Indiscriminately Loving Sticklebacks

A test of assortative mate preferences between two morphologically diverged parapatric populations of threespine stickleback (*Gasterosteus aculeatus* L.) concerning pelvic reduction

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Abstract

Two parapatric populations in western Norway in which one have completely reduced its pelvic complex are tested for assortative mating being an important mechanism in maintaining barriers keeping them from mixing in their contact zone. Three predictions are launched and tested: 1. Females from both populations prefer mating homogeneously with males from their own population; 2. Females may mate with males from both populations, but spawn larger clutches for males from their own populations; 3. Males from both populations prefer mating homogeneously with females from their own population. Weak and contrasting patterns emerge regarding female preferences, while male preferences can indicate homogeneous mate choice. In conclusion, the existence of assortative mating cannot be ruled out, but it seems not to be the most likely candidate to prevent hybridization and preserve the sharp morphological distinction between these two parapatric populations of threespine stickleback.

1. Introduction and Theory

Two morphologically different parapatric populations of threespine stickleback (*Gasterosteus aculeatus*) from the same water system in western Norway conspicuously do not mix in their contact zone. For populations to keep from mixing in the absence of geographic barriers some other mechanism must be responsible for maintaining a barrier between them, and assortative mating i.e. non-random mating is one such mechanism often associated with parapatric divergence of species (Butlin, Beaumont, & Hewitt, 1992; McLain, 1985; Scott, 2004; Ziuganov & Zotin, 1995).

1.1. Assortative Mating in Species Divergence

Early theories on species divergence involve a three-stage process of geographic isolation, divergence, and finally secondary contact causing reinforcement of divergence and reproductive isolation (Freeman & Herron, 2007). Reasoned by Dobzhansky (1937), the logic goes that hybrid offspring should be of lower fitness than their diverged parental populations, thus assortative mating keeping diverged populations from mixing should be strongly favored by selection (Freeman & Herron, 2007). Although reinforcement contributes as a likely explanation to why prezygotic speciation is more common in sympatric species than allopatric (Coyne & Orr, 1997), studies are finding reinforcement not to be a necessity for the evolution of reproductive isolation and assortative mating (McKinnon et al., 2004).The idea of species divergence strictly by the three-stage allopatric process is proving much oversimplified, as species frequently diverge in the absence of isolation by time or space (Barreto & McCartney, 2007; Fisher, 1980; Pryke, 2009). For instance, it has been found that speciation in the cyprinid genus *Barbus* has been initiated by polyploidization (Machordom & Doadrio, 2001). Another important force in evolution of biotic diversity is character displacement (Schluter, 2000). Ecological character displacement has traditionally gained the most attention when considering divergence of sympatric populations (Schluter & McPhail, 1992; Vamosi, 2002), yet effects of interactions across trophic levels are increasingly being investigated (Buckling & Rainey, 2002**;** Hudson & Greenman, 1998; Marchinko, 2009; Vamosi & Schluter, 2002). Predators may affect selection both as a second-hand effect of ecological character displacement through differences in habitat-specific predation regimes (Marchinko, 2009; Vamosi, 2002), and more directly by mediating interactions and divergence between prey species affected by shared predators (Vamosi & Schluter, 2004). Even when lowering competition amongst prey populations, predation might still potentially strengthen selection for character divergence (Rundle, Vamosi, & Schluter, 2003). In general, sources of mortality should have the potential to significantly impact on species divergence, and one such other force is represented by parasites (Rundle et al., 2003). Likewise predators, parasites contribution to divergence can come as a second-hand effect from ecological character displacement and habitat-specific differences in parasite exposure (MacColl, 2009), or more directly through shared pathogens of interacting host populations imposing apparent competition (Hudson & Greenman, 1998). Mortality-inflicting sources such as parasites and predators are increasingly being recognized as important agents of selection in driving divergent selection among parapatric and sympatric populations (MacColl, 2009; Rundle et al., 2003; Vamosi, 2002). Reproductive isolation is required for divergence to complete, the classical view on which achieving involved a radical rearrangement of the genome rendering hybrids highly dysfunctional (Freeman & Herron, 2007). For sympatric divergence in sexual populations assortative mating is considered a prerequisite in establishing reproductive isolation (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998), and while earlier believed only to evolve in situations of severe hybrid fitness deficiency, emerging evidence is suggesting that not only can assortative mating evolve despite hybrid viability, but also in the face of frequent gene flow and without any great extent of genomic divergence between diverging populations (Barreto & McCartney, 2007; Pryke, 2009). Theoretically, assortative mating can evolve and initate speciation both in the absence of geographic barriers and in the presence of high gene flow (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998). Modeling assortative mating as a single force in driving sympatric divergence, Kondrashov and Shpak (1998) find it possible only when there is very strict assortative mating. However, their models assume all genotypes to be on average equally fit, causing no selective mating or sexual selection, thus increasing frequency of matings between similar individuals should facilitate divergence (Kondrashov & Shpak, 1998). A frequency-dependent fitness-landscape might favor individuals of dissimilar morphology, yet if mating is random divergence is prevented by a continual production of intermediates (Dieckman & Doebeli, 1999). Split-trait assortative mating based on similarity in an ecologically neutral marker trait will only evolve under very strong disruptive selection, requiring linkage disequilibrium between loci controlling the different traits (Kondrashov & Shpak, 1998). Sexual selection might provide such a strong selective force for divergence, thus by itself able to initiate divergence in the absence of disruptive natural selection (Turner & Burrows, 1995). In Dieckmann and Doebeli's (1999) models, using populations of finite size, linkage disequilibrium is achieved through genetic drift, yet the likelihood of this occurring lessens with increasing size of populations. However, when genes controlling both traits are located on sex chromosomes, recombination events are reduced, presenting a possible explanation to establishment and persistence of assortative mate-preferences in the face of frequent gene flow among diverging populations (Pryke, 2009). For instance, in interbreeding morphs of Gouldian finches head-colors and preferences for these are important in mediating assortative mating, the genes for which are both linked and located on the sex chromosomes (Pryke, 2009). These findings are consistent with similar studies in suggesting a coupling of traits' genes on sex chromosomes to be of particular importance in evolution of assortative mating (Pryke, 2009). On the contrary, when depending on an ecological similarity-preference, establishment of assortative mating is not hindered by recombination events (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998). Assortative mating based on similarity in an ecological character under divergence may lead to complete divergence and reproductive isolation between derived populations (Dieckmann & Doebeli, 1999), even at relatively weak disruptive selection on the trait diverging (Kondrashov & Shpak, 1998). Compared to when driven by strong selective forces, such as sexual selection, divergence and elimination of intermediate morphotypes by ecological similarity preference under relatively weak disruptive selection typically proceed at a much slower rate, the degree of assortativeness steadily increasing with time (Kondrashov & Shpak, 1998). Interval assortative mating represent yet another form of assortative mating, and can arise as a by-product of genetic variability causing differences in timing and location of mating (Kondrashov & Shpak, 1998). Although not a prerequisite, clearly the existence of strong selection against hybrids would further assist the emergence and persistence of assortative mating through imposing stronger disruptive selection (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998; Turner & Burrows, 1995). However, when populations diverged in allopatry experience secondary contact and hybridization, hybrid elimination is not necessarily the outcome as this might lead to establishment of hybrid zones of varying stability depending on relative fitness to parental generations and fitness concerning the spatial landscape; if hybrids are better suited to a novel environment an entire new isolated population might result (Freeman & Herron, 2007).

The above outlined theories on divergence by assortative mating are based on models and examples considering divergence in sympatry. This is regarded a most conservative scenario, and there is no reason these mechanisms should not apply to parapatric populations, as further restricting gene flow should only facilitate divergence (Turner & Burrows, 1995).

1.2. Threespine Stickleback (*Gasterosteus aculeatus*): Morphological Divergence and Mate Choice

The threespine stickleback (*Gasterosteus aculeatus*) species complex have a holarctic distribution, are easily accessible and reared in laboratory environments, and has throughout the last century become established as a widely used model species within fields such as evolutionary biology, behavioral ecology, parasitology and genetics, and recently the whole genome have been sequenced (Bell & Foster, 1994; Kingsley & Peichel, 2007). Ancestral threespine stickleback are marine, yet the species are found throughout a diverse range of habitats including both marine and freshwater, they possess great morphologic diversity both within and between water systems, and are well renowned for their rapid evolutionary rates (Bell & Foster, 1994). Invasions of freshwater lakes are believed to be mainly independent events, meaning that freshwater populations are mostly independently derived from the ancestral marine form, creating a phylogenetical raceme and giving rise to what is known as the Gasterosteus complex (Bell & Foster, 1994). Threespine stickleback possesses great diversity in expression of morphological anti-predator structures, a trait of the species complex that has gained much attention from evolutionary biologists (Albert et al., 2007; Bell, 1987; Colosimo et al., 2004; Giles, 1983; Hoogland, Morris, & Tinbergen, 1956; Reimchen, 1980; Shapiro et al., 2004).

Marine threespine stickleback are largely monomorphic for extensive external anti-predator structures, consisting of a complete row of approximately 35 bony lateral plates from head to caudal fin on each side, three dorsal spines, two pelvic spines and a calcified pelvic girdle (Bell & Foster, 1994). Both dorsal and pelvic spines can be locked in erect positions and function in post-capture escape by increasing effective size, penetrating mouth tissue and overall complicating the handling of sticklebacks for gape-limited piscivores (Hoogland, 1951; Hoogland et al., 1956; Reimchen, 1980, 1983, 1991). Lateral bony armor plates support and strengthens dorsal spines, and increases chances of surviving attacks by protecting internal organs from damage during capture (Reimchen, 1983, 1992). Further, posterior lateral bone plates directly affect chances of post-capture escape by increasing failure rates of fish predators in handling prey sticklebacks (Reimchen, 2000). The pelvic girdle has important functions in protecting internal organs, supporting pelvic spines, and overlapping with lateral bone plates supporting dorsal spines, thus importantly contributing to the overall strengthening of the spinal defense structure (Reimchen, 1983). Invasion of freshwater is normally associated with a decrease in morphological defensive structures, including smaller pelvic girdle, reduction in lateral plates number and size, and shorter and less supported, hence weaker, dorsal and pelvic spines (Bell, Aguirre, & Buck, 2004; Giles, 1983; Klepaker, 1993; Reimchen, 1983). Reduction of plates and spines in a population can be rapid (Klepaker, 1993), yet reduction of pelvic girdle have not been shown to occur at the same extreme speed (Bell, Travis, & Blouw, 2006).

Populations totally lacking the pelvic girdle and associated pelvic structures represent a form of extreme armor-trait reduction in threespine stickleback (Bell, Orti, Walker, & Koenings, 1993; Reimchen, 1980). Pelvic reduction is a rare phenomenon yet reported from geographically distant areas such as the pacific coast of North America, Iceland, Scotland and Norway (Klepaker, Østbye, & Bell, 2013). In its plesiomorphic state the pelvic complex has a bilateral structure, on each side consisting of one spine, one fin ray and the pelvic girdle, joined ventrally by a median suture (Bell & Harris, 1985). The pelvic girdle is made up of three developmentally separated parts: the ascending branch, the anterior process and the posterior process (Bell & Harris, 1985). Pelvic reduction is expected to have evolved independently several times, yet the process of reduction and sequence in which elements are lost is strikingly homogeneous across populations (Bell, 1987).

There is currently no consensus on causative selective mechanisms of pelvic reduction, although several are suggested. One possible important agent of selection could be predation and differing predation regimes (Reimchen, 1980). Gape-limited vertebrate piscivores are expected to select for maintenance of the plesiomorphic pelvic structure (Reimchen, 1980; Vamosi, 2002; Vamosi & Schluter, 2004). Predatory fish feeding on threespine stickleback in freshwater also prey on and control aquatic insect communities (Reimchen, 1980). In the absence of these fish predators, aquatic insects will proliferate and may very well become the dominating stickleback predators (Reimchen, 1980). Reimchen (1980) hypothesized that external armor structures such as the pelvic complex provides solid grappling points for invertebrate piscivores, and that selection should favor reduction of such traits when predation is mainly due to invertebrates. In the absence of fish predators, aquatic insect communities include larger and more active species (Johnson, 1991; Morin, 1984). Results from Vamosi (2002) and Vamosi and Schluter (2004) support differences in selection pressure from these two predation regimes, and similarly a study on pelvic reduced ninespine sticklebacks (*Pungitius pungitius*) (Ziuganov & Zotin, 1995). However, Foster, Garcia, & Town (1988) find most invertebrate piscivores in a lake free of other piscivorous fish except cannibalistic stickleback mainly to predate on juvenile threespine stickleback (< 25mm SL). Studying threespine sticklebacks polymorphic for pelvic spine length Zeller, Lucek, Haesler, Seehausen, & Sivasundar (2012) find no selective advantage for shorter spines facing predation by dragonfly larvae (*Aeshna* sp.), and likewise with ninespine sticklebacks Mobley, Ruiz, Johansson, Englund, & Bokma (2013) find no selective advantage towards predation by dragonfly larvae found when artificially removing spines. Vamosi (2002) use both backswimmers (*Notonecta* sp.) and dragonfly larvae testing for an effect in armor reduction towards invertebrate predation, and find only predation by backswimmers to be affected by armor differences. Marchinko (2009) also use both backswimmers and dragonfly larvae, and do find evidence for higher survival of individuals with reduced pelvic girdles in invertebrate predator regimes, yet those individuals totally missing the pelvic girdle suffered from predation compared to the rest. Marchinko (2009) argues that these results might be circumstantial due to negative fitness epistasis resulting from crossing two divergent populations of different genetic background, and further highlights the possibility of body size and growth being the target of selection and not the armor structures themselves.

Giles (1983) suggests the reduction of the pelvic complex to be strictly a result of low calcium levels in freshwater lakes, a hypothesis in which predator regimes are of less importance concerning reduction of the pelvic complex. Bell *et al.* (1993) dismiss Giles (1983) hypothesis of calcium concentrations alone controlling pelvic reduction as they find both the predation hypothesis and the calcium limitation hypothesis to partially explain pelvic reduction. As an alternative, Bell *et al.* (1993) propose a threshold relationship between calcium concentrations and pelvic reduction, implying a threshold value of calcium concentration below which the gains in protection from the pelvic complex, related to predation regime, does not outweigh costs imposed by low calcium concentrations. As such, selection would favor reduction of pelvic girdle and enable increased allocation of resources to growth (Bell et al., 1993; Giles, 1983). In lakes with sympatric threespine stickleback species pairs of armor-reduced benthic and armored limnetics, comparisons of limnetics with allopatric populations showed very similar expression of armor traits, thus not giving support to Giles' (1983) hypothesis (Vamosi & Schluter, 2004). Further, the occurrence of such armor-reduced/ armored species pair in Paxton Lake, calcium rich and containing piscivorous fish, conflict with both the calcium limitation hypothesis and the predation hypothesis (Bell et al. 1993).

Another example of sticklebacks conflicting with conventional theory comes from Lake Storvatnet in Norway, which has pelvic reduced threespine sticklebacks, a population of brown trout, and few large aquatic insects (Klepaker, Østbye, Bernatchez, & Vøllestad, 2012). While clearly not supporting Reimchens' (1980) predation hypothesis, it is not likely to strictly result from limiting calcium concentrations either, as this would imply pelvic-reduction to be much more common in Norwegian lakes than it is (Klepaker et al., 2012). Reduced and imposed development of the pelvic complex has been mapped to changes in expression of the Pitx1-gene (Shapiro et al., 2004), and an alternative explanation is discussed in Klepaker *et al.* (2012) suggesting that the necessary regulatory mutations needed for pelvic reduction might be very rare. However, this fails at explaining the lack of pelvic-reduced morphs in lakes downstream of Lake Storvatnet, which is most conspicuous if reductions are mainly limited only by rates of mutation (Klepaker et al., 2012).

Marchinko and Schluter (2007) found reduction of armor i.e. plate-reduction to be beneficial concerning growth rates. Low-plated morphs have higher growth rates than high-plated morphs when raised in freshwater, while little difference when raised in salt water (Marchinko & Schluter, 2007). Selection for armor reduction appears to be a correlated response to selection for faster growth rates (Marchinko & Schluter, 2007). Klepaker *et al.* (2012) also found differences in growth rates with respect to pelvic-reduction, showing that pelvic-reduced individuals had highest growth rates. Furthermore, an ontogenetic shift in morph frequencies was registered, and it appears that while armor structures could provide higher energetic costs while giving little in return for juveniles, at one point during a sticklebacks development the benefit of carrying armor balances the costs, possibly explaining a raising frequency of high-CPS (combined pelvic score) morphs after an age-class' first winter (Klepaker et al., 2012).

