018b).



Appendix 2-2. Repeatability of fertilization scoring to ensure the selected sample accurately represents overall fertilization success. The fertilization success from each capture date and tank group of four randomly chosen fertilization sets were scored twice using different sets of eggs each time. Capture dates (blocks) are indicated by different lines, whereas tank (tank group) by different shapes. The average 2.02% coefficient of variation (CV) between two scorings signifies low relative variability, indicating high repeatability in the selected samples and supporting its reliability as a representation of overall fertilization success.

The average CV is determined by calculating the CV for each combination of capture dates and tank groups separately, based on two scorings for fertilization sets. The overall average CV is then obtained by averaging these individual CVs.

Chapter 3. Can sperm experience prior to fertilization improve offspring performance beyond paternity in an external fertilizing fish?

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Abstract

Recent research has suggested that the environment encountered by sperm post-ejaculation may impact offspring development beyond the transfer of the paternal genome. However, the adaptive significance and mechanisms that underlie such effects remain unclear. Two potential processes have been proposed: 1) haploid selection, whereby stressful conditions act as post-ejaculation pre-fertilization selective pressures on semen, resulting in the fertilization of, on average higher quality sperm and the production of offspring that exhibit superior performance across all environmental conditions that they might encounter; and 2) epigenetic inheritance, where environmental conditions induce changes in sperm that are passed down to offspring, resulting in improved offspring performance, but only under conditions that match those experienced by the sperm. Capelin (*Mallotus villosus*) sperm and embryos are sensitive to salinity and represent a good system to investigate these phenomena. We used a split-ejaculate and split-brood experimental block design to expose capelin semen to benign (25 psu) and stressful (35 psu) salinity prior to egg contact and split each batch of fertilized eggs for incubation at matched and mismatched salinity to those of sperm exposure. Our findings revealed no significant differences

in hatch characteristics between offspring produced by sperm exposed to benign and stressful salinity conditions. A follow-up experiment found the same result with an increased selection gradient at 5 psu and 35 psu. Our study does not support the hypothesis that sperm experiences exert an adaptive influence on the development of offspring characteristics.

Introduction

Offspring experience variable conditions during their ontogeny, particularly during the embryonic phase in species with external development. When these conditions vary in space and across generations, selection encounters challenges in fine-tuning local adaptation (Mérot, 2022). This is complicated by a biphasic life cycle where selection pressures experienced by diploid adults may be very different from that of their haploid gametes (Purchase et al., 2021). Beyond genetic inheritance, parents possess the capacity to influence their offspring's phenotype through other means, a phenomenon referred to as a parental effect (Badyaev & Uller, 2009). If these effects are adaptive, they can help offspring in coping to these varying conditions (Burgess & Marshall, 2014; Chirgwin et al., 2021; Crean et al., 2013; Jensen et al., 2014). Among parental effects, maternal effects hold particular significance in anisogamous reproduction, where the egg, characterized by its larger size and nutrient-rich composition, plays a pivotal role in embryo development. Compared to sperm, the substantial allocation of resources to eggs leads to a more direct and influential impact on the offspring's development, particularly during critical early life stages (Bonduriansky & Head, 2007; Jensen et al., 2014; Mousseau & Fox, 1998; Wolf & Wade, 2009).

However, there is increasing evidence of paternal effects, challenging the conventional notion that fathers solely contribute DNA through sperm (Crean et al., 2013; Crean & Bonduriansky, 2014; Evans et al., 2017; Ragsdale et al., 2022; Simmons et al., 2022). Changes in the father's condition, such as modifications in diet, changes in hormonal profiles, or altered social interactions, can affect the content of their semen. Such modifications in semen content can influence offspring development (Butzge et al., 2021; see review Evans et al., 2019; Ragsdale et al., 2022; Simmons et al., 2022). But sperm also encounter various conditions post-

release, especially in external fertilizers (Purchase et al., 2021). Several studies have proposed that post-release sperm experiences (environment encountered by sperm post-ejaculation) may influence the subsequent phenotype of offspring outside of paternity (see review Crean & Immler, 2021). The literature presents contrasting perspectives on these effects. Some propose that preparing offspring for anticipated conditions could be an adaptive strategy (Immler et al., 2014; Ritchie & Marshall, 2013), while others express concerns about the potential transmission of physiological stress from sperm to offspring (Kekäläinen et al., 2018; Lymbery et al., 2021).

Sperm, beyond their role in transmitting haploid DNA, are equipped with a suit of epigenetic components such as DNA methylation, RNAs, and histone modifications (Immler, 2018). These components can undergo alterations in response to the post-ejaculatory environment and are integral to the process of epigenetic regulation, which affects gene expression without changing the DNA sequence (Immler, 2018; Lettieri et al., 2019b; Lymbery et al., 2020; Pitnick et al., 2020). However, for epigenetics to play a role in the adaptative process, these altered sperm must impact the phenotype of the resulting offspring (Donkin & Barrès, 2018; Marshall, 2015). These alterations in sperm may confer advantages to offspring when they encounter similar conditions to those experienced by the sperm (Figure 3-1 A & B). This introduces the anticipatory hypothesis, suggesting that offspring are primed for anticipated conditions, enhancing their performance in such conditions (Burgess & Marshall, 2014; Graziano et al., 2023; Hosken et al., 2003; Marshall, 2015; Ritchie & Marshall, 2013). However, if conditions differ from those experienced by the sperm, offspring performance may not improve, as the modifications may not prove advantageous in the new context (Lymbery et al., 2021).

In contrast to sperm modifications inducing parental effects in offspring, there is also the potential possibility for haploid selection (Immler et al., 2014; Kekäläinen et al., 2018;

Lymbery et al., 2021; Ritchie & Marshall, 2013). Sperm competition is usually conceptualized as between ejaculates of rival males (Parker 1970; Parker, 2020). However, the presence of numerous sperm cells in an ejaculate, along with the limited availability of eggs for fertilization, naturally gives rise to competition among sperm within an ejaculate (Immler et al., 2014; Sutter & Immler, 2020). This competition is significantly influenced by the phenotypic traits of individual sperm, which include morphology, motility, and viability (Immler et al., 2008). Traditionally, these traits were thought to be regulated only by the male's diploid genotype (all sperm within an ejaculate thus having phenotypes under the same genetic influence), which relegated sperm haploid DNA to a mere carrier of paternal genetic information without influence on sperm phenotypes. However, emerging studies may suggest that these phenotypic traits are partly genetically determined by the sperm's haploid DNA (Alavioon et al., 2017; Borowsky et al., 2018; see review Immler, 2019). This introduces the concept of haploid selection, a process whereby the phenotype of sperm is partially governed by their unique haploid genome (Alavioon et al., 2017; Immler et al., 2014). If this is true, sperm with “high quality” genes can manifest phenotypically superior sperm traits (for example, larger size, higher motility) and may confer a selective advantage that can impact fertilization outcomes.

Conditions faced by sperm post-release further influence the outcome of this competition among sperm within an ejaculate. Post-release under benign conditions, where the selection pressure is lower, a greater number of lower quality sperm can achieve fertilization, lowering the average quality across embryos. This, in turn, can lead to the production of offspring of average lower quality. In contrast, under stressful conditions, due to higher selection pressure, on average, superior sperm are selected (Holt & Look, 2004; Sutter & Immler, 2020). This selective filtration can result in a greater proportion of the remaining superior sperm

carrying 'high-quality' genes than under benign conditions. Consequently, this may result in the production of, on average, high-quality offspring capable of superior performance across diverse environmental conditions (Figure 3-1 C & D).

Therefore, beyond paternity, offspring performance is possibly influenced by sperm experience, encompassing which sperm fertilizes the egg, haploid selection, and what happened to the fertilizing sperm, epigenetics (Purchase et al., 2021). These mechanisms collectively contribute to shaping the genetic and epigenetic inheritance that offspring receive from their fathers, ultimately influencing both offspring genotype and phenotype. Therefore, it is imperative to conduct further research to understand these intricacies and ascertain their relative contributions. Such understanding is of paramount importance in elucidating the potential adaptive mechanisms at play and thereby shedding light on evolutionary processes and local adaptation.

Hence, to explore which potential adaptive mechanisms, haploid selection or epigenetics, have a greater influence on offspring, we exposed the offspring produced from sperm exposed to benign and stressful water chemistries by incubating embryos under salinity conditions that match and mismatch those of sperm exposure. We used the marine beach spawning fish, capelin (*Mallotus villosus*), which is an ideal system as its sperm and embryos are highly sensitive to salinity (Beirão et al., 2018a; Purchase, 2018). Capelin have a unique reproductive strategy for an external fertilizer wherein its sperm are active upon leaving the male's body (Beirão et al., 2015; Beirão et al., 2018a). Capelin embryos, although sensitive to salinity, still manage to develop successfully in ocean salinity in nature (Purchase, 2018). This could be due to the potential transmission of adaptive traits regarding salinity conditions from the post-ejaculation experiences of capelin sperm to embryos, possibly enhancing the embryo's

ability to cope under such conditions. To investigate the existence of such an effect of capelin sperm and, if confirmed, to delineate the underlying mechanisms and their relative contribution, we conducted experiments in which we exposed sperm to benign or stressful salinity conditions and then incubated the embryos under conditions that either corresponded to or differed from the sperm's initial salinity exposure. Specifically, if there are no differences between offspring development sired by sperm exposed to different conditions, it implies there is no impact of sperm experiences on offspring development (Figure 3-2 A). If we observe improved offspring development when the incubation conditions match those experienced by the sperm, it would point toward the inheritance of epigenetic modifications (Figure 3-2 B). Conversely, if we find that offspring development is superior when sired by sperm exposed to stressful salinity conditions, regardless of the salinity conditions during embryo incubation, it suggests the haploid selection process (Figure 3-2 C).

Methodology

General design

The study used a split-ejaculate and split-brood experimental block design to investigate the influence of sperm exposure on offspring development (Figure 3-3). To isolate the influence of sperm post-ejaculation pre-fertilization exposure on offspring, offspring performance sired by sperm exposed to benign or stressful conditions was compared under matched or mismatched conditions to those of sperm exposure. In 2022, 33 experimental blocks (families) were used with a lower exposure gradient, while in 2023, a higher exposure gradient was implemented, employing 9 blocks (families). Each block was based on a different male-

female pairing to control for any systematic variation due to diploid paternal and maternal level effects.

2022 experiment: lower exposure gradient

Capelin collection and gamete collection

Capelin were captured using a cast net in July 2022 as they were spawning on four beaches of Newfoundland, Canada (Middle Cove: 47°39' 2.75" N, 52°41' 45.24" W; Bellevue Beach: 47° 38' 9.276" N, 53° 46' 51.0924"W; Bryant's Cove 47° 34' 18.155" N, 52° 44' 14.862" W; Chapel Arm: 47° 31' 0.6564" N, 53° 40' 10.7508" W). They were transferred to the laboratory and kept in flow-through (simulated natural photoperiod) seawater tanks (males and females separately) at 4 – 8 °C that roughly match the conditions in the natural habitat at the spawning period. Gametes were collected within 48 hours of capture.

To create an experimental block, a randomly selected female capelin was euthanized using an overdose of tricaine methanesulfonate (also known as MS-222) buffered with sodium bicarbonate and then dried using a paper towel. The eggs were collected through gentle pressure on the abdomen (Beirão et al., 2018a; Purchase, 2018), divided into two standard batches of 0.4g (about 2000 eggs) each and placed in flexible teflon trays on ice. Similarly, semen from a male was collected in a teflon tray and split into two 20 µL aliquots, with each aliquot placed in a new tray. This was done to potentially ensure that each aliquot had the same number of sperm. Only males with whiter semen (excellent semen quality – see Chapter 2 for semen quality categorization), indicative of high sperm density (Steyn, 1993), were used to ensure good fertilization success in the experiment.

Sperm exposure

Capelin sperm are motile upon release from the male body and exhibit salinity sensitivity (Beirão et al., 2018a). Hence, without delay, to manipulate the environment that semen (hereafter sperm) experienced, each aliquot was exposed to 40 µL water of either benign (25 psu) or stressful (35 psu) for a period of 4 seconds (Figure 3-3). This quick exposure was achieved by having two people work on each aliquot simultaneously. After the 4 second exposure, the sample was returned to a salinity of 30 psu (midpoint of 25 psu and 35 psu) by exposing it to reciprocal salinity for 1 second. The purpose of returning the sample to 30 psu was to ensure that the eggs received the same salinity level regardless of which salinity exposure the sperm had undergone. This ensured that any effects on the embryos were solely due to sperm exposure. This resulted in two exposed sperm aliquots from the same semen sample.

The exposure gradient, exposure duration, and sperm-water ratio were chosen to optimize their impact on embryo development while carefully considering the trade-off involved in maintaining a sufficient number of embryos produced. Additionally, it should reflect realistic natural conditions. Specifically, 25 psu and 35 psu were chosen as 35 psu is stressful to capelin sperm, closely approximating the salinity levels typically found in ocean water, while 25 psu, though not precisely representative of ocean salinity levels, was chosen as a more benign alternative (Beirão et al., 2018a). Capelin spawn on beaches in between breaking waves, which last 2-3 seconds (Orbach et al., 2020; Templeman, 1948), and their sperm are already active upon release (Beirão et al., 2018). So, 4 seconds of exposure duration was chosen to represent the natural sperm exposure period during fertilization and to optimize the effect on embryos while still yielding a sufficient number of fertilized eggs for measuring various hatch characteristics, recognizing its potential impact on fertilization success. Additionally, a sperm-water ratio of 1:2 was chosen to dilute the sperm enough to change the chemistry of the seminal plasma while

ensuring an adequate number of fertilized eggs. Salinity water used for sperm exposure was maintained at 4 °C.

