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Reproduction in Arctic charr – timing and the need for speed

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Preface

This dissertation is submitted in partial fulfillment of the requirements for the Degree of Philosophiae Doctor (PhD) at The Faculty of Biosciences and Aquaculture (FBA), Nord University (Nord), Bodø, Norway. The presented original research was performed as part of my PhD-project, founded by the Norwegian Government.

The project team consisted of the following members:

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Bodø, November 10, 2017

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Abstract

In many polyandrous species the males compete over fertilizations both before and after copulation. In the pre-copulatory competition, the males can compete by adopting alternative reproduction tactics. The two most common alternative reproductive tactics are dominant (guarding) and subordinate (sneaker) tactics. The biggest males normally use a guarding tactic, which include guarding and courting of female while the smaller males use a sneaker tactic, which include nonaggressive behavior and courting behavior when the guarding male is occupied with chasing away other males. For males with alternative reproductive tactics the pre-copulatory competition is often about two things, (1) to get the opportunity to mate and (2) to spawn in synchrony with the female and release sperm as close as possible to the released eggs.

In **paper 1** a total of 157 spawnings were recorded and analyzed. It is clear that male Arctic charr have alternative reproduction tactics where guarding and sneaker males differ in their reproduction behavior. Every female in the recorded spawning events was protected by a dominant male guarding her from the surrounding sneaker males. The guarding male released milt before the sneaker males in 73 out of 85 spawning events with sperm competition. Further, the guarding male ejaculated on average 0.13 seconds after the female whereas the sneaker males ejaculated in average 0.6 seconds after the female. This shows that the guarding males have an advantage in the pre-copulatory competition.

The females spawned when courted by the guarding males in 125 out of 157 spawning events. It seems like size-dependent dominance among males is the prime driver in the female mate choice in charr.

Males with alternative reproductive tactics may also compete in the post-copulatory competition. Here, sperm competition happens when sperm from several males compete to fertilize the eggs from the same female. Sperm competition can be measured as the risk (the probability of sperm competition) or the intensity (number of competing males)

of sperm competition. In external fertilizers the outcome of sperm competition is often decided by the distance between released sperm and eggs, sperm velocity, and the amount of motile sperm cells in the ejaculate.

Sperm competition is common in Arctic charr. That is, 75.9 % of the 303 ejaculates recorded in **paper 1** experienced sperm competition. The mean number of competing males under sperm competition was 2.69. Sperm velocity and the percentage of motile sperm cells are the overall most important factors predicting the outcome of sperm competition. Further, the sneaker males may compensate for their disadvantaged mating role by producing ejaculates of higher quality, **paper 2**.

In Arctic charr, sperm swimming speed is influenced by ovarian fluid, where there is a status dependent modulation of sperm activity as described in **paper 3**. Although this finding could partly be caused by cryptic female choice exerted by the ovarian fluid for sperm from guarding males, an alternative and more parsimonious explanation is that sperm from guarding males may simply be better designed for swimming in ovarian fluid compared to sperm from sneaker males. Thus, sperm production in the two reproductive roles seems to be adaptively tailored to different external environments.

Abstract in Norwegian – Samandrag på norsk

Reproduksjon hos røya handlar om «timing» og fart

I arter der hoa parer seg med fleire hannar i same paringssesong konkurrer hannane over befruktningar både før og etter paring. Før paring kan hannane konkurrere gjennom bruken av alternative reproduksjonstaktikkar. Dei to mest vanlege taktikkane er dominant og snikar-taktikk. Dei største hannane brukar den dominante taktikken som inkluderer å vakte og kurtisera hoa. Dei mindre hannane brukar snikartaktikken, den inneber ikkje-aggressiv åtferd og kurtisering av hoa mens den dominante hannen er opptatt med å jage vekk andre hannar. For eksternt befruktande arter handlar før-paringskonkurransen i hovudsak om to ting: (1) å få moglegheita til å pare seg med hoa og (2) å ejakulere samtidig som når hoa gyter og å sleppe spermien så nærme egga som mogleg.

157 gytingar blei filma og analysert i **artikkel 1**. Gytingane viser at hos røya så har hannane alternative reproduksjonstaktikkar, der dominante og snikar-hannar visar forskjellige reproduksjonsåtferd. Alle hoene hadde ein dominant hann som prøvde å holde andre hannar borte, noko som resulterte i at den dominante hannen ejakulerte før snikarhannane i 73 av 85 gytingar. Den dominante hannen ejakulerte i gjennomsnitt 0,13 sekundar etter hoa mens snikarhannane ejakulerte i gjennomsnitt 0,6 sekunder etter hoa. Dette visar at den dominante hannen har ein fordel i før-paringskonkurransen.

I 125 av 157 gytefrekvensar gytte hoa mens ho blei kurtisert av ein dominant hann. Det verkar dermed som at det størrelsesavhengige statushierarkiet mellom hannane er den viktigaste faktoren for hoas partnarval hos røye.

Hannar med alternative reproduksjonstaktikkar kan også konkurrere etter ejakulasjon gjennom spermkonkurranse. Spermkonkurranse oppstår når sperma frå to eller fleire hannar konkurrerer om å befrukte egg frå same ho. Spermkonkurranse kan enten målas som risiko for spermkonkurranse (sannsynet for at spermkonkurranse oppstår) eller som intensiteten av spermkonkurranse (mengd hannar som konkurrerer i spermkonkurransen).

Artikkel 1 viser at spermkonkurranse er vanlig hos røya, 75,9 % av 303 ejakulat opplevde spermkonkurranse og gjennomsnittsmengd hannar som deltok i spermkonkurranse var 2,69. **Artikkel 2** viser at symjehastigheita og part av mobile spermceller er dei to faktorane som i størst grad påverkar utfallet av spermkonkurransen. Vidare viser det seg at snikarhannane kan kompensera for at dei gyt etter og lengre vekke frå hoa med å produsere ejakulat av høgare kvalitet.

Artikkel 3 visar at symjehastigheita til sperma blir påverka av ovarievæska og at denne påverknaden er statusrelatert ved at den aukar hastigheita til sperma frå dominante hannar samtidig som den redusera hastigheita til sperma frå snikarhannar. Dette kan tyde på eit kryptisk partnerval det ovarievæska favoriser sperma frå dominante hannar. Ei alternativ og enklare forklaring er derimot at spermien til den dominante hannen er betre tilpassa til å symje i ovariavæska enn sperma frå snikarhannar. Det verkar som hannar frå dei to reproduksjonsrollane skreddarsyr sperma til å symje i forskjellige miljø.

1. Introduction

1.1. Sexual selection

Sexual selection arose with anisogamy, where females produce large eggs and males produce small sperm. Since females have a limited number of eggs they can produce during a lifetime and males can produce a nearly infinite amount of sperm, the females have a lower reproductive potential (Bateman, 1948; Trivers, 1972). This results in different sex roles with caring females and competitive males. The mechanism behind the sexual differences are sexual selection on males, loss of paternity because of multiple female matings, male's uncertainty around own paternity and mortality patterns that generate female-biased adult sex ratios (Kokko and Jennions, 2008). In general, males with high future reproduction success provide less parental care (Duckworth et al., 2003; Jennions and Polakow, 2001; Mitchell et al. 2007; Robertson and Roitberg, 1998). Since the selection can only act upon the parenting traits of the reproductively successful males, you will in species with strong sexual selection on males (i.e. species with a high variance in mating success between males) find that males invest less in parental care (Kokko and Jennions, 2008). When a female mates with multiple males, each male has lower expected relatedness to the brood than the female, making it more likely that the females provide care (Queller 1997; Trivers, 1972). Males, contrary to females, often face an uncertainty in the paternity and has therefore a higher risk of decreasing their fitness by investments in unrelated offspring. Mauck and co-workers (1999) showed that the amount of parental care given by a male's depended on the male ability to predict paternity and that a male should decreased their parental care with decreasing ability to predict parentage. Finally, there is empirical evidence supporting that a change in adult sex ratios can result in sex role reversal (Donald, 2007; Forsgren et al., 2004; Heinsohn et al., 2007; Jiggins et al., 2000).

When Darwin introduced his theory of sexual selection in 1859, he indirectly proposed two distinct mechanisms. Selection "...*depends, not on a struggle for existence, but on a struggle between males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring."* He continued "*I can see no good reason to doubt that female birds, by selecting, during thousands of generations, the most melodious or beautiful males according to their standard of beauty, might produce a marked effect.*"

These mechanisms are today known as "male-male competition" and "female choice", or alternatively as "intrasexual- and intersexual competition", respectively (Bateman, 1948; Darwin, 1859; Trivers, 1972). Intrasexual competition arise when members of one sex, usually males, compete with one another for access to the other sex, usually females, and the outcome of this competition is often decided by differences in body size or weaponry between the competitors (Møller, 1998). The intersexual competition arises when individuals of the two sexes differ in the reproductive potential, where the sex with the lowest reproduction potential (usually females) is the choosier (Clutton-Brock and Parker, 1992; Lawrence, 1986). Females choose mates for their immediate benefits (direct fitness benefits) and/or because they provide genes that increase fitness for future generation (indirect fitness benefits) (Burke et al., 1989; Davies, 1992; Jennions and Petrie, 2000). Secondary sexual traits signal fitness and such traits can therefore by used by the females in their assessment of potential mates. Secondary sexual traits linked directly to fitness benefits are traits that reflect the quality of a territory, the quality or quantity of a male's parental care or the male's ability of preventing predation (Conner, 1988; Packer, 1983). Mate choice based on direct fitness benefits can explain size dimorphism and some exaggerated traits that produce particular fitness. However, they cannot explain extravagant traits such as the long, coloured tail-feathers of the peacock that led Darwin to nausea and later to his theory of sexual selection. Ronald Fisher introduced the concept of indirect fitness benefits in 1930, he suggested that mate

preferences and male secondary traits, provided that both have a heritable variation among them, to co-evolve to more exaggerated versions until they reach equilibrium between costs and benefits. There are two main theories of how the female preferences for male secondary sexual traits of genetic quality should evolve:

1. Female should have preferences for males displaying honest and costly signals that suggest they have superior survival abilities. These can be explained by the good genes theory (Anderson, 1994), which stresses the fitness advantages, or the handicap theory (Hamilton and Zuk, 1982), with focus on the costliness of the honest signal.
2. The attractive son's theory states that females will increase their long-term reproductive success by selecting males with heritable traits that make their sons attractive to females in the next generation (Fisher, 1930).

1.2. Male-male competition

1.2.1. Pre-spawning competition

In many polyandrous species the males compete over fertilizations both before and after copulation (Dominey, 1984; Parker, 1990; Taborsky, 1994; Yeates et al., 2007). In the pre-copulatory competition the males can compete directly through contents competition or by adopting alternative reproduction tactics (Neff et al., 2003; Oliveria et al., 2001). The two most common alternative reproduction tactics are dominant (guarding) and subordinate (sneaker) tactics (Taborsky, 1998). The different tactics can be distinguished by behavioural and morphological traits (Liljedal and Folstad 2003). The guarding tactic includes guarding, territory defence behaviour or weaponry. Whereas, the sneaker tactic often includes nonaggressive behaviour, yet also courting behaviour of the female when the protective dominant male is occupied with chasing away other males. Sneaker behaviour also includes sperm competition with the guarding male (Sørum et al., 2011). The most common morphological traits that differ between the two

reproductive tactics are the size of the body and the size or presence of sex traits such as weaponry.

For external fertilizers the pre-copulatory competition are mainly the opportunity to mate with the female, and to spawn in synchrony with the female and release sperm as close as possible to the released eggs. In external fertilizers, the gametes are viable for a short period (Billiard et al., 1986) and can quickly be scattered in the water column (Pennington, 1985). In Atlantic salmon a two seconds delay in sperm release under sperm competition decreases the paternity by approximately 40 % (Yeates et al. 2007). The sneaker Japanese medaka male experience a 20-41 % reduction in paternity if spawning out of synchrony with the female (Koya et al., 2013).

1.2.2. Post-copulatory competition

Males with alternative reproductive tactics may compete not only in the pre-copulatory competition but also in post-copulatory competition. In the pre-copulatory competition the guarding male uses courting and aggressively guards the mate to gain benefits by spawning in synchrony with the female and close to her eggs. The sneaker males are usually smaller, and therefor they are often forced to spawn out of synchrony with the female and further away from the eggs. This behaviour often results in sperm competition where sperm from several males with different reproduction tactics compete to fertilize eggs from the same female (Parker, 1970). Sperm competition is widely common within fishes, typically in species where males have alternative reproductive tactics.

"*Sperm competition is a central part of Darwin's theory of sexual selection. Sexual selection does not stop at copulation, and the fact that females in virtually every animal group copulate with several males means that sperm competition is a central and ubiquitous part of sexual selection."* (Birkhead and Møller, 1998)

"*There is every reason to suppose that selection acts on individual sperm – those which physiologically "outdo" the sperm from other ejaculates in competition for the fertilization of a given ovum would confer a selective advantage upon the male which produced them.*" (Parker, 1970).

The study of sperm competition, as a field in science, did not really take off before Geoff Parker published his groundbreaking paper on sperm competition within insects in 1970. All models used by Parker and co-workers have an evolutionary stable strategy (ESS) approach where the optimal ejaculate strategy of a male depends on the strategy adopted by the competing males (Petersen and Warner, 1998). These models, or sperm competition games as Parker terms them, bring into questions such as; "how much energy should be spent on each ejaculate?" or "what are the optimal number of sperm and sperm size in the ejaculate?" One example is that it may be advantageous for males mating in a disfavoured role to invest more in sperm production than males mating in favoured role. This has been tested in Atlantic salmon (*Salmo salar*) revealing that sneaker males invest more in sperm production than the larger guarding males (Gage et al., 1995). Further, males experiencing high risk of sperm competition should invest more in sperm production than males experiencing low risk of sperm competition. There is empirical evidence supporting that sperm production is costly (Wedell et al., 2002), thus males are expected to strategically allocate resources to sperm competition according to their mating opportunities (Gasparini et al., 2009). Further, there are trade-offs between ejaculate investments and other life sustainable processes, such as defence against pathogens. Thus, males should differ their investments in sperm competition depending on age, social status and infection levels (Parker et al., 2010).

Sperm competition occurs among internal and external fertilizing species (Birkhead and Møller, 1998). Among species with internal fertilization sperm competition occurs

when more than one male inseminate a female within a single fertile period, while for external fertilizers, ejaculates from several males may interact in the external environment before the spawned eggs are fertilized.

1.3. Cryptic female choice

Cryptic female choice (CFC) is defined as female-mediated mechanisms that operate to bias fertilization towards sperm of specifics males (Eberhard, 1996). CFC gives polyandrous females better control over paternity, especially when pre-copulatory choice is difficult such as for species with broadcast spawning where females have less control over which male fertilize the eggs (Firman et al., 2017). There are several potential mechanisms for CFC. Females may influence the timing and order of competing inseminations/ejaculates and in internal fertilizers the sperm storage organs can potentially influence the degree of which sperm to be stored and/or displaced (Pilastro et al., 2004; Ward, 2000; Xu and Wang, 2010). Additionally, female reproductive fluid, such as ovarian fluid, may have an effect on sperm swimming speed (Barnett, 1995; Urbach et al., 2005; Gasparini and Pilastro, 2011; Oliver and Evans, 2014) and females may also produce eggs that select sperm non-randomly (Holt and Fazeli, 2015; Stapper et al., 2015).

To demonstrate CFC, a female trait or behaviour that affects sperm is needed and it must be shown that this trait or behaviour favours or disfavours sperm of certain males (Firman et al., 2017). Several empirical studies indicate that CFC is identified in different species (Alonzo et al., 2016; Lüpold et al., 2016; Pilastro et al., 2007; Pizzari and Birkhead, 2000). However, it is difficult to find the specific mechanism behind CFC in most of these studied species and Firman et al., (2017) points out that CFC has seldom been clearly demonstrated. Additionally, studies on CFC can be confounded by male adaptions, such as differences in sperm performances between alternative reproduction tactics.

1.4. Arctic charr - Study population

The Artic charr (*Salvelinus alpinus*) is an external fertilizer with a lek-like mating system. Females get only genes from the male and neither males nor females provide any form of parental care (Fabricius and Gustafson, 1954). According to Anderson (1994), lek-like species are of special interest in sexual selection. Since the females receive only sperm from males, the females in lekking species will be selected for their ability to choose mates with high offspring fitness, including the offspring ability to attract females. Additionally, in such species, the mating success among the males often varies greatly, and since they show no parental behaviour the sexual selection and dimorphism are expected to be higher in lek-like species (Darwin 1871; Payne 1984).

All fish used in this thesis' work are from Lake Fjellfrøsvatn in northern Norway. The spawning period in Lake Fjellfrøsvatn starts in the beginning of September when males start to aggregate at the spawning grounds. When sexual mature females arrive at the spawning ground, the male-male competition gets rapidly more intense and, depending on the competition, the males may shift between guarding and sneaker reproductive tactics. The large males guard the females with aggressive behaviours towards the smaller sneaker males. At the same time, the guarding males court the females by gliding alongside them while quivering with high frequency, low amplitude waves (Fabricius and Gustafson 1954; Sigurjonsdottir and Gunnarsson, 1989). Since the spawning ground provides no physical protection, sneaker males rush into the spawning site and release their own milt and participate in sperm competition with the guarding male (Sørum et al., 2011).