As outlined above, assortative mating might also be of importance in explaining initiation and maintenance of diverged morphotypes (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998; Turner & Burrows, 1995) e.g. size-based assortative mating in stream-anadromous threespine stickleback populations (McKinnon et al., 2004). Mate-choice in threespine stickleback is assumed to be a female choice (Foster, 1994; Luttbeg, Towner, Wandersforde-Smith, Mangel, & Foster, 2001; Wootton, 1976). As breeding season emerge, males develop their nuptial coloration consisting of red throats and fore-bellies, blue eyes, and an overall iridescent blue-to-black dorsolateral body surface (McLennan & McPhail, 1989a), and start establishing territories and nests in the shallow waters of the littoral zone (Foster, 1994). Territories are vigorously defended against all sorts of intruders, and during the nest-building phase males act aggressively towards all females as well (Wootton, 1976). The sexual phase starts when the male for the first time tunnels through his nest, from which point on the male is ready to lead females back to the nest for spawning (Sevenster, 1961). Females leave feeding aggregations shortly after ovulation and individually starts assessing potential mates; with the opportunity to assess multiple males before choosing (Foster, 1994). Threespine stickleback courtship behavior has been extensively described in Wootton (1976), and divided into distinct phases (Goldschmidt & Bakker, 1990). Following here is a short version of stickleback courting behavior and how it progresses. For more extensive reviews on threespine stickleback courting behavior, see Wootton (1976) and Goldschmidt and Bakker (1990). As a female approaches a male or as a male approaches a female entering his territory, courtship is initiated by the male either by a characteristic zigzag display or by a more direct approach. This may result in the female taking on a head-up posture, followed by the male leading the female towards his nest. It also happens that entering females are stopped by the male, as he prickles her abdomen with his dorsal spines. Such dorsal pricking might result in meandering, in which case the pair might start swimming around in slow circles, unless she takes off thus ending courtship. Following this, the male usually takes off back to its nest to perform activities assumed to increase his sexual motivation. The next step of courtship followed by any of these approaching scenarios and their outcomes is to lead the female towards the nest and present to her the nest entrance. In a process of which the female is still assumed to be assessing whether or not to mate with the respective male, she puts her snout into the nest entrance. The final step of courting involves the female entering the nest, and while inside, the male repeatedly taps the female on her tail, stimulating her to spawn. The male then goes through the nest to fertilize the eggs, and afterwards chase the female off his territory. Tinbergen (1951) described such sequential courtship as a reaction chain, in which each behavioral act stimulates a response in the cofactor of the courtship. The process is described here as straightforward in progress; however, most often deviations occur in which steps might be omitted or repeated (Wootton, 1976).

The extensive courting in threespine sticklebacks might serve several purposes. Mainly it is assumed to have orientating and distinguishing effects, ensuring that females spawn at the right spot and mate with the correct species; thus enhancing reproductive isolation between closely related species (Wootton, 1976). Throughout the breeding season males are forced to repeatedly switch between aggressive and sexual behavior, and the extensive courting has in relation to this been suggested to have a homeostatic function in maintaining males in the correct motivational state (Wootton, 1976). Van den Assem (1967) find a direct, positive relationship between the size of males territories and their success in getting females into their nests, thus aggressiveness seems to favor mating success (Wootton, 1976). However, highly aggressive males might not be able to immediately adjust their behavior towards emerging gravid females, and behavior such as that seen in relation to dorsal pricking could have evolved in order for males to readjust into a more sexual state (Wootton, 1976). The nest directed activities described in relation to dorsal pricking occurs at regular intervals even when there are no females present, and there are reasons to believe these activities to have effects on balancing behavioral motivations with respect to aggressive and sexual behavior (Wootton, 1976). Being able to control and maintain behavioral homeostasis in which neither aggression or sexual motivation is too strong might be an important function in fish courtship behavior, as a too strong drive in either might potentially reduce the success of courtship (Wiltz, 1972). Evidence points towards a mutually inhibitory relationship between courtship and aggressive behavior at least in the short term, although if considered over a longer time span, aggressive behavior might actually prove to be neutral or even stimulating with respect to sexual behavior (Wootton, 1976).

Fertilization is normally carried out immediately after female spawning, followed by a refractory period which includes chasing the female away (Wootton, 1976). However, it does occur that the male chases the female away before fertilizing the eggs, leaving the eggs unprotected for some amount of time until he returns to fertilize them (Wootton, 1976). As pointed out by van den Assem (1967), although males holding the largest territories are the most successful in getting females to enter their nest, the function of this relation might not be entirely the same if considering who is fathering the offspring. Males holding small territories might compensate for lower rates of female visits by sneaking into other males nest and cuckold them (van den Assem, 1967), a tactic with good potential for males unable to compete for mates early in the spawning period (Jamieson & Colgan, 1992). In close relation to sneak fertilizations, males are also known to steal other male sticklebacks' eggs (van den Assem, 1967; Jamieson & Colgan, 1992). At a point during courtship the female have her nose stuck into the males nest, assumingly still deciding whether or not to mate (Wootton, 1976). It has been suggested that females might prefer to mate with males having nests containing eggs, as it could provide safety for own offspring being part of a big egg-aggregation or serve as a sign of high male quality (Ridley & Rechten, 1981; Rohwer, 1978). Results from Ridley and Rechten (1981) indeed show females to prefer mating with males having eggs in their nests compared to those without. Female preferences for eggs in male nest might have evolutionary implications in explaining egg-theft among threespine stickleback males (Ridley & Rechten, 1981).

Different sexual cues and signals used by threespine stickleback during mate-assessment and mate-choice processes are likely functioning as multiple cues having an additive informative effect rather than being merely back-up cues (Candolin, 2003; Heuschele, Mannerla, Gienapp, & Candolin, 2009). Female sticklebacks have been found exhibit preferences towards larger males (Rowland, 1989a, 1989b), and also there is vast evidence pointing at a preference for males' intensity of expressed red nuptial coloration (Braithwaite & Barber, 2000; McLennan & McPhail, 1990a). Intensity of male red nuptial coloration reflect male dominance status (Bakker & Sevenster, 1983) and a positive correlation between males' condition and expression of red nuptial coloration suggest that females are able to avoid males of poor condition e.g. parasitized males through actively choosing the most intensively red colored males (Milinski & Bakker, 1990). Males are required to defend eggs from cannibalistic raiding conspecifics and survive throughout the period it take to hatch and nurse fry while performing their parental duties, thus female preference for good condition males might likely be a preference for good fathers; if in addition there is additive genetic variance for parasite resistance she will simultaneously be choosing genes favoring parasite resistance (Milinski & Bakker, 1990). There exist a positive genetic correlation between males' intensity of red and female preferences for red, thus females select genes positively influencing offspring attractiveness (Bakker, 1993). Braithwaite and Barber (2000) points out that while there is much evidence favoring the importance of male red nuptial coloration in female choice, many studies have also demonstrated exceptions and lack of this preference. Testing females' ability to sequentially compare between males when allowed only visual contact, Bakker and Milinski (1991) show females to rate males according to brightness of previously encountered ones, the previous male effect, yet of lower choosiness at increased cost of sampling. Using a design allowing both visual and olfactory contact and in which females actively can decide on approaching males, costs imposed by time and energy both lowers female choosiness (Milinski & Bakker, 1992). Costs imposed by time and energy restriction affects different parts of the courtship sequence, thus potentially affecting the relative importance of different male sexual ornaments and behaviors (Luttbeg et al., 2001). Braithwaite and Barber (2000) suggest a threshold-value in differences between males with respect to red nuptial coloration should be exceeded in order for females to actively discriminate between males on behalf of red coloration. However, female sticklebacks do not only rely on visual cues, and are furthermore known to discriminate between males based on odor-cues as well; one of its main function believed to be a process of counting alleles in the major histocompatibility complex (MHC), thus optimizing offspring immunocompetence (Reusch, Häberli, Aeschlimann, & Milinski, 2001). Peptides likely make up part of the MHC-related odor signals used by females in mate-assessment, suggesting a molecular link between MHC-polymorphism and individuals body odor (Milinski et al., 2005). However, MHC-signals insufficiently explain female odor-preferences and the presence of a second, costly odor cue, honestly signaling about males' condition, is suggested (Sommerfeld, Boehm, & Milinski, 2008).

Costly male cues are important to females in honestly signaling male condition (Milinski & Bakker, 1990; Sommerfeld et al., 2008). Male territory size is important to males in attracting females (van den Assem, 1967), leading to male-male interactions in establishment of territories and maintenance of these, which further ensures the honesty of the condition-indicative signals displayed by males (Candolin, 1999, 2000). Red nuptial coloration intensity has a social cost for males competing with superior males by increasing their risk of getting attacked, thus color intensity honestly signal male parental ability (Candolin, 2000). Although females are not selecting for competitive ability in itself (Östlund-Nilsson & Nilsson, 2000), competition between males is important in ensuring the accuracy and reducing cost of females assessment of male quality (Candolin 1999, 2000). On the contrary to intensity of red, courtship intensity by itself is found to be a poor indicator of males' condition (Milinski & Bakker, 1990). Courtship is of low honest signal value in the absence of male competition, yet when facing competition, courtship intensity might add to the dominance signaling function of red color (Candolin, 1999).

The relative importance of cues differs with environmental conditions, and given circumstances with increased turbidity females pay more attention to olfactory cues than visual, possibly relying most heavily on the most easily assessed cue (Heuschele et al., 2009). Increasing pH also result in heavier reliance on olfactory cues and is likely adaptive as increases in pH in nature often coincide with eutrophication events; also it could be coupled to direct effects of increased pH on the chemical nature of the cues themselves (Heuschele & Candolin, 2007). Neglecting availability of visual cues effect female mate-choice, supporting that information given by visual and olfactory cues has a multiple informative effect rather than being back-up cues (Heuschele et al., 2009). Sympatric benthic/ limnetic threespine stickleback relies on visual and olfactory cues to different extents (Rafferty & Boughman, 2006). Benthic females significantly favor conspecific male odor, while limnetic females show no such preference and instead show a preference in male color not found in benthic females; possibly resulting from ecological differences (Rafferty & Boughman, 2006).

Differences in body shapes might also affect mate-choice (Vines & Schluter, 2006). The *Ectodysplasin* (*Eda*) gene region has direct effects on expression of lateral bone plates in threespine sticklebacks (Colosimo et. al., 2004, 2005), in addition to being located nearby other QTL associated with body shape (Albert et. al., 2007) and pelvic spine length (Shapiro et. al., 2004). Marchinko (2009) find predation by aquatic insects to select for the low-morph version of *Ectodysplasin* (Eda^L). Exact pleiotropic effects of *Eda* on body shape are not known, but given that there are effects, and the predation is in fact the underlying selective agent causing changes in frequency of the Eda^L allele, predator-driven selection could lead to assortative mating and potentially play an important role in establishing reproductive isolation (Marchinko, 2009). Size-, color- and odor-based assortative mating is found in sympatric benthic/ limnetic threespine stickleback (Nagel & Schluter, 1998; Rafferty & Boughman, 2006). Morphs of either extreme in these sympatric species pairs likely experience a fitness advantage compared to individuals of intermediate phenotypes, giving a reproductive advantage for phenotypically extreme individuals thus facilitating assortative mating and reproductive isolation (Schluter & McPhail, 1992). Although sympatric benthic/ limnetic threespine stickleback likely have resulted from two separate invasions and as such do not represent sympatric divergence (McPhail, 1992a), assortative mating still appear of importance in keeping them from subsequently mixing (Schluter & McPhail, 1992). Size-and color-based assortative mating has been demonstrated among stream-anadromous threespine stickleback (McKinnon et al., 2004, 2012; Scott, 2004), assortatively mating sympatric threespine stickleback from Iceland are suspected at least to some degree to discriminate with respect to nests (Ólafsdóttir, Ritchie, & Snorrason, 2006), and parapatric ninespine stickleback from Russia mating assortatively possibly based on differences in expression of pelvic complex (Ziuganov & Zotin, 1995). Studies from Norway find assortative mating indicative of sympatric divergence in a population showing pelvic reduction (Klepaker et al., 2012). This population has a stable bimodal distribution with respect to pelvic-developed morphs that might have resulted from opposing selection pressures creating two adaptive peaks; a fitness landscape of which could facilitate emergence of assortative mating and subsequent reproductive isolation (Klepaker et al., 2012). Genetic analyses reveal associations between allelic composition and variations in pelvic spines, and some degree of reproductive isolation is indicated by phenotypically similar individuals appearing parts of similar genetic clusters compared to more dissimilar individuals (Klepaker et al., 2012).

1.3. Study Case, Hypothesis and Predictions

Two parapatric populations of threespine stickleback found in western Norway show great differences in pelvic reduction. The population in Lake Nesavann express completely reduced pelvic complex (percentage normal/vestigial/lost – 0.0/15.4/84.6) while Lake Vigdar population express fully developed pelvic complex (percentage normal/vestigial/lost – 92.3/5.9/1.8) (Klepaker et al., 2013, see for overview of pelvic scores and phenotypes, including Lake Liavann). In Lake Liavann, interspersing between these two other lakes, threespine sticklebacks are found polymorphic in expression of pelvic complex. The two populations from Lake Nesavann and Lake Vigdarvann are parapatrically connected via Lake Liavann by a one-way allowed migration from Lake Nesavann into Lake Vigdarvann, yet hybrids and pelvic reduced individuals rarely occur in Lake Vigdarvann. Instead an abrupt change in morphotypes is found when going downwards the water-channel leading from Lake Liavann into Lake Vigdarvann, from polymorphic to nearly monomorphic fully developed pelvic complex. This is most conspicuous, and some mechanism must be responsible in keeping these two populations from mixing. Assortative mating being of importance in other pelvic-diverged stickleback populations (Klepaker et al., 2012; Nagel & Schluter, 1998; Ziuganov & Zotin, 1995), it is expected to be of similar importance in keeping the two populations from Lake Nesavann and Lake Vigdarvann from mixing.

This study attempts to show the existence of assortative mating between these two populations by launching and testing three predictions:

1. Females from both populations assortatively prefer to mate homogeneously with males from their own population
2. Females may mate with males from both populations, but spawn larger clutches for males from their own population

3. Males from both populations assortatively prefer to mate homogeneously with females from their own population

These three predictions are tested through a mate choice experiment in order to test the hypothesis of assortative mating being an important mechanism in keeping these two populations from mixing.

2. Material and Methods

2.1. Sampling of Threespine Stickleback: Geographic Location and Sampling Methods

The two threespine stickleback populations of interest come from two lakes belonging to the same watershed. These lakes constitute parts of a greater water system named Vigdar – water system, consisting of more than 30 connected lakes and ponds and known to be residence for at least 22 morphologically different populations of threespine stickleback (Tom O. Klepaker, 2012, personal communication). Lake Nesavann (59°33’19.01”N, 5°25’57.13”Ø) is the uppermost elevated of the two (16 MAMSL), and drains into Lake Vigdarvann (59°30'37.10"N, 5°25'59.09"Ø) via Lake Liavann. See figure 1 for an overview of lakes sampled from. A threshold in the creek connecting Lake Liavann and Lake Nesavann facilitates only downstream migration, while the water channel between Lake Liavann and Lake Vigdarvann enables both upstream and downstream migration of fish. Table 1 show an overview of characteristics of these three lakes.

Table 1: Lake characteristics

	Lake Vigdarvann	**Lake Liavann**	**Lake Nesavann**
Elevation	10 MAMSL	10 MAMSL	16 MAMSL
Surface area	7.36 km^2	0.23 km^2	0.66 km^2
Avg. pH value	6.6	6.7	6.4
Calcium mg/l	2.39	4.54	2.88
Fish fauna	Brown trout (*Salmo trutta*), Arctic char (*Salvelinus alpinus*), Eel (*Anguilla anguilla*), Threespine stickleback (*Gasterosteus aculeatus*), and in Lake Vigdarvann there is in addition Atlantic salmon (*Salmo salar*).		
Bird fauna	Gulls (f. Laridae), Cormorants (f. Phalacrocoracidae), Ducks, Geese and Swans (f. Anatidae), Grey Heron (*Ardea cinerea*).		

All three lakes have populations of brown trout (*Salmo trutta*) and arctic charr (*Salvelinus alpinus*), although densities of both are likely to be lower in Lake Nesavann (Tom O. Klepaker, 2013, personal communication). Stomach content analyses indicate that the trout population in Lake Nesavann include threespine stickleback in its diet to a greater extent than trout from the other two lakes; data on char of no indicative value as only 2 char were caught from Lake Nesavann. A wetlands bird reserve in close proximity to all three lakes adds to the diversity of avian piscivores in the area.