In-vitro wet fertilization

Immediately after sperm exposure, within each block, to create two sets of fertilized eggs of benign and stressful salinity, each exposed sperm aliquot was mixed with a standard batch of eggs (i.e. 0.4g of eggs, ~ 2000 eggs) using a toothpick. Care was given to ensure that both standard batches of eggs underwent the same procedure and fertilization was done at the same time. Capelin eggs are sticky when they come in contact with water, so immediately after fertilization, a solution of 600 mg/L tannic acid (see Purchase (2018)) mixed in 30 psu water was added and swirled for 30 seconds to remove stickiness. This was then poured off and rinsed thrice with 30 psu water, making the observation of individual eggs possible.

For each block, two control batches underwent the same process as the fertilized eggs described above but did not have sperm added. These unfertilized eggs were used as a reference when assessing fertilization success.

Incubation

The two sets of fertilized eggs per block were divided into four fertilization subsets based on two sperm salinity × incubation salinity combinations, each consisting of approximately 1200 eggs. The subsets were then subjected to incubation at either matched or mismatched salinity to that of sperm exposure, resulting in four distinct conditions: benign-benign, benign-stressful, stressful-benign, and stressful-stressful (see Figure 3-3). Additionally, the two control batches per block were incubated, one at benign and the other at stressful salinity. All four fertilization subsets and two control batches were incubated at 4 °C.

After 20 hours, approximately 100 eggs from each fertilization subset were examined under a microscope to determine fertilization success by counting the number of fertilized (8 and 16-cell differentiation stages) and unfertilized eggs (no cell division) (Beirão et al., 2018b). To ensure successful hatches and produce enough data for the analysis of offspring performance, families with > 25% average fertilization success (n=33 in 2022) were chosen (Appendix 3- A1) to proceed with the experiment. The fertilization rates of two families were assessed twice using different eggs within each subset to measure the repeatability of our assessment (Appendix 3- A2).

Following previous protocols to transfer the eggs to replicate incubation beakers (Purchase, 2018), a small group of eggs was removed from a fertilization subset, placed in a petri dish and counted. Additional eggs were added or removed until the final count reached 50 eggs. A picture was taken to validate the count. The eggs were then transferred to a replicate beaker containing 40 mL of water of the same salinity as that of the subset. This process was repeated until each family was transferred to 16 beakers, with four replicate beakers for each subset (4 subsets × 4 replicate incubation beakers = 16 beakers).

These replicate incubation beakers were kept in the same incubator, but the temperature was raised from 4 °C to 10 °C, which took ~15 minutes. This temperature was chosen to produce the highest hatch success rates across all salinity levels (Purchase, 2018). This choice was important because it ensures a higher sample size to measure other offspring development parameters, which rely on hatch success. All replicate beakers from the same family were placed in the same incubator. The water in each replicate beaker partially was replaced with new water of the same salinity every other day, with half of the volume being decanted off before the addition of new water. Experimental saline water was prepared in bulk by

dissolving an appropriate amount of Instant Ocean Sea Salt™ in dechlorinated tap water and stored at 10 °C.

Replicate beakers were checked daily at 10 AM for hatched larvae, and the larvae were removed immediately and recorded by replicate beaker. All larvae were preserved in buffered formalin solution (2.2%), and hatch size (body length- from snout to end of the tail fin) was determined by selecting five larvae at random, if available, using digital pictures taken from a dissecting microscope. For a given replicate incubation beaker, once hatching started, if no new larvae hatched for three consecutive days, it was assumed that no more would hatch, and the replicate beaker was discarded (Purchase, 2018).

2023 experiment: higher exposure gradient

In 2022, we found no significant effect of sperm exposure on offspring traits (see result section). So, in 2023, we increased the exposure gradient from the previous experiment in the hope of gaining more insights into the impact of sperm exposure on offspring development and measured the length of the starvation time to assess the potential effects of sperm exposure on offspring in later development stages.

The fertilization and incubation process followed the protocol of the 2022 experiment, with some modifications. Gametes were collected from fish within 6 hours of capture. To increase exposure gradient, sperm aliquots were exposed to 5 psu (benign) and 35 psu (stressful) at a sperm-to-salinity water ratio of 1:5. The tannic acid solution was prepared using 20 psu water (midpoint of 5 psu and 35 psu). To assess fertilization success, approximately 300 eggs were scored. Consistency in our scoring was evaluated by scoring two sets of eggs twice (see Appendix 3 - A2).

The initial five larvae hatched from each beaker were preserved in a 2.2% buffered formalin solution for hatch size measurement. Subsequently, to measure the length of starvation time, a minimum of ten larvae were collected from each beaker carefully using a wide-mouth disposable pipette, either on the same day or spread across subsequent days, based on availability (Purchase, 2018). These larvae were then transferred to 50 mL glass vials filled with the same salinity water as the beaker from which they were collected and kept at 10 °C. The number of days required for all larvae to die from the day of hatch was recorded as starvation time. Each vial held a maximum of five larvae, and new vials were used daily. Larvae were checked daily, and survivors were counted and recorded (Purchase, 2018). The remaining hatched larvae were preserved in the same formalin solution along with the initial first five larvae. From these preserved larvae, five larvae were randomly selected for hatch size measurements.

Calculations and statistical analysis

The calculation of the hatch time involved computing a weighted average by multiplying the number of larvae hatched on a given day by the number of days it took them to hatch, summing the values across all hatching days, and dividing by the total number of hatched eggs (Purchase, 2018). As we had four values from four replicate beakers for all hatch characteristics for each salinity treatment (except in some treatments in hatch time, hatch size and starvation time in 2023), a data quality control approach was taken where any experimentally produced outliers were identified using a coefficient of variation above 30% to address possible measurement errors (Brown, 1998). Outliers were only observed in the hatch success data, with no outliers detected in the datasets for hatch time and hatch size in both 2022 and 2023 (Appendices 3- B1, B2, C1, D1, D2, E1, E2). This approach was not extended to the starvation time data due to limited data availability (Appendix 3-F).

Hatch characteristics of four replicate beakers, if available, containing the same salinity water from each fertilization subset were averaged to generate one value (per fertilization subset) and used in data analysis (Figure 3-3). Starvation time was determined only in 2023; the values were averaged first between vials (if available) and then between four replicate beakers. The data for hatch and starvation time had a resolution of a whole day.

Each hatch characteristic was analyzed separately for the years 2022 and 2023, as treatment levels varied between years. Hatch success was analyzed using a generalized linear mixed model (GLMM) with binomial error distribution ('lme4' package; Bates et al., 2015) utilizing the function 'cbind(hatched, unhatched)' as the response variable. The model included fixed-effect categorical variables of sperm salinity, incubation salinity, and their interaction, as well as a random effect of family. Additionally, only in 2023, fertilization success was added as a covariate to the model and reanalyzed. Due to low precision in scoring fertilization success in 2022, this step was not taken during that year's analysis. However, all hatch success models exhibited overdispersion, so they were re-analyzed with a beta-binomial error distribution ('glmmTMB' package; Brooks et al., 2017; Lymbery et al., 2021). The same model parameters were used for hatch time, starvation time and hatch size analyses but utilizing a general linear mixed model with a normal error distribution. Statistical analyses were performed in R v. 4.3.0 (R Core Team, 2023).

Results

If offspring development improves when the incubation conditions match those experienced by the sperm, it would support the epigenetic hypothesis with a significant interaction between sperm salinity and incubation salinity. Our results (Tables 3-1 & 3-2)

indicate no significant interactions on all hatch characteristics in both 2022 and 2023. If offspring sired by sperm exposed to stressful salinity condition exhibit improved development across all salinity conditions, then it would support the haploid selection hypothesis with significance in sperm salinity in the results. We found that sperm exposure did not influence development at either salinity (Tables 3-1 & 3-2). As expected, there was a positive relationship between fertilization rate and hatch success (Table 3-1, 2023a). We observed that benign incubation salinity conditions led to higher hatch success (~1.12 times), shorter hatch time (~1.02 times) and hatch at a larger size (~1.03 times) in 2022 when compared to the stressful incubation salinity condition. Notably, this contrast was more pronounced in 2023 (~4.5 times for hatch success calculated from both total and fertilized eggs, 1.13 times for hatch time and 1.13 for hatch size) than in 2022 (Figure 3- 4 A-D and Figure 3-5 A-C). Starvation time was only measured in 2023 and showed the same pattern: ~2.19 times longer time to starve in benign incubation salinity than in stressful incubation salinity (Figure 3-4 E) but non-significant.

Discussion

Juveniles may encounter unpredictable environmental conditions during development due to spatial or temporal variation in the landscape or environmental stochasticity across generations. This creates a challenge for selection to fine-tune local adaptation in development. Parental effects may bridge this gap by transmitting information to offspring about conditions they are likely to encounter, which can influence their development. In external fertilizers, prior to fertilization, sperm are exposed to conditions that embryos will likely experience. Sperm experiences may have the potential to alter embryo development in an adaptive way. We tested this in beach spawning capelin, where development occurs across widely varying salinities.

Capelin sperm swimming performance and embryo development are both highly sensitive to external salinity, however, our results indicate that sperm exposure to benign or stressful salinity conditions does not influence offspring development (i.e. hatch time and size, and starvation time). This suggests that, contrary to some previous studies in other species, sperm experiences do not serve as a conduit for parental effects in capelin; seemingly, a sperm's only role is transferring the paternal genome. Further research is required to clarify whether sperm experiences, beyond the salinity exposure in general, exert an influence on offspring development in capelin.

The influence of sperm post-ejaculation experiences on offspring phenotype is a subject of ongoing research yielding a range of results. Lymbery et al. (2021) revealed that exposure of sperm to high temperatures, indicative of stressful conditions, exerted adaptive effects on embryos of mussel (*Mytilus galloprovincialis*) only when the embryos were incubated at ambient temperatures, considered benign conditions. However, when embryos were incubated at high temperatures, those sired by sperm treated at high temperatures exhibited inferior performance. In contrast, Ritchie and Marshall (2015) and Graziano et al. (2023) suggested that the adaptive effects of tubeworm (*Galeolaria gemineoa*) and salmon (*Salmo salar*) sperm exposure when the offspring condition aligns with the condition of sperm exposure, hinting at the epigenetic mechanisms at play. On the contrary, Kekäläinen et al. (2018) present evidence of maladaptive effects on European whitefish (*Coregonus lavaretus*) offspring resulting from sperm experiences, further complicating the overall picture. It is important to note that our study represents a departure from these findings, as it reports no discernible impact of sperm experiences on the offspring phenotype.

We propose two primary interpretations for our findings. Firstly, it is well-established that sperm possess a group of epigenetic components (Donkin & Barrès, 2018; Immler, 2018) and undergo changes due to exposure to post-release conditions (Lymbery et al., 2020; Pitnick et al., 2020). But, for any post-release alterations in these epigenetic components to affect the embryo, they must have a functional role during the embryo's development. Our study suggests that salinity exposure either does not alter the epigenetic component of capelin sperm or that if it does; the changes do not translate into developmental effects on the embryo. This implies that the post-release epigenetic condition of sperm does not affect the embryo, possibly because these changes do not persist through fertilization or are not influential during the critical stages of embryonic development. Secondly, the post-release condition may selectively influence the average phenotype of sperm that is able to fertilize eggs (Alavioon et al., 2017; Marshall, 2015). However, our results suggest no effect of sperm salinity experiences on embryo development; therefore, it appears that phenotypic variation among sperm within a single ejaculate may not be influenced by their haploid genome. Taken together, these findings align with the traditional belief that the function of sperm is predominantly to deliver the paternal genome and that the experiences of sperm do not impart developmental directives to the embryo, at least in capelin and in the context of salinity.