2. Main objectives

The general objective of this doctoral project was to examine the pre- and post-copulatory competition in Arctic charr – an external fertilizer. An observational study was conducted to describe the differences in behaviour, risk and intensity of sperm competition between males with alternative reproductive tactics. An experimental study was also conducted to measure the importance of spawning synchrony, sperm quality and sperm quantity in sperm competition. Additionally, for males with alternative mating tactics, the effect of ovarian fluid on sperm swimming speed was analysed.

The specific objectives of the 3 papers were:

Paper 1:

- To describe the mating behaviour of Arctic charr, with focus on female choice and the competition between males with alternative reproductive tactics.
- To compare the gamete release synchrony between the female and the guarding and sneaker males.
- To investigate the differences in risk and intensity of sperm competition between the guarding and the sneaker males.

Paper 2:

- To investigate the importance of synchrony in gamete release, sperm number and sperm motility for the reproductive success of guarding and sneaker males under sperm competition.

Paper 3:

- To evaluate the potential modulating influence from the ovarian fluid on sperm swimming speed from guarding and sneaker males. Guarding and sneaker Arctic charr have different sperm swimming speed when measured in water. It is not known if this difference in swimming speed is maintained when sperm is swimming under the influence of ovarian fluid.

3. General discussion

The numerical order of papers in the dissertation is based on theme and not on chronology. In **paper 2,** descriptive data from Sørum and co-workers (2011) was used as a basis for the fertilization trails. Since **paper 1** is an extended version of Sørum and co-workers (2011) (i.e., it includes a larger dataset and additional analyses) it is natural to use the manuscript from 2016/2017 as **paper 1**.

This PhD project main aim was to describe the pre- and post-copulatory competition in an external fertilizer – the Arctic charr - and to evaluate how the outcome of this competition effects the male reproduction success. Action cameras were used to film spawning charr on the spawning ground (**paper 1**). These videos made it possible to describe and analyse the behaviour of males with alternative reproduction tactics and to analyse the risk and intensity of sperm competition in the population. Additionally, it was also possible to investigate if the females spawned more often with the guarding male or with the sneaker males. In **paper 2** the observed synchrony in gamete release on the spawning ground was used to investigate if spawning synchrony has an effect on the fertilization success. Furthermore, the effect of sperm quality and sperm quantity on fertilization success was examined. In **paper 3** the effect ovarian fluid has on sperm swimming speed was investigated.

3.1. Pre-copulatory competition between males

In the 157 recorded spawnings in **paper 1**, every female was guarded by one large male from the surrounding sneaker males. The sneaker males spawned by either stimulating the female to release her eggs in the temporary absence of a guarding male, or by releasing their milt over the eggs after the guarding male had stimulated the female to spawn. The latter resulted in sperm competition. The dominant guarding male was easily identified by the larger body size and behavioural traits like positioning himself

above the female, swimming slowly nearby the female or attacking other males. It is clear that the pre-copulatory competition between males in the study population is between males using alternative reproductive tactics, and that males with different tactics differ in their behaviour (**paper 1**).

For external fertilizers the pre-copulatory male-male competition is mainly about (1) getting the opportunity to mate with the female and (2) to spawn in synchrony with the female in order to release sperm as close as possible to the released eggs. How often a male succeeds to mate versus how often he fails is difficult to measure, because it demands observations of every spawning attempts of a male throughout the spawning season. However, it is possible to measure the synchrony in gamete release between males with different reproductive tactics and females. In **paper 1** the guarding male released milt before the sneakers in 73 out of the 85 spawning with sperm competition. The guarding male ejaculated in average 0.13 seconds after the female. The sneakers, on the other hand, ejaculated in average 0.6 seconds after the female. In Atlantic salmon, a 2 seconds delay in sperm release has been shown to reduce paternity by approximately 40 % in spawning events with sperm competition (Yeates et al., 2007). Thus, it might be that the guarding males, by spawning more in synchrony with the female, has an advantage in the post-copulatory male-male competition.

3.2. Female choice

In **paper 1**, the females spawned when courted by the guarding male in 125 out of 157 spawning events. For salmonids, male size is a well-known female mate choice criterion (Bolgan et al., 2016) also male size is known to be an important factor for eliciting the behaviour leading to spawning (Gaudemar et al., 2000). It seems like size-dependent dominance among males is the prime driver in the female mate choice in charr. Salmonid males do not provide parental care, but larger males are better egg defenders and

females might derive direct benefits from spawning with large males through lower egg predation (Berejikian et al., 2000).

3.3. Sperm competition

Sperm competition can be measured as risk (probability of sperm competition) or intensity (number of competing males) of sperm competition (Parker, 1970). Sperm competition is common in Arctic charr, i.e. 53.5% of the 157 analysed spawning events were with sperm competition and 75.9% of 303 ejaculates experienced sperm competition. The mean number of competing males in sperm competition (i.e., intensity) was 2.69 (**paper 1**).

Sperm velocity and the percentage of motile sperm cells are the overall most important factors predicting the outcome of sperm competition in charr (**paper 2**). These results are in line with previous findings for charr (Liljedal et al., 2008) and for other salmonids (Gage et al., 2004; Lahnsteiner et al., 1998). Further, it seems like the sneaker males can compensate for their disadvantaged mating role (the guarding males spawns more in synchrony with the female) by producing ejaculates of higher quality. Previous studies on Arctic charr, and other salmonids, have shown that sneakers have sperm that swim faster in water (Flannery et al., 2012; Lehnert et al., 2017; Liljedal and Folstad, 2003; Rudolfsen et al., 2006) and that ejaculates of sneakers also contain a larger fraction of fast swimming sperm cells (Haugland et al., 2008; Vaz Serrano et al., 2006). Sneaker males becoming guarding males, on the other hand, reduce their sperm velocity compared to levels previously held as sneakers (Rudolfsen et al., 2006). This velocity reduction is in line with Parker's (1990) theoretical model which suggest that male in the disfavoured roles should invest more in sperm production.

The sperm competition experiment in **paper 2** was the first experiment to disentangle the effects of naturally occurring adjustments in sperm quality, sperm quantity and

spawning synchrony and their interactions for reproduction success in sperm competition among sneaker and guarding males of an external fertilizing species. Yet, the experiment in **paper 2** mimicked a situation where the guarding and sneaker males have the same distance to the spawned eggs. The new and improved spawning records from our high-definition videos in **paper 1**, reveal that the guarding males ejaculate directly into the stream of female gonadal products where the concentration of ovarian fluid are high and the distance between the milt and eggs is short. The sneaker males on the other hand, spawn after the females have shed their eggs and also further away from the eggs. Therefore, it seems like the guarding male, in addition to having an advantage by spawning in synchrony with the female, also gets a positive positional effect in the pre-spawning competition. As mentioned above, sperm swimming speed is one of the most important factors predicting paternity under sperm competition and recent evidence suggest that both ovarian fluid (**paper 3**) and seminal fluid (Bartlett et al., 2017, reviewed by Pizzari, 2017) may affect swimming speed of sperm. In **paper 3** there was a status dependent modulation of sperm activity in ovarian fluid. That is, ovarian fluid increased the sperm swimming speed of guarding males while reducing the sperm swimming speed of sneakers compared to that seen in water. Bartlett and coworkers (2017) has reported that sperm from both guarding and sneaker males swam faster in seminal fluid from a sneaker males than in seminal fluid from guarding males. It seems like the sneaker males have seminal fluids of higher quality, and that seminal fluids from males with different reproductive tactics can affect sperm swimming speed differently. Rudolfsen and co-workers (2015) found that in Arctic charr the seminal fluid had no effect on sperm swimming speed but there were an effect on the activation of sperm. However, since the reproductive tactic of the males was not taken into consideration in the models (they used random males, regardless of reproductive tactic), this study does not exclude the possibility of a status depended variation in the quality of seminal fluid. In sum, both ovarian fluid and seminal fluid can, through their effect on sperm swimming speed, affect the outcome of sperm competition. To conclude, future fertilization trials should, in

addition to mimic the differences in synchronized spawning, also test the positional effect of mate guarding, the status dependent modulating of sperm swimming speed in ovarian fluid and the status dependent difference in quality of seminal fluid.

3.4. Cryptic female choice or male adaption?

To demonstrate cryptic female choice you need to identify a female trait or behaviour that affects sperm and you need to show that this trait or behaviour favours or disfavours sperm of certain males (Firman et al., 2017). Ovarian fluid has been shown to have an effect on swimming speed of sperm and that this effect differ between sperm from different males, suggesting ovarian fluid may act as a medium where cryptic female choice can occur (Alonzo et al., 2016; ; Dietrich et al., 2008; Nordeide, 2007; Rosengrave et al., 2016; Urbach et al., 2005). However, it has been difficult to separate effects of varying quality of sperm and differing ovarian fluids on fertilization success and offspring quality under sperm competition. Some authors have demonstrated positive effects of ovarian fluid on sperm velocity (Alonzo et al., 2016; Evans et al. 2012; Gasparini and Pilastro, 2011; Oliver and Evans, 2014) while Lumley and co-workers (2016) found no effect of ovarian fluid on relative offspring fitness. Moreover, Evans et al. (2013) reported no overall effect of ovarian fluid on paternity under sperm competition and no evidence for male-female interactions. **Paper 3** reveals that sperm velocity is influenced by ovarian fluid in Arctic charr and that there is a status dependent modulating of sperm activity. This suggests that ovarian fluid can act as a medium of cryptic female choice. However, this is probably a male adaption and the observed differences in sperm velocity in ovarian fluid do not need to be a result of cryptic female choice. That is, there must be something with the gonadal products from guarding males that separate them from sneakers' gonadal products. This difference between tactics must be a prerequisite for any female medium that should manage to influence sperm from guarding and sneaker males differently. If there had been no difference in sperm from guarding males and sneakers, ovarian fluid would have nothing to act upon. Yet, cryptic female choice might still occur

in ovarian fluid, but this additional rationale is not needed for explaining the results in this study.

4. Conclusion

In Arctic charr the males compete both pre- and post-copulatory. In the pre-copulatory competition, the guarding male gains an advantage under ejaculation in both time and space through guarding and courting the females. Mate guarding is the prevailing factor for paternity in Artic charr. That is, mate guarding affect accessibility to females, synchrony of gamete release and subsequent egg predation. By tailoring sperm production and synchronized milt release, the guarding male's sperm have increased chances of fertilizing the eggs. However, a synchronized gamete release requires good communication. Charr seem to have developed signals that facilitate synchronize gamete release, but such signalling comes with a cost of increased detectability by surrounding males. Thus, the need for synchronisation increases the risk of sperm competition and the sneaker males with their high proportion of motile sperm cells and sperm that swim fast in water have high paternity in sperm competition. Finally, a status dependent modulating of sperm swimming speed in ovarian fluid promoted the swimming speed of sperm from guarding males. This is probably a result of male adaption; however, cryptic female choice cannot be excluded.

5. Further Perspectives

The use of action cameras with microphones in **paper 1** revealed that vibrational communication might exist between the female and the courting male. This communication can be important for the timing of gamete release between the female and guarding male, but the sound signal the vibrations produce can also alert other males of the place and time for spawning. Playback experiments of the sound of courting individuals on the spawning ground, while monitoring the behaviour of nearby fish, would help to understand how sneaker males are able to anticipate time of female gamete release.

The recordings from **paper 1** showed that spawning fish occasionally foraging on "own" eggs. It would be interesting to test whether this phenomenon of foraging occurs more frequently under sperm competition events where the density of males around the spawning site are higher and therefor a higher risk of egg predation.

The sperm competition experiment described in **paper 2** mimics a situation where the distance from males with different reproduction tactics, to eggs are the same. It would be interesting to conduct a sperm competition experiment where the positional advantage of the guarding males where incorporated. In the same experiment, also effect of ovarian fluid by "letting" the guarding male release milt in an environment with a higher concentration of ovarian fluid could by studied.

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Paper I

Spawning behaviour of Arctic charr (*Salvelinus alpinus*): spawning synchrony, vibrational communication and mate guarding

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Abstract

A mismatch between male and female gamete release in external fertilizers can result in reduced or failed fertilization, sperm competition and reduced paternity. Here, spawning behaviour of free-living Arctic charr (*Salvelinus alpinus*) was video recorded, and their reproductive behaviour was analysed. From evaluating 157 spawning events we observed that females mainly spawned with a guarding male and that a high level of synchrony in timing of gametes released occurred between the female and the guarding male even under sperm competition. Although sneakers spawned with higher synchrony than the guarding male in single male spawning events, the average sneaker released his milt 0.6 seconds after the spawning female under sperm competition. Approximately 50% of the recorded spawning events occurred under sperm competition, where each event included an average of 2.7 males. Additionally, sneakers were more exposed to sperm competition than guarding males. An influx of males, in close proximity to the female, occurred during the behavioural sequences leading up to egg release, but this influx seemed not dependent on egg release, suggesting that there is something else than gonadal product that attracts sneaker males to the spawning female. Just before and during the actual release of gametes the spawning couple vibrates their bodies in close contact and it seems likely that vibrational communication between the spawning couple reveals time of gamete release to surrounding sneaker males. This might explain the relative high level of synchrony in gamete release between the female and the males from both reproductive tactics under sperm competition. Thus, vibrational communication between the guarding male and the female comes with the cost of higher detectability from surrounding males and may represent a "double-edged sword" for the guarding male.

Keywords: Arctic charr, *Salvelinus alpinus*, reproductive behaviour, spawning synchrony, sperm competition, female choice, mate guarding, quivering, vibrational communication, signal, acoustic communication

Introduction

In a blink of an eye, hundreds of eggs and millions of sperm are released in open water when external fertilizers pass on their genes to the next generation. In salmonids, the micropyle stays open for approximately 40 seconds before osmotic swelling blocks the micropyle and prevents sperm from fertilizing the egg (Billiard, 1992; Ginsburg, 1963; Hoysak and Liley, 2001). Unlike mammalian egg cells, the first sperm cell to reach the egg (i.e., that enter the micropyle) fertilizes the egg (Hoysak and Liley, 2001; Kobayashi and Yamamoto, 1981; Yanagimachi et al., 1992). Given these constraints, a mismatch between male and female gamete release can result in reduced or failed fertilization. Additionally, given sperm competition, the blocking of the micropyle by foreign sperm might result in reduced paternity for other males (Kobayashi and Yamamoto, 1981). Synchrony in gamete release is therefore particularly important for external fertilizing species with eggs equipped with micropyles (Mjølnerød et al., 1998; Yeates et al., 2007).

Annually, breeding Arctic charr (*Salvelinus alpinus*) gather on specific spawning grounds to reproduce by shedding their gonadal products into the external environment. Here, on shallow waters, females ready to release their eggs seem to attract males to their desired spawning site. The spawning males often adopt different mating tactics, either dominant (guarding) or subordinate (sneaker), according to their hierarchical status (Figenschou et al., 2007; Sigurjónsdóttir and Gunnarsson, 1989). Their differing status and tactics is easily distinguished by recognizable behavioural and morphological traits (Sigurjónsdóttir and Gunnarsson, 1989). The male spawning tactic may be conditional (Liljedal and Folstad, 2003; Rudolfsen et al., 2006) and body size seems to be an important factor in the choice of spawning tactic (Sigurjónsdóttir and Gunnarsson, 1989). Bigger dominant males often acquire a guarding tactic, protecting and defending the spawning female against other surrounding males by aggressive behavioural traits like biting and chasing (Sigurjónsdóttir and Gunnarsson, 1989). In the presence of a guarding male, smaller subordinate males often adopt a sneaking spawning behaviour. Here, sneakers circulate the spawning female and occasionally try to court the female in an inadvertent moment of the protective guarding male. The sneakers may also try to fertilize the female gametes by rushing into the spawning site and releasing their milt shortly after the guarding male and the female have spawned (Sigurjónsdóttir and Gunnarsson, 1989). The males spawning tactics seem to be highly plastic as they can shift between guarding and sneaker behaviour depending on interacting males (Rudolfsen et al., 2006).

Conflicts between males trying to fertilize the eggs are common (Sørum et al., 2011, unpublished data). Bigger guarding males have the advantage of spawning close to and in synchrony with the spawning female. Smaller sneaker males, on the other hand, are forced by the aggressive bigger male to spawn out of synchrony and further away from the released gonadal products of the female (Sørum et al., 2011). This may leave fewer unfertilized eggs available for the sneaker male and the eggs will also be more dispersed and difficult to fertilize. As a consequence of these behavioural characteristics, sperm competition occurs with sneaker males try to fertilize a limited number of dispersed, unfertilized eggs (Birkhead and Møller, 1998; Egeland et al., 2016).