Both populations were sampled in mid-April, using stickleback traps made of plexiglas. Live specimens from both populations were short after sampling brought in separate tanks to the research facilities on Austevoll. No mortality was observed during transportation. Figure 1 gives an overview of sample sites in both Lake Vigdarvann and Lake Nesavann.

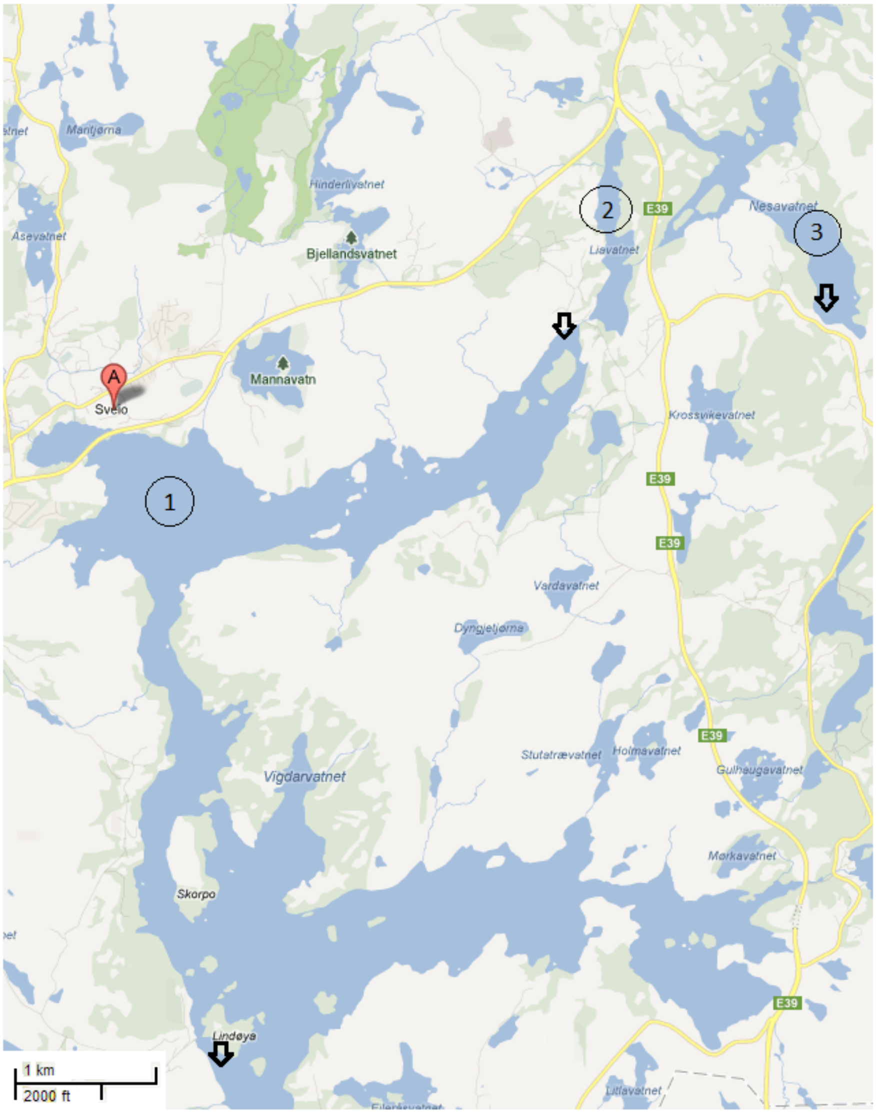

Figure 1: 1=Lake Vigdarvann, 2=Lake Liavann, 3=Lake Nesavann. Sampling sites indicated by arrows. Taken from google maps.

Female threespine sticklebacks used as stimulation for nest-building males were sampled from the local lake next to the facilities where the experiment was carried out, Lake Kvernavann on Storebø, Austevoll. Sampling of these occurred continuously throughout the experimental period.

2.2. Fish Holding

All caught threespine sticklebacks were held at Lerøy Vest's old smolt facilities (formerly known as Kvernsmolt), on Storebø, Austevoll; the two populations kept separately in two tanks of 3.5m diameter and 1.20m water height; a total of 300 specimens from Lake Vigdarvann and 700 specimens from Lake Nesavann. Halfway throughout the period of the experiments, fish from both populations were transferred to separate salmon runs (3.6m x 0.42m), in order to reduce stress imposed during catching and selection of fish for mate choice trials. All healthy individuals from Lake Nesa population were transferred (30 males and 60 females), and an equal amount from Lake Vigdar population. A continuous flow-through ensured adequate level of oxygen. All holding tanks used were thoroughly cleaned with S1-Extra, and disinfected with Vircon S before use.

Feeding done *ad libitum;* at first twice a day with frozen chironomid larvae, yet from the midst of June once a day live zooplanktons from the local lake. Zooplankton was caught using a plankton-net of 180µm towed behind a boat for an hour each day, and mainly consisted of daphnia, copepods and amphipods**.** Alongside daily feeding routines, tanks were checked on a regular basis and cleaned for dead fish.

Both populations held were externally checked for parasites, and prior to entering the experiment all fish selected were checked once more. The pseudophyllidean cestode *Schistocephalus solidus,* infecting through a cyclopoid copepod intermediate host (Clarke, 1954), and the microsporidian *Glugea anomala*, infecting directly through free spores or through infected aquatic invertebrates (Weissenberg, 1968), are both common parasites of threespine sticklebacks (Barber & Scharsack, 2010; Ward, Duff, Krause, & Barber, 2005). Parasites may affect the outcome of threespine stickleback mate-choice (Milinski & Bakker, 1990). In particular so infections by *S. solidus,* as it may functionally castrate its threespine stickleback host (Barber & Scharsack, 2010). Infections by *S. solidus* are recognized by an unsymmetrical ventral distention of the abdomen (Barber & Scharsack, 2010), often more anteriorly positioned compared to the symmetrical bulging ovaries characterizing gravid females. Proglottids and movements of the cestode can sometimes be seen as imprints on the skin of infected individuals, leaving no doubt of infection status. The microsporidian *G. anomala* is easily spotted as white cysts on the skin and inside of opercula of infected individuals (Ward et al., 2005). Specimens sampled from Lake Vigdarvann showed low rates of parasite infection (3.9% scored as infected/ unsure); infections found only due to *S. solidus*, no infection by *G. anomala* was detected. Specimens sampled from Lake Nesavann had high rates of both *G. anomala* and *S. solidus* (73.1% scored as infected/ unsure). These scores do not directly represent parasite intensity of the two lakes, as 90 healthy individuals from each population were taken out prior to this parasite count for other purposes. It does however represent a difference between the two populations in intensity of parasite infections. Before experimental start up, this left the following amounts of healthy usable fish from the two populations: Lake Vigdarvann – 102 males/ 95 females; Lake Nesavann – 74 males/ 87 females. However, the extent of parasite infections among stored fish is expected to have been underestimated as parasites might not yet have been fully developed; further supported by later observations.

Water supply to storage tanks come in from the local lake, Lake Kvernavann. Lake Kvernavann houses its own population of threespine stickleback, thus risk of introducing local parasites must be considered. Copepods from the local lake constitute an important part of the food given, yet former investigations of Lake Kvernavann have found very low rates of *S. solidus* infected fish since 1981, and pr. 2012 the lake is considered free of *S. solidus* (Jakobsen, Johnsen, & Larsson, 1988; Per. J. Jakobsen, 2012, personal communication). Threespine sticklebacks sampled from Lake Kvernavann throughout the study period showed no sign of *S. solidus* infections. On the contrary, *G. anomala* infections are common on the local threespine stickleback population. Although occurring throughout the whole water column, free infective spores of *G. anomala* are mainly found in sediments (Per J. Jakobsen, 2012, personal communication). Given that water supply is taken from 17m depth in hypolimnion and not in contact with sediments, risk of introducing *G. anomala* is considered low.

2.3. Experimental Setup and Execution

Salmon runs are reorganized into two test compartments (1.6m each), each compartment enabling one parallel run at a time. Each mate choice parallel run includes one male from each of Lake Vigdarvann and Lake Nesavann populations, and one randomized female from either of these two populations. Males are placed separately in enclosures in either end of a test compartment, and when both males have readied nests, one female is introduced into the middle of the test compartment in a transparent plastic box, at which point male enclosures are opened and recording starts; all parallels are recorded the first 3 hours from insertion of female box. Figure 2 gives an overview of a reorganized salmon run, showing two test compartments, male enclosures and insertion of experimental female.

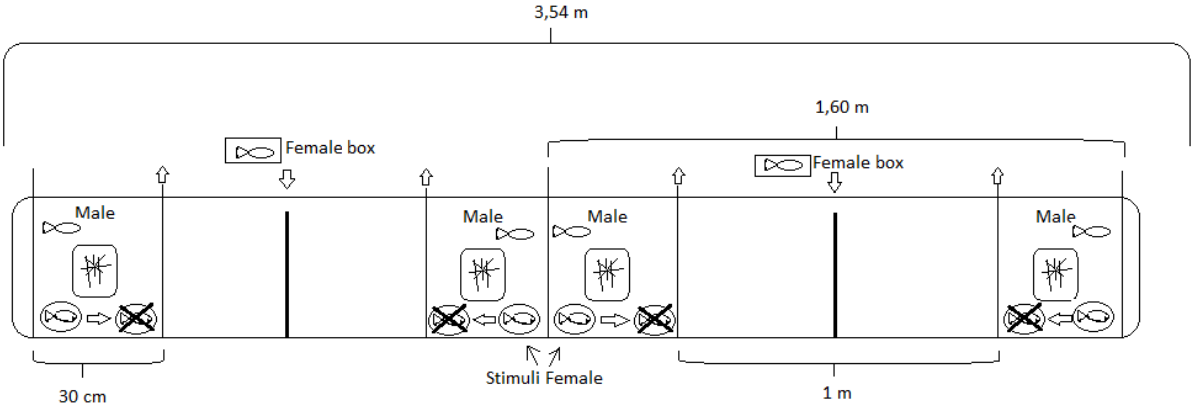

Figure 2: Schematic view of one reorganized salmon run, i.e. two test compartments. At the time of experimental female-box insertion (placed between partitions), walls keeping males within enclosures are raised and males stop receiving stimuli females; this change is indicated by thick arrows. Recording starts when inserting experimental female.

The first 10-15 minutes after female insertion she is confined within the plastic box in order for her to acclimatize (adaption phase), and afterwards released (main phase). Female threespine stickleback are known to relent on several cues in the process of choosing mates, thus both visual cues (McLennan & McPhail, 1990; Milinski & Bakker, 1990; Rowland, 1989a; Vines & Schluter, 2006) and olfactory cues (Reusch et al., 2001; Sommerfeld et al., 2008) are important, and likely have an additive informative effect (Candolin, 2003**;** Heuschele et al., 2009). Different phases through courtship involve assessment of different cues, thus when testing for a mate-preferences one should enable a most natural situation and allow courtship to go as unhindered as possible (Östlund-Nilsson & Nilsson, 2000). The experimental setup used allow all three fish to freely interact, allowing females to assess and choose between males, and consequently also potentially confounding male-male interactions (Bakker & Sevenster, 1983; Östlund-Nilsson & Nilsson, 2000); confounding male-male interactions are corrected for using video analysis. Parallels belonging to the same camera position tend to be started simultaneously as parallel rounds, of course depending on males speed at establishing nests and territories. In order to ensure equal number of females tested from both populations in total, females used are randomized with respect to population on parallels within a round, but with ratios of females evening out total number of females tested from both populations thus far.

Salmon runs (3.6m x 0.42m) are cleaned and disinfected with S1-Extra and Virkon S, respectively, and reorganized into two test compartments of 1.6m in length using hard black plastic plates attached with sikaflex; before first-time usage all runs are filled with water for 24 hours to rid sikaflex leftovers, water changed before start-up. A total of 5 salmon runs are reorganized in this fashion, but only 4 ever used at the same time due to spatial limitations; the 5th kept as backup. Experiments are carried out in a small shelf, isolating from outer disturbances. Illumination provided by 4 stand-lamps of 400W each, turned towards the wall with an upward tilt to avoid sharp light contrasts; lamps equally placed with respect to experimental tanks (reorganized salmon runs) on both sides to avoid differences. 2 camera stands are mounted on a wooden beam stretching across the width of the room, enabling recording of 4 parallels by one camera simultaneously; camera positions indicated by blue stars (figure 3). Water used standardized in order to avoid confounding factors from differing water qualities (Heuschele and Candolin, 2007; Heuschele et al., 2009), always taken from Lake Kvernavann, treated with UV light, and filtrated through a plankton net (180µm) to avoid potential external pathogens, micro-organisms and accumulation of algae and debris in test compartments. Experimental tanks checked to be horizontal in order to avoid differences in water height due to slanting. Water height set to 12.5 cm, yet varying some throughout tanks (+/- 0.5 cm) as bottoms are not totally even. Figure 3 give an overview of all experimental tanks lined up together, indicating camera positions and lamp stands.

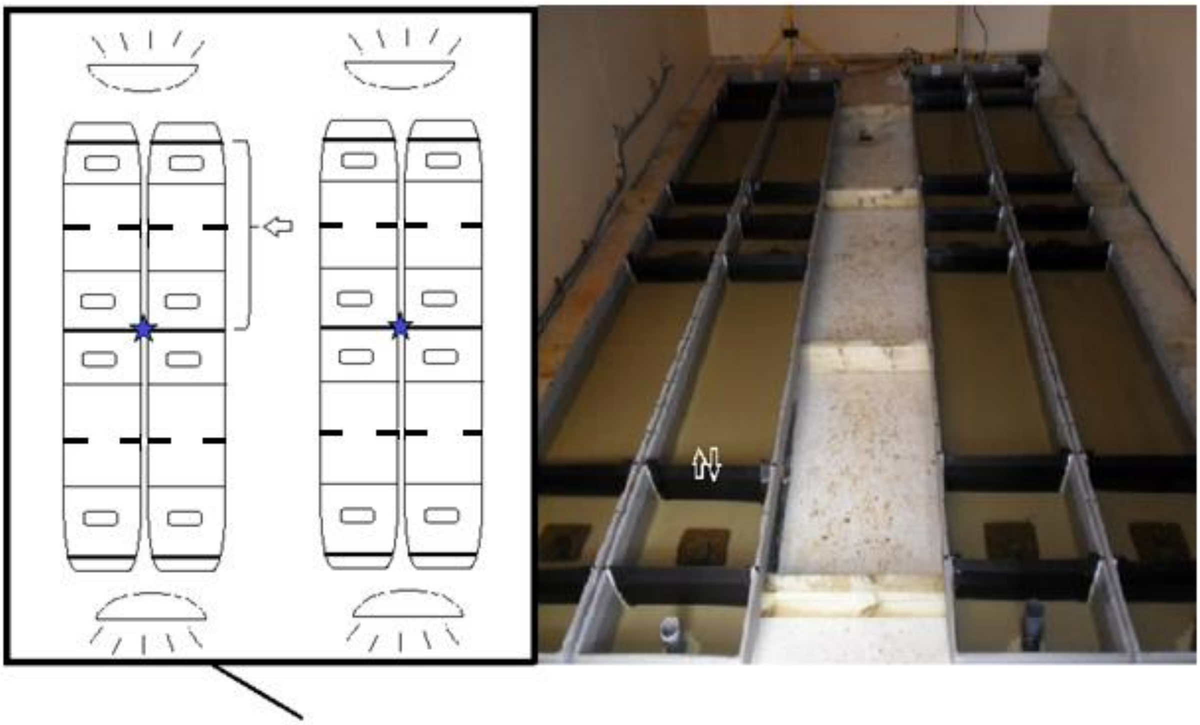

Figure 3: Schematic and real picture overview of shelf and runs (before insertion of experimental female). Horizontal arrow indicate one test compartment, vertical arrows indicate movable enclosure wall. Blue stars show camera positions. Partition walls are not shown in the picture.

Great care is taken to avoid parasite infections confounding results (Milinski & Bakker, 1990); all fish infected or suspected infected (by *S. solidus*, *G. anomala*, or other), or appearing to be of general poor condition, are excluded. To avoid size-differences confounding results (Rowland, 1989a, 1989b), males are roughly classified into size-groups through a scaling.

- Small – shorter or equal to 3.5 cm
- Medium – larger than 3.5 cm and up to/ equal to 4.0 cm
- Large – larger than 4.0 cm and up to/ equal to 4.5 cm

Only sexually mature males are used, recognized by red nuptial coloration on their chin and fore-bellies, and blue eyes (Foster, 1994). Placement of males within parallels is randomized by coin toss before male selection. Each enclosure of test compartments is designated heads or tails, a coin is dedicated one of the populations, and then tossed individually for each of the parallels to be started that round. Capture of sticklebacks is done with either stickleback plexiglas traps from the main holding tanks, or a small landing net from holding salmon runs.