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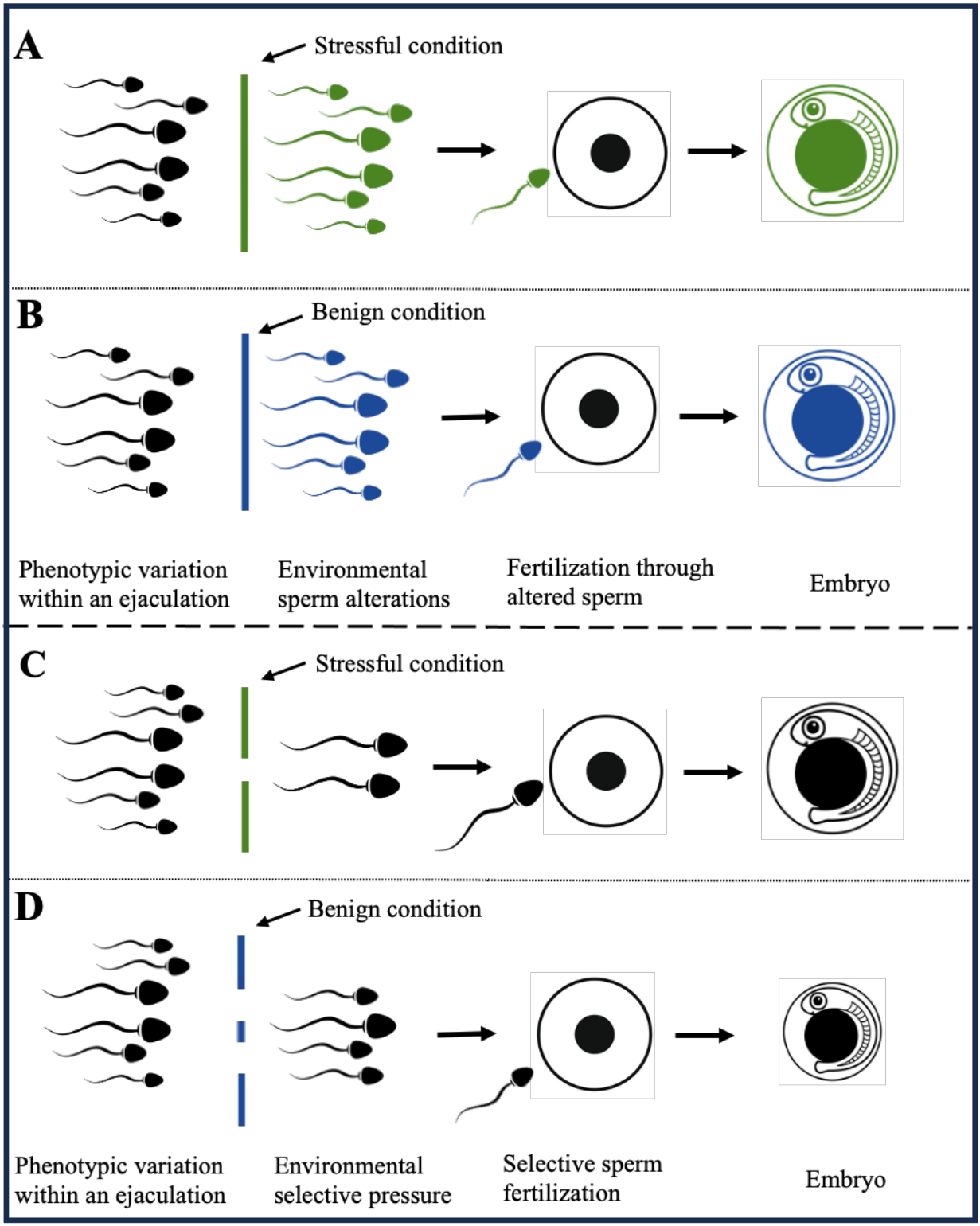
Chapter 3 tables

Table 3 - 1. Type III Wald $\chi 2$ tests for the effects of sperm exposure salinity, incubation salinity and their interaction (S × I) on hatching success in capelin, fitted using generalized linear mixed effects models (GLLM), in 2022 and 2023 (without fertilization covariate) and 2023a (with fertilization covariate). Removing the interaction term from the models did not change the result of odds of hatching in either year (results not shown). The results also remained consistent when re-running the models with the data without removing outliers, with no change observed upon excluding the interaction term (results not shown). * Indicates p-value < 0.05.

Effects	2022			2023			2023a		
	$\chi 2$	d.f.	p-value	$\chi 2$	d.f.	p-value	$\chi 2$	d.f.	p-value
Sperm salinity (S)	2.05	1	0.151	0.013	1	0.909	0.020	1	0.886
Incubation salinity (I)	9.021	1	0.002*	46.627	1	< 0.001*	59.480	1	< 0.001*
S × I	0.221	1	0.638	0.000	1	0.999	0.083	1	0.773
Fertilization (covariate)							9.557	1	0.001*

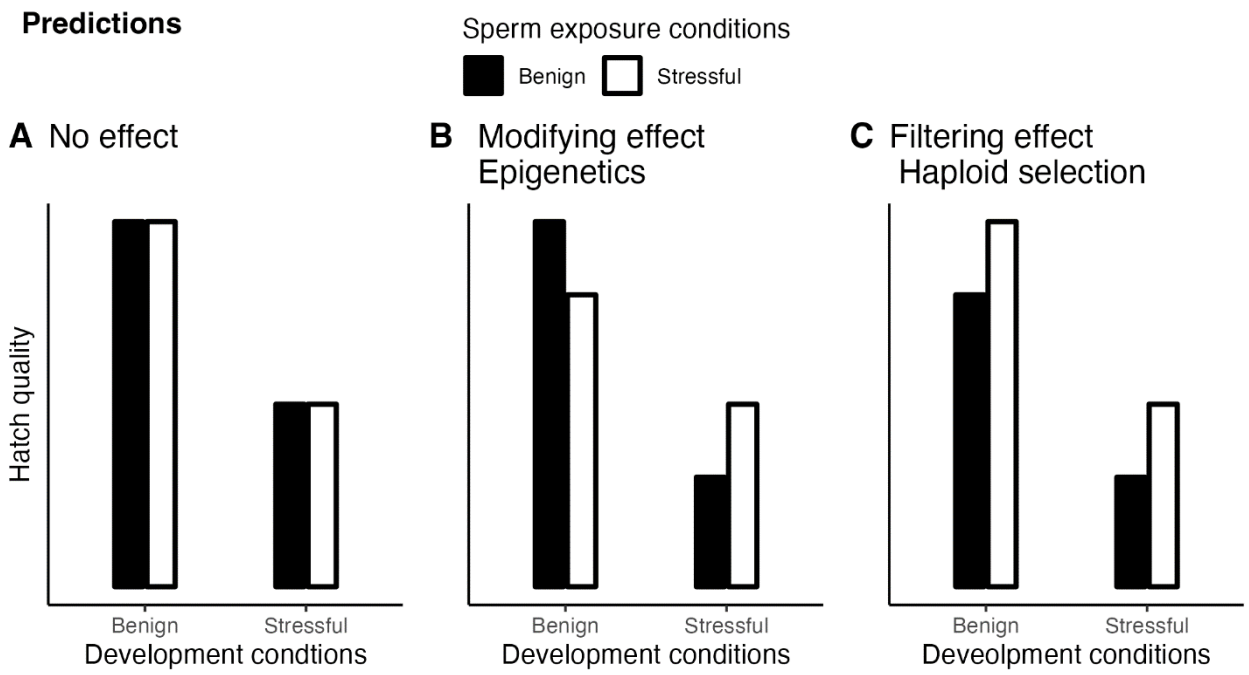
Table 3 - 2. Type III Analysis of variance table with Satterthwaite's method for the effects of sperm exposure salinity, incubation salinity and their interaction (S × I) in 2022 on a) hatch time, b) hatch size in capelin. Additionally, in 2023, the analysis included c) starvation time. Linear mixed effect models (LLM) fit by residual maximum likelihood (REML) in both 2022 and 2023. Removing the interaction term from the models did not change the results for any hatch characteristics in either year (results not shown). * Indicates p-value < 0.05.

Effects	2022			2023		
	F-value	d.f.	p-value	F-value	d.f.	p-value
a) Hatch time						
Sperm salinity	2.175	1, 96	0.144	0.442	1, 21.82	0.512
Incubation salinity	87.790	1, 96	< 0.001*	48.923	1, 22.08	< 0.001*
S × I	0.039	1, 96	0.845	0.0084	1, 22.08	0.927
b) Hatch size						
Sperm salinity	1.271	1, 96	0.262	0.038	1, 21.26	0.847
Incubation salinity	13.899	1, 96	< 0.001*	18.692	1, 18.20	< 0.001*
S × I	0.420	1, 96	0.528	0.007	1, 18.43	0.935
c) Starvation time						
Sperm salinity				0.103	1, 16.34	0.752
Incubation salinity				1.640	1, 17.85	0.216
S × I				1.827	1, 16.34	0.194

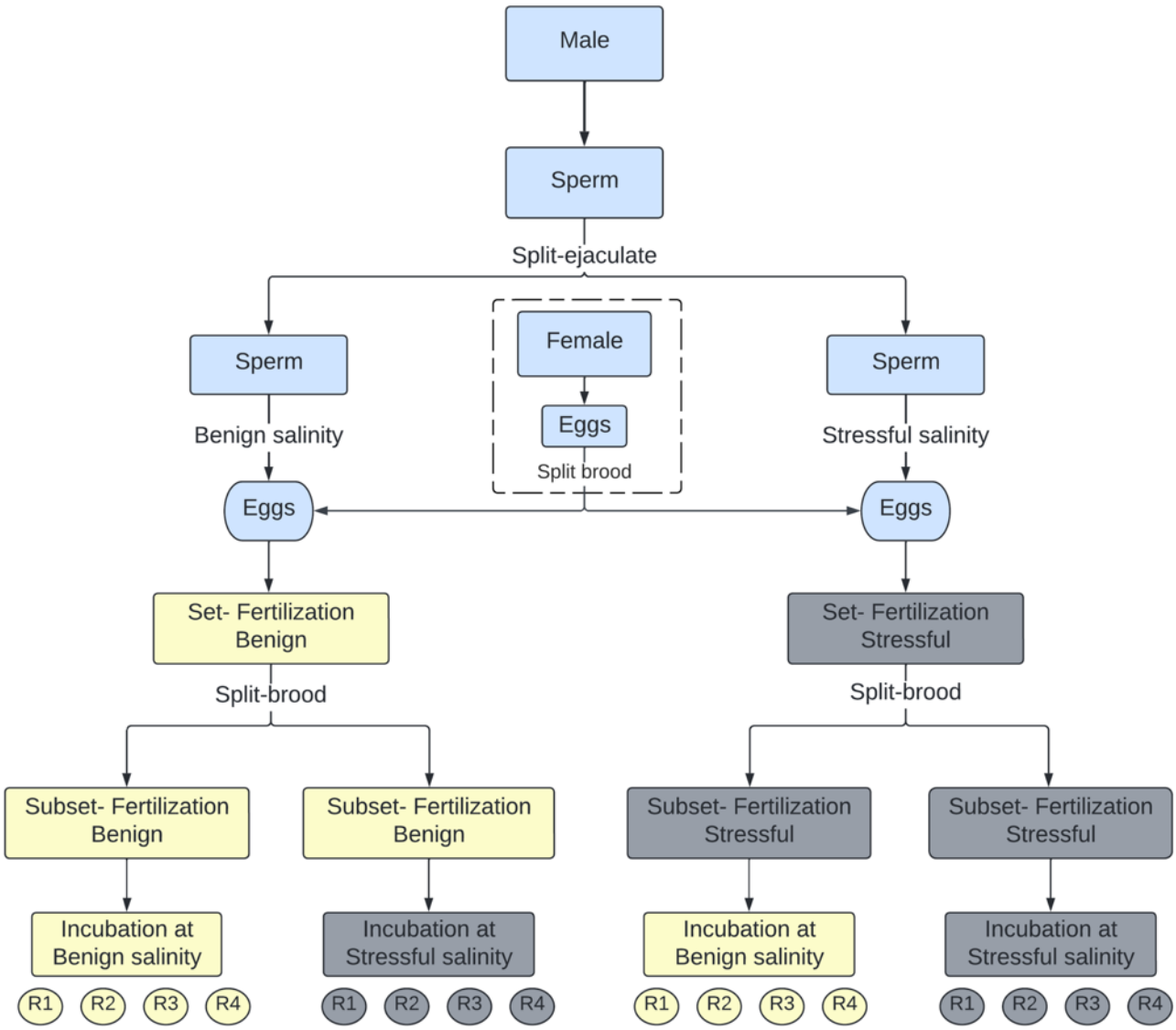

A
Stressful condition
B
Benign condition
Phenotypic variation within an ejaculation
Environmental sperm alterations
Fertilization through altered sperm
Embryo
C
Stressful condition
D
Benign condition
Phenotypic variation within an ejaculation
Environmental selective pressure
Selective sperm fertilization
Embryo

1625

Figure 3-1. Impact on embryo development resulting from sperm experiences through two potential non-mutually exclusive mechanisms: epigenetic alterations (panels A and B) and haploid selection (panels C and D). In panels A and B, stressful condition (A) and benign condition (B) induce alteration in sperm (indicated by colour), affecting all sperm. These altered sperm influence embryo performance in such a way that embryos show optimal performance when exposed to condition matching to those of sperm exposure (same colour of sperm and embryo), as opposed to mismatched condition. This effect on embryo due to the alteration of sperm can also be called as modifying/anticipatory effect. Whereas, in panels C and D, under stressful condition (C), a higher selection pressure acts upon sperm, leading to the preferential selection of, on average, higher-quality sperm. These selected higher-quality sperm contribute to superior embryo performance across all conditions (as indicated by embryo size), whereas in benign condition (D), lower selection pressure permits, on average, lower-quality sperm pass to through, resulting in embryos of reduced overall quality. This effect on the embryo due to the selection of sperm can also be called a filtering effect.