In species where the males show alternative reproductive tactics, reproductive behaviour is of particular interest (Hoysak and Liley, 2001; Taborsky, 1998). These different behaviours are tailored to increase a male's chance to fertilize the eggs, and physiological adaptations to each tactic would involve adjustments of reproductive organs, spermatozoa and other seminal products (Parker, 1984; Taborsky, 1998). Increasing the chance of fertilization by expressing one trait may also reduce the investment in an alternative trait, therefore a trade-off between different traits might be expected (Taborsky, 1998). For spawning Arctic charr, sneaker males are disfavoured because of their "delayed gamete release" and increased distance to the already dispersed eggs. Yet, sneakers seem to compensate for these disadvantages by producing more sperm, and sperm that also swim faster in water than the sperm from guarding males (Rudolfsen et al., 2006). However, sperm from sneakers swim slower in water diluted ovarian fluid, compared to sperm from guarding males, suggesting that sperm cells of guarding males are tailored to swim in a different environment than sperm from sneakers (Egeland et al., 2016). Thus, sperm competition in charr seems to be a "loaded raffle" (Parker, 1990).

High synchrony in gamete release relies on good communication. Many species of fish are reported to use vibrational signals to synchronize spawning (Satou et al., 1991). For the landlocked red salmon (*Oncorhynchus nerka*) the vibrational signals, made by trunk muscle activity during courtship between male and female, are detected and processed by the literal line system to elicit the synchronized spawning behaviour (Satou et al., 1994a). These vibrations act as timing cues to enabling synchronicity of the gamete release. As shown by Sørum and co-workers (2011), guarding and sneaker males of Arctic charr may differ in how synchronous they manage to ejaculate with the spawning female, both in situations with and without sperm competition. Additionally, the average time delay in gamete release under sperm competition between the guarding male and the first sneaker was shown to be 0.68 seconds (Sørum et al., 2011). Females also initiated spawning with guarding males

in 73.3% of all observed events, and 55.6% of the spawning events occurred under sperm competition. Yet, only 45 spawning events were included in their study. In order to increase the knowledge about spawning behaviour among free-living charr, more and improved data are needed to be able to conduct an experiment that closely mimics the actual spawning situation (see Egeland et al., 2015 for a first attempt). In this study, further observations were conducted on spawning individuals of the same population, using underwater cameras aimed at stationary females. Although replicating previous observations are relevant (Ioannidis, 2005; Van Bavel et al., 2016), observations on whether the quivering of the spawning individuals could be detected as sounds was included in the present study. Such sound emission might explain the influx of males in the proximity to a female right before egg release, as observed by Sørum and co-workers (2011).

Methods

Some of the data presented in this study have previously been analysed and described in Sørum and co-workers (2011). In this former study, conducted in 2006-2007, spawning behaviour was recorded for 69 hours and 40 minutes, showing 45 spawning events. To increase the sample for the present study, recording of spawning behaviour was conducted for 284 hours and 28 minutes during the 2016 spawning season, using the same approach as Sørum and co-workers (2011) but with improved camera quality enabling a more detailed evaluation of charr behaviour. In total, 110 hours and 42 minutes of the 2016 recordings were analysed. Here 112 new spawning events were analysed, and data from 2006-2007 and 2016 were pooled. This summed up to 180 hours and 22 minutes of analysed videos resulting in 157 spawning events.

The quivering from the courtship behaviour of a spawning couple made a distinguishable sound which was recorded by the recording camera. 32% of the videos from 2016 were analysed by using the sound files only to identify spawning. This resulted in identification of 33 spawning events. The remaining 68% were analysed by watching the video, resulting in identification of 79 additional spawning events. To control the accuracy of using sound files only to identify a spawning, we matched the spawnings, first identified from watching the videos, with those identified (by a different person) from the sound file only. The match between the two separate methods to identifying spawning events was 100%, (n = 33).

Study site and video recordings

The study was carried out during the spawning period from mid-September to early October in Lake Fjellfrøsvatnet, Troms, Norway (69°08’N 19°34’E). Video monitoring of

spawning Arctic charr on their lek sites was conducted at known locations in and around spawning site 3 (see Figenschou et al., 2004). Low spawning activity at other spawning grounds prevented the use of other locations. Camera used in the survey varied in technical specifications, but all were "action sport cameras" equipped with watertight housing and a wide-angle lens. All eight cameras belonged to the GoPro brand including models GoPro Hero 3 and 4 (types plus, silver and black). Chosen settings for video quality was 1080p with 60 frames per second. The camera recorded both image and sound, and there were only minor technical differences in camera design and housing.

When arriving at the spawning grounds, the first 5 – 10 minutes were spent studying the charr in order to identify stationary females. Once identified, cameras mounted on tripods were deployed aiming towards the stationary females that appeared to be preparing to spawn. The distance from the camera to the spawning female was approximately 0.3 to 1 meter. Recording lasted as long as the battery capacity allowed (from about 90 to 270 minutes), and the capacity of the memory card was only rarely a limiting factor. The recording cameras were left undisturbed on the spawning site for minimal human interference until they were replaced by new cameras. The procedure often resulted in an exchange of cameras in the early morning, before midday and in the afternoon. Recorded videos were immediately copied to hard drives and the batteries recharged.

The spawning events took place in shallow waters (0.2 - 2 meters deep), often near land or on a spawning site about 100 m from land. The preferred spawning habitats consisted of small to intermediate sized rocks covered in algae interspersed with areas of gravel. Females ready to release their gametes hover a few centimetres above their chosen spawning site while being guarded by a dominant male. Females seem to get more stationary the closer they are to spawning and this increases the chance of recording the actual spawning event. All recordings had to be carried out under daylight conditions, yet night and sunset hours might be the periods with the most spawning activity (Bolgan et al., 2017).

Spawning located by soundwave

The high amplitude quivering of the courtship behaviour of a female and a male Arctic charr could be recorded and identified as a distinct sound curve (see figure 1) and this soundwave was easy distinguishable from other sounds in the videos. By placing a camera close to the spawning female, the camera would record vibration as sound from spawning individuals as far as 5 to 6 meters away. Since the recording camera occasionally registered

soundwaves from spawning individuals located in a blind angle of the camera, video was used to verify the observed soundwave, and used to locate spawning events. By using the WavePad Audio Editing Software (version 6.59) to visualize and analyse the extracted sound files from a recorded spawning video, it was possible to pinpoint the exact time of a spawning. Compared to watching videos in search for spawning events, observing the sound tracks minimizes the time used to discover spawning events from the videos.

The spawning event and its definitions

In accordance with Sørum and co-workers (2011), a spawning event is defined when the following 4 different types of spawning behaviour (adapted from Fabricius, 1954; Fabricius, 1953; Sigurjónsdóttir and Gunnarson, 1989; Sato, 1991; Flemming, 1996) take place:

1. The female lay stationary close to the bottom substrate with an erected anal fin and with the upper body slightly pointing upwards.
2. The male (both guarding male and sneaker) courts the female as he approaches the female from behind, and in the moment his head touches the female's tail slowly initiating quivering. The males quivering increases as he glides forward close up to the female's body. The female often responds by quivering shortly after the quivering males touches her body.
3. Quivering increases in strength until both the male and the female gapes. The female often gapes first. Gamete release occurs at maximal mouth opening. Males milt can be visible as a cloud in the water and eggs can be seen both soaring in the water and lying on the bottom substrate. Male and female propel slightly upwards and forwards with an open mouth and a lifted head.
4. The male and the female separate and quickly return to the spawning spot where they start to chase away other fish from the spawning location.

In cases of reproductive competition, the sneaker would either dart into the spawning site and release its milt in sperm competition with the guarding male, or a single sneaker may court the female to spawn without sperm competition.

Guarding and sneaking tactics

Stationary females tend to be more aggressive against smaller sneaker males than against bigger males employing the guarding tactic (Bolgan et al., 2016a). Additionally, the guarding male is recognized by, besides bigger body size, a less dark colour dorsally, and behavioural traits as laying above the female, swimming slowly nearby the female or attacking other males (Sigurjónsdóttir and Gunnarsson, 1989). The sneaker, on the other hand, is typically characterized by his smaller body size and by approaching and swimming

slowly near the female (Sigurjónsdóttir and Gunnarsson, 1989). Identifying the type of mating tactic of a male in proximity to the female in a pre-spawning behaviour is therefore easy. In the 157 recorded spawning events, every female was protected by one dominant male guarding her from the surrounding sneaker males. Competing males would spawn by either stimulating the female to release her eggs in the absence of a guarding male, or by releasing their milt over the female eggs immediately after the guarding male has stimulated the female to spawn with him.

Spawning synchrony

The Avidemux 2.6 video processing program (version 2.6.18), enabled analysis of spawning synchrony and time of maximal mouth opening defined gamete release. Not all the spawning females were appropriately recorded, and in 16 of the total 157 recorded spawning events the females spawned with her head pointed away from the camera or other individuals masked the gaping fish, impeding the exact measurements needed. These spawning events were excluded when estimating spawning synchrony.

Male density, sperm competition and gamete release

In accordance with Sørum and co-workers (2011), male density was defined as the number of surrounding males within a radius of a fish length distance (approximately 25 cm, see figure 2) from the spawning female. The density was recorded at specific points in time from five seconds before to five seconds after female gamete release. Sperm competition was defined to occur when more than one male released milt at the same spawning event. Asynchrony in gamete release was estimated by noting time of milt release relative to time of egg release at a precision of 16.6 milliseconds (60 frames per second).

"Near" spawning: Male density and vibrational communication

Examination of the videos revealed some events where the female and male(s) did not release any gametes, despite demonstrating all pre-spawning behaviours. Such events are hereafter termed "near spawning events". Density of neighbouring males at near spawning events was examined in a similar way as real spawning events (see above). Egg release, which did not happen in near spawning events, was estimated to "occur" after a quivering period comparable to that recorded from actual spawning events. That is, we used average length of the quivering period leading up to real spawnings to estimate the likely spawning time at the near spawning events. The events were carefully chosen to fulfil the spawning criteria. In total 20 near spawning events were analyzed, using one random sample for each female.

Statistical analysis

All statistical analyses were performed using R v. 3.4.2 (R Core Team, 2015). Binomial tests (to compare two proportions) was used to examine if females spawned equally often with guarding and sneaker males. Since we were not able to fit a generalized linear mixed model (GLMM) when including all spawning events (i.e., where we used all data regardless of whether the spawning was in competition or not), spawning synchrony between female and males was tested with one sample t-test. Spawning synchrony in sperm competition and single spawning events was examined by generalized linear mixed models (GLMM) using the lmer function in the lme4 package in R (Bates et al., 2014). In these models time since female egg release was used as response variable, male status as fixed factor and female id as random factor. Risk (i.e., probability of experiencing sperm competition) and intensity (i.e., number of competing males) of sperm competition was tested using binomial tests. GLMM with the glmer function in the lme4 package (Bates et al., 2014) was used to analyse the male density around the spawning female. Here we used a poisson distribution with number of males as response variable, time and spawning type as fixed factors and female id as random factor. Finally, Spearman's rank tests was used to examine the potential correlations between the length of the quivering period and (i) number of males releasing milt, (ii) density of males around female and (iii) the relative increase of males in the vibrational timespan.

We recorded multiple spawning events of several of the females, and in order to reduce problems with pseudoreplication (Hurlbert, 1984) in the binomial and Spearman's rank tests we used the average values from the observations of each individual female. In the t-tests we corrected the degrees of freedom according to the number of females we had recorded spawning events from instead of the number of actual spawning events recorded. In the GLMMs pseudoreplication is not a problem since female id was included as a random factor. We checked the model fit using visual examination of normal probability plots and residual plots.

Results

Courtship

The numbers of female spawning events occurring when courted by a guarding male, a sneaker male or when courted by both simultaneously were 124 (78.9 %), 30 (19.1 %) and 3 (1.9%), respectively. Female spawned more often when courted by guarding males than by sneaker males, both under sperm competition (Binomial test comparing two

proportions, n = 32, $x^2 = 92.3$, $p < 0.0001$), and under single spawning events (Binomial test to compare two proportions, n = 29, $x^2 = 34.0$, $p < 0.0001$).

Gamete synchrony, sperm competition and different male tactics

The guarding male ejaculated on average 0.13 seconds (SD ± 0.18, n = 97) after, and significantly later than the spawning female (one sample t-test, $t_{26} = 7.2$, $p < 0.001$). The first sneaker, on the other hand, ejaculated on average 0.41 seconds (SD ± 0.47, n = 75) after the spawning female (one sample t-test, $t_{20} = 7.6$, $p < 0.001$). By pooling all the values of spawning sneaker's, the average sneaker was also observed to spawn significantly later than the female (one sample t-test, $t_{20} = 10.8$, $p < 0.001$), with a delay of 0.6 seconds (n = 106).

The guarding male released milt before the sneaker males in 73 (89.1 %) of the 85 analysed spawning events with sperm competition. The difference in timing of milt release between the guarding male and the first, second and third sneaker was significant (see figure 3, table 1). Yet, in single spawning events, sneaker milt was released more in synchrony with the female egg release than milt released by the guarding males in single spawning events (see figure 4, table 2). In 72.8 % of the spawning events, the female was the first to release gametes.

Intensity and risk of sperm competition

Sperm competition can be expressed as intensity (number of competing males) or risk (probability of experiencing sperm competition) of sperm competition. The percent of sperm competition was 53.5% (n = 157). The mean, median and range number of competing males were 2.69, 2, and 2-6, respectively (n = 58). When including the single spawning events, the numbers of males releasing milt during egg release decreased to 1.93, 2, 1-6 (mean, median, range, n = 157). In total, 303 male ejaculates were released through the 157 recorded spawning events. 230 ejaculates experienced sperm competition (75.9%), compared to 73 in single male spawning events (24.1%). Thus, more ejaculates were released in sperm competition than in single spawning events (Binomial test to compare two proportions, $x^2 = 160.63$, $p < 0.0001$). The number (n = 171) of sneaker ejaculates experiencing and not experiencing sperm competition was 152 (88.9%) and 19 (11.1%), respectively, that is, more ejaculates were released in sperm competition (Binomial test, $x^2 = 206.2$, $p < 0.0001$). The corresponding numbers of ejaculates from guarding males were 79 (60.8%) with sperm competition and 51 without sperm competition. That is, also among dominants more ejaculates were released in sperm competition (Binomial test, $x^2 = 8.79$, $p < 0.0001$).

Male density when females spawn

The male density in proximity to the spawning female started to increase a few seconds before the gamete release (figure 5). In spawning events with sperm competition, the density of males reached its maximum 1.5 s after egg release (mean = 4.63 males per female, median = 4, range 1-9). At the time of egg release, the mean number of surrounding males was 2.64 (median = 2, range 1-7). Males released milt from 0.7 s before egg release to 2.5 s after egg release. During this time window, there was a mean increase of 2.2 males (120%) in the proximity of the female. When only one male spawned, the density of males reached its maximum 2 s after egg release (mean 3.17 males per female, median = 3, range 1-9) and at the time of egg release the mean number of males was 1.74 (median = 1, range 1-5). Overall there were fewer surrounding males in single spawning events then in spawnings with sperm competition ($p < 0.0001$, table 3) and the increase of males over time was also smaller in single spawning than in spawning with sperm competition ($p < 0.0001$, table 3).

Male density when females do not spawn

In "near" spawning events there was a significant increase in density of males in the four seconds preceding estimated female "gamete release" (Pearson correlation test, r = 0.374, $p < 0$ .0001, n = 220, see figure 5). However, compared to spawning events, "near" spawning events had on average fewer males present in the timespan from 2 to 0.75 seconds before "female gamete release" (fig. 5). At estimated time of "egg release" the mean number of males in proximity to the female was similar to of spawning events with egg release (Mean ± SD, "near" spawning: 2.5 ± 1.15, real spawning 2.64 ± 1.27). There was no relationship between the length of the quivering period and (i) number of males releasing milt (Spearman's rank test, S = 660.5, $p = 0.46$), (ii) number of males in proximity to the female at egg release (Spearman's rank test, S = 790.4, $p = 0.91$), or (iii) with the relative increase of males in the vibrational timespan (Spearman's rank test, S = 645.9, $p = 0.422$). Quivering length of the courting male was measured in 71 spawning events with 17 different females.

Discussion

Females spawned more frequently when courted by the guarding males than when courted by the sneaker males. Additionally, like Sørum and co-workers (2011), we found that the spawning female experienced a high level of synchrony in the timing of gamete release with the courting male. The females, which most often released gametes first, were shortly followed by the guarding or sneaker(s) ejaculation. The majority of ejaculates were released under sperm competition. However, ejaculates from guarding and sneaker males

differed in the risk of sperm competition with a higher intensity of sperm competition among sneaker males ejaculates. Additionally, as density of males in proximity to the female increased right before eggs were "shed" in both real and "near" spawning events, there must be some form of communication involved in a spawning synchronization occurring which is unrelated to gamete release.