Start selecting from the one population at each time assumed to be most limiting with regards to males. Males are caught and selected from one population, and counterparts of the same size-group have to be found from the other population. A male caught as the first of a male-pair is designated the next parallel by number. Each male selected is put individually into a plastic box for transport. The process of capture, examination (parasites, condition, size and sexual maturity) and transport likely induce high amounts of stress, and care is taken to minimize the amount of time spent in these plastic boxes for each fish; no more than two complete pairs of males are to be kept in plastic boxes before being transported to and released into designated test compartments and enclosures. Males are released in temporal enclosures in each end of the test compartment, each enclosure standardized with respect to size (30 cm x 42 cm). This is important, as male territory size may directly affect female mate choice (van den Assem, 1967). The enclosures contain a nest building facility; a shallow box filled with sand, 1 goose-egg sized stone and nesting material. Sand boxes are placed in the exact middle of the enclosure to ensure that the distances between both male nests and female release point are the same. 60 green sewing threads of 3 cm length are dropped into each male starting area to serve as nesting material. It is important to keep nesting area and materials simple and standardized because of stickleback preferences for substrate (Rowland, 1994) and nesting material (Östlund-Nilsson & Holmlund, 2003). Washing the sand used removes potential externally introduced nesting material. Threespine stickleback prefers building their nests in close proximity to objects such as stones (Rowland, 1994); hence the stone placed at the backmost part of the sandboxes. Two partitions are placed in the middle of the compartment, keeping males partly visually isolated from each other. Males are fed once a day 1 ml live zooplankton each in their enclosures. To stimulate male nest and territory establishment (McLennan & McPhail, 1990; Milinski & Bakker, 1990) females sampled from Lake Kvernavann are introduced into male starting areas in clear plastic bottles, representing a novel population for both males. Water in these bottles is oversaturated with oxygen to ensure female survival. Both males within a parallel always receive the same amount of stimuli before introduction of the experimental female, and stimulation ends when both males in a parallel have readied their nests. When both males have finished their nests, they are ready to encounter the experimental female. Randomization of female population is done by coin toss before catching and selecting females. Capture, parasite- and condition-examination, and transport carried out as for males. Females are judged to be gravid and ready to spawn based on abdominal distention and dilation of gonopor (Luttbeg et al., 2001). Imprint of eggs in females' skin are also a good indicator of this, and effective in avoiding *S. solidus* infected individuals. Simultaneously with female box insertion and onset of recording, male enclosures are opened by raising the plastic walls and allowing males to move freely in the test compartment. Thus, males are able to assess and court the female during adaption phase, while she is given a fair chance to assess both males before her release. From this point onwards, an additional 1 ml zooplankton is fed in the mid-area of the test compartment; males are still fed at their initial starting ends. Main phase is initiated by removing the plastic lid confining the female within her box. Parallels are ended after female spawning, assessed either by behavioral observation through 30min recordings or directly observing that she have become slim. All three fish are then killed with a lethal dose of the fish anesthetic benzoak. Both males and the female from each parallel are measured in length (maximum total length), photographed, dissected and checked for infection by *S. solidus* and *G. anomala*. Both nests from the parallel are sampled and examined, eggs counted. All three fish from each parallel are frozen, alongside eggs for males who have received this.

Between each round of parallels water is changed and runs washed with UV treated water. Stones and sandboxes washed in UV treated water as well, sand and sewing threads exchanged for new. Washing of tanks, lids and stones ensure removal of leftover olfactory cues from former parallels, while sand and nesting material in addition have to be changed due to the possible presence of leftover eggs (Ridley & Recthen, 1981).

The initial plan was to run a total of 40 parallels, as this was estimated to be required to statistically test the predictions of assortative mating. However, practical challenges mainly with mortality and establishing males greatly reduced the final number of successfully run parallels. Evident from parallel numbers enlisted in results, 23 parallels were in fact run (only 14 of which were successful), and more were attempted started. In starting the first parallels, some challenges were related to inexperience in determining infection of *S. solidus* and consequently parallels were run that could not be used. It was evident when transferring fish from initial storage tanks to salmon storage tanks that there were far less healthy males than first assumed; likely resulting from an underestimating of infections and some mortality since last check. Thus when transferring ,only 30 healthy Lake Nesa males were found, and slightly more from Lake Vigdar population; equal amounts were transferred from both populations to new storage tanks to ensure similar density-conditions. Approaching the end of July the rate of Lake Vigdar males failing to establish nests increased. Males failing at establishing nests prolonged the whole procedure of running parallels as they could not simply be exchanged because of experimental standardization; having two test compartments pr. experimental tank, one would have to end both started parallels. Also, mortality increased greatly towards end of July among Lake Vigdar males kept; during one single weekend more than 2/3 of all remaining Lake Vigdar males died. At the onset of August there were no Lake Vigdar males left. Of the last 14 parallels attempted started, only two were run; the remaining 12 failed due to Lake Vigdar males failing at establishing nests or dying.

2.4. Video Data Sampling and Analysis

From insertion of female box the following 3 hours of every parallel run is recorded for subsequent video data sampling of behavior of the three fish involved. Two types of behavioral data are sampled from video recordings: fish positions in the test compartment and behavioral scores. Only the first 90 minutes of recordings are sampled from. Going through video material is a tedious process, and compromises are made with respect to length sampled, frequency of sampling intervals, and what is assumed sufficient for the subsequent analyzes of mate choice behavior. Sampling of fish positions is done with 20 second intervals for the 90 minutes analyzed and behavioral analysis for 40 of these 90 minutes (10 minutes x 4, see below). Besides consisting of two types of data sampling, adaption phase and main phase are analyzed separately.

The image processing program ImageJ 1.46r (Abramoff, Magalhaes, & Ram, 2004) and the media player program VLC media player are used synchronously in order to get fish position coordinates. Following procedures described in Haverflock Guide to Image Analysis with ImageJ (Zamani, Tousley, & Kane, 2012) video files are converted to pictures and opened as stacks in ImageJ. Scaling based on known distances and defining of origin is done individually for all parallels, necessary because experimental tanks are not placed precisely on the exact mid-point of camera view, thus the effect from wide-angle recording varies slightly. Video files are played in VLC media player and stopped at sampling intervals (every 20 second), and while visually keeping track of all fish as one watch the whole video sequence, coordinates are gathered from the picture-sequence open in ImageJ corresponding to that same moment in the video. Coordinates are found using the point-selection tool and written manually over to a spreadsheet. When sampling coordinates from exact time moments, one might encounter sampling moments in which identification of individuals is close to impossible. In cases of uncertainty, no coordinates are noted, and instead fish are designated NA (not available) in the spreadsheet. An exception are situations were coordinate sampling occur in the middle of male-male fights and exact identification is not possible, in which both males are given the same coordinate corresponding to a mid-point between them.

Each parallel have 3 reference points: the coordinate values corresponding to mid-point of both male nesting boxes and female introductory box. Reference points coordinate values are set independently for each parallel run. Fish are plotted at these reference points' coordinates if some part of the fish bodies touches the reference point's area, e.g. snout of stickleback in over sandbox. An exception to this way of denoting coordinates is made for female introductory box in adaption phase, as males approaching this box from the side furthest from camera position will experience a greater zone in which they are plotted as on reference point; the lid of the box blocks the view. This is compensated for by plotting fish on the camera side of the box as "on reference point" when they are within 1 fish length of the box; based on individuals own length in each recording. In main phase the lid is removed and plotting for this reference point is done similar to the other two.

Camera position might slightly change when changing batteries. For most parallels the 90 sampled minutes belong to the same battery recording rendering this unproblematic. However, parallel 18-21 experience an unfortunate long adaption phase, and between adaption phase and main phase camera battery is changed. Because of this these 4 parallels have two sets of scaling and defined origins, one each for adaption phase and main phase, and obviously two sets of set reference points; one for each scaling.

Sampling of behavioral scores is done in 4 intervals, lasting 10 minutes each: adaptation phase (female acclimatization phase), first 10 minutes after releasing female (main phase I), 50 – 60 minute of total recording (main phase II), and last 80 – 90 minutes (main phase III). Behavior maintained over time is repeatedly scored for every 20 second. This adding effect justifies situations were males maintains an activity continuously over a longer time span. A behavior is stopped being scored for if the male in question switches to one of the other behavioral definitions, or if the behavior ceases to exist for 10 seconds. Following are definitions of behavioral categories fish were registered on:

First approach female box – time for this; first fish to approach the FM-box – touching the point of getting registered on the FM-box' reference coordinates.

Aggression – When one male swim towards the other male thus forcing the second male to change its speed and/or course, resulting in one attacking and one fleeing fish, this is scored as aggressive behavior. Aggression is divided into two subclasses depending on where in the parallel compartment the aggressive display is performed:

- Attack – when initiating attack on the other males half part of the experimental tank, including attacks on the midline between the partitions.
- Defense – when initiating attack on own half part of the experimental tank, not including attacks on the midline between the partitions.

It should be noted that if one male starts chasing the other as a result of a defensive act, the prolonged scoring of this will only be notified as attack; defense is scored as a first-hand act. However, if one male first gets chased by the other, and then immediately strikes back from his own half, this is a firsthand act from this male and duly noted as defense. Situations in which changes of which male's in charge changes too rapidly to keep track, only the first initiative strike, and the finale ending strike is scored for.

Shelter – Fish passively hiding at or behind objects and structural irregularities of the tank, following aggressive male-male interaction, are scored for hiding in shelter; objects may be FM-box, alongside partition walls or alongside sliders for compartments walls. Sandboxes and FM-box (in adaption phase) are exceptions at which males are not scored as hiding. Seeking shelter at sandboxes most likely happens, however there is no way of telling if they are hiding or performing sexually motivated activities while staying at sandboxes as they are hard to observe when staying over the brown sand. When hiding at FM-box during adaption phase one cannot always see the fish hiding and it is not possible to know to which extent it might actually switch to courting the female.

<u>Male – Female interactions</u> – When one male and the female actively interact.

First approach female box and Male-Female interactions are not further used in results. First approach female-box proved of little information, registration of male-female interactions suffers from recordings detail level. Recordings do not allow visual confirmation of all females entering nests. Furthermore, camera distance (in height), wide-angle effects coupled with inexperience in observing sticklebacks made it complicated to distinguish different kinds of male-female interactions i.e. different kinds of courtship and refraction behavior. Thus no distinction was made between different kinds of interactions, and therefore these data were subsequently discarded.

Short time periods occur with human presence in the room, for instance when releasing females. In order to avoid such presence being a confounding factor, no data are sampled from these periods (Milinski, 1997). Recording is mainly done with a GoPro Hero 2 camera. There are two exceptions to standard recording: Parallel 23 is filmed from a different camera position than all other parallels, placed at the longitudinal middle of recorded test compartment. This enables only two parallels to be recorded at the same time but with a closer and better view, using a more narrowly set wide-angle; Parallel 13 is recorded using another camera, GoPro Hero.

Coordinates sampled are affected by wide-angle recording. On the rim of each experimental tank lines are drawn with known distances between them, the width of the tanks is known as well. The change in relationship between measured distances in ImageJ and known distances are used to correct for the wide-angle effect in both length and width of the tanks. Coordinates are duly converted using individually found relationships for each parallel. To correct coordinates in the length-axis (x) a second-degree equation describing the wide-angle effect is found and used. To correct the width-axis (y) of the tanks, the corrected x-values are used to express the change in measured width (z) along the length of the tank (x). The relationship in change of measured z-values pr. corrected x-values is found and expressed as a second-degree equation, and used to find the true width-value for coordinates, y: $y = (K / z) * y_i$, where K represent the true width of the tank, z is the second-degree relationship describing change of measured z_n pr. x_n, and y_i is the registered coordinate value to be corrected.

Individuals' activity levels, proximities to each other, and distances to set reference points can be found with a vector formula using corrected coordinate values. Individuals' activity and location over the 90 sampled minutes are displayed by timelines; one minute corresponds to three time-points on timelines. Adaption phase and main phase are split into two separately displayed timelines, and for each phase males are made separate timelines for each of the two parameters activity and location. Females are made three timelines representing female activity and proximity to both males and their respective nests. Data from timelines coupled with behavioral scores are used to infer about interactions and relationships between fish during the run of the parallels. Mainly these behavioral data are used to investigate the extent of which females have a free choice of males or if dominant males deprive them of this. Timelines are too extensive to include in the appendix, but can be provided by request.

Investigating male-male relationships, it is preferable to know if spawning occurs during the period analyzed, as spawning affects behavioral patterns for all three fish (Wootton, 1976; Kraak & Bakker, 1998). Anticipated time of spawning is studied for all parallels by using timelines for main phase and comparing changes in female activity levels with female proximity to males and their nests, female-male interactions, and patterns indicating changes in female-male interactions from courting behavior to male refraction behavior. Time of spawning is denoted as minutes into main phase. Only video data sampled before spawning is used for subsequent analysis of male-male interactions. In those parallels were no indication of spawning is found, spawning is assumed to have occurred after the sampled period allowing the whole sampled main phase to be used.

Parameters used to determine male-male dominance relationships are the behavioral scores (attack/defense/shelter), chances to freely court female, interference of opponent male courting opportunities, activity (mean distance moved, cm), mean proximity to female/female box, and mean home range (mean distance to own nest, cm). Definitions and criteria upon which the behavioral scores (attack/defense/shelter) are sampled are outlined above. Chances to freely court female and courting interferences are found based on activity and location timelines. Chances for male courting are registered whenever males spend at least two consecutive time-points within 20cm of female, on that males' half of the parallel compartment, and without interference from opponent male. Threshold values for opponent male interferences are set to being one out of two values, whichever occur first: not crossing over onto other half of parallel compartment, or no closer than 30cm to female. For situations in which the above criteria are met except the one regarding opponent male interference, the interfering male is scored for interference. Both scores might also be given consecutively if one male first have sufficient time to court the female freely before opponent male interferes, thus ending the free courtship. Activity and distance parameters are calculated directly from the corrected coordinate values. Because of camera placement always being at one end of the test compartments, a difference in effective tank-size measured exists as a result of water height and refraction; fish on camera side are biased towards a relatively higher score in activity and distance parameters as this side appear effectively larger when measured on images. The wide-angle correction does not compensate for this. By comparing the measured lengths on images between male nesting boxes and female box (indicative of parallel middle), a difference relationship in effectively measured tank-size is found and used to correct activity and distance values; reference points are submerged thus affected by the same camera-side effect. As this relationship is based on both nests distance to the middle, it represents an extreme correction and assume that both males spend most of their time in close proximity to their own nests. If this is untrue, strictly correcting by this means erroneously result in exaggerated correction. Differences in activity and distance parameters are tested for significance ($p < 0.05$) using a two-sample t-test, both before and after correcting for camera-side effect. Also when interpreting timelines one must consider the difference in effectively measured lengths due to camera-side effect.

Total time sampled from varies depending on time of spawning in the different parallels, and some variation due to minor differences in adaption phase; meaning that data used to judge male-male relationships corresponds to varying time-spans between parallels. Behavioral scores represent 10 minute intervals through the totally sampled 90 minutes of video. In adaption phase this roughly overlaps with the overall coordinate sampling, as adaption phases are rarely much more than 10 minutes long. In main phase, the behavioral scores used corresponds to minimum one sampling interval (10 minutes), thus extending past time of spawning for those parallels in which spawning is anticipated to occur within the first 10 minutes. For all other time-intervals sampled for behavioral scores, none are used for determination of dominance unless the whole interval is included within time of spawning. Data used from coordinate sampling and their displayed timelines are adjusted the time of spawning precisely.

Parallel 18-21 deviates from the rest in having an unfortunately prolonged adaption phase, lasting close to two hours. For investigation of adaption phase, only the last 10 minutes of this phase is used.

Determining male-male relationships in adaption phase and main phase is done separately and by differently set criteria; necessary as a stationary female in the middle of the parallel compartment creates a different scenario from a males' point of view as opposed to when she is freely swimming around. Dominance relationships are set to be one out of four possible situations; total domination, strong domination, weak domination and no domination. Regarding behavioral scores, shelter score is seen as the most indicative of male dominance, followed by attack score, and last defense score; attack and defense score are collectively referred to as aggression scores. Distance and activity parameters, and their corrected values, are mainly used by a worst-case scenario principle i.e. most conservative approach. The worst case here is that there are in fact differences in males' activities; that established dominance relationships exist**.** Differences in distance to own nest will always be more affected by the camera-side effect, unless males have average distances into one another halves, which is unlikely and if necessary dealt with in results part; thus corrected average distance to own nest combined with average distance to female box give an impression of an individual's average location in test compartment, and can be used to infer if camera-side correction is likely to be suitable. If both males have average locations far from their own nests, the difference in distances measured is likely to be less than suggested by the camera-side correction factor. Average distances to female box and own nest for adaption phase are given uncorrected for camera side in results (with t-test output for both corrected and uncorrected differences), being used to determine dominance relationships for this phase. An overview of average distances to nests and female box is given in appendix A; listing both corrected and directly measured values for both phases.