1641 Figure 3-2. Predictions on the effect of pre-fertilization sperm exposure to benign and stressful
1642 conditions on hatch quality under matched and mismatched incubation conditions to those of
1643 sperm exposure. Shown are three panels representing different possible outcomes: (A) No effect,
1644 where exposure of sperm to different conditions has no impact on hatch quality; (B) Modifying
1645 effect, where exposure of sperm induces modifications within the sperm themselves, resulting in
1646 better hatch quality under matched versus mismatched conditions to those of sperm exposure and
1647 (C) Filtering effect, where exposure of sperm to stressful conditions filters out more of the low-
1648 quality sperm, resulting in better hatch quality in all conditions due to, on average, to fertilization
1649 from sperm of higher quality.
1650



1651 Figure 3-3. Split-ejaculate and split-brood experimental design to investigate the effect of capelin
1652 pre-fertilization sperm exposure to two salinities on offspring development at matched and
1653 mismatched salinity level to those of sperm exposure. Shown is the procedure for 1 family
1654 (block) created from 1 male and 1 female chosen at random. Eggs from the single female were
1655 split into two standardized batches. A male's ejaculate was then divided into two standardized
1656 aliquots, with one exposed to benign salinity and the other to stressful salinity for 4 seconds prior
1657 to egg contact. Each brood of fertilized eggs was further split into two subsets and incubated at
1658 matched and mismatched salinity to those of sperm exposure. The design controlled for male and

female variation and allowed the isolation of the effect of sperm exposure to different salinities on offspring development.

R1, R2, R3, and R4 are replicate beakers.

2022 experiment - Lower exposure gradient: Benign (25 psu), Stressful (35psu), n = 33 blocks/families

2023 experiment - Higher exposure gradient: More benign (5 psu), Stressful (35psu), n = 9 blocks /families

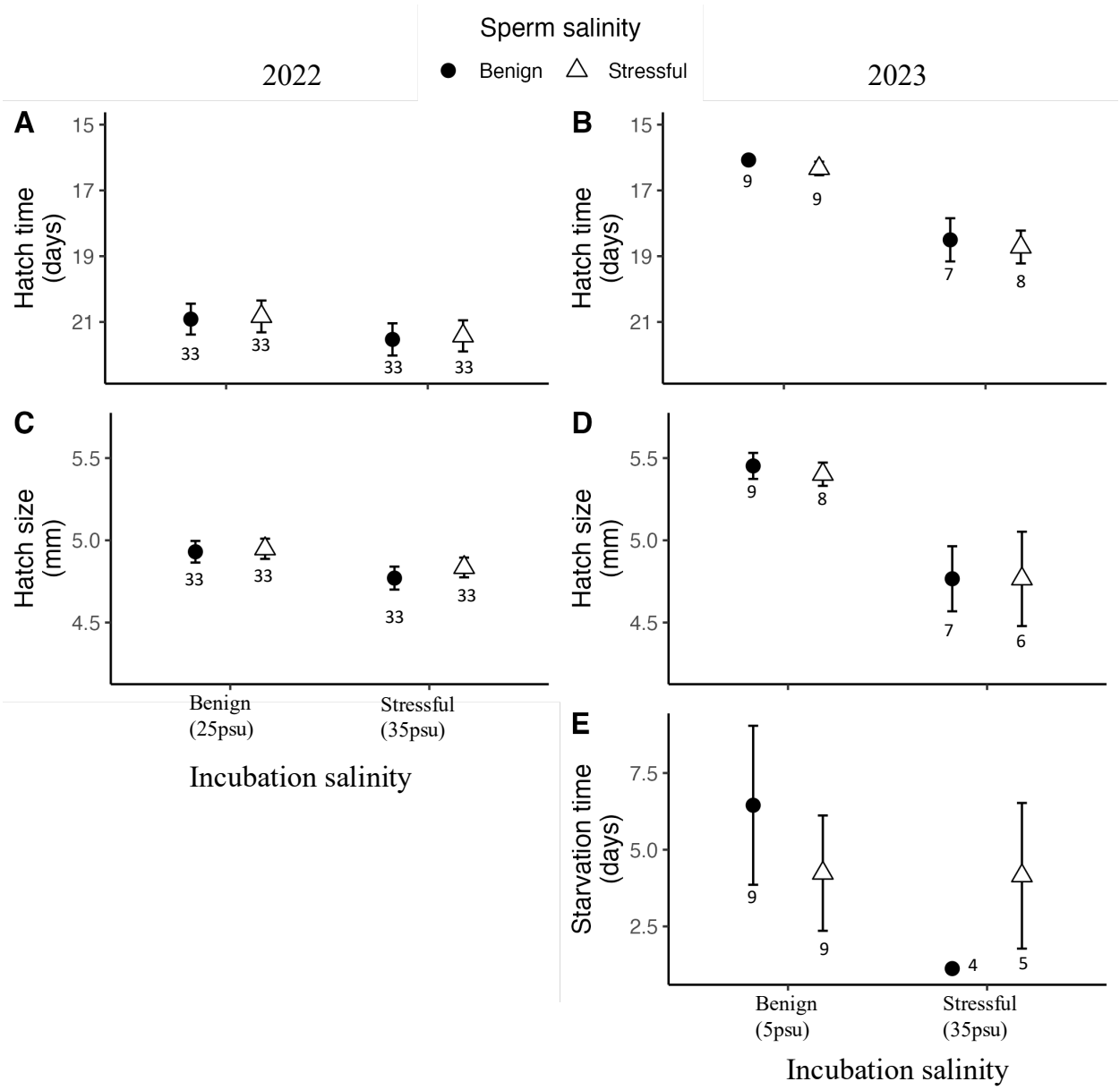


Figure 3-4. The effects of sperm and incubation salinity using a split-ejaculate and split-brood experimental design on hatch qualities of capelin. The left panel presents hatch time (A) and hatch size (C) from 2022, while the right is hatch time (B), hatch size (D), and starvation time (E) from 2023. Data are shown as means, averaged within families and treatments, calculated giving equal weight to each replicate incubation beaker. For starvation time, equal weighting was first done within vials and then across incubation beakers, treatments, and families. Error bars

are standard errors among families, with the number next to each point indicating the number of families. Family number differences in hatch characteristics, apart from hatch success, in some treatments in 2023 are due to no hatching in those treatments. Y-axis of hatch time is inverse, indicative of its negative association with hatch quality. Salinity conditions in 2022 were benign (25 psu) and stressful (35 psu), while in 2023, benign at 5 psu and stressful at 35 psu (Purchase, 2018).

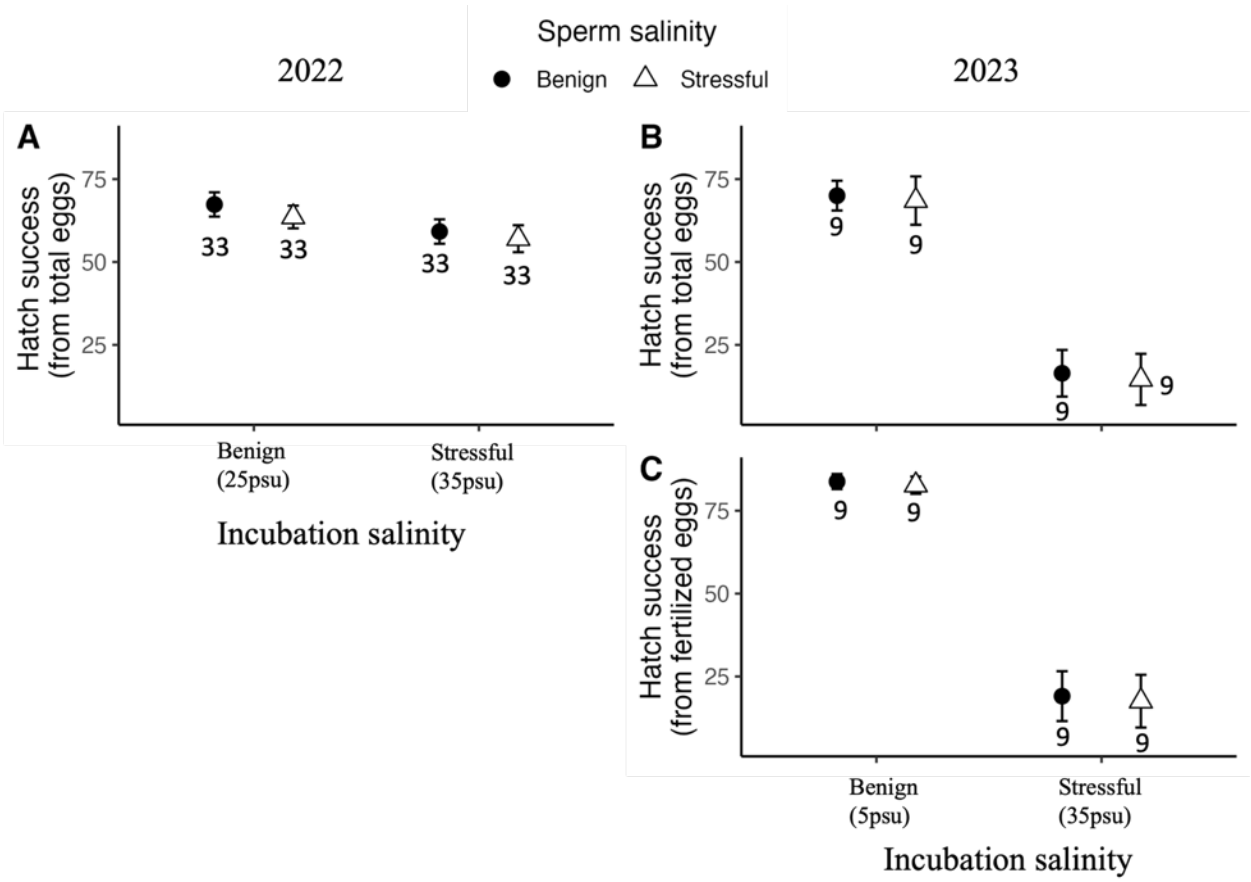
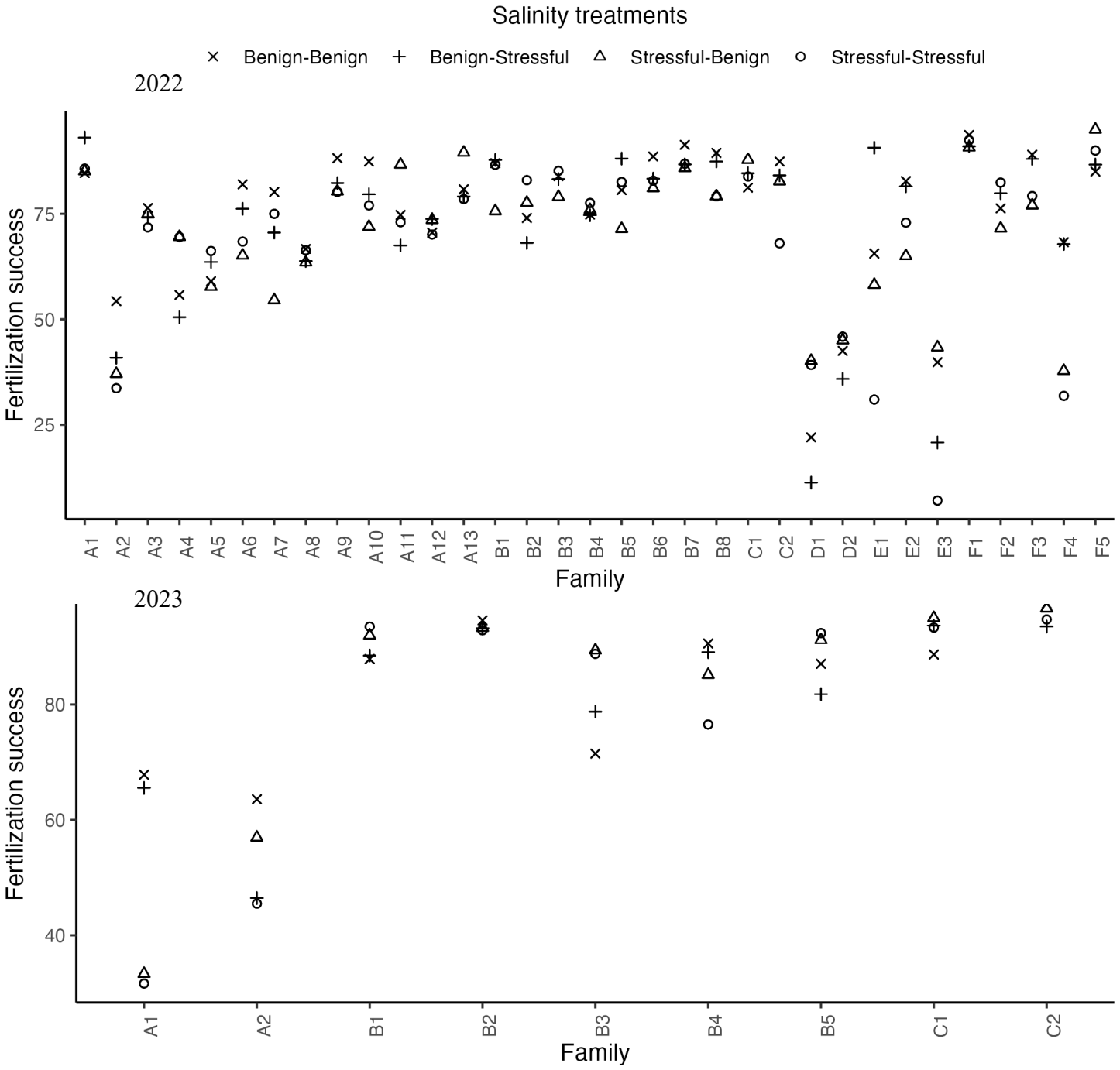


Figure 3-5. The effects of sperm and incubation salinity using a split-ejaculate and split-brood experimental design on hatch success. The left panel presents hatch success, calculated from total eggs (A) from 2022, while the right is hatch success, calculated from total eggs (B), and hatch success, calculated from fertilized eggs (C) from 2023. Data are shown as means, averaged within families and treatments, calculated by giving equal weight to each replicate incubation beaker. Error bars are standard errors among families, with the number next to each point indicating the family numbers. Salinity conditions in 2022 were benign (25 psu) and stressful (35 psu), while in 2023, benign at 5 psu and stressful at 35 psu (Purchase, 2018).