Female preference

In the present study, the majority of females spawned when courted by the larger guarding males (in 125 out of 157 events). Size is a well-known mate choice criterion in salmonids (Bolgan et al., 2016a), and females have in the presence of small males been shown to delay their spawning allowing larger males to displace the small males (Blanchfield and Ridgaway, 1999; Gaudemar et al., 2000). Male size is also known to be an important factor for eliciting the behaviour leading to spawning. A study of Atlantic salmon (*Salmo salar*) indicated that relative mate size seemed to be important for female mate choice, and in the absence of courtship behaviour, male size alone increased the spawning behaviour of the female (Gaudemar et al., 2000). We observed that females occasionally also spawned with sneaker males. Benefits to females in these cases may arise from exposing eggs to sperm from other males than the dominate one, resulting in higher genetic variation among offspring (Jennions and Petrie, 2000; Reichard et al., 2007). It is not unlikely that female charr also incorporate a passive mate choice, yet actively chose spawning ground and "nest" site. In this scenario, the outcome of the competition between the males in the proximity of the selected "nest" site decides which male the female spawns with. In case, mate guarding and social dominance among males becomes paramount. Thus, it seems like size-dependent dominance among males including direct choice for male size might drive selection among males, but the two mechanisms may be hard to disentangle.

Salmonid males do not provide parental care, but larger males are better egg defenders. Thus, females might derive direct benefits from spawning with large males through higher egg survival (Blanchfield and Ridgway, 1998; Berejikian et al., 2000). Yet, in the present study both the female and the guarding male were observed foraging on eggs after own spawning (unpublished data, video). This result is contrary to other observations among spawning charr were guarding male never foraged on eggs (Sigurjónsdóttir and Gunnarsson, 1989). Analysis of stomach contents have, however, shown that charr may eat eggs during the spawning period (Malmquist et al., 1992). Although intuitively maladaptive, eating own eggs is not uncommon among fish (review by Manica, 2002). Such filial cannibalism has been explained by either by removal of unfertilized, malformed or diseased eggs, or by

energy-based arguments in species which have very high energy expenditures and limited foraging opportunities (Manica, 2002). The behaviour observed in the present population is most likely related to high predation pressure, causing males and females to forage on eggs that apparently will not evade predation by conspecifics anyway.

Synchrony

In sperm competition events, females experienced higher synchrony of gamete release with the guarding male than with the sneaker male(s). By synchronizing the ejaculation with female egg release, the courting male can reduce the effect of sperm competition. In Atlantic salmon, a 2 seconds delay in sperm release reduced paternity by approximately 40% in spawning events under sperm competition (Yeates et al., 2007). The average charr sneaker ejaculate their milt only 0.47 seconds after the guarding male, and the effect of sperm competition is necessarily not comparable in the two species. That is, unlike charr, which spawn in still water, salmon spawn in flowing water, rendering the physical properties of the two fertilization environments quite different. Close imitations of natural sperm competition in charr shows that when sneaker males release ejaculates 0.68 s after the guarding male there is no difference in fertilization success (Egeland et al., 2015). That is, the initial higher sperm velocity and higher sperm numbers among sneakers may partly compensate for their lack of synchrony. Yet, this benefit might be outweighed by the sneaker's lower sperm velocity in water-diluted ovarian fluid compared to that of guarding males (Egeland et al., 2016). In single male spawning events, on the other hand, the sneaker males released their gametes with significantly higher synchrony than guarding males. By releasing milt in high synchrony with the female, eggs are forced to pass through a cloud of milt in the water (Fitzpatrick and Liley, 2008). The high synchrony exhibited by the sneakers suggest that sneakers lack of synchrony under sperm competition is caused by the mate guarding of the guarding male, rather than by the sneakers lack of ability to synchronize gamete release (Sørum et al., 2011). Thus, mate guarding seems to have an effect on sneakers ability to synchronize their ejaculation with the egg release by the female.

Sperm competition

Even though the female was protected by one guarding male in the lead-up to every spawning situation, the bigger male could not prevent sperm competition. Approximately 50% of the observed spawning events occurred with sperm competition, and in these cases around 3 males participated on average. Yet, compared to guarding males, sneakers experience a higher intensity of sperm competition, suggesting that there is an effect of guarding on the likelihood of experiencing sperm competition. Although females also show

aggressive behaviour towards sneaker males (unpublished data), females might have benefits from sperm competition. That is, eggs spawned under sperm competition are observed to achieve a higher fertilization success and a higher offspring survival relative to eggs fertilized by a single male (Keil and Sachser, 1998; Liljedal et al., 1999; Shapiro et al., 1994). Exposing eggs to sperm from several males may also result in higher genetic variation among offspring (Jennions and Petrie, 2000; Reichard et al., 2007). Yet, approximately 50% of the observed spawnings were single male spawning events. These events may have occurred either when the density of surrounding males was low, or when the surrounding males were occupied in intrasexual interactions resulting in a late arrival to the spawning female. Thus, aggressive behaviour from both the guarding male and the female may reduce the intensity of sperm competition, but the estimated number of interacting males in all spawning events (close to 2) hints to a situation where the ejaculates investments should be at the highest (Parker et al., 1996). Thus, it is not surprising that extreme adaptations to sperm competition (i.e., tailoring of sperm production to the different fertilizing environments) are found in this species (Egeland et al., 2016).

Male density

There was a clear increase of males in proximity to the spawning female seconds before female egg release. Additionally, a similar increase is observed in ‘’near spawning events”, where there is no release of neither male nor female gametes. This indicates that there is some other factor than gonadal products, or its associated chemical components, that is attracting males to the spawning couple. Signals between the spawning pair are thought to be perceived visually or by tactile sensation (Uematsu and Yamamori, 1982). It is unlikely that the attractor for sneaker males are visual cues only. That is, individuals heading away from the pre-spawning pair are sometimes observed to turn, and rapidly head for the spawning pair when the courtship quivering begins and before the actual spawning occurs (unpublished data). Additionally, the spawning individuals in a pair would not be able to see gamete release from the partner (i.e., it occurs in a dead angle of his/her visionary field). Thus, communication signals related to spawning synchrony are most likely not visual, but rather vibrational. In captive experiments of spawning behaviour of landlocked red salmon (*Onchorhynchus nerka*), visual patterns were not alone essential for eliciting the male spawning behaviour. Yet, the vibrational and visual cues had to spatially coincide with each other to elicit the male spawning behaviour (Satou et al., 1994b). From our videos, it seems like the spawning pair use vibrational communication to synchronize the gamete release (video) and that this vibrational communication produces waves in the water column that can be recognizes as sound (Figure 1). This is, to our knowledge, the first time sound

producing communication has been reported in Arctic charr. On the contrary, Bolgen et al. (2016b) found no evidence of sound producing communication during courtship in Arctic charr. Thus, the observed pre-spawning increase in density could be caused by surrounding males picking up the vibrational signal used between the spawning pair. Vibrational signals could be informing the sneakers about time and space of gamete release, possibly explaining the relatively short delay in sneaker's milt release and the observed influx of males close to egg release. If noticeable vibrations attract males to the courting couple, it might be argued that a long vibration should attract more males than a shorter vibration. Yet, no correlation was found between vibrational length and the number of males related to the spawning event. Thus, rather than length of vibration, frequency might be the important component of vibrational communication. This concurs with findings in landlocked red salmon where the male behaviour was clearly influenced by the vibrational frequency of the model female (Satou et al., 1994b). Consequently, the frequency of vibrational signals could be the main signal to how the spawning pair synchronizes their gametes release and surrounding males may eavesdrop on these signals for synchronizing their spawning.

Throughout this study, mate guarding seems to be the prevailing factor for paternity in Arctic charr. Mate guarding affects accessibility to females, sperm competition, synchrony of gamete release and subsequent egg predation. Mate guarding influences the outcome of the spawning situation, affecting fertilization and paternity. By obstructing competition, advantageous positioning, tailoring of sperm production and synchronized milt release, a guarding male's sperm have increased chances of reaching the micropyle. Yet, a synchronized gamete release requires good communication, and charr seem to have developed signals to synchronize gamete release with the cost of increased detectability by surrounding males. Thus, the need for synchronization comes at the cost of sperm competition, making vibrational communication a "double-edged sword".

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591 Figures

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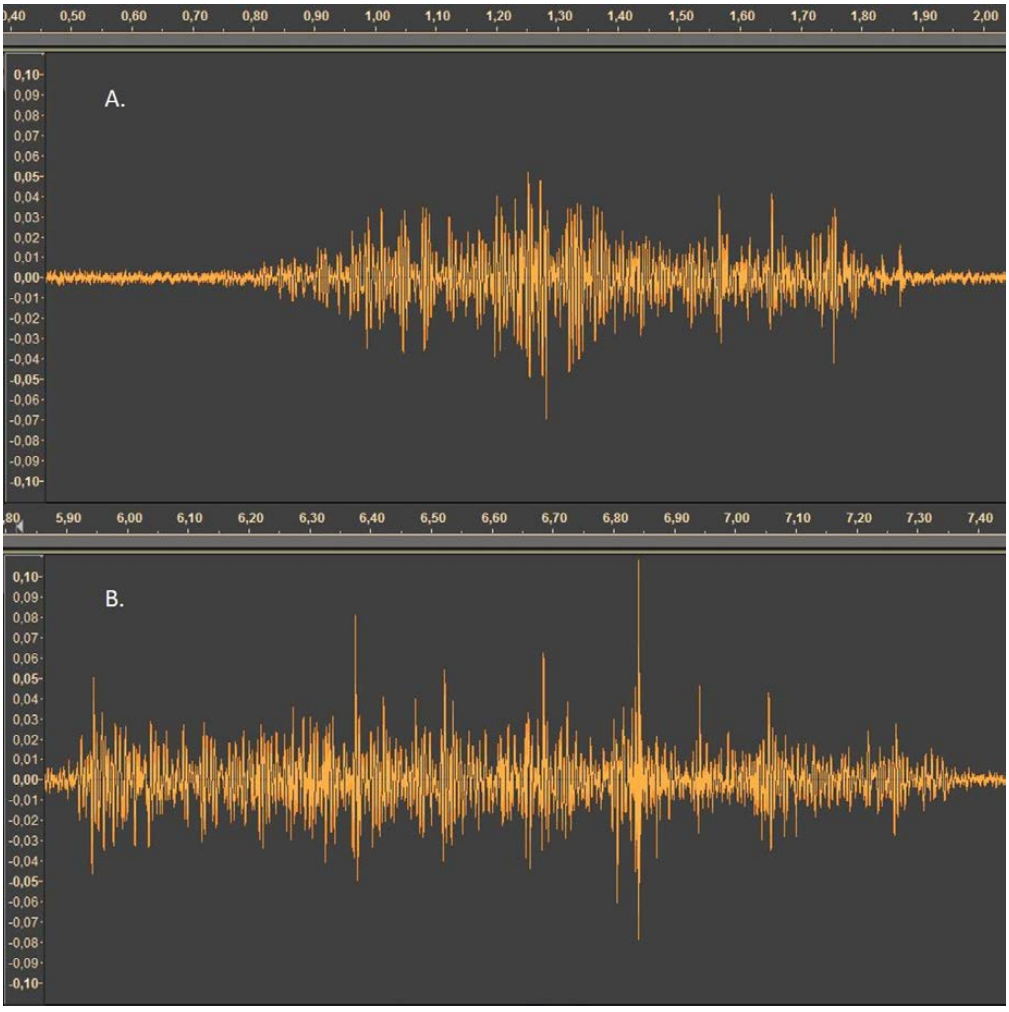

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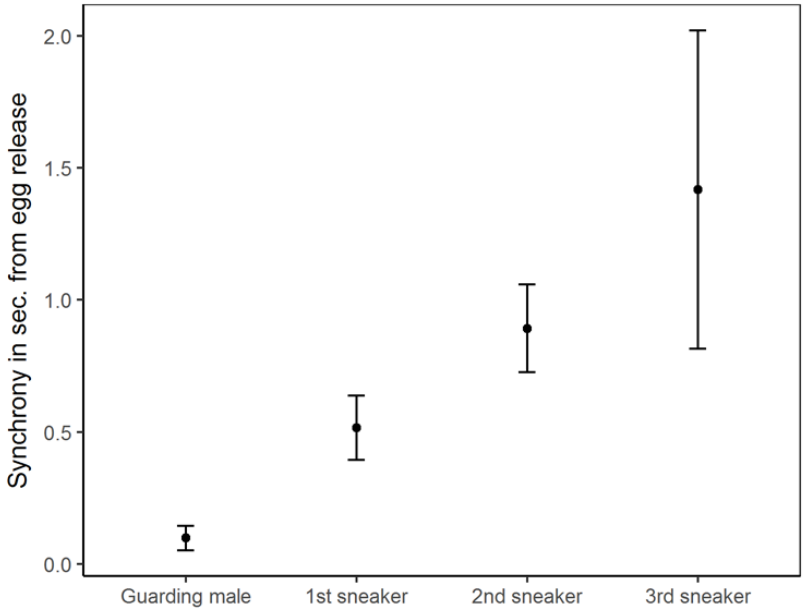
594

595 **Figure 1**: Oscillogram of recorded sound from spawning Arctic charr: **A**: Oscillogram
596 recorded during a spawning event without sperm competition. **B**: Oscillogram recorded
597 during a spawning event with sperm competition including, four spawning males (X-axes:
598 time in ms, Y-axes: linear scale amplitude).

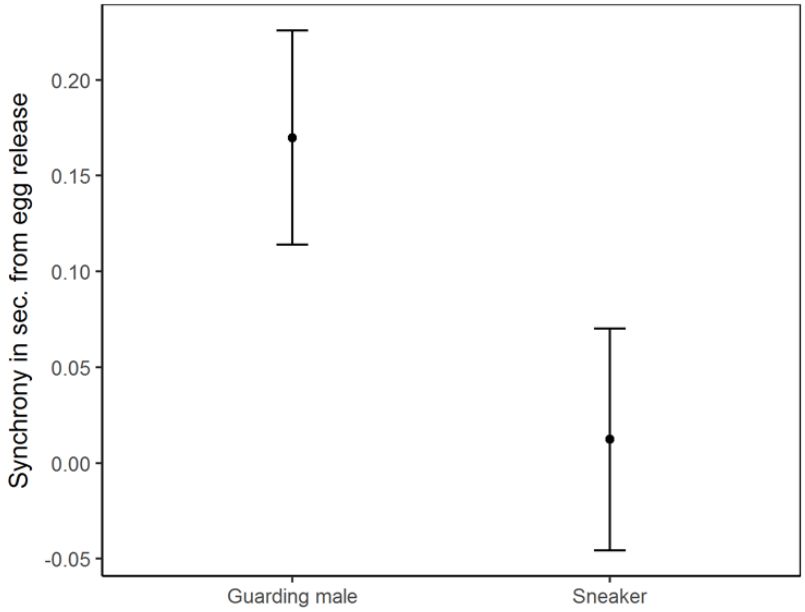
599
600

601 **Figure 2**: Male density was estimated as number of males in proximity to the spawning
602 female (one fish length, ca. 25 cm, is illustrated in the picture).



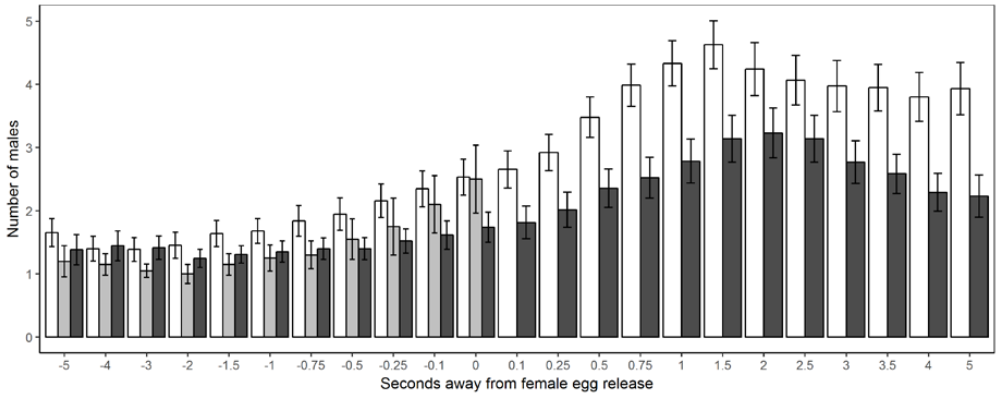
603

604 **Figure 3**: Time delay (mean ± 95% CI) between time of egg release (0) to time of milt
605 release under sperm competition (N=85, but sample size differs among male spawning
606 tactics).



607
608 **Figure 4**: Time delay (mean ± 95% CI) for guarding (n = 41) and sneaker (n = 15) male
609 milt release in single male spawning events, relative to female egg release (0).

610



611
612 **Figure 5**: Number (mean ± 95% CI) of males in the proximity of the spawning female in
613 spawning events with sperm competition (white bars, n = 84), in near spawning events
614 (grey bars, n = 20) and in single spawning events (black bars, n = 73). Zero seconds
615 indicates time of female egg release.

Tables

Table1: Results from a linear mixed effects model for spawning synchrony between the female and guarding male; 1st sneaker; 2nd sneaker; 3rd sneaker in spawning events with sperm competition. Fixed effects are presented with estimate parameters including, standard error (St. error), 95% confidence intervals (95% CI) and *p*-values (*p)*, (n = 146).