Criteria used for determination of dominance relationship in adaption phase are as follows:

> <u>Total dominance</u> –Absolute differences in shelter score vs. aggression scores between two males of a pair is indicative of a very strong dominance relationship. Males average proximity to female box and own nest gives a good indication of males' placement in the tank throughout the period analyzed, and given that females in this period are locked in boxes in the middle of the parallel compartment, differences in males' location in the tank may further support the dominance relationship seen from the behavioral scores. If both distance parameters significantly favor a male dominance relationship indicated by behavioral scores, male dominance relationship is set to total domination. However, a suppressed male might take shelter close to female box, thus scoring low average distance to this reference point; potentially creating contradicting indications from different parameters. Such cases of doubt are investigated closer by comparing parameter values directly with patterns emerging from timelines; especially considering suppressed males' location. Differences in activity levels might also shed light on male-male situation, as a dominant male should be expected to be more active than a suppressed one during adaption phase.
>
> <u>Strong dominance</u> – Absolute differences in shelter score vs. aggression scores are highly indicative of a skewed male dominance relationship, yet the distance and activity parameters might not significantly favor the assumed dominant male; given absolute differences and distance parameters not significantly contradicting what's indicated by behavioral scores, a strong male-male dominance relationship is assumed. Parallels having conspicuously contradicting parameter values are further investigated through timelines. Differences in shelter score vs. aggression scores

might not be absolute, yet still strongly indicative of a highly dominant male, having absolute differences in shelter score between the two males. If further backed by a significant difference in distances to female box, or a high and significant difference in distances to own nest, a strong male-male dominance relationship is assumed.

Weak dominance – A less strong dominance relationship indicated by behavioral scores, not containing one-way indicative shelter scores coupled with indications of an extremely intruding male, yet backed by some significant difference in activity scores and/or average distances.

No dominance – If none of the above categories are met. Males might appear equal in all parameters measured, or a weak trend given by behavioral scores might totally contradict what's given by distance and activity parameters.

Timelines are used as an accessory to interpret situations were parameter values occur conclusively unclear and contradicting. Differences in parallels length of adaption phase gives different amount of data to test for differences upon, thus in parallels having the shortest adaption phases it will be harder to find significant differences.

Criteria used for determination of dominance relationship in main phase are as follows:

Total dominance – Given by absolute differences in shelter score vs. aggression scores, and further indicated by comparing location and activity graphs for the male pair. Abnormalities in male activity patterns are further revealed by timelines, measured activity levels, and average distances to female; a totally dominant male is expected to have both higher activity levels and less average distance to female. Opportunity to freely court female is also to be expected.

Strong dominance – If one male has no courting opportunities because of opponent male interference, or if the one male having significantly most female possession is also the most aggressive one; with equal males the male not having the female would be expected to be somewhat more intruding and aggressive, attempting to get the female himself.

Weak dominance – Given by differences in behavioral scores, rates of interfering opponent male courting opportunities, and differences in activity and female proximity; must be taken into consideration that if one male has significantly less average distance to female, the opponent should be expected to be somewhat more aggressive as a direct result of this. Indication of a male dominance relationship not fitting into one of the above categories is determined a weak dominance relationship.

No dominance – none of the above categories are found to fit the situation as males appear equal in their aggressive displays. Males are assumed equal, no dominance relationship between males is assumed. Males' chance to freely court the female does not by itself inform about a male-male relationship, unless males' absence of courting opportunities is solely due to opponent male interference. If one male have no scored chances for freely courting, and its opponent none scores for interference, it cannot be concluded based on this that the lack of courting is due to dominance; it might result from a lack of interest from the female.

While significant difference in means of activity and distance relationships is well and indicative of a difference between males, one must consider the actual difference in these means; a significant but very small difference in means might not be very indicative of a dominance relationship. Especially in main phases having no suspected spawning, the huge amount of data tests are based on might make very small differences in means significant still.

Determined dominance relationships for adaption phase and main phase are summed up, and validity of female choice evaluated for each parallel. A new check for indications of assortative mating is performed using data corrected for male dominance; excluding parallels lacking free female choice as non-valid. Furthermore, using dominance relationships for valid parallels, indicative strengths of female preferences based on choices made are found and summed up. Parallels in which a total or strong dominant male is also the one receiving eggs, are excluded as not having a valid female choice; assuming a worst-case situation. On the contrary, if a suppressed male is the winner, female choice is assumed valid. Strength and direction of female choice is scored based on dominance regime and whichever male she mated with. Females mating homogeneously with males from own population are scored positive, and heterogeneously with males from foreign population negative. Further, the indicative strength of female choice regarding male preferences are scored numerically; choice of suppressed male = 2, choice in a no-domination regime = 1, choice of weakly dominant male = 0.5. In assessment of parallels validity and indicative strengths, dominance relationship in main phase is weighted most heavily, being based on males' actually chances of courting a free female. However, if dominance relationship between the two phases deviates by 2 categories or more, the mid-point between categories is used, rounded up towards the trend from main phase if needed; e.g. strong dominance is the mid-point category between weak and total dominance by one given male. Some main phases span over a very short amount of time because of early spawning, and this is taken into account and further commented in results if relevant.

Male behavioral data are further used to infer males' preferences for females. Using dominance relationships from main phase and changes in relationships as one goes from adaption phase to main phase, males' interest in females is investigated. If homogeneous assortative mating occurs, males are expected to be disproportionately more interested in females of their own population. As one goes from adaption phase to main phase, a change in dominance relationship category is expected to reflect a change in males' interest in female, as female recognition becomes easier and more accurate when female is released. Furthermore, assuming males to be of equal condition, males dominance status in main phase should also reflect their interest in the female; although less reliable than changes in dominance relationships. If males prefer mating homogeneously with females from their own population, this should be reflected in changes of dominance status between phases, and male dominance relationships in main phase generally. Male mate-preferences are determined as follows: A dominant male in main phase accompanied by a positive change in dominance relationship going from adaption phase to main phase, is strongly indicative of a male mate-preference. In parallels having no dominance in main phase, only the change from adaption phase to main phase is used to decide on males' preferences; moderately indicative of male preferences. Some parallels might not experience a change between phases, in which case dominance relationships alone are used to infer male interest, weakly indicative of a male mate-preference. If no change and no domination regimes exist, there is seemingly a lack of disproportionate preference from the homogeneous male. It must be mentioned that this only measure a correlational effect, as males are not given a choice between females.

2.5. Male Red Nuptial Coloration

Using photographs taken at dissection after ended experiments, male nuptial coloration for males from each parallel is checked and scored for intensity based on a numerical scale of 1-4; in which 1 represent most dull and 4 represent most intense. Several ways of scoring color is found throughout literature (McLennan & McPhail, 1989a; Östlund-Nilsson & Nilsson, 2000), for instance Milinski and Bakker (1990) use several people scoring on a 10-point scale. However, being only a test for confounding effects in this study, a rougher 4-point scale assessed by one person is assumed sufficient.

2.6 – Statistics

The statistical program R 3.0.0 (R Core Team, 2013) is used for statistics. Two sample t-tests are run testing for significance in activity and distance parameters when checking for dominance relationships, and differences in clutch sizes spawned are tested using ANOVA.

2.7 – Citation Style

APA citation style is used for writing this thesis (Publication Manual of the American Psychological Association (6th ed.), 2009).

3. Results

Out of 40 initially planned parallels, a total of 14 successful parallels were run. This results mainly from practical challenges with male mortality and establishment, further outlined in Material and Methods, 2.3. Experimental Setup and Execution.

3.1. Time of Spawning

Times of spawning are given in table 2, as minutes into main phase. Criteria used in determining occurrence of spawning are described in Material and Methods, 2.4. Video Data Sampling and Analysis.

Table 2: Time of spawning. Egg receiving male given as winner (population).

Parallel number	**<- time of spawning; minutes into main phase (winner male)**	**Parallel number**	**<- time of spawning; minutes into main phase (winner male)**
1	18 min 20 sec (Vigdar)	**17**	Not Occurring
2	Not Occurring	**18**	Not Occurring
9	Not Occurring	**19**	8 min 20 sec (Vigdar)
11	5 min 40 sec (Nesa)	**20**	Not Occurring
13	36 min (Vigdar)	**21**	Not Occurring
14	6 min 40 sec (Nesa)	**22**	Not Occurring
16	8 min (Vigdar)	**23**	39 min 20 sec (Nesa)

Parallel 2, 17 and 18 have behavioral patterns challenging to interpret according to used criteria for occurrence of spawning. For all three of these, mating is assumed as to not occur during video-sampled main phase.

In parallel 2, the female undergo a clear change in activity, from active to stationary, yet this change do not directly relate to a clear spawning opportunity followed by patterns of male refraction behavior.

In parallel 17 there is a longer period in which the female stays within the nesting-box belonging to the Lake Nesa male. However, throughout this period this males activity level is very high and not indicative of courting behavior; rather this male appear as preoccupied with hassling its opponent male as with courting the female. No clear changes in female activity levels or patterns of male refraction behavior are evident.

In parallel 18, female activity pattern coupled with location might indicate an early spawning for Lake Vigdar male, yet this males' subsequent behavior do not support this. This is further supported by sampling of eggs, as these were sampled from Lake Nesa males' starting area.

3.2. Male-Male Dominance Relationships

Effects of male-male interactions are studied from video recordings, using only data sampled up to the point of spawning; when occurring.

3.2.1. Adaption phase

Male-male dominance relationships for adaption phase are determined by parameters outlined in table 3. Descriptions of data and definitions upon which the different parameter values are based, as well as definitions for determining dominance relationships, are given in Material and Methods, 2.4. Video Data Sampling and Analysis. Activity and distance values enlisted are not corrected for camera-side effect. Parallel 18-21 differ from the rest in having a prolonged adaption phase (see material & methods), only the last 10 minutes of these adaption phases are used. For corrected distance values, see appendix A.

Parallel 1 and 18 have a total dominant male-male relationship. Parallel 1 fit criterion well, parallel 18 less clearly. For parallel 18, behavioral scores are poorly indicative, and contradicted by distance parameters; directly looking at timelines reveal male-male interaction patterns more clearly. The determined difference between males is strengthened by a significant difference in activity levels, combined with males' patterns of activity and location shown by timelines. Parallel 18 is amongst those four parallels experiencing a prolonged adaption phase, and as sampling interval starts (the last 10 minutes), Lake Nesa male is already hiding, thus partly explaining low aggression scores from Lake Vigdar male. However, as Lake Nesa male presumably hides at female box, this is not noted as such in behavioral scores; explaining low activity levels and low average distance to female box.

Table 3: Adaption phase – male behavior. Summing up parameter values used to determine male dominance relationships in adaption phase. P. Nr = Parallel number;
A/D/S = Attack/Defense/Shelter; Activity = mean distance moved, cm (CS, if on camera side).

*Corr. factors, used to correct for camera-side effect. Significant difference before camera-side (CS) correction is denoted *, after correction another* $^{(*)}$*. Adaption phase data are not available for parallel 20 and 22.*

		Lake Vigdar male			**Lake Nesa male**		
P. Nr	**Corr. factor**	**A/D/S**	**Activity, cm (cam side):**	**Avg. distance to female box / own nest, cm**	**A/D/S**	**Activity, cm (cam side):**	**Avg. distance to female box / own nest, cm**
1	1,089	10/2/0	27,0	27,6*$^{(*)}$ / 53,2*$^{(*)}$	0/0/8	25,6 (CS)	50,5*$^{(*)}$ / 34,6*$^{(*)}$
2	1,086	0/2/1	12,8 (CS)	45,4 / 21,5*$^{(*)}$	3/0/0	13,9	37,9 / 64,4*$^{(*)}$
9	1,087	1/2/0	29,8 (CS)	33,1*$^{(*)}$ / 42,6*	2/1/7	20,6	47,1*$^{(*)}$ / 23,7*
11	1,076	0/0/3	20,9	39,8 / 30,1	3/2/0	25,6 (CS)	31,6 / 36,4
13	1,138	4/0/3	35,4 *$^{(*)}$	32,1*$^{(*)}$ / 53,8*$^{(*)}$	0/5/1	13,3 (CS) *$^{(*)}$	55,0*$^{(*)}$ / 12,1*$^{(*)}$
14	1,145	0/1/0	35,6 (CS) *$^{(*)}$	27,4$^{(*)}$ / 37,5*$^{(*)}$	1/2/0	19,0 *$^{(*)}$	36,5$^{(*)}$ / 20,8*$^{(*)}$
16	1,140	4/4/2	22,7	34,6 / 33,6	2/3/4	32,2 (CS)	36,6 / 37,2
17	1,113	1/6/0	29,3 (CS)	48,8*$^{(*)}$ / 26,4*$^{(*)}$	3/4/1	33,6	27,4*$^{(*)}$ / 52,3*$^{(*)}$
18	1,110	1/0/0	59,6 *$^{(*)}$	48,8*$^{(*)}$ / 78,5*$^{(*)}$	0/0/0	7,0 (CS) *$^{(*)}$	8,2*$^{(*)}$ / 57,9*$^{(*)}$
19	1,103	0/0/0	21,1 (CS)	17,0*$^{(*)}$ / 50,8*$^{(*)}$	0/1/0	14,6	39,0*$^{(*)}$ / 20,6*$^{(*)}$
20	1.105	-	-	-	-	-	-
21	1,106	4/0/0	11,5 *	7,7*$^{(*)}$ / 48,4*$^{(*)}$	0/4/0	16,6 (CS) *	38,3*$^{(*)}$ / 26,4*$^{(*)}$
22	1.188	-	-	-	-	-	-
23		1/0/0	15,8 (cam mid)	28,6* / 36,1*	0/4/0	20,3 (cam mid)	39,8* / 48,9*

Parallel 2, 9, 11, 13 and 23 have a strong dominant male-male relationship. Parallel 2, 9 and 11 fit criterion well, having either absolute difference in shelter score vs. aggression scores, or one-way indicative shelter score backed by aggression score, coupled with significant differences in distances to reference points. Parallel 13 have less clear indications from shelter score, yet strong indication from aggression score, activity, distance parameters, and closer investigation of timelines showing that Lake Vigdar male intrude well onto Lake Nesa males half thus suppressing Lake Nesa male, suggests a strong domination relationship. Distance parameters appear conspicuous when one male have both high distance to own nest and to female box; requiring further investigation of male location patterns from timelines. Parallel 23 have weak indicative behavioral scores, yet adaption phase last for 19 minutes, meaning that the last 9 minutes are not covered by behavioral score sampling. Distance scores inquire further investigation of male movement patterns as Lake Nesa male have both high average distance to its own nest and to female box. Males' activity and location timelines reveal a heavily intruding Lake Nesa male halfway into adaption phase.

Parallel 17 and 21 have weak dominant male-male relationship. In parallel 17 indications by shelter score contradicts indications by aggression scores, yet distances significantly supports Lake Nesa male as the most intruding of the pair. Parallel 21 have no shelter score, clear indications by aggressive scores, further backed by a high and significant difference in female box proximity; also a significant difference in distance to own nest in favor of the dominant Lake Vigdar male, yet coupled with distance to female box there are no indications of heavy intrusion onto opponent males' half.

Parallel 14, 16 and 19 have no male-male dominance relationship; based on contradicting and/or weak indications not further backed by remaining parameter values.

3.2.2. Main phase

Male-male dominance relationships are determined by parameters outlined in table 4. Descriptions of data and definitions upon which the different parameter values are based, as well as definitions for determining dominance relationships, are given in Material and Methods, 2.4. Video Data Sampling and Analysis. Activity and distance values enlisted are not corrected for camera-side effect. For distances regarding male location, see appendix A.

<u>Table 4: Main phase – male behavior.</u> Summing up parameter values used to determine male dominance relationships in main phase. P. Nr = Parallel number; A/D/S = Attack/Defense/Shelter;

IOC = Interference of Opponent male Courting, nr. of times; CFC = Chances to Freely Court female, nr. of times; Activity = mean distance moved, cm (CS, if on camera side); MDF = Mean Distance to Female, cm.