The difference in hatch success between the years 2022 and 2023 under stressful incubation conditions may be due to variations in the gamete quality of capelin between these respective

fish. This aligns with the different hatch successes reported by Purchase (2018) when incubated at 30 psu in the years 2011 and 2012.

Chapter 3 appendices



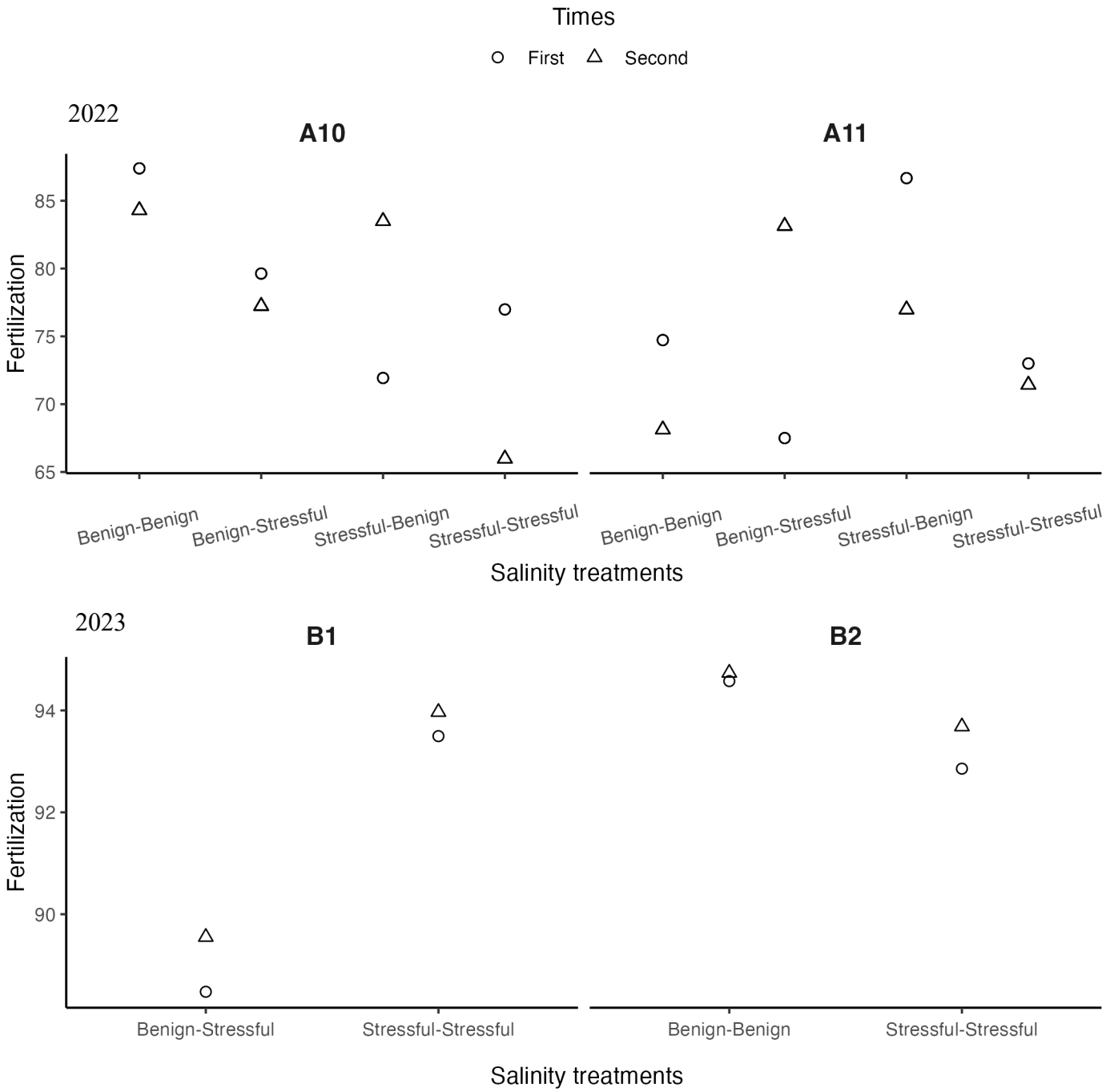
1694 Appendix 3-A1. Assessment of fertilization success across 33 capelin families in 2022 and 9

1695 capelin families in 2023. The effect of sperm and embryo exposure to salinity, with four salinity

1696 treatments (Stressful- Stressful, Stressful-Benign, Benign Stressful, and Benign-Benign)

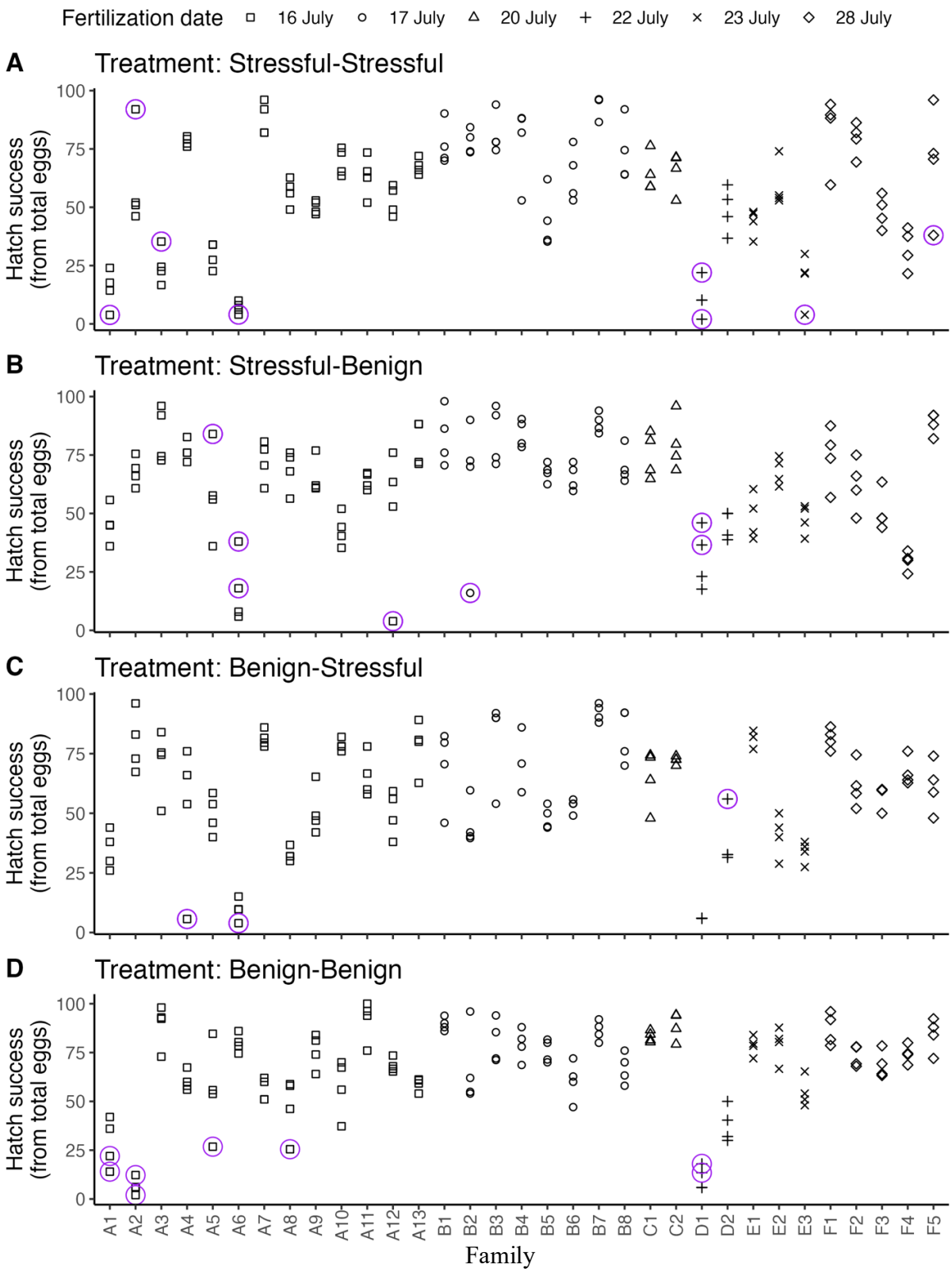
represented by different shapes. The fish were picked at random, but the results are not from every fish. In both 2022 and 2023, only a subset of families are shown, with approximately 39% and 35%, respectively, being excluded on day 1 due to poor fertilization rates. Additionally, any male and female gametes that appeared poor quality were not used and are not represented in this figure in both 2022 and 2023. The overall mean fertilization success was 71.92% in 2022 and 80.26% in 2023.

Salinity conditions in 2022 were benign (25 psu) and stressful (35 psu), while in 2023, benign at 5 psu and stressful at 35 psu (Purchase, 2018).



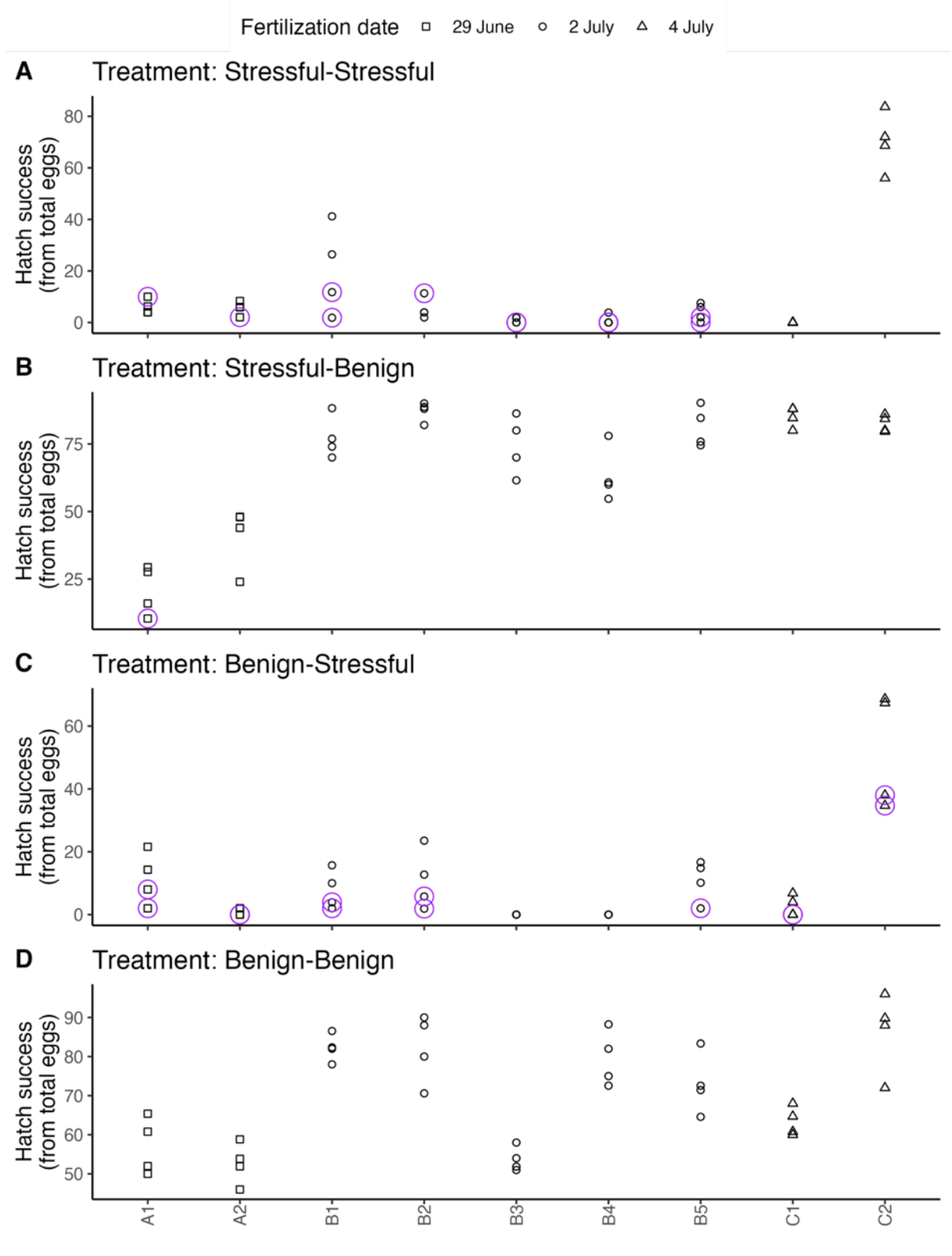
Appendix 3-A2. Repeatability of fertilization scoring to ensure the selected sample accurately represents overall fertilization success. The fertilization success of two capelin families (A10 and A11 in 2022, B1 and B2 in 2023) was scored twice using different sets of eggs each time and are represented by different shapes. In 2022, fertilization assessment was done using approximately 100 eggs, whereas in 2023, it was done using approximately 300 eggs. The data indicates that the fertilization assessment in 2023 demonstrated high precision compared to the low precision

observed in scoring fertilization success in 2022. Salinity conditions in 2022 were benign (25 psu) and stressful (35 psu), while in 2023, benign at 5 psu and stressful at 35 psu (Purchase, 2018).