Response	Predictor	Estimate	St. error	95% CI	*p*
Time since female egg release	Intercept	0.07	0.06	-0.04-0.19	0.21
	1st Sneaker	0.45	0.07	0.31-0.58	<0.0001
	2nd sneaker	0.82	0.09	0.65-1.00	<0.0001
	3rd sneaker	1.31	0.15	1.03-1.60	<0.0001

Table2: Results from a linear mixed effects model comparing spawning synchrony of the guarding males versus sneaker males in solitary spawning situations (i.e., without sperm competition). Fixed effects are presented with estimate parameters including, standard error (St. error), 95% confidence intervals (95% CI) and *p*-values (*p)*, (n = 56).

Response	Predictor	Estimate	St. error	95% CI	*p*
Time since female egg release	Intercept	0.17	0.02	0.12-0.21	<0.0001
	Sneaker	-0.16	0.05	-0.26--0.06	<0.0001

Table3: Results from a generalized linear mixed effects model for number of males in close proximity to the female over time (std Time) in spawning with sperm competition, "near spawning events (Near) and single spawning events (Single). Fixed effects are presented with estimate parameters including, standard error (St. error), 95% confidence intervals (95% CI) and *p*-values (*p)*, (n = 157).

Response	**Predictor**	**Estimate**	**St. error**	**95% CI**	***p***
Number of males	Intercept	0.89	0.05	0.79-0.99	<0.0001
	std Time	0.73	0.03	0.67-0.79	<0.0001
	Near	-0.16	0.11	-0.34-0.07	0.21
	Single	-0.2	0.03	-0.25--0.14	<0.0001
	std Time x near	0.42	0.24	-0.6-0.89	0.08
	std Time x single	-0.18	0.05	-0.28--0.09	<0.0001

Paper II

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On the relative effect of spawning asynchrony, sperm quantity, and sperm quality on paternity under sperm competition in an external fertilizer

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How much of a fitness benefit is obtained by dominant males of external fertilizers from releasing ejaculates in synchrony with female egg-release when engaging in sperm competition, and what is the most important sperm trait for paternity in these situations? The Arctic charr (*Salvelinus alpinus*) is an external fertilizer experiencing intense male-male competition over reproductive opportunities including sperm competition. To compensate for their disadvantage the sneaker males, which often spawn out of synchrony with the female, produce more and faster sperm than the guarding males. We used controlled *in vitro* fertilization trials with experimentally produced dominant and subordinate, sneaker males to test what effect relative synchrony in gamete release, sperm quality (i.e., motility and velocity) and sperm quantity have on a male's fertilization success in pair-wise sperm competitions. When the sneaker males released ejaculates after the guarding male there was no overall difference in fertilization success. The quality (i.e., motility and velocity) of a male's sperm relative to that of the competing male was the best predictor of male fertilization success regardless of their mating tactic and spawning synchrony. The relative number of sperm cells also had an effect on fertilization success, but mainly when the dominant and sneaker male ejaculated synchronously. Our close imitation of natural sperm competition in charr shows that the sneaker males of external fertilizing species may fully compensate for their disadvantaged mating role by producing ejaculates of higher quality—an adjustment strangely not met by dominants.

Keywords: sperm competition, costly sperm production, delayed ejaculation, loaded raffle, reproductive behavior, ejaculate characteristics

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Introduction

Sperm competition occurs when spermatozoa of two or more males have the opportunity to fertilize the same ovum (Parker, 1970) and it occurs amongst species practicing both internal- and external-fertilization (Birkhead and Møller, 1998). Among species with internal fertilization sperm competition may occur when more than one male inseminates a female during a single fertile period, while for external fertilizers, ejaculates from several males may interact in the external environment before the spawned eggs are fertilized. Sperm competition

allows the male–male competition to continue after ejaculation.

For many external fertilizers the eggs and sperm are viable for a rather short period (Billard et al., 1986) and for species without nests gametes can quickly be scattered out in a large area (Pennington, 1985; Denny and Shibata, 1989; Yund, 1990; Levitan et al., 1992; Babcock et al., 1994; Levitan, 2005). Thus, it is important that the release of gametes for males and females occur at the same site. This may, in non-sessile species, lead to an intensive pre-spawning site competition between males, often of various reproductive tactics, for a position close to the egg releasing female (see e.g., Dominey, 1984; Kodric Brown, 1986; Taborsky, 1994; Alonzo and Warner, 2000; Oliveira et al., 2001; Neff et al., 2003). Furthermore, following the pre-spawning site competition, males must synchronize ejaculation with female egg release in order to reduce the effect of sperm competition. This is documented in Atlantic salmon (*Salmo salar*), where a 2 s delay in sperm release under sperm competition decreased paternity by approximately 40% (Yeates et al., 2007). Moreover, among sneaker male Japanese medaka (*Oryzias latipes*) paternity dropped from 41 to 20% when spawning out of synchrony rather than in synchrony with the dominant male and the female. The latter findings made the authors (Koya et al., 2013) suggest that the reproductive success in medaka was primarily determined by the timing of sperm release corresponding to egg release, something certainly possible given ejaculates of equal sperm numbers and a "fair raffle" (Parker, 1990).

However, ejaculates in competition are seldom composed of equal sperm numbers and the "raffle" is often "loaded" (Parker, 1990). That is, natural adjustments of both sperm quantity and sperm quality (i.e., motility, viability, longevity, velocity, and size) have been documented important for an ejaculate's competitive ability. The importance of sperm numbers for reproductive success is shown in several external fertilizers (Lahnsteiner et al., 1998; Rurangwa et al., 2001; Neff et al., 2003; Ottesen et al., 2009). For example, in the walleye (*Sander vitreus*) the number of sperm cells in the ejaculate is positively related to fertilization success under sperm competition (Casselman et al., 2006). The importance of sperm motility, on the other hand, is illustrated in the Atlantic halibut (*Hippoglossus hippoglossus* L.) where the male with the highest proportion of motile sperm cells under multi-male sperm competitions has the highest fertilization success (Ottesen et al., 2009). Studies on the importance of sperm velocity have been conducted in several species (Gage et al., 2004; Casselman et al., 2006; Rudolfsen et al., 2008; Evans et al., 2013) producing equivocal results. For example, in the myobatrachid frog (*Crinia georgiana*) the fertilization success of the focal male increases as his relative sperm swimming speed decreases (Dziminski et al., 2009), while for the walleye sperm velocity is positively related to fertilization success (Casselman et al., 2006). Yet, in sperm competition it intuitively seems best not just to ejaculate many sperm cells close to and in synchrony with the spawning female, but also to maximize sperm velocity and the number of motile sperm cells—at least for a "Darwinian demon" (Law, 1979).

The arctic charr (*Salvelinus alpinus*) is an external fertilizer where neither males nor females provide any form of parental care after spawning (Fabricius, 1953; Sørum et al., 2011). Free-living charr are easily observed during spawning activity and thus represent a suitable model species for studying pre- and post-copulatory (i.e., spawning) competition among males. Males compete intensely throughout the approximately one-month long spawning season and their social status can easily be identified (Sigurjonsdottir and Gunnarsson, 1989; Liljedal and Folstad, 2003; Sørum et al., 2011; http://naturweb.uit.no/amb/evolution/). Large dominant males use aggressive behaviors toward smaller subordinate males (i.e., chase them away) when trying to guard the females (Sigurjonsdottir and Gunnarsson, 1989). Yet, the spawning area provides no physical protection for the spawning pair and when the female releases eggs the dominant males often spawn in competition with the subordinate males (Sigurjonsdottir and Gunnarsson, 1989; Sørum et al., 2011). Since the subordinate males often employ a sneaking behavior they experience a higher risk of sperm competition, spawn out of synchrony with the female and further away from the released eggs than the dominant males (Sørum et al., 2011). In our study population sneaker males on average ejaculate approximately 0.7 s after the guarding males, 76.5% of the ejaculates experience sperm competition and the mean number of males releasing milt (i.e., the ejaculate of a fish) in each competition is 2.6 (Sørum et al., 2011). Thus, sperm competition is common and in order to compensate for their disadvantages the subordinate males increase their investments in sperm production and sperm velocity (Liljedal and Folstad, 2003; Rudolfsen et al., 2006; Vaz Serrano et al., 2006; Haugland et al., 2011). Sperm velocity and sperm density have also been shown to influence fertilization success under sperm competition in our studied charr population (Liljedal et al., 2008).

Previous studies within the salmonida (*Oncorhynchus tshawytscha*) have shown that ejaculates from sneakers may outcompete ejaculates from dominant males under synchronized fertilizations (Young et al., 2013). Yet, can natural ejaculate adjustments make subordinates ejaculates successful under sperm competition also when released after the synchronized spawning between the dominant male and the female? In the present study we closely mimic natural spawning in charr and use *in vitro* fertilizations to evaluate the hypothesis that naturally occurring adjustments within an ejaculate allow males adopting a disfavored spawning strategy to successfully compete with the spawning strategy of males trying to monopolize access to the spawning females. That is, we investigate, for the first time, the relative importance of asynchrony in gamete release, sperm number, and sperm motility for the reproductive success of subordinate and dominant males under sperm competition. Will the allocation of resources to larger sperm numbers with higher velocity, as previously observed among subordinate charr, be sufficient to compensate for their asynchronous spawning under sperm competition from dominants?

Methods

Study Site, Fish Sampling, and Tagging

We carried out the fieldwork in our study population at Lake Fjellfrøsvatn at 69°08′N 19°34′E in northern Norway from the

8th to the 27th of September 2008. The gametes used in this experiment came from reproductively active fish (16 males and 16 females) caught with gill nets from the same spawning ground (see Figenschou et al., 2004 for details on spawning grounds). Individuals were continuously removed from the nets in order to minimize stress. Males included in the experiment were transported to the field laboratory where they were anesthetized using benzocaine. Then, they were stripped for all available milt and length was measured (29.6 cm ± 2.2, mean ± SD) before each male was id tagged in the dorsal fin with similar sized, yet easily distinguishable plastic tags (approximately 0.5 cm^2), attached with Floy's elastic vinyl filament. Thereafter the males were size-matched and caged in pairs with a maximum length difference within each of the 8 pairs established of 4 mm (minimum 2 mm). Previous studies have shown that males entering a dominant position under these experimental conditions do not initially differ in ejaculate characteristics, size or ornamental development from males taking up a subordinate strategy (Rudolfsen et al., 2006). The cages (40 × 60 × 90 cm, made of chicken wire) were placed at about 1.5 m depth, 2–3 m apart and the fish were left undisturbed for 24 h before our behavioral observations started (see below). On day four, the fish were again anesthetized, and stripped for all available milt which had been produced during social interactions as either dominant or subordinate. The milt volume was measured to the nearest 0.1 ml (overall: 0.70 ± 0.34, dom: 0.78 ± 0.37, sub: 0.62 ± 0.31, mean ± SD, respectively) and then stored on ice for further analyses (see Sperm Analysis) and for fertilizations (see Fertilizations). Females were caught immediately prior to our planned fertilizations and stored in plastic containers before they were brought to the field-laboratory, anesthetized and stripped of their eggs. A small amount of ovarian fluid, later used for measurements of sperm velocity in ovarian fluid, was separated from eggs using a pipette and stored at lake water temperature (6°C). All fish used in this study were released back into the lake (see Haugland et al., 2011 for a more detailed description of capture and caging methods).

Behavioral Observations

Status roles are highly dynamic in charr and individuals employing a subordinate spawning strategy at natural leks, readily takes up dominant strategies during experimental trials lasting less than 1 day (own observations). In order to determine social rank, we recorded male behavior twice a day for 5 min during the last 3 days of the four-day caging period. Bathyscope underwater viewers were used for observing the individual number of aggressive acts (e.g., an initiation of a chase) and the male performing most aggressive acts within a pair was considered the dominant. The presence of an observer during such behavioral observations does neither significantly alter fish activity nor the within pair hierarchical position (see Liljedal and Folstad, 2003 for details).

Sperm Analysis

To minimize handling time all the measurements were conducted as fast as possible by skilled personnel and at temperatures similar to lake temperatures. The handling of males was also conducted randomly with respect to the male's social position and without knowledge about the individual's social position. Spermatocrit (i.e., the percentage of the ejaculate consisting of sperm cells) was measured by centrifuging 10 µl homogeneous milt (i.e., milt gently shaken in an Eppendorf tube) in a capillary tube for 195 s at 11 500 rpm (Compur-electronic Gmbh, Munich, Germany) and used as a measure of sperm number (overall: 14.5% ± 9.3, dom: 8.17% ± 0.82, sub: 20.86% ± 9.64, mean ± SD, respectively). Thereafter, the entire milt sample produced during the 4 days was split in 20 subsamples (average volume 0.032 ml). Each subsample of milt is thus represented with a volume giving an ejaculation frequency of 5 per day, a frequency within the range of that observed in our free-living population (own observations, see also Sørum et al., 2011). Of these, 16 subsamples were used for fertilizations and 4 for evaluations of sperm quality. That is, for each male we quantified sperm motility and velocity in water and in water diluted ovarian fluid (1:2) from the focal female used in each fertilization. Measurements of sperm behavior were taken 10 s following activation of the sperm cells and later analyzed using CASA (HTM-CEROS v.12). Each motility measurement lasted 0.5 s and the parameters assessed were mean curvilinear velocity (VCL) (water overall: 140.0 µm/s ± 31.6, and VCL ovarian fluid overall: 139.3 µm/s ± 23.0, mean ± SD, respectively) and percentage motile cells (water overall 89.3 ± 14.7, and ovarian fluid overall 81.2 ± 16.9, mean ± SD, respectively) using the methods described in Vaz Serrano et al. (2006).

Our statistical model (see Statistics) is only valid over the range of values in our pair-wise comparisons of velocity, motility and spermatocrit observed in our eight pairs of dominant and subordinate males. That is, sperm velocity estimates in water range from dominants having velocities 24.3 µm/s faster than subordinates and subordinates having velocities 15.5 µm/s faster than dominants (average difference 2.9 µm/s, SD ± 24.3 µm/s). Sperm velocities in ovarian fluid range from dominants having sperm swimming 58.4 µm/s faster than subordinates to subordinates having sperm swimming 52.2 µm/s faster than dominants (average difference is 10.8 µm/s, SD ± 25.9 µm/s). Motility range in water from dominants having 27% more motile sperm than subordinates to subordinates having 2.5% more motile sperm than dominants (average difference is 6.4%, SD ± 10%). Motility in ovarian fluid range from dominants having 54% more motile sperm cells than subordinates to subordinates having 27.5% more motile sperm cells than dominants (average difference is 6.4%, SD ± 24.8%). Spermatocrit values range from subordinates having 25–3% more sperm in their ejaculates than dominants (average 12.7%, SD ± 9.6%). Differences in spermatocrit between dominants and subordinates were also controlled for in our experimental design (see Fertilization).

Fertilization

We examined the relative paternity of dominant and subordinate males competing to fertilize eggs in *in vitro* fertilization trials using the following approach: An approximately equal numbers of mature eggs (20–30) from each female were distributed in marked plastic beakers and fertilizations were conducted by manually "ejaculating" [from pipettes aimed with the same angle toward the eggs and located at same distance (3 cm) from the

eggs] sperm from the two competing males into the beakers immediately after adding 50 ml water to the eggs. The amount of water added to the eggs before "ejaculating" the sperm was just enough to cover the eggs, giving the dominant male low sperm dilutions when ejaculating before the subordinate. To investigate the effect of sperm numbers on paternity we used two kinds of sperm competition trials: One where the two competing males (i.e., ejaculates) had different sperm numbers (i.e., not controlling for initial differences in sperm cell density by adjusting ejaculate volume) and one where they had a similar number of sperm cells (i.e., after controlling for initial differences in sperm cell density by adjusting ejaculate volume). The average ejaculate volumes used in these to different trials were the same (18.5 μl), yet the standard deviation naturally differed (± 9.1 and 3.9 μl, respectively). Nested within each of these two types of sperm competition we either added the sperm from the two males synchronously or with a time delay to the subordinates "ejaculation" (i.e., asynchronous fertilizations). As the asynchrony in gamete release between dominant and subordinate male in natural spawning from the same population is on average 0.68 s (Sørum et al., 2011), we used that time as a reference to perform the asynchronous fertilizations. In order to "ejaculate" the sperm from the pipettes at the right time, we used a metronome guiding the "ejaculator" (TBE) and we also video recorded the actual "ejaculation" of milt from the pipettes. The latter enabled an *a posteriori* analysis of the timing of milt release to the nearest 0.01 s, showing that subordinate "ejaculations" in our fertilizations were 0.67 ± 0.04 s (mean ± SD, $n = 64$) after the dominant. Investigations of sperm-egg interactions were enabled by letting every pair of caged males fertilize eggs from two different females. Every treatment was done with replications, giving 16 independent fertilizations for each combination of 2 males × 2 females. After ejaculation the beakers with the mixture of eggs, water and milt were gently stirred and approximately 15 s after ejaculation 0.5 l of water was slowly added to dilute the sperm densities (to avoid possible polyspermy), which also occur under natural spawning.

Hatchery

The fertilized eggs were stored at 6°C over night and then transported carefully to the hatchery. The hatchery contained six 600 l tanks with 6°C water continuously flowing through. The eggs, which are demersal, were randomly distributed into plastic cups (4 cm^3) with a bottom made of nylon net with water flowing through each cup (family). Unfertilized, infected and dead eggs, dead eyelings and dead fry were removed every week to avoid fungus growth. After 120 days, living fry were anesthetized and killed, using benzocaine, and stored on ethanol. The mean survival through the experimental period was 64% and (because of monetary limitations) 1255 of the 5688 fry were included in paternity analysis representing 128 families (i.e., an average of 9.8 fry per family, $SE \pm 0.11$). Our experiments conform to the relevant regulatory standards in Norway.