*Significant difference before camera-side (CS) correction is denoted *, after correction another $^{(*)}$. Parallel 23 has camera in mid-position (mid), hence no camera-side correction. Correction factors are given in adaption phase table.*

	Lake Vigdar male					**Lake Nesa male**				
P. Nr	**A/D/S**	**IOC, nr of times**	**CFC, nr of times**	**Activity, cm (cam side?)**	**MDF, cm**	**A/D/S**	**IOC, nr of times**	**CFC, nr of times**	**Activity, cm (cam side?)**	**MDF, cm**
1	2/3/0	0	1	21.3	20.2 *(*)	0/0/3	3	0	15.1(CS)	61.6 *(*)
2	1/3/0	5	6	20.6(CS) *(*)	52.6 *	1/5/0	4	9	15.7 *(*)	44.8 *
9	1/7/0	0	7	16.5(CS) *(*)	38.4 *(*)	8/2/0	7	1	27.4 *(*)	51.9 *(*)
11	0/0/20	0	0	18.1	78.4 *(*)	7/3/0	0	1	40.2(CS)	35.7 *(*)
13	0/2/0	0	4	14.4 *(*)	44.0	1/0/0	2	2	24.6(CS) *(*)	49.9
14	0/0/0	0	0	15,7(CS)	96.8 *(*)	1/1/0	0	1	7,5	7.5 *(*)
16	0/1/0	0	2	6.5 *(*)	6.3 *(*)	0/0/0	1	0	25.9(CS) *(*)	99.2 *(*)
17	0/14/0	0	1	13.4(CS) *(*)	62.1 *(*)	12/1/0	3	10	32.8 *(*)	36.2 *(*)
18	5/6/0	0	2	27,0 *(*)	45,5 *	0/0/19	3	0	15,3(CS) *(*)	53,8 *
19	0/1/0	0	1	11.6(CS)	5.8 *(*)	0/0/0	0	0	8.4	108.0 *(*)
20	0/7/0	0	2	11.4(CS) *(*)	47.9 *(*)	4/0/0	1	2	27.4 *(*)	39.1 *(*)
21	14/4/0	4	1	21.2 *	40.1 (*)	4/3/0	4	6	24.7(CS) *	36.4 (*)
22	12/3/0	2	5	22.6 (*)	28.5 *(*)	1/5/0	0	2	20.7(CS) (*)	49.3 *(*)
23	0/0/33	1	1	8.2(mid) *	47.2	11/5/0	5	1	49.2(mid) *	43.6

Parallel 1, 11, 18 and 23 have a total dominant male-male relationship. Neither parallel 1 nor parallel 11 have significant differences in activity, although both differences in means favor the dominant male; Lake Vigdar male and Lake Nesa male, respectively. Parallel 11 have early spawning (at 5 min 40 sec), thus sampling of behavioral scores extend past coordinates used. However, timelines clearly indicate male-male interactions within time of spawning, reflecting the same pattern as behavioral scores. In parallel 18, distance to female favors a totally dominant Lake Vigdar male, although not significantly after correcting for camera-side effect. However, as both males keep a very high average distance to their own nests, correcting for camera-side effect is likely to erroneously exaggerate i.e. over-correct. The significant difference found in distance to female before camera-side correction is likely what is most correct, and furthermore the most conservative approach when interpreting this parallel. In parallel 23 males have no significant difference in average distance to female, yet the overall parameter values favor a total dominant Lake Nesa male. Lake Vigdar male stay hidden in shelter for most of the 70 minutes sampled, revealed by timelines, and scores gained for interference and courting opportunity are likely to be circumstantial to criteria used.

Parallel 17, 20 and 22 have strong dominant male-male relationship. In all three parallels the one male having most female possession is also the most aggressive one; never are suppressed males derived of free courting opportunities strictly as a result of suppression. Parallel 17 and parallel 20 have all parameter values significantly favoring the dominant male; both dominant males are from Lake Nesa. In Parallel 22, activity levels favor the dominant Lake Vigdar male significantly only after camera-side correction, yet as both males keep on average reasonably within their own halves, the corrected significance in difference is likely to be most correct. In addition taking a most conservative approach, the corrected significant difference is the one used here.

Parallel 9 and 13 have a weak dominant male-male relationship. In parallel 9, the dominant Lake Nesa male is significantly most active, have significantly highest distance to female and is the most aggressive of the two males, yet more aggressive than expected given that both males have a relatively high average distance to female. Parallel 13 show no significant difference in female proximity levels, yet the significantly most active male, Lake Nesa male, is the only one of the male-pair having any attack- or interference score.

Parallel 2, 14, 16, 19 and 21 have no male-male dominance relationship. Parallel 2 have no notable differences in male aggression displays. Significant differences in activity and female proximity are found and assumed likely to arise as a result of female behavior. Parallel 14 have an absolute difference in aggression scores, however the real difference in scores are minimal. Spawning is assumed to occur very early (at 6 min and 40 sec into main phase), and female spends the entire period analyzed within close proximity of Lake Nesa male. Lake Vigdar male score no courting opportunities, yet this male is known to have a chance at courting before video data sampling starts (observed on video); sampling delayed due to human presence in the room. Given the early time of spawning, behavioral sampling extends past coordinate sampling, and timelines indicate that the noted aggression scores most likely stem from activities after suspected time of spawning. Both parallel 16 and parallel 19 have minimal levels of male aggressive display. In both of these two parallels one of the males lack courting opportunity, but not as a result of opponent male interference. Both parallels have spawning within the first 10 minutes, resulting in behavioral score sampling extending past the point of coordinate sampling. However, aggressive display is barely noted for either parallel throughout these 10 sampled minutes, rendering this of no concern. P.21 have marked differences in behavioral score values, yet both males appear more aggressive than not. As both males stay on average within close proximity of own nest, camera-side correction applies, rendering activity levels non-significantly different, while female average distance become significantly different in favor of Lake Nesa male; potentially explaining differences in aggressive display seen between the two males. Taking a most conservative approach, a significant difference in distance to females also applies. While significant, the actual difference in average proximity to female is low, yet definitions behind attack and defense scores might explain the differences in scores if the female stays on average just within Lake Nesa males' half. Chances of free courtship further back a more aggressive Lake Vigdar male.

3.3. First Prediction: Females Choose Homogeneous Males

3.3.1. Nest Sampling

Results from sampling of nests are given in figure 4. The distribution shown is based on sampling of eggs from male starting areas and assuming that males fertilize eggs sampled in their own original territories.

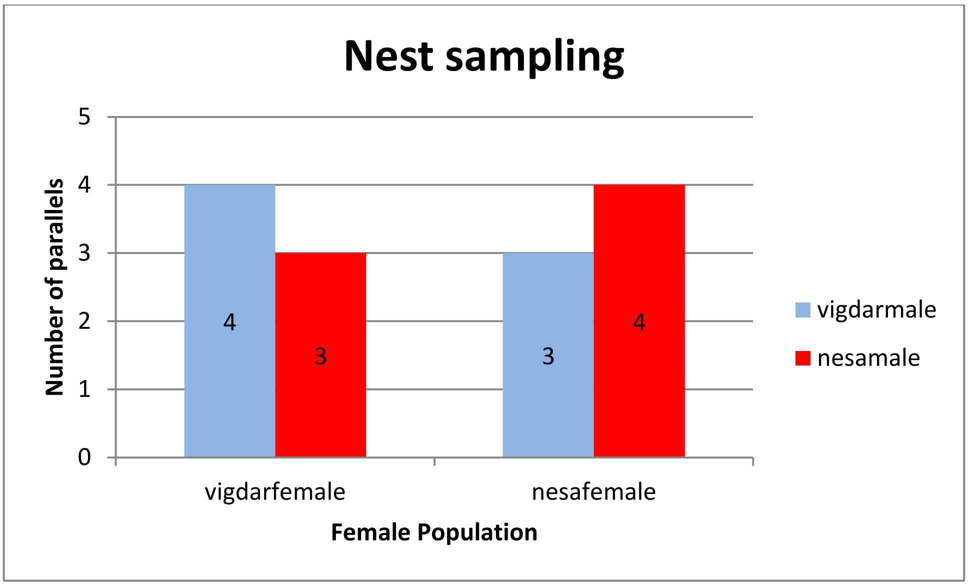

Figure 4: Distribution of males receiving eggs from females of both populations

For both populations' females, there is only a difference of one male chosen in favor of homogenous mate preference, thus no indication of assortative mating is found.

3.3.2. Female Choice adjusted for Confounding Male Behavior

Table 5 sums up dominance relationships from adaption and main phase, combined with female population and actual winner male, assessing validity of female choice in each parallel, and furthermore indicative strengths of female preferences based on choices made; instructions on assessing parallel validity and indicative strengths are outlined in Material and Methods, 2.4. Video Data Sampling and Analysis.

Table 5: Validity and indicative strength of female choice. *Dominance relationships from adaption phase and main phase are summed up to infer validity of females' choice in each parallel, and furthermore an indicative strength of mate-preferences from choices made.*

P. Nr = parallel number; DR = Dominance Relationship: Total, Strong, Weak or No dominance.

P. Nr.	Adaption phase: DR (dominant male, population)	Main phase: DR (dominant male, population)	Female pop.	Actual Winner Male	Valid Female Choice	Assortative mating indication: positive/negative? Strenght (0.5/1/2)
1	Total (Vigdar)	Total (Vigdar)	Vigdar	Vigdar	No	0
2	Strong (Nesa)	No dominance	Vigdar	Vigdar	Yes	+2
9	Strong (Vigdar)	Weak (Nesa)	Vigdar	Vigdar	Yes	+1
11	Strong (Nesa)	Total (Nesa)	Nesa	Nesa	No	0
13	Strong (Vigdar)	Weak (Nesa)	Nesa	Vigdar	Yes	-1
14	No dominance	No dominance	Vigdar	Nesa	Yes	-1
16	No dominance	No dominance	Vigdar	Vigdar	Yes	+1
17	Weak (Nesa)	Strong (Nesa)	Nesa	Vigdar	Yes	-2
18	Total (Vigdar)	Total (Vigdar)	Vigdar	Vigdar	No	0
19	No dominance	No dominance	Nesa	Vigdar	Yes	-1
20	-	Strong (Nesa)	Nesa	Nesa	No	0
21	Weak (Vigdar)	No dominance	Nesa	Nesa	Yes	+1
22	-	Strong (Vigdar)	Vigdar	Nesa	Yes	-2
23	Strong (Nesa)	Total (Nesa)	Nesa	Nesa	No	0

In parallel 18, eggs were sampled from the determined suppressed males' initial starting area. However, given the total domination situation and behavioral patterns seen in the dominant Lake Vigdar male, it is highly likely that this dominant male is the one that fertilized the eggs.

Parallel 1, 11, 18, 20 and 23 are excluded as having no free female choice. In parallel 22, the female used was found to have an asymmetrically shaped pelvic complex during dissection, possibly a hybrid of the two morphs. Parallel 22 is excluded from the conclusive evaluation of all three predictions.

Excluding non-valid parallels leave 8 valid parallels, of which 4 females choose males of own population (homogeneous choice, total strength of 5), and 4 females choose males of foreign population (heterogeneous choice, total strength of 5).

Taken from adaption phase, both total domination predicts outcome correct, 4/5 strong domination predicts outcome correct, both weak domination predict wrong, and those having no domination relationship consequently do not predict any winner. Summing, 6/12 outcomes are correctly predicted based on dominance relationships in adaption phase.

Figure 5 give an overview of female choice corrected for confounding male behavior, excluding non-valid parallels lacking free female choice.

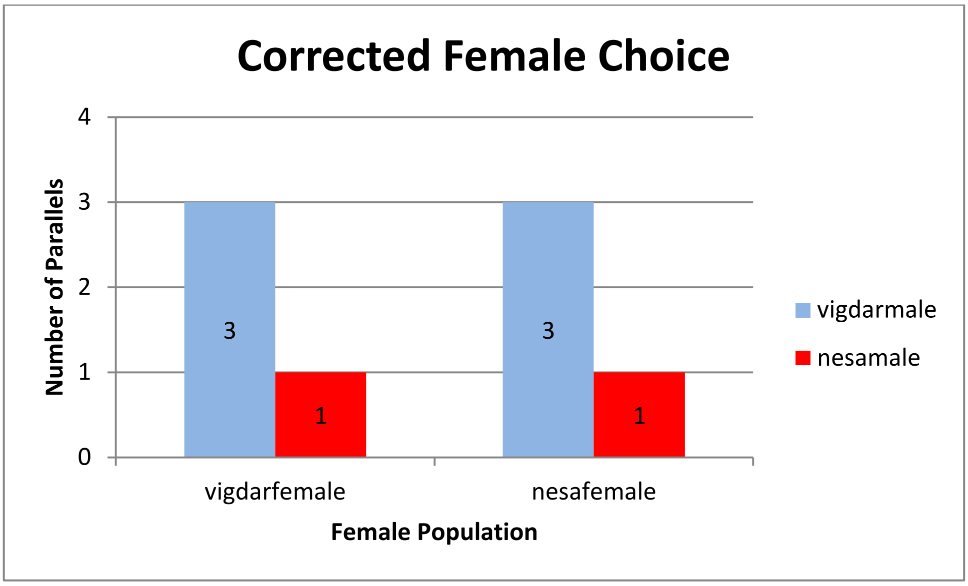

Figure 5: Females choice of males. Non-valid parallels due to male dominance are excluded as having no free female choice.

Figure 6 give an overview of preference-indicative strength in females' choice of males, excluding non-valid parallels lacking free female choice.

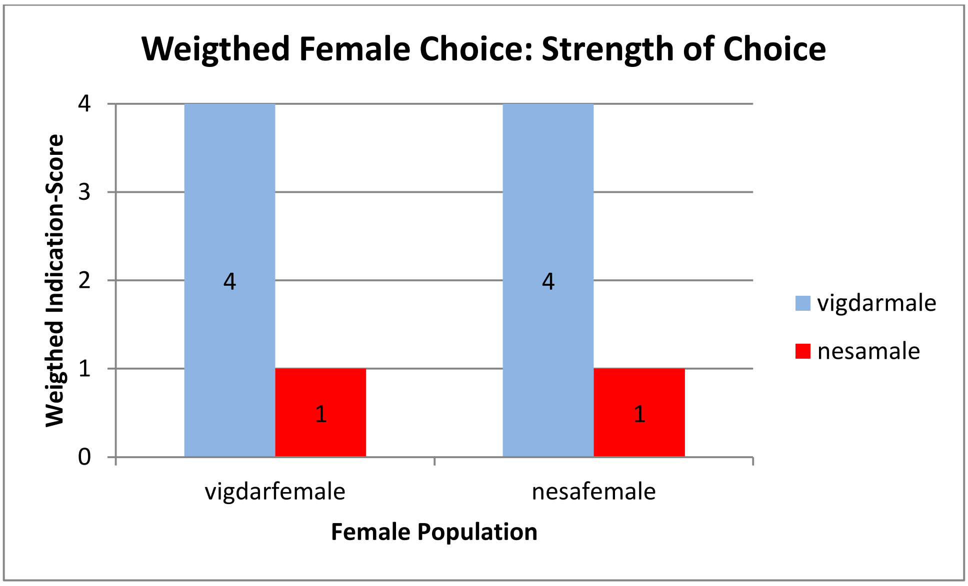

Figure 6: Indicative strength of females' choice. Non-valid parallels are excluded as having no free female choice.

There are no indications of homogeneous assortative mating between the two populations based on females' choice of males. Rather, both populations' females seem to prefer Lake Vigdar males. Considering indicative strength of females' choice, this trend is strengthened even more.

3.4. Second Prediction: Females Spawn Larger Clutches for Homogeneous Males

Females' mate-preferences are studied with regard to differences in number of spawned eggs i.e. clutch size. Parallel 22 is excluded due to possibly hybrid female. Parallel 19 is excluded due to an accident during spawning in which the female ruined the males nest in the process of spawning. Eggs were subsequently observed spread around in the parallel compartment, and also all fish were found to have consumed a great deal of eggs during dissection afterwards. Only 10 eggs were found in this males' nest during sampling.

Differences in clutch sizes spawned for males with respect to homogeneous preferences are shown in figure 7.