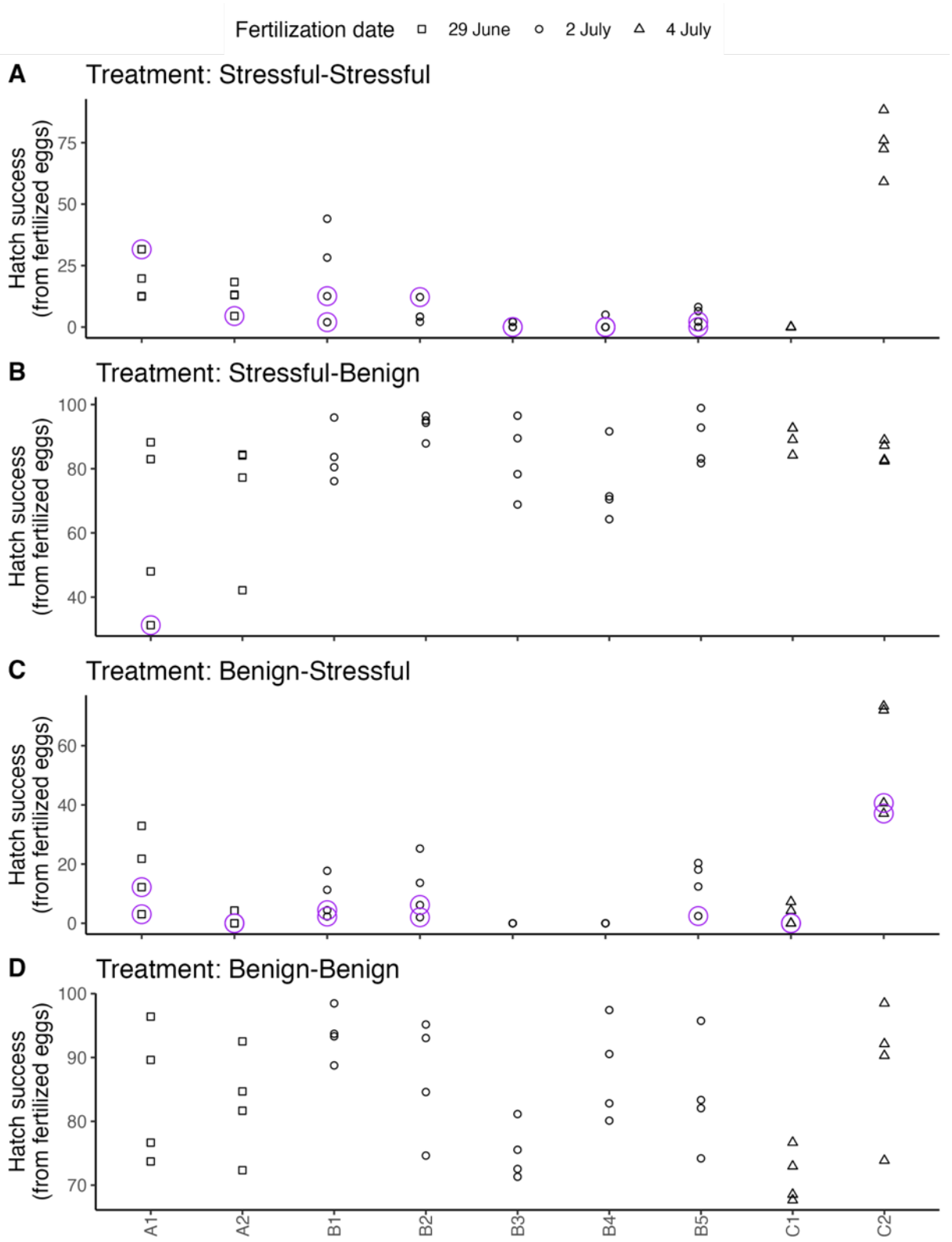
Appendix 3-B1. Effect of sperm and embryo exposure to salinity on hatch success calculated from total eggs assessed across 33 capelin families in 2022. Four panels showing the results of

four salinity treatments (A: Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization dates are represented by different shapes. Each panel features four points per family, representing the four incubation replicate beakers under the corresponding salinity treatment. Outliers were detected using a CV threshold above 30% to address possible measurement errors. This involved removing the most deviant data point iteratively until the CV of the remaining data points was below 30% or if only two data points remained. These identified outlier data points are marked with blue circles in the figures. Data remaining after the removal of outliers were used for analysis. Hatch success was calculated based on the total number of eggs. Benign: 25 psu and Stressful:35 psu (Purchase, 2018).



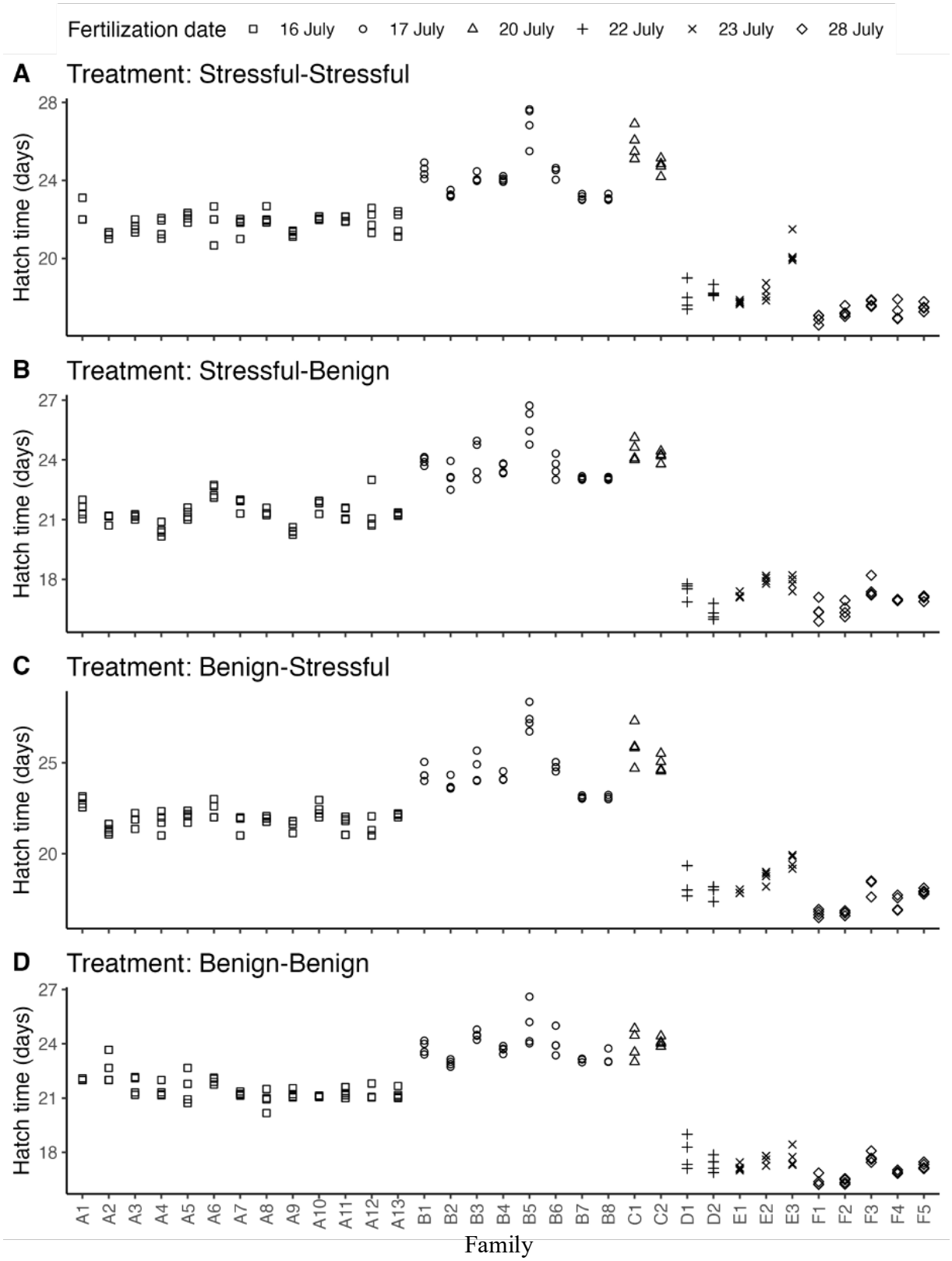
Appendix 3-B2. Effect of sperm and embryo exposure to salinity on hatch success calculated from total eggs assessed across 9 capelin families in 2023. Four panels showing the results of

four salinity treatments (A: Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization dates are represented by different shapes. Each panel features four points per family, representing the four incubation replicate beakers under the corresponding salinity treatment. Outliers were detected using a CV threshold above 30% to address possible measurement errors. This involved removing the most deviant data point iteratively until the CV of the remaining data points was below 30% or if only two data points remained. These identified outlier data points are marked with blue circles in the figures. Data remaining after the removal of outliers were used for analysis. The calculation of hatch success was based on the total number of eggs. Benign: 5 psu and Stressful: 35 psu (Purchase, 2018).



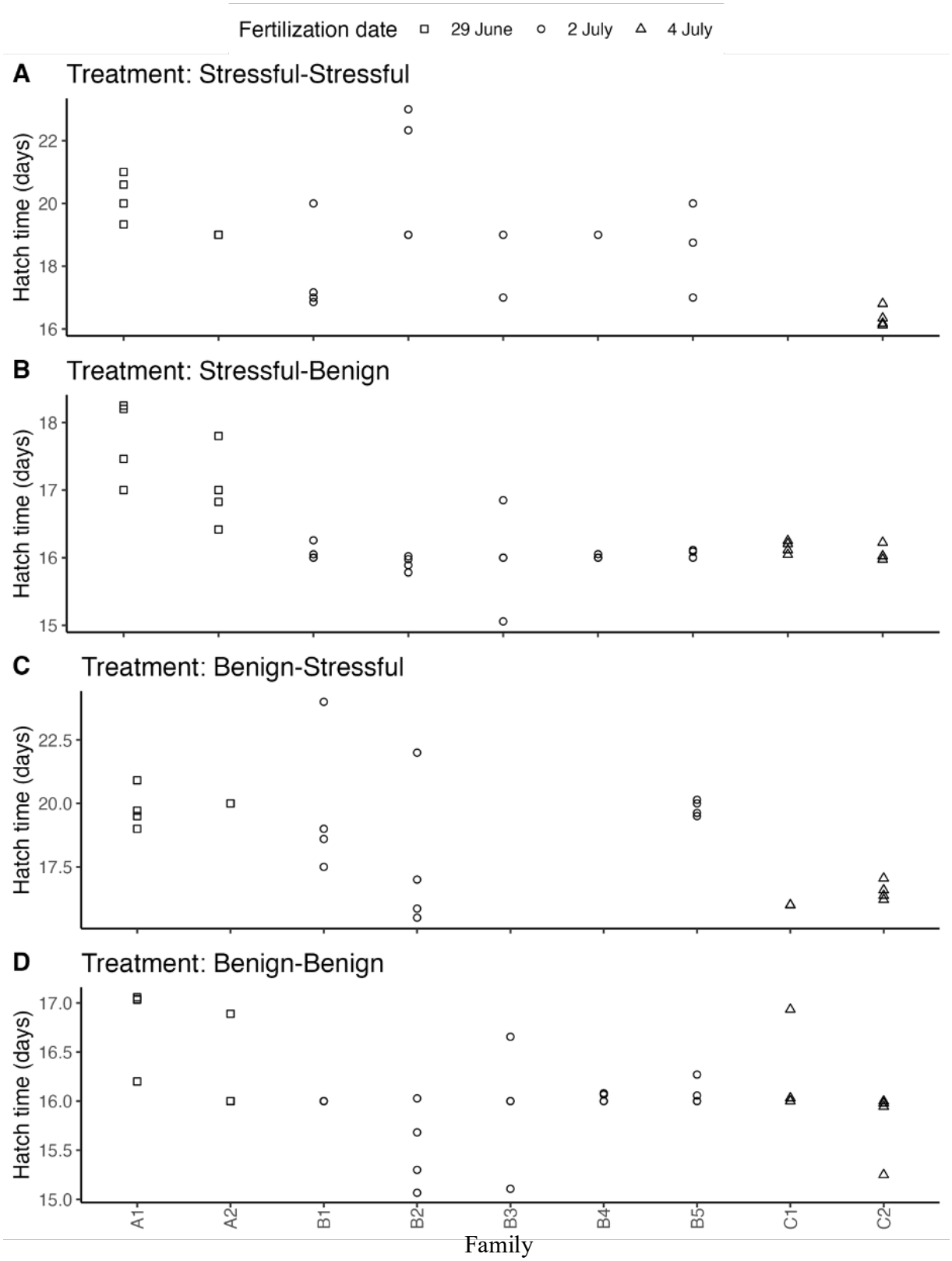
Appendix 3-C1. Effect of sperm and embryo exposure to salinity on hatch success calculated from fertilized eggs assessed across 9 capelin families in 2023. Four panels showing the results

of four salinity treatments (A: Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization dates are represented by different shapes. Each panel features four points per family, representing the four incubation replicate beakers under the corresponding salinity treatment. Outliers were detected using a CV threshold above 30% to address possible measurement errors. This involved removing the most deviant data point iteratively until the CV of the remaining data points was below 30% or if only two data points remained. These identified outlier data points are marked with blue circles in the figures. Data remaining after the removal of outliers were used for analysis. The calculation of hatch success was based on the estimated number of fertilized eggs. Benign: 5 psu and Stressful: 35 psu (Purchase, 2018).