DNA-extractions

We obtained tissue for DNA extraction by cutting the caudal fin of the larvae and a small part of the dorsal fin for the adults. The tissue samples were then stored in 96-well PCR plates. DNA was isolated using a modified procedure of Miller et al. (1988). Cell Lysis Buffer and Proteinase K were added in a relationship 3:2 (i.e., 22.5 μl Cell Lysis Buffer and 15 μl Proteinase K) using a Gilson Pipetman Concept multichannel (Gilson, Middleton, WI, USA). After incubating the plates overnight, at 55°C and 150 rpm, they were heated to 80°C for 15 min using an Eppendorf Mastercycler (Eppendorf HQ, Hamburg, Germany) followed by a short centrifugation, using a Labofuge 400 R (Heraeus, Buckinghamshire, England) and then stored in a freezer at −20°C.

PCR

Paternity was examined using microsatellites and polymerase chain reactions (PCR) on an Eppendorf Mastercycler (Eppendorf HQ, Hamburg, Germany) and a C1000 Thermal Cycler (BIO-RAD HQ, Hercules, CA, USA). The PCR's were carried out in 10 μl reaction volumes containing: 0.4 μl (50–100 ng) Arctic charr genomic DNA, 0.2 μl (0.2 μM) of each of the forward and reverse fluorescently marked primers, 0.2 μl (0.05 mM) dNTP, 1.0 μl (2.5 mM) buffer, 0.08 μl (0.04 units/μl LaTaq) enzyme and 7.92 μl distilled H_2O. The PCR profile was: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 59°C for 30 s, 72°C for 25 s with a final 72°C extension for 7 min. Later analysis of the PCR-products was carried out at the DNA-Sequencing Laboratory at the University of Tromsø on an Applied Biosystems (ABI) 3130×l Genetic Analyzer with ROX 500 and HiDi Formamide from ABI. Allele size was examined with the software GeneMarker v. 1.6 (SoftGenetics, PA, USA). Paternity was unambiguously assigned manually. We used the primers Smm_22 and Smm_24, which were isolated and characterized from other salmonid species by Crane et al. (2004). We chose these primers based on a previous study from the same population, which demonstrated high polymorphism at these loci (**Table 1** in Westgaard et al., 2004). This analysis of the 32 parents gave 16 and 13 different alleles at Smm_22 and Smm_24, respectively, whereas the numbers of different alleles counted in males only were 15 and 12 at the two loci, respectively. Smm_22 and Smm_24 from all potential parents gave consistent allele sizes when examined twice.

Statistics

As sperm velocity and percentage of motile sperm cells in water and ovarian fluid were correlated ($0.27 < r < 0.62$), we quantified the parameters of sperm behavior (i.e., quality) by using a principal component analysis. This reduced the four variables to one statistically independent component (from now on termed motility PCA, Eigenvalue = 2.63, variance explained = 66%, the PCA's respective r-values to motility in water and ovarian fluid were 0.9 and 0.5, and the corresponding numbers for the velocity in water and ovarian fluid were 0.9 and 0.5). In order to test what effects sperm motility, sperm number and synchrony of gamete release have on the fertilization success we ran a mixed-effects model with the proportion of eggs fertilized by the focal male as the response variable (using the cbind function in R, see Crawley, 2013 p. 628 for further details). Since the response variable is a proportion, we ran the model

TABLE 1 | Parameter estimates (SE, 95% CI and *p*-values) for the effects of sperm velocity and motility in water and ovarian fluid (i.e., motility PCA), sperm quantity (i.e., number of sperm cells), ejaculate release delay (i.e., asynchrony) and the interactions on proportion of larvae sired ($n = 128$).

Response	Predictor	Estimate	St. error	95% CI	*p*
Proportion	Intercept	0.71	0.51	−0.48–1.9	0.24
Sired	Motility PCA	1.6	0.82	−0.009–3.2	0.051
	Number of sperm cells	0.0009	0.0007	−0.0004–0.002	0.21
	Asynchrony	0.19	0.27	−0.33–0.71	0.48
	Motility PCA × number of sperm cells	0.00008	0.001	−0.002–0.002	0.94
	Motility PCA × asynchrony	−0.06	0.49	−1.01–0.89	0.91
	Number of sperm cells × asynchrony	0.0023	0.001	−0.0001–0.005	0.066
	Motility PCA × number of sperm cells × asynchrony	0.007	0.002	0.003–0.01	0.0016

with a binomial distribution. As fixed factors we entered sperm motility PCA and the two manipulated traits (sperm number and synchrony). Batch of eggs from the two females per male pair and female ID were included as random factors, with female ID nested in batch (i.e., 8 pairs of males, 2 females per pair, a 2 × 2 full factorial design, 2 replicates per pair × female combination). Motility PCA and number of sperm cells were entered as relative measures, which are the measures for the focal male minus the measures of the competing male in the pair. Asynchrony was entered as the relative time difference in milt release between the two competing ejaculates. For example, if the focal male's ejaculate was released 0.67 s before the competing male's ejaculate it was entered as 0.67 s. Model fitting and estimates were obtained with the linear mixed-effects (lmer) package lme4 (Bates et al., 2014) in R (version 3.1.3, R Development Core Team, 2015) using restricted maximum likelihood estimates (REML). Model fit and significance were tested using Akaike's Information Criterion corrected for sample size (AICc) (d'Auvergne and Gooley, 2003) and log-likelihood ratio statistics (LLR $\lambda 2$) (Bates, 2005). Finally, the model fit was checked using visual examination of normal probability plots and residual plots. The three-way interaction between all predictors was included in the final model, thus as higher order interactions were significant the lower order interactions and main factors also had to be included. In order to visualize results we used plots from the R libraries languageR (Baayen, 2013), hexbin (Carr, 2014), akima (Akima, 2013), and latticeExtra (Sarkar and Andrews, 2013). The parameter estimates in the plots are back-transformed to proportional scale for better interpretation and visualization.

Results

No association was apparent between the proportion of eggs surviving and the relative paternity of the males as revealed by microsatellites ($r_s = 0.068$, $p = 0.44$, $df = 128$, Spearman). This suggests that it is unlikely that the actual fertilization success we measure was caused by differential mortality or differential ability to develop by the embryos sired by the two males (for a detailed discussion see García-González, 2008). The finding is in agreement with results previously reported using individuals from the same population, similar experimental design, the same rearing equipment and housing, and the same time period for embryonic development as in the present study (Liljedal et al., 2008).

Increased motility PCA (i.e., the percentage of motile cells and sperm velocity in water and water diluted ovarian fluid) had the strongest independent positive effect on the proportion of larvae sired under sperm competition (**Table 1**). Increased sperm cell numbers, on the other hand, had no independent effect on paternity and there was also no obvious independent effect of ejaculation asynchrony (**Table 1**). That is, whether the ejaculate of the focal male was released before, after or in synchrony with the competing male had no overall effect on proportion of larvae sired.

There was however, a positive interaction effect between synchrony in gamete release and number of sperm cells in the ejaculate on the proportion of larvae sired ($p = 0.066$, **Table 1**). That is, the relative number of sperm cells seem to influence the proportion of larvae sired more for a male that ejaculated "last" (i.e., the subordinate) than for a male that ejaculated "first" (i.e., the dominant, see **Figure 1**). Yet, there was no significant interaction between the relative motility PCA and relative number of sperm cells on the proportion of larvae sired ($p = 0.94$, **Table 1**). Additionally, there was no significant interaction effect between relative motility PCA and gamete release synchrony on paternity ($p = 0.91$, **Table 1**).

There was however a highly significant positive three-way interaction involving relative motility PCA, relative amount of sperm cells and asynchrony (**Table 1**). That is, when ejaculating in synchrony both high motility PCA and high sperm numbers are important for paternity (**Figure 2**). Yet, when there is a time delay in the gamete release between the two mating tactics (the subordinate is always last) the fertilization success is much more dependent on a high motility PCA for the dominant male (**Figure 3**) than for the subordinate male (**Figure 4**). **Figures 2–4** also illustrate how the effect of the motility PCA varies depending on whether the males spawn first, in synchrony or last.

Discussion

This is the first study to disentangle the effects of naturally occurring adjustments in sperm quality, sperm quantity and spawning synchrony and their interactions for paternity in sperm competition among subordinate and dominant

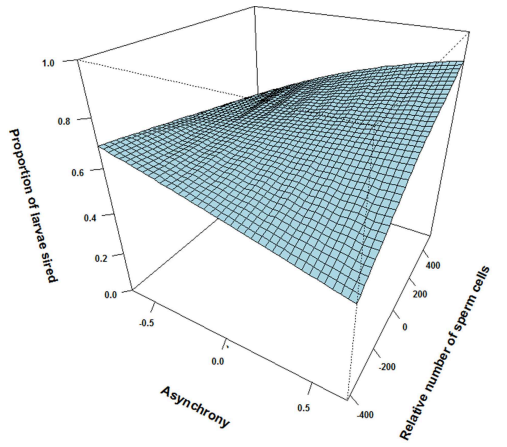

FIGURE 1 | The interactive effect of relative number of sperm cells and ejaculation asynchrony on proportion of larvae sired between pairs of males in sperm competition. An asynchrony value of −0.67 indicates that the male releases milt 0.67 s before the competing male and 0.67 indicates that the male release milt 0.67 s after the competing male. In our experiment dominant males either released their ejaculate before or in synchrony with the subordinate male, while subordinates either released their ejaculate in synchrony or after the dominant male. Although, a subordinate male may ejaculate later than a dominant male, he might compensate for the delay by increasing sperm numbers.

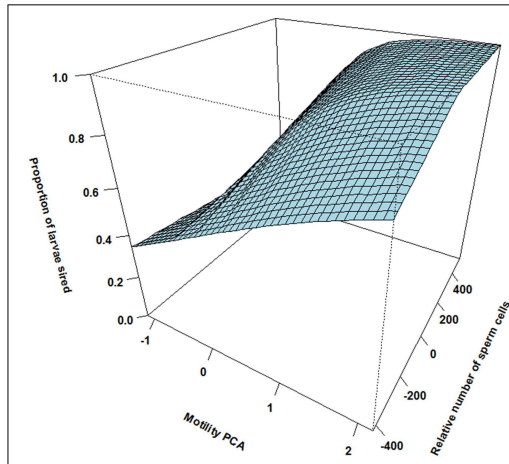

FIGURE 2 | The interaction between relative motility PCA (i.e., per cent motile sperm cells and sperm velocity in water and ovarian fluid) and relative number of sperm cells on proportion of larvae sired for the males that ejaculated in synchrony (simultaneously). When the two males have similar sperm numbers there is a sharp increase in paternity with increasing difference in motility PCA. At similar motility PCA there is also a slight increase in paternity with increasing difference in sperm numbers.

individuals of an external fertilizing species. Our results show that sperm motility and number of sperm cells are the overall most important variables influencing paternity under our experimental conditions. Whether the ejaculate of the subordinate is released after or in synchrony with the competing male, showed no overall effect on paternity. However, ejaculate characteristics in synchronized and asynchronized spawning affect paternity of the dominant and subordinate males differently. That is, dominants ejaculating in synchrony with the female egg release and (on average 0.67 s) before the subordinate male, show a rapid increase in paternity with increasing sperm motilities—a relationship not much influenced by relative sperm numbers. Subordinates, ejaculating out of synchrony (0.67 s after the dominant male), show a slow increase in paternity with increasing sperm motility and need high sperm numbers to outcompete the dominant male.

This experiment mimicked the situation with ejaculations from one dominant and one subordinate male given an equal distance to the eggs. The proximity of the female to the male during spawning may also be of large importance for the outcome of reproductive activities and our study is, consequently, not a complete description of all factors influencing reproductive success under sperm competition in charr. The spawning in our population of charr has been studied in some detail showing a large variance in the behavioral repertoire (Sørum et al., 2011). From one to nine males may release ejaculates from very close positions to the released eggs within 1.9 s after spawning between a female and the dominant male. Dominant males and subordinates may also spawn with the female without sperm competition (Sørum et al., 2011). Additionally, as the spawning site provide no protection for the dominant male and the female, there is an increase in the density of males positioning themselves at the spawning site before the actual spawning occur between the dominant male and the female. That is, the positional advantage for the dominant male during spawning is in our population somewhat unclear, and our mimic is well within the behavioral repertoire observed (i.e., it is not a mimic of a constructed artificial situation). To get a complete understanding of all factors influencing male reproductive success in our population, female proximity and more intense sperm competition (more than two males) should also be experimentally evaluated.

In bluegill (*Lepomis macrochirus*), sneakers on average release their sperm approximately 0.46 s after parental (Stoltz and Neff, 2006a). Yet, when mimicking this delay in *in vitro* sperm competition trials, ejaculates from sneaker males outcompete those from parentals (Stoltz and Neff, 2006b). Although sperm numbers influence the outcome, the advantage for sneakers is larger than that accounted for by differences in sperm numbers. The authors conclude that some other aspect than flagellum length, curvilinear speed and path linearity, the three quality measures of sperm included in the study, must contribute to the increased competitiveness of sperm from sneakers (Stoltz and Neff, 2006b). Thus, for bluegills the cause for the "loaded raffle" seems still unclear.

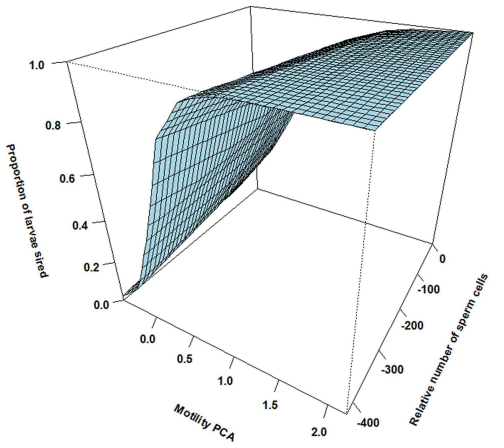

FIGURE 3 | The effect of relative motility PCA (i.e., per cent motile sperm cells and sperm velocity in water and ovarian fluid) and the relative number of sperm cells on proportion of larvae sired for dominant males ejaculating before the competing subordinate male (asynchrony). Although dominant males show a sharp increase in paternity with increasing sperm motility PCA independent of sperm numbers, dominants show low sperm velocity compared to subordinates under natural reproduction.

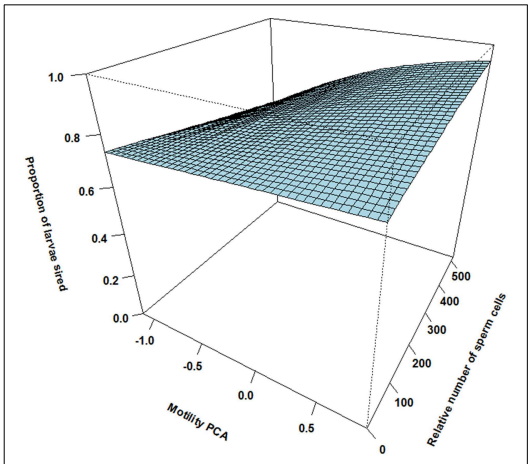

FIGURE 4 | The effect of relative motility PCA (i.e., per cent motile sperm cells and sperm velocity in ovarian fluid) and relative number of sperm cells on proportion of larvae sired for subdominant males ejaculating after the competing dominant male (asynchrony). Here, the effect of increased motility and increased sperm numbers is weak.

Our overall results are, on the other hand, in line with earlier studies revealing sperm velocity and sperm motility as good predictors of a male's fertilization success both in the absence (Lahnsteiner et al., 1998; Froman et al., 1999; Rurangwa et al., 2001; Kupriyanova and Havenhand, 2002; Gomendio et al., 2007) and in the presence of sperm competition (Birkhead et al., 1999). These results are also in line with previous findings in charr (Liljedal et al., 2008) and in closely related species like salmon (*S. salar*) (Gage et al., 2004) and trout (*Salmo trutta*) (Lahnsteiner et al., 1998). Salmonids are known to have one of the briefest fertile windows among fish and the period of sperm survival is short when observed in water (Vladic and Järvi, 1997, own observations). Yet, the ovarian fluid represents a protective environment (Litvak and Trippel, 1998) and Arctic charr ejaculates have a higher percentage of motile sperm cells in ovarian fluid than in water (Turner and Montgomerie, 2002). Moreover, the ovarian fluid seems to represent a selective environment for sperm (Yeates et al., 2013), and there is a strong interaction effect of ovarian fluid on sperm swimming speed in charr with certain female fluids stimulating swimming speed of sperm from some males over others (Urbach et al., 2005). Eggs are fertilized after a sperm cell enters the micropyle, which is barely wide enough to allow entry of one sperm cell (Ginsburg, 1963; Kobayashi and Yamamoto, 1981; Yanagimachi et al., 1992). Thus, in salmonids, where up to 80% of the eggs can be fertilized within the first 5 s of egg and sperm interactions (Hoysak and Liley, 2001), the first sperm cell to enter the micropyle fertilizes the egg (Kobayashi and Yamamoto, 1981; Yanagimachi et al., 1992). These female evolved characteristics may help enforce cryptic choice and may explain why sperm motility, in this study also measured in ovarian fluid, is so important for the observed fertilization success under sperm competition in Arctic charr.