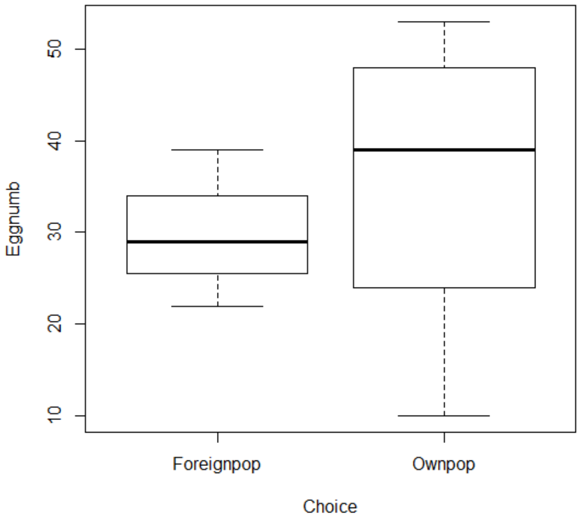

Figure 7: Clutch size distribution considering homogeneous mate-preferences. Choice; winner male: Ownpop = homogeneous spawning, Foreignpop = Heterogeneous spawning

Mean clutch sizes spawned for own and foreign population are 34.9 eggs and 30.0 eggs, respectively. Output from ANOVA give an F-statistic of 0.272, df = 1 and 10, and p-value = 0.613. There is no significant difference in clutch sizes spawned for males of own or foreign population.

A difference here exists in the amount of data the two choices clutch sizes are based on. As both excluded parallels represent heterogeneous male choice, heterogeneous spawning is represented here by only 3 parallels, while homogeneous spawning is represented by 9 parallels.

An interesting point worth noting is a difference seen in the greatest clutch sizes spawned. While 4 homogeneous spawning's resulted in clutch sizes larger than 40 eggs, none of the heterogeneous spawning's did so. However, dissection does not reveal any evidence of female egg retention.

The corrected results regarding female preferences through males chosen indicate a preference for Lake Vigdar males, and a test of differences in eggs spawned based on male population, regardless of females' population, is appropriate. Figure 8 show a boxplot display of clutch sizes spawned for males of the two populations.

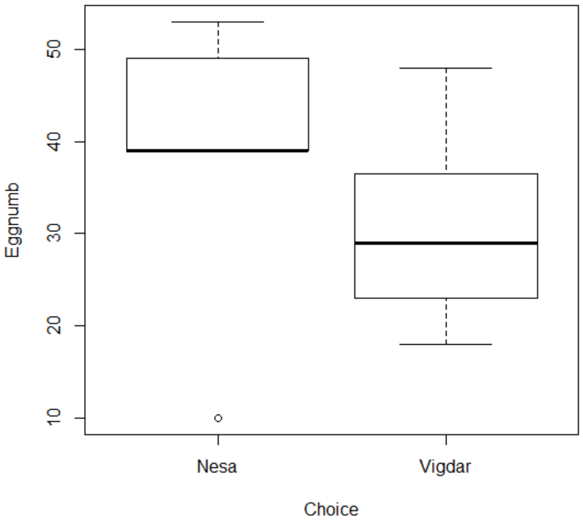

Figure 8: Clutch size distribution considering population-preferences. Choice; winner male: Lake Nesa male, Lake Vigdar male.

Mean clutch sizes spawned for Lake Vigdar males and Lake Nesa males are 30.6 eggs and 38.0 eggs, respectively. Output from ANOVA give an F-statistic of 0.862, df = 1 and 10, and p-value = 0.375. There is no significant difference in clutch sizes spawned for males of the two populations. Both of parallel 19 and 22 is excluded also here. Lake Nesa data based on 5 parallels, Lake Vigdar data based on 7.

Females of parallel 17 and parallel 20 were found to have some minor infection in dissection after ended experiment. This could possibly have limited energy allocated to egg production. Excluding these two parallels give mean clutch sizes spawned for homogeneous and heterogeneous males equal to 38 eggs and 30.5 eggs, respectively. ANOVA output gives an F-statistic of 0.564, df =1 and 8, and p-value = 0.474, meaning no significant difference in mean clutch sizes spawned. Data for homogeneous choice based on 8 parallels, heterogeneous choice based on 2. Excluding these two parallels from test of a general population preference give mean clutch sizes spawned for Lake Vigdar male and Lake Nesa male equal to 30.8 eggs and 45.0 eggs. ANOVA output gives an F-statistic of 4.36, df = 1 and 8, and p-value = 0.070, meaning no significant difference in mean clutch size spawned. Data for Lake Nesa males based on 4 parallels, data for Lake Vigdar males based on 6 parallels.

3.5. Third Prediction: Males Choose Homogeneous Females

Table 6 outline parameters used to assess the extent of male preferences for homogeneous females, based on male aggression levels. Instructions on assessing male mate-preferences are outlined in Material and Methods, 2.4. Video Data Sampling and Analysis.

Table 6: Male Choice. Dominance relationships from main phase and changes from adaption phase to main phase used to assess male female-preferences.

P. Nr. = Parallel Number; DR = Dominance Relationship: Total, Strong, Weak or No dominance; Biggest male, difference in size, mm; Fm = female; pop = population.

P. Nr	**Adaption phase: DR (dominant male, population)**	**Main phase: DR (dominant male, population)**	**Biggest male / dif. mm**	**Fm, pop.**	**Fm, size, mm**	**Adaption phase -> Main phase: change in favor Homogen. Preference?**	**Indication of Male Homogen. Preference?**
1	Total (Vigdar)	Total (Vigdar)	Nesa/ 5	Vigdar	46	Equal	Yes
2	Strong (Nesa)	No dominance	Nesa/ 1	Vigdar	52	Yes	Yes
9	Strong (Vigdar)	Weak (Nesa)	Vigdar/ 2	Vigdar	46	No	No
11	Strong (Nesa)	Total (Nesa)	Vigdar/ 2	Nesa	48	Yes	Yes
13	Strong (Vigdar)	Weak (Nesa)	Vigdar/ 5	Nesa	37	Yes	Yes
14	No dominance	No dominance	Nesa/ 1	Vigdar	44	Equal	No
16	No dominance	No dominance	Nesa/ 3	Vigdar	38	Equal	No
17	Weak (Nesa)	Strong (Nesa)	Nesa/ 2	Nesa	40	Yes	Yes
18	Total (Vigdar)	Total (Vigdar)	Vigdar/ 0.5	Vigdar	47	Equal	Yes
19	No dominance	No dominance	Nesa/ 1	Nesa	41	Equal	No
20	-	Strong (Nesa)	Equal	Nesa	43	-	Yes
21	Weak (Vigdar)	No dominance	Vigdar/ 1	Nesa	40	Yes	Yes
22	-	Strong (Vigdar)	Nesa/ 1	Vigdar	37	-	Yes
23	Strong (Nesa)	Total (Nesa)	Nesa/ 5	Nesa	46	Yes	Yes

9/13 parallels show homogeneous preference by males, 4/13 does not; only 1 of which has a male with heterogeneous preference. Parallel 22 is excluded from evaluation of male preference due to possibility of hybrid female.

Parallel 11, 13, 17 and 23 are strongly indicative of male homogeneous mate-preferences, as dominant males in main phases belong to the same population as these respective parallels' females, and changes in dominance categories from adaption phase to main phase favor these males. Parallel 9 is likewise strongly indicative of male heterogeneous mate-preference.

Parallel 2 and 21 are moderately indicative of male homogeneous mate-preferences, as both experiences a change in dominance relationship from adaption phase to main phase favoring homogeneous preference, while lacking male dominance relationships in main phase.

Parallel 1, 18 and 20 are weakly indicative of male homogeneous mate-preferences, having dominance relationships in main phase favoring homogeneous mate-preference, yet no change between adaption phase and main phase.

Parallel 14, 16 and 19 have no dominance relationships given for any of the two phases, and are scored as having a lack of homogenous preference.

3.6. Other Confounding Effects

3.6.1. Size Effects

Males' size does not confound the dominance relationships and changes seen, and no patterns emerge from males' aggression levels coupled with females of different sizes.

Comparing male size differences given in table 6 with actual winner male from table 5, it is evident that in 6 / 14 parallels the biggest male won, and in 3 of these 6 the difference between males is less than or equal to 1 mm.

3.6.2. Parasite Infections

All fish were checked for parasites before start-up of each parallel, yet dissection after ended experiments revealed some discrete infections still. An overview of infections found during dissection is given in table 7.

Table 7: Parasite infections. Enlisting parasite infections found during dissection after ended experiments.

P. Nr. = Parallel Number.

	Parasite Infection		
P. Nr.	**Lake Vigdar male**	**Lake Nesa male**	**Female (population)**
2		*G. anomala* in gill	
16		*G. anomala* in gill	
17			Tiny *S. solidus* (Nesa)
18	Tiny *S. solidus*		
20		Small white dots on skin surface	Small white dots on skin surface and opercula (Nesa)
21	Tiny *S. solidus*	*G. anomala* in gill	
22		Small white dots on skin surface	
23		*G. anomala* in gill	

In parallel 2, 16, 21 and 23 Lake Nesa males were found to have *G. anomala* infection in gills. Data sampled through video recordings does not imply any indications of this affecting males' behavior in terms of aggression or courting intensity. Furthermore, two of these four males ended as winning males, one of them totally dominant.

In parallel 20 and 22 both Lake Nesa males were found to have small, white dots covering their skin surface. Both of these two males ended up receiving eggs.

In parallel 18 and 21 both Lake Vigdar males were found to have one small worm each in their abdomens, possibly small *S. solidus*, too small for manipulation. Both males readied territories and nests, attempted courtship and had more intensive dominance display than their opponents; Lake Vigdar Male from parallel 18 totally dominating its opponent Lake Nesa male.

In parallel 17 the female was found to have a small worm-like parasite in the abdomen, possibly a small schisto. In parallel 20 the female had opercula and nearby skin covered by a large amount of small, white dots (> 100), and some few spread over the rest of her body surface. Both females spawned and were clearly not castrated by infections. However, female from parallel 20 spawned a conspicuously small clutch, consisting of only 10 eggs.

3.6.3. Male Red Nuptial Coloration

A comparison of male red nuptial coloration within parallels is shown in table 8. Brightness of red nuptial coloration is expressed by number 1-4, in which 1 indicates a dull male and 4 a very bright male.

Table 8: Male Nuptial Coloration. Male intensity of red nuptial coloration is given by a 4-point scale, at which 1 = dull, 4 very bright/intensely colored. Scoring done based on photographs taken directly after ended experiments. The rightmost column enlists winner males in those parallels having valid female choice.

P. Nr. = Parallel Number; DR = Dominance Relationship: Total, Strong, Weak or No dominance

P. Nr.	**Lake Vigdar male**	**Lake Nesa male**	**Adaption phase: Dominance (dominant male, population)**	**Maine Phase: Dominance (dominant male, population)**	**Valid Female Choice: Winner Male**
1	3	1	Total (Vigdar)	Total (Vigdar)	-
2	3	2	Strong (Nesa)	No dominance	Vigdar
9	4	3	Strong (Vigdar)	Weak (Nesa)	Vigdar
11	1	3	Strong (Nesa)	Total (Nesa)	-
13	4	2	Strong (Vigdar)	Weak (Nesa)	Vigdar
14	3	3	No dominance	No dominance	Nesa
16	2	2	No dominance	No dominance	Vigdar
17	4	4	Weak (Nesa)	Strong (Nesa)	Vigdar
18	3	2	Total (Vigdar)	Total (Vigdar)	-
19	2	2	No dominance	No dominance	Vigdar
20	4	3	-	Strong (Nesa)	-
21	3	3	Weak (Vigdar)	No dominance	Nesa
22	4	4	-	Strong (Vigdar)	Nesa
23	2	3	Strong (Nesa)	Total (Nesa)	-

Excluding non-valid parallels based on male dominance and lack of female choice. In three parallels females chose the brighter male; all of these Lake Vigdar males. In 6 parallels there is no difference between males. Amongst valid parallels, never does a female choose a duller male of a pair. Including the excluded parallels, in 4 of these the brightest male did also receive eggs, yet in parallel 20 the dominant and egg receiving Lake Nesa male is less brightly colored than Lake Vigdar male, scored 3 and 4 respectively.

In 6 parallels the male having brightest red nuptial coloration is also the most dominant in adaption phase. In those three parallels having no dominance relationship between males in adaption phase, there is neither any difference between males in intensity of red nuptial coloration. In three parallels the intensity of red nuptial coloration fails at explaining dominance relationships seen in adaption phase.

In 4 parallels the male having the brightest red nuptial coloration is also the most dominant in main phase. In four of the parallels having no dominance relationship, males are equal by intensities of red. In 6 parallels the intensity of red nuptial coloration fails at explaining dominance relationships seen in main phase.

3.7. Summing Up Mating Preference Results

Outcomes from the three tested predictions are summed up in table 9.

Table 9: Summed indications of mating preferences

	Indications of mating preferences				
Test	**Homogeneous mating**	**Heterogeneous mating**	**No pref.**	**Lake Vigdar population**	**Lake Nesa population**
Female choice of male				X	
Egg distribution			X		
Male preferences	X				

There is not a consistent pattern from the experiment with regard to the three predictions in individuals mating preferences. Female choices show no preference for males from same population, but indicate a stronger preference for Lake Vigdar males. Clutch sizes indicate a female preference for Lake Nesa males, yet lack of egg-retention or spreading of clutches leave this non-indicative. Male choice seems to give a preference of females from their own population; however, data for this last prediction are correlational of nature given that males have no choice between two females.

4. Discussion

4.1. Predicting Reality?

This study aims at studying if morphological differences between two parapatric populations are maintained through assortative mating. The threespine stickleback populations of Lake Nesavann and Lake Vigdarvann are connected through Lake Liavann, and the population from Lake Nesavann is free to migrate into Lake Vigdarvann. Both morphotypes of pelvic complex is found in Lake Liavann, accompanied by a range of intermediate hybrids. The abrupt change in pelvic morphology in the water-channel leading from Lake Nesavann via Lake Liavann into Lake Vigdarvann is highly conspicuous, changing from polymorphic for different degree of pelvic expression to monomorphic completely developed pelvic complex. This difference in morphology among these two parapatric populations has to be maintained by some sort of mechanism, and one plausible is reproductive isolation. One reproductive isolation mechanism is assortative mating.

Assortative mating might arise and drive divergence of populations even in the face of frequent gene flow, either through mating preferences for similar-based ecological traits, sexual selection, or the emergence of interval-based assortative mating (Barreto & McCartney, 2007; Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998; Pryke, 2009; Turner & Burrows, 1995;). Although assortative mating might emerge and proceed even at relatively weak disruptive selection, increasing hybrid fitness deficiencies further facilitates divergence and fixation of assortative mating traits and preferences (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998). In situations of which assortative mating cannot drive the divergence, it might still prevent already diverged populations from merging during secondary contact (Kondrashov & Shpak, 1998). While much theory presented here represents divergence in sympatry, there is no reason it should not apply to parapatric divergence as this further restricts gene flow and expectedly should facilitate divergence (Turner & Burrows, 1995). Examples of assortative mating preventing morphologically diverged populations from mixing are many. Representing some are parapatric populations of soldier beetle *Chauliognathus pennsylvanicus* mating assortatively based on a size-dependent morphological trait (McLain, 1985), color pattern-based assortative mating between natural populations of *Hypoplectrus* sea basses (Fisher, 1980), and populations of fruit-parasitizing flies with assortative mate preferences resulting from natural selection on ecotypes and niche divergence despite gene flow (Freeman & Herron, 2007). Also within the Gasterosteidae assortative mating is commonly found, e.g. morphologically diverged parapatric ninespine sticklebacks in Russia (Ziuganov & Zotin, 1995), sympatric populations of threespine sticklebacks on Iceland (Ólafsdóttir et al., 2006), sympatric species pairs of limnetic/ benthic threespine sticklebacks from Canada (Nagel & Schluter, 1998; Rafferty & Boughman, 2006), and assortative mating in populations of stream-anadromous sticklebacks (McKinnon et al., 2004; Scott, 2004).

The sudden change in morphotypes seen in the water-channel leading from Lake Nesavann into Lake Vigdarvann could result from assortative mating, and three predictions are made and tested in order to check for this.