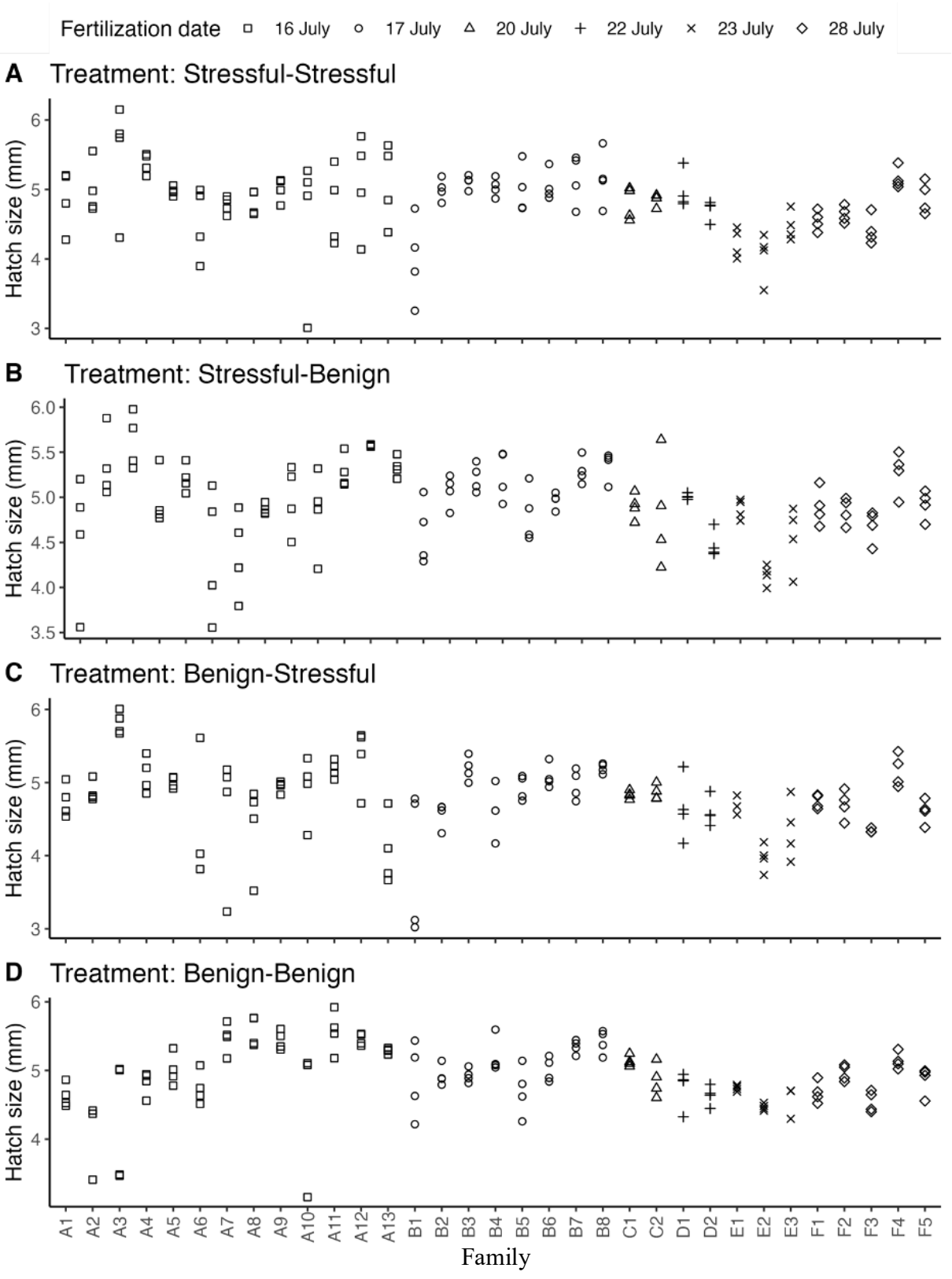
Appendix 3-D1. Effect of sperm and embryo exposure to salinity on hatch time assessed across 33 Capelin families in 2022. Four panels showing the results of four salinity treatments (A:

Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization dates are represented by different shapes. Each panel features four points per family, representing the four incubation replicate beakers under the corresponding salinity treatment. Capelin used in this study were collected from different locations and dates and may have been handled differently. Eggs were also incubated in different incubators, which may have slightly different temperatures. However, a self-controlled block design was employed, wherein all four replicate beakers from each salinity treatment were incubated in the same incubators across all capelin families, to minimize the impact of potential confounding factors. Benign: 25 psu and Stressful:35 psu (Purchase, 2018).



Appendix 3-D2. Effect of sperm and embryo exposure to salinity on hatch time assessed across 9 capelin families in 2023. Four panels showing the results of four salinity treatments (A:

Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization dates are represented by different shapes. Each panel features four points per family, representing the four incubation replicate beakers under the corresponding salinity treatment. Capelin used in this study were collected from different locations and dates and may have been handled differently. Eggs were also incubated in different incubators, which may have slightly different temperatures. However, a self-controlled block design was employed, wherein all four replicate beakers from each salinity treatment were incubated in the same incubators across all capelin families, to minimize the impact of potential confounding factors. Benign: 5 psu and Stressful: 35 psu (Purchase, 2018).



Appendix 3-E1. Effect of sperm and embryo exposure to salinity on hatch size assessed across 33 capelin families in 2022. Four panels showing the results of four salinity treatments (A:

1909 Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the
1910 first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization
1911 dates are represented by different shapes. Each panel features four points per family, representing
1912 the four incubation replicate beakers under the corresponding salinity treatment. Benign: 25 psu
1913 and Stressful: 35 psu (Purchase, 2018).
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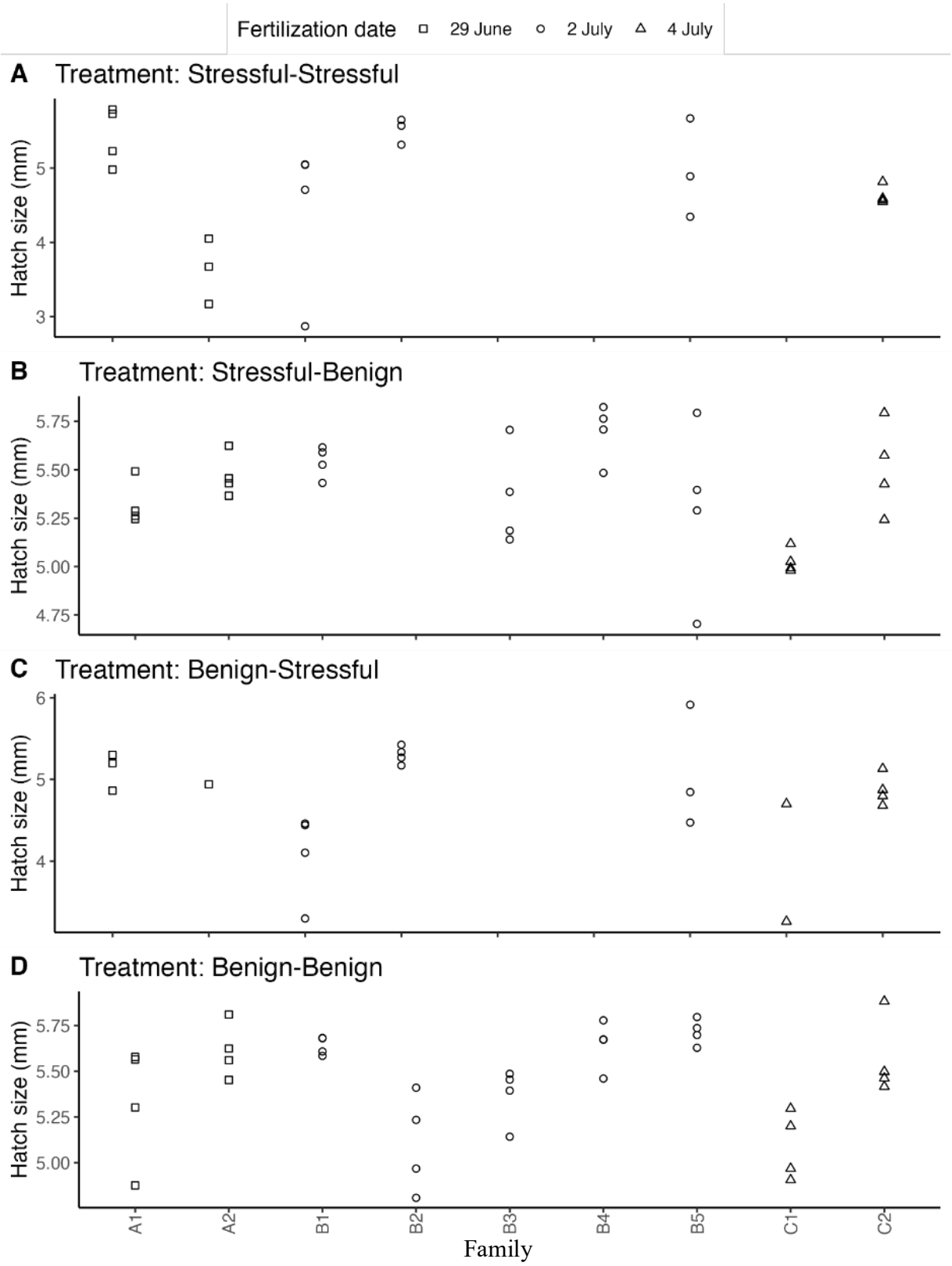
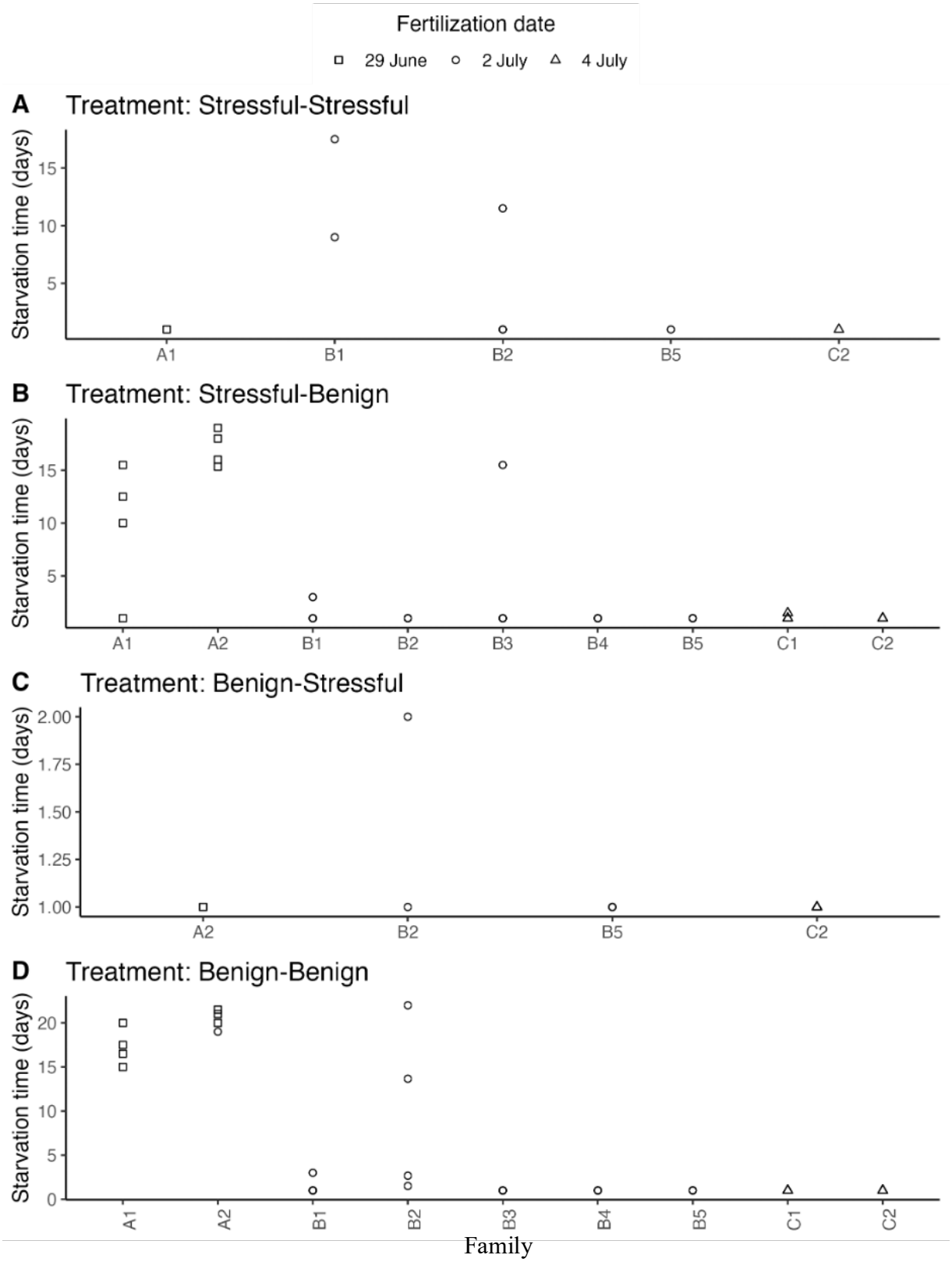


Appendix 3-E2. Effect of sperm and embryo exposure to salinity on hatch size assessed across 9 capelin families. Four panels showing the results of four salinity treatments (A: Stressful-

Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization dates are represented by different shapes. Each panel features four points per family, representing the four incubation replicate beakers under the corresponding salinity treatment. Benign: 5 psu and Stressful: 35 psu (Purchase, 2018).