In previous studies where the dominance status of males is experimentally manipulated, individual male Arctic charr becoming subordinates increase average sperm velocity compared to males becoming dominant (Liljedal and Folstad, 2003; Rudolfsen et al., 2006). Ejaculates of subordinates also contain larger fractions of fast sperm cells, those most likely to fertilize the eggs, than ejaculates of dominants (Vaz Serrano et al., 2006; Haugland et al., 2008). Subordinate males becoming dominant, on the other hand, reduce their sperm velocity compared to levels previously held as subordinates (Rudolfsen et al., 2006). This velocity reduction among males becoming dominants is puzzling given the large importance of sperm motility for number of offspring sired among these individuals. Yet, the velocity reduction is in accordance with Parker's (1990) theoretical model of ejaculate investments under sperm competition which suggest that "..if mating order is non-random, the favored male should expend less on sperm." One might speculate that the metabolic resources for sperm production and sperm velocity, are traded-off differently in dominant and subordinate males over the entire spawning season (Jeulin and Soufir, 1992; Burness et al., 2004, but see Burness et al., 2005) and that dominants in need of more energy for guarding activities, potentially resulting in positional advantages under synchronized spawning, might have to reduce energy investments in sperm. This explanation, which relies heavily on energy being a limited resource for reproductively active males, fits the observations that ATP-levels in sperm of charr is positively related to sperm velocity and negatively related

to measures of high social status (i.e., dominance) (Figenschou et al., 2013). An explanation based on energy limitations also correspond with the recent suggestion that the adipose fin may have evolved as a signal of energy stores in salmonids (Haugland et al., 2011). That is, adipose fin size may actually be indicative of the energy available for reproductive activities in salmonids.

The overall positive relationship between relative sperm number and fertilization success in the present study is also in agreement with empirical evidence from other external fertilizers experiencing sperm competition (Dziminski et al., 2009; Ottesen et al., 2009). It also corresponds with results showing an inter-specific relationship between the intensity of sperm competition and sperm production in external fertilizers (Byrne et al., 2002). While the ejected distance of an ejaculate may be longer then 20 cm (own observations), sperm cells from charr are only able to swim about half of the circumference of the egg, i.e., 0.5 cm (Billard and Cosson, 1992). Thus, given a large difference in sperm numbers between the two competing males, the male with most sperm cells can scatter his sperm in a larger area and therefore reach the spawned and widespread eggs before the competing male. Moreover, the generally held assumption that a gradual dilution of seminal fluid in general is initiating sperm activity seem to be violated in charr, as activity of sperm cells here are only influenced by individual specific dilution of own seminal fluid in water and unaffected by the presence of seminal fluids from other males (Rudolfsen et al., submitted). This latter finding clearly illustrates the fine-tuned adaptions to sperm competition in charr.

Our close experimental imitation of sperm competition under natural spawning asynchrony, natural variation in sperm number and natural variation in sperm velocity show that a high degree of synchrony in gamete release between the female and the dominant male may result in a high paternity share of the dominant male. Yet, this benefit might to a large extent be offset by a rapid (within 4 days) increase in sperm cell production and sperm motility among subordinate, sneaker males. The latter is however dependent upon a rapid response to female egg release by subordinate males. In our studied population the number of subordinate males at the spawning site increases through the 0.25 s period elapsing right before egg release (Sørum et al., 2011), suggesting that subordinates may perceive the forthcoming spawning. Yet, although the first sneaker males are ejaculating on average 0.68 s after the guarding male, subordinates are also seen releasing milt as late as 1.9 s after female egg release (Sørum et al., 2011).

Although natural spawning in charr includes a large range of spawning behaviors including a high frequency of highly synchronized spawning between the female and a dominant male, our close natural mimic adds parameter estimates to the outcomes most commonly observed (Sørum et al., 2011). At this intensity of sperm competition, i.e., when approximately two males compete, allocation of resources to sperm production should be at its most intense (Parker et al., 1996) rendering our system ideal for studies on behavioral and physiological adaptations to sperm competition. We are however currently unable to quantify the fitness effects of resource investments in sperm quantity and quality for the different spawning tactics throughout the 1-month long spawning period. Is it possible that the relative low investment in sperm quantity and sperm quality observed in dominant males compared to that of subordinates under such long-term scenario prove beneficial because of benefits from synchronized spawning and positional effects? Additionally, how does ejaculate investments trade-off against the obvious costs of mate guarding and courting? Our results suggest that constraints on investments in sperm number and motility among dominant guarding males should be considerable given the large potential fitness benefits from such investments when ejaculating in synchrony with the female—and before the subordinates.

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Paper III

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Status Specific Tailoring of Sperm Behavior in an External Fertilizer

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Why dominant males experiencing intense sperm competition sometimes show low investments in sperm production is not always obvious. One well-documented example is that of the external fertilizing teleost, the Arctic charr (*Salvelinus alpinus*), where individuals becoming dominant reduce sperm production and sperm swimming speed in water compared to subordinates. Here, we report how ovarian fluid differentially influences sperm velocity of dominant and subordinate male Arctic charr. That is, sperm from dominant males increase their velocity in water diluted ovarian fluid compared to that observed in water, while sperm from subordinates, on the other hand, decrease velocity in ovarian fluid compared to that observed in water. Thus, subordinates, who invest more resources in their sperm and usually show the highest sperm velocity in water, have lower gains from their investment than dominant males when sperm are swimming in ovarian fluid. In sum, our result suggests that ovarian fluid increase sperm velocity more in dominant males than in subordinate males. Although this finding could partly be caused by cryptic female choice exerted by the ovarian fluid for sperm from dominant males, an alternative and more parsimonious explanation is that sperm from dominant males may simply be better designed for swimming in ovarian fluid compared to sperm from subordinate males. Thus, sperm production in the two reproductive roles seems to be adaptively tailored to different external environments.

Keywords: sexual selection, cryptic female choice, sperm competition, sperm selection, sperm velocity, ovarian fluid

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INTRODUCTION

Polyandry leads to conflict between males over fertilizations resulting in both pre- and post-copulatory male adaptations (Birkhead and Møller, 1992, 1998; Andersson, 1994; Andersson and Iwasa, 1996; Eberhard, 1996; Alonzo and Warner, 2000; Simmons, 2001; Chapman et al., 2003). This is easily seen in species with external fertilization where adorned dominant males gain fitness benefits by spawning in synchrony with the female and close to her eggs after courting and aggressive mate guarding. Less competitive males, on the other hand, are often forced by the dominant male to spawn out of synchrony with the female and further away from the eggs (Taborsky, 1998). This behavior often results in sperm competition where sperm from two or more males co-occur at the site of fertilization (Parker, 1970; Simmons, 2005). When there is risk of sperm competition, males may produce more sperm, larger sperm or sperm that have higher velocity than would be required to fertilize the eggs in absence of competition, at least in theory (Parker, 1970, 1998; Ball and Parker, 1996). Recent empirical studies have, in line with theory, also shown

that increased risk of sperm competition leads to a higher investment in sperm velocity (Burness et al., 2004; Rudolfsen et al., 2006) and that such sperm velocity increases may be important for fertilization success (Levitan, 2000; Al-Qarawi et al., 2002; Kupriyanova and Havenhand, 2002; Gage et al., 2004; Liljedal, 2005; Schulte-Hostedde and Burness, 2005; Egeland et al., 2015).

Inference about the importance of sperm velocity for fertilization in external fertilizers stems in general from evaluations of sperm velocity measurements obtained from activation in water (Lahnsteiner et al., 1998; Levitan, 2000; Gage et al., 2004; Liljedal, 2005). However, eggs of external fertilizers are embedded in ovarian fluid, and in certain species the amount of ovarian fluid released together with the eggs is up to 30% of the total egg volume (Lahnsteiner et al., 1999). Ovarian fluid is suggested to compensate for the sub-optimal environmental conditions for the sperm in water (Lahnsteiner, 2002), and has been shown to enhance overall sperm longevity and velocity compared to that of water (Hayakawa and Munehara, 1998; Lahnsteiner, 2002; Turner and Montgomerie, 2002). Thus, the characteristics of ovarian fluid in external fertilizing species is likely to have evolved, at least partly, to increase the probability of fertilizing the eggs (Lahnsteiner, 2002).

Females of external fertilizers which experience strong sperm competition are expected to evolve mechanisms to enhance paternity of favorable males at the cost of unfavorable males, and should not be regarded as only providing an arena for sperm competition (Thornhill, 1983; Eberhard, 1996; Olsson et al., 1996; Zeh and Zeh, 1996; Birkhead, 1998). Ovarian fluid has been shown to favor swimming speed of sperm from certain males over others, suggesting that ovarian fluid may act as a medium where female-mediated cryptic selection processes can occur (Urbach et al., 2005; Nordeide, 2007; Dietrich et al., 2008; Rosengrave et al., 2008; Alonzo et al., 2016). However, disentangling the separate effects of varying quality of sperm and differing ovarian fluids on fertilization success and offspring quality under sperm competition is challenging. Some authors have demonstrated positive effects of ovarian fluid on sperm velocity (Gasparini and Pilastro, 2011; Evans et al., 2012; Oliver and Evans, 2014; Alonzo et al., 2016; Rosengrave et al., 2016), while Lumley et al. (2016) revealed no effect of ovarian fluid on relative offspring fitness. Moreover, the only published intraspecific study exchanging ovarian fluid between eggs from different females documented no overall effect of ovarian fluid on paternity success under sperm competition and no evidence for male-female interactions (Evans et al., 2013).

The Arctic charr (*Salvelinus alpinus*) has external fertilization with males aggregating annually at specific spawning areas. Dominant males attract and guard arriving females, yet spawning can hardly occur isolated from other males as the spawning area offer no form of protection from sneakers (Sigurjonsdottir and Gunnarsson, 1989; Sørum et al., 2011; http://naturweb.uit.no/amb/evolution/). Moreover, males show high plasticity in reproductive behaviors, and social status seems to be conditional depending on other interacting males (Fabricius and Gustafson, 1954; Sigurjonsdottir and Gunnarsson, 1989; Cutts et al., 2001). Observational studies of reproductively active male charr show, in accordance with that predicted from theoretical models (Parker, 1990; Parker et al., 2013), that social status is negatively related to sperm velocity (Figenschou et al., 2013). Additionally, males experiencing a change in mating roles have repeatedly been found to rapidly adjust sperm production. That is, compared to males in subordinate mating roles, males attaining dominance reduce sperm production and velocity of sperm cells in their ejaculate within 4 days in their new mating role (Liljedal and Folstad, 2003; Rudolfsen et al., 2006; Vaz Serrano et al., 2006; Haugland et al., 2008). Additionally, this difference in sperm velocity between dominant and subordinate individuals is most predominant among the fastest sperm cells—those most likely to fertilize the eggs (Vaz Serrano et al., 2006; Haugland et al., 2008). Moreover, sperm velocity is documented to be of major importance for fertilization success under sperm competition in Arctic charr (Liljedal, 2005; Egeland et al., 2015) and carefully controlled *in vitro* sperm competition trials, including a realistic time-lag to subordinates ejaculation, have shown that subordinate males may fully compensate for disadvantages in their unfavorable mating role (i.e., ejaculating out of synchrony with the female) by having more and faster sperm than dominants (Egeland et al., 2015).

So, why do males becoming dominant reduce sperm numbers and sperm velocity in their ejaculates when they have large fitness benefits under sperm competition by maintaining high sperm production and high sperm velocity (see Figure 3 in Egeland et al., 2015)? In the present study, we reanalyze data from Egeland et al. (2015) (See first paragraph in Material and Methods) in order to evaluate the potential modulating effect from ovarian fluid on sperm velocity from dominant and subordinate male charr. Dominant and subordinate Arctic charr have different sperm velocity when measured in water, but whether this difference in velocity is maintained when sperm is swimming under the influence of ovarian fluid is not known.

MATERIALS AND METHODS

The data used in this study have partly been analyzed and presented for other purposes in Egeland et al. (2015). In the former publication, we used eight pairs of males and females to test the effect of spawning asynchrony, sperm quantity, and sperm quality on paternity. To increase the sample size in the present study we use those eight pairs in addition to eight more pairs of males and females (i.e., in total 16 pairs, 32 males, and 32 females) caught and analyzed during the same spawning season in 2008.

Fish Sampling and Handling

During mid-September 2008, in Lake Fjellfrøsvatn northern Norway (69° 4′ N, 19° 20′ E), we gill netted reproductively active charr at one spawning ground (i.e., males and females came from one naturally interbreeding population; see Figenschou et al., 2004). To minimize stress the fish were continuously removed from the gill nets. The 32 males included in the experiment were transported to the field laboratory where they were anesthetized using benzocaine. The length was measured (29.7 cm ± 2.1, mean ± *SD*) and the males were then stripped for all available

milt before id tagging (see Egeland et al., 2015). Thereafter the males were size-matched and caged in pairs, with a maximum length difference of 5 mm within each of the 16 pairs. Rudolfsen et al. (2006) showed, using the exact same procedures that males entering a dominant position in pair-wise interactions do not initially differ in ejaculate characteristics, size or ornamental development from males taking up a subordinate position. The cages (made of chicken wire, 40 × 60 × 90 cm) were placed 2–3 m apart at about 1.5 m depth and left undisturbed for 24 h before the first behavioral observation started (see below). After 4 days, the fish were again anesthetized and stripped for all available milt produced during social interactions as either dominant or subordinate. The collected milt was stored on ice for further analysis (See Sperm Analysis). Females were caught on the fourth day and stored separately from the males before they were anesthetized and stripped for all their eggs and ovarian fluid. Ovarian fluid was separated from the eggs using a pipette and stored at lake temperature (6°C). Troms County Governor's environment department gave permission to catch the fish (see Haugland et al., 2011 and Egeland et al., 2015 for more details about capture and handling methods). At the time of commencement, ethical approval was not required for this study as per the legislation in Norway.

Social Position

Although the social rank between males is highly dynamic at the spawning site over the nearly 1 month long spawning period, the status roles have never changed during our behavioral observations. That is, when status roles are established (after 1 day) they are maintained the next 3 days (see Liljedal and Folstad (2003) for more information). On day 2 we started the observation period in order to determine dominance. We observed the pairs twice a day during the last 3 days of the 4-day caging period. Observation periods lasted for 5 min. For observing the individual number of aggressive acts (e.g., an initiation of a chase) we used Bathyscope underwater viewers and the males performing most aggressive acts were considered dominants. Subordinate individuals are usually stationary at the bottom of the cage and are hardly seen conducting aggressive acts at all. Dominant males, on the other hand, roam around in the cage, and sometimes initiate interactions. The average number of aggressive acts for subordinates and dominants was, respectively, 0.1 ± 0.2 (mean ± *SD*) and 6.1 ± 6.1 (mean ± *SD*) during the 5 min long observation periods. Liljedal and Folstad (2003) found that the presence of an observer does not significantly alter fish activity or the within pair hierarchical position under such experimental conditions.

Evaluating Sperm Behavior

All sperm sampling was done by one skilled person and the measurements were done as fast as possible and randomized without the experimenter knowing the fish's social position. For each male in a pair we quantified sperm motility and velocity in water and in water diluted ovarian fluid (1:2, OF:water) from the same two females. The ovarian fluid:water ratio was chosen under the assumption that the sperm of salmonids are only able to swim around half the circumference of the egg (Billard and Cosson, 1992) and that males must therefore shed sperm in the immediate proximity of the eggs where the ovarian fluid concentration is likely to be high. We evaluated sperm in ovarian fluid solutions from two females per male pair. For measurements of sperm motility and velocity, we placed <0.12 µl of sperm on a pre-cooled chamber and initiated motility by adding 4.5 µl of either water or ovarian fluid dilution (termed "ovarian fluid" throughout). Measurement were taken 10, 20, 30, and 40 s following activation and lasted 0.5 s. Measurements of sperm behavior, including curvilinear velocity (VCL), were later analyzed using CASA (HTM-CEROS v.12) using the methods described in Vaz Serrano et al. (2006).

Data Analysis

For statistical analyses, we used R (version 3.3.1, R Development Core Team, 2016). To make the results easier to interpret we ran four different linear mixed models, based on model simplification, using four different subsets. Model fitting and estimates were obtained with the linear mixed-effects package lme4 (version 1.1–12, Bates et al., 2016). In all four models sperm velocity was entered as the response variable, and male pair and female ID were included as random factors, with female ID nested in male pair (i.e., 16 pairs of males, 2 females per pair, and 2 replicates per pair × female combination). To assess if the change in sperm velocity over time depended on activation medium we entered time and activation medium as fixed factors (**Table 1**, Model 1). In order to test the effect of status on sperm velocity we ran two separate models, one model for sperm velocity in water and another model for sperm velocity in ovarian fluid. We ran the two models with status and time as fixed factors (**Table 1**, Model 2 and 3). To assess the effect of activation medium on sperm velocity for the dominant and subordinate male we used data from 10 s and entered status and activation medium as fixed factors (**Table 1**, Model 4). The formula $ICC = \frac{\sigma^2_{u0}}{\sigma^2_{u0} + \sigma^2_e}$ (σ^2_{u0} = the variance of the random intercept, σ^2_e = the variance of the residuals) were used to calculate interclass correlation coefficients. To visualize the results we used the ggplot2 package (version 2.1.0, Hadley and Winston, 2016). We checked the model fit using visual examination of normal probability plots and residual plots, the qq plot showed no marked deviations from linearity.