Appendix 3-F1. Effect of sperm and embryo exposure to salinity on starvation time assessed across 9 capelin families in 2023. Four panels showing the results of four salinity treatments (A:

1967 Stressful-Stressful, B: Stressful-Benign, C: Benign-Stressful, and D: Benign-Benign), where the
1968 first and last words denote the salinity exposure to sperm and embryo, respectively. Fertilization
1969 dates are represented by different shapes. Each panel features four points per family, representing
1970 the four incubation replicate beakers under the corresponding salinity treatment. Data quality
1971 control to address potential measurement errors wasn't applied due to limited data availability.
1972 Benign: 5 psu and Stressful: 35 psu (Purchase, 2018).
1973

Chapter 4. General discussion

Capelin - a strange but interesting species

Capelin is a small marine fish species that inhabits cold waters and typically swims in schools (Jeffers, 1931). During their spawning season, they migrate towards the coast to reproduce (Carscadden & Vilhjálmsson, 2002; DFO, 1991). They have two spawning habitats, one on beaches and the other offshore demersally (Nakashima & Taggart, 2002). In beach spawning, males strategically position themselves near the beach awaiting females. When females approach males, they spawn in duos or trios (one female and two or three males) (Jeffers, 1931; Templeman, 1948; Orbach et al., 2019). Adult male capelin are larger than females, but there is no evident male-male competition for mating and female mate choice is not based on male size, so it is suggested that the mating system is based on endurance rivalry (Orbach et al., 2019). Despite being a marine species, their sperm and embryos are highly sensitive to salinity (Purchase, 2018). They also release “unique sperm”, being the only known external fertilizing vertebrate to release pre-activated sperm (Beirão et al., 2018). Additionally, they have unusually small testes with a gonadosomatic index of around 1% (Ressel et al., 2020; Orbach et al., 2020), which is rather low among fish species (Stockley et al., 1997; Tsikliras et al., 2010). Due to these characteristics, capelin is an interesting study system for reproductive biology in the context of evolution.

Chapter - 2

Male-biased sexual size dimorphism in capelin is suggested to aid in endurance rivalry (Orbach et al., 2019), an interaction-independent male-male competition, which served as the focal point in Chapter 2 of this thesis. The research chapter examined the dynamics of semen

quality in relation to the concept of endurance rivalry. Despite having unique sperm, capelin had remarkable semen regeneration capacity. Also, delayed spawning for six days had no adverse effect on gamete fertilizing ability. This implies that male capelin can continually be ready with their semen to seize multiple mating opportunities, which aligns with the concept of endurance rivalry.

Regarding temporal trends, I found no clear temporal pattern in semen quality. But for semen quantity, there seemed to be a trend, but only in the excellent semen quality – increasing at the start, peaking in the middle and gradually decreasing towards the end. However, caution is warranted in drawing conclusions regarding the presence or absence of a trend from the data due to limited sampling resolution.

Chapter 2 research is limited by a number of aspects, which can be addressed in future research to get a stronger conclusion. One could address the limitation associated with subjective categorization of semen quality to enhance precision and reliability by using quantitative methods in assessing semen quality and quantity, such as sperm motility, swimming speed, concentration, volume and morphology (Cabrita et al., 2014; Fauvel et al., 2010; Kowalski & Cejko, 2019). I advise expanding the sampling resolution to improve statistical robustness and reliability by sampling capelin from diverse temporal and spatial contexts during the spawning season (Cornish, 2006). Additionally, I only kept capelin in the tank for six days to observe the effect of delayed spawning on gamete fertilizing ability. Longer delays exceeding six days (e.g., 10 days) in spawning may negatively affect gamete ability for fertilization, which needs to be investigated. Future research also needs to investigate the impact beyond gamete fertilizing ability by including additional metrics of impact assessment, such as embryo development (Gasparini et al., 2017; Hay, 1986), as it was observed that although longer delays

in spawning in capelin had no effect on fertilization success, there was an effect on embryo development (Purchase, unpublished).

During the semen regeneration experiment, my sampling approach assessed capelin semen regeneration capacity after only one post-stripping (potentially equivalent to one mating). Consequently, the semen regenerative potential following multiple matings (repeated strippings of the same fish) remains unexplored. To address this limitation, future studies should consider a sampling approach that allows assessing the semen regeneration ability of each fish at each successive interval, possibly by tracking individual fish (for e.g., Hajirezaee et al., 2009; Suquet et al., 1992). Moreover, the semen regeneration ability of capelin beyond six days also needs to be investigated.

Further investigation into the concept of endurance rivalry in capelin could utilize the condition factor, bioelectrical impedance analysis (BIA), and scaled mass index (SMI) as valuable metrics for assessing energy reserves (Chellappa et al., 1995; Wuenschel et al., 2019). This approach can offer valuable insights into the relationship between energy reserves and semen quality. Additionally, the utilization of individual fish tagging methods can present a promising avenue for understating capelin mating behaviour (Thorstad et al., 2013). Using tagging techniques such as fluorochrome dye mass marking (Hongjian Lü & Yao, 2020; Leblanc & Noakes, 2012; Solomon-Lane & Hofmann, 2018), researchers can ascertain the duration males remain at a given site. This can also provide insights into whether larger fish possess an advantage in the prolonged waiting periods for mating opportunities. Furthermore, these tagging methods can also facilitate the collection of data on the spawning habitat preferences of capelin, elucidating patterns of habitat preference or if they switch between two spawning habitats during

a spawning season, along with associated influencing factors (see Crook et al., 2017; Davoren, 2012).

Another interesting way to study mating behaviours is to record video footage, particularly before, during, and after the mating (Rowland, 1999). While capturing footage of individuals or couples of capelin while spawning may be challenging, obtaining just a few video clips could be sufficient to elucidate their mating behaviours. For example, one hypothesis is that the male anal fin plays a role in guiding the semen towards the eggs (Orbach et al., 2019). This hypothesis can be tested by capturing video footage of a few capelin, say 10 or 15 couples, during the mating and observing the behaviour and movements of the male anal fin. Additionally, capturing video footage can also offer valuable insights into the difference in mating strategies that capelin may employ in beach spawning versus deep water spawning habitats.

Chapter - 3

Chapter 3 of this thesis investigated the effect of sperm experiences post-ejaculation, but prior to fertilization, on embryo development. Traditional understanding held that sperm merely transmit the paternal genome to offspring (Crean & Bonduriansky, 2014). However, recent research has challenged this notion, suggesting that sperm experiences affect embryo development outside of paternity (see review Crean & Immler, 2021; Pitnick et al., 2020). But discrepancies in findings exist, with some studies suggesting an adaptive influence (Graziano et al., 2023; Ritchie & Marshall, 2013) and others indicating a maladaptive effect (Kekäläinen et al., 2018; Lymbery et al., 2021). Thus, the chapter aimed to elucidate this influence and explore the underlying mechanism.

I used capelin as the model organism for this research, as their sperm and embryos are highly sensitive to salinity (Beirão et al., 2018; Purchase, 2018). I employed a split-ejaculate and split-brood experimental block design. Capelin sperm were exposed to two salinity conditions (25 and 35 psu) prior to fertilization with eggs. Embryos, thus produced, were incubated at salinity similar to or different from those of sperm exposure. The initial experiment did not show any discernible effect of sperm experiences on embryo development (i.e. hatch time and size), so to get further insights, I increased the exposure salinity gradient (5 psu and 35 psu) and measured starvation time as an additional hatch characteristic metric to detect the impact on later phases, allowing more time for the sperm exposure influence to manifest. Despite these adjustments, the results consistently showed that capelin sperm post-ejaculation exposure to salinity had no effect on the aspects of embryo development studied.

The results of Chapter 3 led me to conclude that the haploid genome in sperm has no influence on its phenotype, and the epigenetic components in sperm do not play a substantive role in influencing embryo development, hinting at the function of capelin sperm being the sole conveyance of the paternal genome, at least in the context of salinity exposure. But, given capelin's unique sperm, which is pre-activated sperm and its salinity sensitive on physical characteristics such as swimming velocity (Beirão et al., 2018), it is possible that the “no effect” result may be due to some kind of resilience of sperm’s epigenetic and genomic component to salinity exposure. And even if there are alternations in sperm, there is also a possibility that eggs/embryos may have some mechanism to withstand such changes, ensuring unaffected embryo development. Hence, future research using capelin as a model organism for sperm experiences could focus on testing other environmental variables, e.g. temperature. Additionally, Beirão et al. (2018) suggested that sperm of offshore spawning capelin populations may exhibit

higher salinity tolerance compared to those from beach-spawning populations. However, this remains unclear (Purchase, 2018), necessitating an investigation into whether salinity tolerance aligns more closely with beach or offshore populations. Thus, the effect of sperm exposure to salinity on embryo development may vary depending on the capelin population and spawning habitat due to their potential difference in sperm and embryo sensitivity to salinity, which could be investigated in future. I also propose future research to consider long-term effects beyond the context of starvation and also use an experimental design that can detect intergenerational effects for multiple generations. However, detecting such long-term effects in capelin may present challenges due to the absence of established protocols for captivity beyond starvation, needing innovative approaches. Along with measuring phenotypes, molecular-level insights through techniques such as transcriptomics and epigenome-wide association studies (see Fitz-James & Cavalli, 2022; Immler, 2019; Labbé et al., 2017) can be carried out to get finer resolution by examining gene expression patterns and epigenetic modifications. These techniques enable the identification of key regulatory genes and epigenetic signatures associated with specific embryonic phenotypes. Such molecular techniques can uncover haploid selection and epigenetic mechanisms and may provide insights into the capelin's unique pre-activated sperm. In the context of epigenetics, molecular biology techniques can uncover the function of different epigenome components present in sperm. Another interesting area to study, besides using molecular techniques, would be to compare the morphology of capelin sperm with that of vertebrate sperm with varying fertilization modes (Beirão et al., 2015; Kahrl et al., 2022).

Given the inconsistent findings across the literature (including my results), there is potential for species-experience-specific effects (Graziano et al., 2023), thus necessitating examination of whether such results occur across diverse species and environments such as

temperature, contaminants and pH. In this regard, I propose alternative model organisms with advantages for sperm exposure research. Zebrafish can be an alternative model organism as they are easy to house in aquaria, short-lived, and quickly reproduce (Hoo et al., 2016). These attributes facilitate longitudinal studies across multiple generations. Marine invertebrates such as *Diplosoma listerianum*, *Celleporella hyalinacan* and *Botryllus schlosseri* can also be used as alternate species due to their prolonged sperm motility compared to fish (Bishop & Pemberton, 2006), facilitating extended sperm exposure. Mosses, lycopods and ferns produce genetically identical gametes, as all gametes are produced by haploid parents (Haig, 2016; Purchase et al., 2021). So, these species can eliminate genetic diversity associated with meiotic division, allowing for a more controlled experimental design of sperm exposure experiments (Purchase et al., 2021).

Recent research has found that seminal fluid also has an impact on embryo development (Kekäläinen et al., 2020; Simmons et al., 2022; Simmons & Lovegrove, 2019), indicating a potential dependency of sperm phenotype on seminal fluid constituents or its direct influence on embryo development. This presents a compelling avenue for further investigation. Moreover, given the significant role of both sperm and egg in embryo formation, it is crucial to explore how egg experiences influence embryo development (Graziano et al., 2023).

The author advocates for research across a wide range of species to understand the impact of different environmental variables on gamete function and their potential impact on offspring, as even the slightest alteration in the gamete can have far-reaching consequences in the offspring.

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