RESULTS

Sperm Velocity in Water Vs. Ovarian Fluid

There was a significant main effect of activation medium. That is, sperm swim in general faster in ovarian fluid than in water (**Table 1**, **Figure 1**). Furthermore, the decrease in sperm velocity from 10 to 40 s after activation was highly significant (**Table 1**), but the velocity decrease was much larger in water than in ovarian fluid (**Table 1**).

Sperm Velocity in Water

There was a significant decline in sperm velocity over time (**Table 1**, **Figure 2**). Although the effect of male status on sperm velocity in water did not reach significance at this sample

TABLE 1 | The four models fixed factors including estimate (*B*), 95% confidence intervals (*CI*), *p*-values (*p*), random factors including the model's group count (*N*), intraclass correlation coefficient (ICC), and observations.

	Model 1			Model 2			Model 3			Model 4		
	Water vs. ovarian fluid			Water			Ovarian fluid			10 s		
Fixed factors	***B***	***CI***	***p***	***B***	***CI***	***p***	***B***	***CI***	***p***	***B***	***CI***	***p***
Intercept	130.08	124.5 to 135.7	**<0.001**	127.43	117.35 to 137.51	**<0.001**	135.4	128.95 to 141.85	**<0.001**	135.4	123.94 to 146.85	**<0.001**
Time 20	−6.74	−12.65 to −0.86	**0.025**	−24.15	−36.82 to −11.5	**<0.001**	−10.75	−18.47 to −3.03	**0.008**			
Time 30	−18.86	−24.75 to −12.96	**<0.001**	−52.63	−65.29 to −39.98	**<0.001**	−25.37	−33.09 to −17.65	**<0.001**			
Time 40	−34.78	−40.68 to −28.88	**<0.001**	−73.31	−85.97 to −60.64	**<0.001**	−42.00	−49.72 to −34.28	**<0.001**			
Status (Sub)				11.52	−1.14 to 24.18	0.084	−10.64	−18.36 to −2.92	**0.008**	−10.64	−23.21 to 1.93	0.095
Medium (Water)	3.11	−5.11 to 11.34	0.466							−7.97	−23.73 to 7.97	0.31
Time 20 × Status (Sub)				−13.76	−31.66 to 4.15	0.143	8.00	−2.92 to 18.91	0.157			
Time 30 × Status (Sub)				−12.97	−30.87 to 4.94	0.167	13.02	2.1 to 23.94	**0.022**			
Time 40 × Status (Sub)				−11.2	−29.10 to 6.71	0.233	14.34	3.52 to 25.35	**0.011**			
Medium (Water) × Time 20	−24.29	−34.5 to −14.08	**<0.001**									
Medium (Water) × Time 30	−40.25	−50.47 to −30.04	**<0.001**									
Medium (Water) × Time 40	−44.13	−54.34 to −33.91	**<0.001**									
Medium (Water) × Status (Sub)										22.16	0.39 to 43.93	**0.045**
Random factors												
N_{pair}		16			16			16			16	
$N_{FemaleID:pair}$		48						32			48	
ICC_{pair}		0.13			0.21			0.14			0.24	
$ICC_{FemaleID:pair}$		0.09						0.07			0.02	
Observations		384			128			256			96	

Significant fixed factors are indicated in bold.

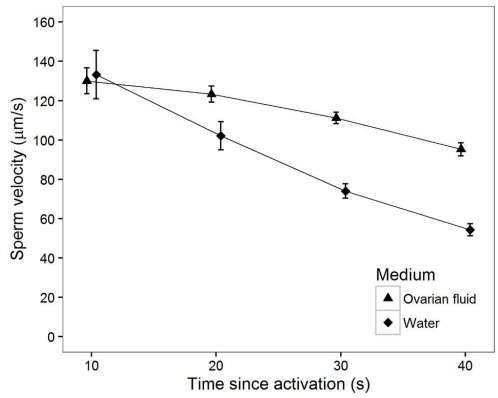

FIGURE 1 | Mean sperm velocity (VCL) in water (squares) and ovarian fluid (triangles) measured at different times (s) after activation. Vertical bars are 95% confidence intervals.

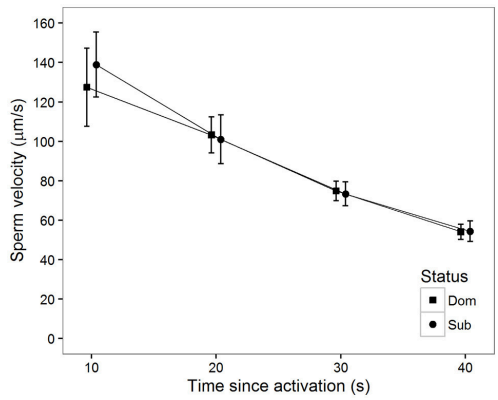

FIGURE 2 | Mean sperm velocity (VCL) in water after social status was established among subordinate ($n = 16$, circles) and dominant ($n = 16$, squares) males measured at different time (s) after activation. Vertical bars are 95% confidence intervals.

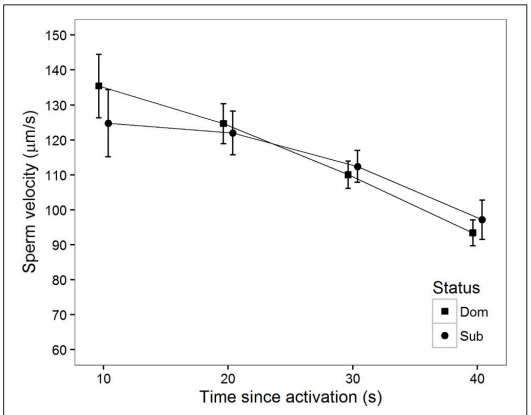

FIGURE 3 | Mean sperm velocity (VCL) in ovarian fluid after social status was established among subordinate ($n = 16$, circles) and dominant ($n = 16$, squares) males measured at different time (s) after activation. Vertical bars are 95% confidence intervals.

size (**Table 1**), the general pattern of a higher sperm velocity among subordinates in the initial period after activation was also apparent in this sample (see Haugland et al. (2008) for a meta-analysis of previous data). Additionally, there was no significant status-specific decline in sperm velocity over time (**Table 1**).

Sperm Velocity in Ovarian Fluid

There was also a significant decline in sperm velocity over time in ovarian fluid (**Table 1**, **Figure 3**). Additionally, sperm from dominant males swam faster than sperm from subordinate males at 10 s (**Figure 3**). Contrary to what was observed in water, there was a tendency for a status-specific decline in sperm velocity with a larger velocity decrease for the dominant than for the subordinate males (**Table 1**, **Figure 3**). That is, sperm from dominant males show a significantly more rapid velocity decline in the latter part of our 40 s long observation period compared to subordinates.

Sperm Velocity in the Two Media

Ten seconds after activation there was a significant interaction between activation medium and social status (**Table 1**, **Figure 4**). That is, sperm from dominant males increase their velocity in water diluted ovarian fluid compared to that observed in water, while sperm from subordinates, on the other hand, decrease velocity in ovarian fluid compared to that observed in water. There were no significant interactions between activation medium and social status at any other time after activation (20 s: $B = 0.4$, $p = 0.93$, 30 s: $B = -3.8$, $p = 0.31$ and 40 s: $B = -3.5$, $p = 0.36$).

DISCUSSION

In accordance with our previous reporting, we show that male Arctic charr occupying a subordinate social position produce sperm that initially tend to swim faster than sperm from dominant males in water, a status specific adjustment. Yet, more important for our present reporting, ovarian fluid seems to have a status specific effect on enhancement of sperm velocity favoring sperm originating from dominant males. That is, sperm from dominant males increase their velocity in ovarian fluid compared to that observed in water while sperm from subordinate decrease velocity in ovarian fluid compared to that observed in water. Additionally the dominant males show the most rapid decrease in sperm speed in ovarian fluid through our 40 s observational period.

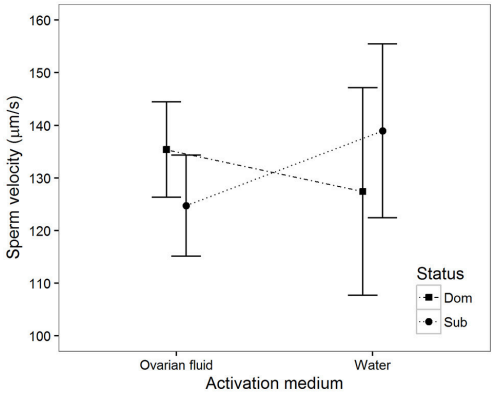

FIGURE 4 | Mean sperm velocity (VCL) 10 s after activation among subordinate ($n = 16$, circles) and dominant ($n = 16$, squares) males measured in ovarian fluid and in water. Vertical bars are 95% confidence intervals.

In accordance with theoretical models (Parker, 1970, 1998; Ball and Parker, 1996; Parker et al., 2013), sperm from males mating in disfavored roles tend to have higher velocity in water than the sperm from males mating in favored reproductive roles. This difference in sperm velocity between dominant and subordinate males is mainly manifested in the initial period after activation and in water only. Additionally, there is no status specific difference in the velocity decline through our observation period. These results are similar to that previously well-documented in Arctic charr (Rudolfsen et al., 2006; Vaz Serrano et al., 2006; Haugland et al., 2008) and also in Bluegill (*Lepomis macrochirus*; Burness et al., 2004). ATP stored in spermatozoa prior to ejaculation provides the necessary chemical energy to sustain sperm motility (Jeulin and Soufir, 1992), and in Bluegills, sperm from subordinates have about 1.5 times more ATP than sperm from dominants (Burness et al., 2004). Further, sperm ATP is positively associated with sperm velocity (Burness et al., 2004; Figenschou et al., 2013) and could be the proximate explanation for the differences in sperm velocity observed previously between males in the two mating roles when activated in water (see Haugland et al., 2008). Moreover, as sperm velocity in water has been found to predict fertilization under sperm competition (Gage et al., 2004; Liljedal, 2005; Schulte-Hostedde and Burness, 2005, see also Egeland et al., 2015), this investment could compensate for mating in a disfavored mating role when ejaculating out of synchrony and further away from the egg releasing female and the dominant male (Sørum et al., 2011; Egeland et al., 2015). That is, unlike dominant's that may spawn directly into the stream of released gonadal products of the female, subordinate's ejaculate is met by an environment more dominated by water and the adaptation to high velocity in water among subordinates thus seems reasonable. In this context it should be noted that the distance covered by self-propulsion of sperm cells represent approximately half the circumference of the egg (Billard and Cosson, 1992) while the ejected distance of gonadal products often exceed 10 cm (own observations from videos of spawning events). Thus, the ability of subordinates to eject the sperm correctly into the gonadal products released from females must be paramount.

Recent studies have shown that there can be considerable female-male interaction in offspring survival among external fertilizing species (Welch et al., 1998; Wedekind et al., 2001; Welch, 2003; Rudolfsen et al., 2005; Evans et al., 2007), suggesting that there might be larger fitness benefits from female choice than the 5–10% increase suggested from estimating variance in fitness and comparing selected and unselected populations (see Burt, 1995). Thus, the female's role in determining which sperm fertilize her eggs, either through her own preferential mate selection or through her cryptic choice, may be important. In accordance with this contention, we found that sperm velocity was influenced by ovarian fluid in charr. This is not surprising as the ovarian fluid of Arctic charr contains a variety of compounds for the sperm to metabolize (Lahnsteiner et al., 1995) and the fluid is also known to increase sperm velocity (Turner and Montgomerie, 2002) depending on individual male-females interaction (Urbach et al., 2005). Yet, the results from current intraspecific studies on the importance of ovarian fluid as a medium for cryptic female choice in external fertilizers are not unambiguous (See Introduction). However, our present documentation of a status dependent modulation of sperm activity, increasing the sperm speed of dominant males while reducing the speed of sperm from subordinates compared to that seen in water, suggest that ovarian fluid could act as a medium for cryptic female choice. That is, as dominant males have less ATP in their sperm cells than subordinates (Figenschou et al., 2013), ovarian fluid seems selectively promoting swimming of sperm from dominant males. Yet, if it were a general tendency for ovarian fluid to "prefer" sperm from dominant males, one would probably not predict a more rapid decline in sperm velocity for sperm from dominant males. Sperm from dominant males show, however, a significantly more rapid velocity decline in the latter part of our 40 s long observation period compared to sperm from subordinates. This suggests that the higher sperm velocity in ovarian fluid of dominants, compared to subordinates, is a male adaption rather than an effect of cryptic female choice. Alonzo et al. (2016) suggested something similar: "The differences between the male types in sperm characteristics and the effect of ovarian fluid on male sperm characteristics are likely the result of male adaptation to selection arising from the environment provided by the female's ovarian fluid during sperm competition." Thus, both the Alonzo et al. (2016) study and our study indicate that increased velocity of sperm in ovarian fluid observed among males mating in a favored mating role must involve a male adaption. That is, there must be something with the gonadal products from dominants that separate them from gonadal products from subordinates. This difference must be a prerequisite for any female medium that should manage to influence sperm from dominant and subordinate males differently. If there had been no difference in sperm from dominants and subordinates, ovarian fluid would have nothing to act upon. Yet, cryptic female choice might still

occur in ovarian fluid, adaptively promoting swimming speed of sperm from dominant males, but this additional rationale is not needed for explaining our results. Thus, status specific tailoring of sperm behavior is the most parsimonious explanation for our observation (Beck, 1943). On the other hand, our study is a retrospective study, and it was not designed to disentangle the importance of the two models of male and female adaptations. We can, consequently, not exclude that cryptic female choice may also be operating in ovarian fluid (see also Simmons et al. (2008) for an example within Anuran).

Recent evidence suggests that when dominant and subordinate charr compete in pairwise sperm competitions over fertilizing eggs embedded in ovarian fluid, subordinates seem to be fully able to compensate for their delayed ejaculation by increasing sperm numbers and sperm speed (Egeland et al., 2015). However, as the authors of the latter study also wrote: The ".. experiment mimicked the situation with ejaculations from one dominant and one subordinate male given an equal distance to the eggs. The proximity of the female to the male during spawning may also be of large importance for the outcome of reproductive activities and our study is, consequently, not a complete description of all factors influencing reproductive success under sperm competition in charr." If sperm from dominant males had been given the advantage of entering the ovarian fluid influenced environment immediately after ejaculation, something that under natural spawning normally would occur for dominant males (when gametes are released in synchrony and in close proximity to the released female spawning products), a different outcome might have been produced. Thus, a better mimicking of a natural spawning with an immediate mix of ovarian fluid and sperm following ejaculation might have given sperm from dominant males an immediate access to the environment to which they were better adapted and produced different results to those of Egeland et al. (2015).

So, why do dominant males reduce sperm production? We believe that the benefits observed by tailoring sperm production to a specific fertilization environment combined with a synchronized spawning and positional effects might compensate for low sperm numbers and low energy content of sperm throughout the annual spawning season. Our results suggest that future sperm competition experiments should be very sensitive to positional effects as sperm production may be adapted to different fertilization environments. In charr, sperm competition does not seem to be a "fair raffle."

ETHICS STATEMENT

The fieldwork was carried out in 2008 in accordance with the ethical guidelines stated by the Norwegian Ministry of Agriculture through the Animal Welfare Act from 1996. All fish used in this study were released back into the lake.

AUTHOR CONTRIBUTIONS

TE, GR, JN, and IF have contributed to the design of the work, sampling of the data, labwork, and statistics. TE, GR, JN, and IF have worked on the manuscript and have approved the submitted version. TE, GR, JN, and IF have all agreed to be accountable for all aspects of the work.

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The reproduction in Arctic charr is about synchrony of gamete release between the spawning male(s) and female and the need for speed in the race towards the eggs. Charr males have two reproduction tactics, guarding and sneaker tactic. Through mate guarding and vibrational communication the bigger males get an advantage in the pre-spawning competition and can as a result spawn more in synchrony with the female and closer to the released eggs. However, the need for synchronisation comes at the cost of sperm competition and the sneaker males with their high concentration of fast swimming sperm in water have relative high paternity in sperm competition. The sperm from guarding males, that spawn directly into the stream of ovarian fluid from the female, swim in an environment with relative high concentration of ovarian fluid, whereas sperm from sneaker males swim in a more water based environment. As a result, the guarding males tailor their sperm to swim fast in ovarian fluid while the sneaker males tailor their sperm to swim fast in water. The presented work illustrate the complexity of natural reproduction and can be a useful resource for industries that work with artificial fertilizations of fish.

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