The first prediction predicts females to strongly prefer to mate homogeneously with males from their own population. The extensive courtship found in the threespine stickleback is regarded to be important in ensuring conspecific mating and enhancing reproductive isolation between closely related species (Wootton, 1976). The examples of assortative mating in ninespine and threespine stickleback from Russia and Canada involve population pairs of which one show a complete reduction of the pelvic complex (McPhail, 1992b; Ziuganov & Zotin, 1995). Male ninespine sticklebacks usually have bluish-white colored ventral spines very visible in water and known to be of importance in courtship display (Wootton, 1976), and also in the blackspotted stickleback (*Gasterosteus wheatlandi*), males are known to use ornamented pelvic spines in courtship (Rowland, 1994). Several populations of threespine stickleback have been found to possess reddishly ornamented pelvic spines (McLennan & McPhail, 1989a; Nordeide, 2002); observed excessively expressed during breeding season (Nordeide, 2002). Rowland (1994) argues that ecological differences experienced by different populations might likely facilitate divergence of courtship behavior. The function of pelvic spines in male threespine stickleback courtship display is poorly documented, yet pelvic spines have been shown to be of some importance in aggressive displays (McLennan & McPhail, 1990). In general, mixing of morhologically diverged populations might involve varying sorts of fitness deficiency for hybrids (Naisbit, Jiggins, & Mallet, 2001; Rogers & Bernatchez, 2006; Rundle & Nosil, 2005), e.g. Gow, Peichel, & Taylor (2007) find ecological selection against hybrids to be of importance in sympatric species pairs of limnetic/ benthic threespine sticklebacks. Observed populations with high occurrence of hybrid individuals and lack of assortative mating could merely represent an initial stage of diverging populations (Dieckmann & Doebeli, 1999; Ziuganov & Zotin, 1995). Sampling of male nests and comparing which males receive eggs for all successfully run parallels give no support to the predicted homogeneous assortative mate-preferences. The absence of pelvic spines clearly does not result in communication breakdown during courtship between these two morphs. However, there is reason to believe that directly determining female choice and mate-preferences based on comparison of all nests sampled might not be all there is to it, as the experimental design allowed free interaction of all fish. Male threespine sticklebacks are known to be highly aggressive throughout the breeding season (Wootton, 1976). Size of territory positively affects male's chances of getting females into their nest, thus acquiring, maintenance and expenditure of territories are vigorously fought for (van den Assem, 1967). Male-male interactions can lead to male suppression, deriving females of a choice between males (Bakker & Sevenster, 1983; Östlund-Nilsson & Nilsson, 2000). While males were confined within isolated enclosures up to the point of female insertion, adaption phases in particular provide opportunities for males to interact, settle dominance relationships, and expand/restrict territory boundaries between them. This of course goes further on throughout main phase. Male dominance relationships are corrected for going through video recordings and data sampled from this. By correcting for dominance relationships and excluding those parallels in which there is seemingly a lack of free female choice, a more realistic picture of female mate preferences can be found. Correcting for dominance relationships, no indication of homogeneous assortative mating is found between Lake Vigdarvann and Lake Nesavann populations of threespine stickleback. Instead, a trend of females from both populations preferring Lake Vigdar males emerges.

The second prediction state that assortative mating could occur even if females mate with males from both populations, by females spawning larger clutches for males of their own population. Females are expected to be the choosiest sex, both from theory and shown in nature, and threespine sticklebacks are no exception (Foster, 1994). Females of sexually reproducing species might engage in multiple matings to increase the genetic compatibility of offspring (Krebs & Davies, 1993). Female zebra finches actively engage extra-pair matings with males of higher quality than their initial mates (Houtman, 1992), and similarly female blue tits shopping for good genes (Kempenaers, Verheyen, & Dhondi, 1997). The female European bitterling *Rhodeus amarus* prefer spawning in the presence of multiple males, although males are territorial, and guppy females, having internal fertilization, are able to adjust number of sperm transferred during copulation thus favoring sperm of more attractive males (Candolin & Wong, 2008). Female cuckoldry has been reported from largemouth bass (Avise, Jones, Walker, DeWoody, & collaborators, 2002), and multiple paternity occur in mouthbrooding cichlids (Sefc, Hermann, & Koblmüller, 2009); in addition to intraspecific brood mixings between females holding them (Avise et al., 2002). Both inter- and intraspecific brood mixing occur in cichlids, and one of many suggested explanations for this is that farming out it ensures the survival of at least some of the young (Kellogg, Markert, Stauffer, & Kocher, 1998). Female sticklebacks are able to spawn clutches of several hundred eggs every 3-5 days throughout a 2-3 month long breeding season when kept in laboratory conditions, yet in field this is likely to be highly restricted because of cost-inflicting factors, for instance food limitation, predators and parasites (Whoriskey & FitzGerald, 1994). In populations experiencing relatively short breeding season females might only manage to spawn once or twice during their whole lifetime (Whoriskey & FitzGerald, 1994). Mating with a sub-optimal mate is costly to males, but more so to female sticklebacks having to build up a new clutch of eggs. Female sticklebacks assess males on behalf of multiple cues (Candolin, 2003; Heuschele et al., 2009), and assuming some degree of hybrid deficiencies from mixing the two studied populations (Naisbit et al., 2001; Rogers & Bernatchez, 2006; Rundle & Nosil, 2005), males being unequally attractive based on other factors than general population differences could force females into trading off population preferences against general male preferences. Furthermore, females are believed to decrease risk of filial cannibalism through spawning with males already having eggs in their nests (Ridley & Rechten, 1981; Rohwer, 1978). In some stickleback populations large aggregations of cannibalistic conspecifics are normal (Shaw, Scotti, & Foster, 2007). In such situations, spreading of risk through spawning with multiple males might be adaptive. If female threespine sticklebacks are able to discriminate between males with respect to amount of eggs spawned, male preferences should be detectable from of differences in clutch sizes, and females spawning in both males nests should occur. No significant difference in mean clutch size spawned for males of own and foreign population is found, nor is there any direct evidence from nest-samplings indicating that females spread their clutches by mating with both males. No clutch sizes greater than 40 was found from heterogeneous spawning's, while 4 clutches from homogeneous spawning's exceeded 40 eggs. Observations from dissections do not, however, support an idea of females retaining eggs for later spawning. Retaining eggs could prove costly if no mating opportunity presents itself within short time and eggs would have to be dropped or females would risk becoming egg bound (Guderley, 1994; Wootton, 1976). Males prefer females with great abdominal distention (Kraak & Bakker, 1998) and for females to attain such a second mating might be both more costly and challenging than beneficial. Low clutch sizes found might be resulting from egg theft or cannibalism (Ridley & Rechten, 1981; Rohwer, 1978). While filial cannibalism is unlikely to have occurred given that they were fed throughout experiments, theft by opponent male could be a possible explanation for some of the smallest clutch sizes found. However, there is little direct evidence for this, as only once was there found eggs in both nests; in that one case 1 egg was found in the loosing males egg. Furthermore, in only one instance was eggs found devoured by individuals in the post-experimental dissection.

As the outcome of testing the first prediction show a potential higher preference for Lake Vigdar males from females of both populations, this second prediction is tested for the same. There is, however, no indication of a similar preference to be found based on clutch sizes spawned. Quite contrary, there is a tendency for Lake Nesa males to receive larger clutches.

The third prediction predicts males to homogeneously prefer females from their own population. Male threespine sticklebacks are restricted with respect to immediate refraction periods after receiving spawning, the amount of eggs they can fit into their nests and parental periods lasting 2-3 weeks (Östlund-Nilsson, 2007; Wootton, 1976). It has been demonstrated that males actively choose between females, discriminating with respect to female quality (Kraak & Bakker, 1998; Rowland, 1982). Male mate-preferences could potentially drive selection for female ornamentation, e.g. male gobies' mating-preferences have been suggested to drive selection for female ornamentation (Amundsen & Forsgren, 2001). In some populations female threespine sticklebacks possess dorsal barring-patterns likely to have an effect in signaling sexual receptiveness, yet differences in predation pressures have been suggested to possibly affect selection for this trait as well (Rowland, Baube, & Horan, 1991). Pelvic girdle lengths have been found to vary with respect to sex, in which females have larger pelvic girdle than males, possibly adaptive regarding anti-predation (Reimchen & Nosil, 2006). And, while ninespine stickleback display of colorful pelvic spines is a male trait (Wootton, 1976), the reddishly ornamented pelvic spines reported from threespine stickleback populations concern both males and females; however, it was found to have a negative effect on males i.e. males preferred drab non-ornamented females (Nordeide, 2002). It is not known if Lake Vigdar population shows any degree of pelvic spine ornamentation as this was never checked for, nor is it known if these two populations differ with respect to female barring patterns. Yet, if hybrids of these two morphs suffer from any sort of negative hybrid fitness (Naisbit et al., 2001; Rogers & Bernatchez, 2006; Rundle & Nosil, 2005), it should be selectively advantageous also for males to choose females to mate with from their own populations (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998; Turner & Burrows, 1995). Male-male competition might affect the extent of male choice, in which case only males in good condition might afford being picky (Candolin & Salesto, 2009); males of poor condition were avoided when selecting fish for the experiments and fish were fed constantly throughout the period of the trials. Indications from male domination regimes support that males fight harder for females of their own population, indicating a male preference for homogeneous mating. However, the experimental design does not allow males to discriminate between females from either population, and these data regarding male preferences are thus only correlational. Although indicative, no conclusion can be made stating that it is in fact a strong preference for homogeneous females creating the observed pattern.

The Lake Vigdar female used in parallel 22 was later found to have an asymmetrical pelvic complex, highly indicative of a hybrid between the two morphs; thus excluded from the conclusive evaluation of all three predictions. Another possibility is that she had been damaged at some stage during life, in which case excluding this parallel from the outlined analyses of assortative mating would be wrong. Being used as a Lake Vigdar female, this female chose a Lake Nesa male, with an indicative strength of 2. Including this parallel would have made the overall pattern of a general female preference for Lake Vigdar males vague; only 1 choice in difference for type of male chosen by Lake Vigdar females. Furthermore she spawned 30 eggs for this Nesamale. Including parallel 22 in the analysis of egg distribution does not change indications from this. Regarding male preference, there was a noted homogeneous male preference, assuming she was a Lake Vigdar female, yet including this parallel does not change the outcome of this prediction either.

4.2. Confounding Effects

No consistent pattern of assortative mating between the two studied populations is found from testing three indicative predictions. This can be interpreted as no existence of assortative mating between the populations, or that confounding effects conceal its existence. This will be discussed below.

4.2.1. Size Effects

Adult females are generally larger than adult males (Kitano, Mori, & Peichel, 2007), and males have been shown to prefer larger females providing them larger and higher quality eggs (Kraak & Bakker, 1998). Female preferences for large males have been shown (Rowland, 1989a; Rowland 1989b), and might be adaptive considering nesting success and paternal care (Kitano et al., 2007). Larger males are found to have a competitive advantage when exceeding 15% difference in weight compared to a smaller male (Rowland, 1989b), yet small males have advantages through earlier maturation and acquiring better territories simply because they occupy these before larger males are matured (Kitano et al., 2007). Size have been found to be an important cue mediating assortative mating in both sympatric benthic/ limnetic (Nagel & Schluter, 1998) and stream-anadromous populations of threespine stickleback (McKinnon et al., 2004, 2012), and it is natural to study if any pattern of female or male preferences can be explained by size differences. However, as the largest male won in less than half of the parallels, and the size difference within most of these male-pairs was small, nothing indicates that the largest males were consistently chosen. Size does not explain dominance relationships seen among males, upon which males preferences for females are tested. Further, no indication emerges of larger females eliciting more fighting and aggressive display amongst male sticklebacks.

4.2.2. Parasite Infections

Parasite infections might repel conspecifics through the direct risk of parasite transmission (Dugatkin, FitzGerald, & Lavoie, 1994) i.e. *G. anomala* in this case (Ward et al., 2005), and further correlates with male condition (Milinski & Bakker, 1990). Females commonly avoid males of poor condition, representing neither high quality genes nor good parental ability (Milinski & Bakker, 1990; Reusch et al., 2001; Sommerfeld et al., 2008). Avoiding parasitized males through choosing brightly colored males indirectly selects for offspring with high parasite resistance (Barber, Arnott, Braithwaite, Andrew, & Huntingford, 2001). The commonly found cestode in threespine stickleback populations, *S. solidus*, functionally castrates its host when exceeding a given size (Barber & Scharsack, 2010), highlighting the importance of avoiding parasite infections when testing for mate preferences. Therefore, if a female in the test was presented for a parasitized male from the same population, a preference for this male might have been overridden by a negative preference for infected and perhaps castrated males. To control for this all fish were examined for parasites and general condition before being chosen for the experiment, and the fish were dissected after the experiment to assess parasite status. Some minor infections were found from dissections, but male behavior seems not to be affected by this and since many of the males with minor infections ended up receiving the eggs, it did not appear to influence mating success. Excluding unsuccessful infected males did not strengthen any of the predictions supporting assortative mating. Two of the females used were found to have minor infections from dissection; one of these a tiny *S. solidus*. Parasite infections could potentially have affected size of clutches (Barber & Scharsack, 2010). Excluding these from analyses of clutch sizes spawned does not significantly affect any of the two analyses of egg distribution. It does slightly strengthen an indication of larger clutch sizes spawned for homogenous males, and strengthens the difference in clutch sizes spawned for males of the two populations in general, in favor of Lake Nesa males in general receiving larger clutch sizes.

4.2.3. Male Red Nuptial Coloration

Intensity of male red nuptial coloration correlates with male dominance status (Bakker & Sevenster, 1983). Several studies have shown a female preference for male red nuptial coloration (McLennan & McPhail, 1990; Milinski & Bakker, 1990), although later studies are suggesting the importance to be overrated (Braithwaite & Barber, 2000; Östlund-Nilsson & Nilsson, 2000). Competition between males further enhances differences in males condition thus differences in intensity of red nuptial coloration (Candolin, 1999), which serves as an honest signal of male parental ability (Candolin, 2000). Differences in males with respect to condition and attractiveness to females are expected to be reflected in males' coloration after ended experiments. In those parallels having males differing with respect to red nuptial coloration, this proved to be an accurate predictor of male success. In 3 out of those 9 parallels interpreted as free female choice based on video analysis, there is a difference in brightness favoring Lake Vigdar males. In all of these three parallels the brighter Lake Vigdar male ends up receiving eggs. Excluding these three weakens the above indicated trend of females in general preferring males from Lake Vigdarvann. Only once did a female choose a duller male, however, this male was likely dominant based on video analysis. Intensity of red coloration correlates less with determined dominance regimes based on video analysis. Female spawning affects behavior of all three fish in a parallel (Wootton, 1976), thus females' choice could affect male-male relationships following spawning; considering that pictures were taken after ended experiments this might explain deviation between color-intensity and registered behavior. The stated correlation between intensity of red and dominance status have later been found to not always apply equally well in predicting outcome of male-male encounters, especially when difference between males' color intensity is low (Bolyard & Rowland, 1996; McLennan & McPhail 1989b; Rowland, 1989b; Rowland, Bolyard, & Halpern, 1995). In fact, in all except one, parallels having conflicting data from video analysis and male nuptial coloration considering dominance, males differ no more than 1 point on the color-scale. Furthermore, populations might differ in condition-dependent expression of red nuptial coloration (Boughman, 2007). An alternative explanation would be that parameters used from video analysis in this study to determine male-male dominance relationships poorly reflect reality.

4.3. Conclusion

In total there is no clear evidence towards assortative mating being an important mechanism in keeping these two morphs from mixing in Lake Vigdarvann. Testing the initial predictions, only weak and contrasting patterns emerge regarding females preferences. Male preferences can indicate homogeneous mate choice, yet is of little conclusive value given the correlational nature of the data. The strength of the conclusions from the experiment suffers from the number of parallels becoming lower than planned, this as a consequence of males unsuccessful of building nests or unexpected male mortality after onset of trials. Ending up with fewer parallels than planned, the only chance for a conclusive result would be to find an absolute reproductive barrier between the two populations.

Failing at producing evidence of assortative mating between these two populations does not mean a tendency for preferring a mating partner from the same population may not exist. The simplest explanation would be the lack of statistical power because of the low number of successfully run parallels. However, it is hard to argue for this, lacking even weak patterns indicative of homogeneous assortative mating, not to mention the lack of consistency among patterns found.

Another possibility is that the experimental setup fails to include important factors working under free-living conditions, thus failing to detect existing assortative mating. Laboratory conditions can lead to different expression of phenotypically plastic traits and behavior to what occurs in nature, in which case it may very likely affect outcomes of experiments testing assortative mating (Shaw et al., 2007). Benthic/ limnetic species pairs are known to have different microhabitat preferences for spawning (Boughman, 2001), and a sympatric species pair from Iceland is suspected to have differing preferences regarding nest (Ólafsdóttir et al., 2006). Sticklebacks are known to vary in respect to preferred substrate for nest building (Rowland, 1994), as well as having specific nest material preferences (Östlund-Nilsson & Holmlund, 2003). It could be that the experimental design, placing both males in similarly standardized environments, prevents the females from distinguishing between males to the same extent as in nature. However, as fish from both lakes were sampled from very similar areas, there are no mentionable differences in vegetation between lakes, nor any great differences in lake characteristics suggesting such diverged preferences, this seems unlikely. Furthermore, no notable differences in nest structure were observed.

The conclusion drawn from my experiment is that existence of assortative mating cannot be ruled out, but it seems not to be the most likely candidate to prevent hybridization and preserve the sharp morphological distinction between these two parapatric populations of threespine stickleback in the Vigdar watershed.

4.4. Possible Explanations and Suggestions for Future Research

Working with soldier beetles, McLain (1985) suggest a possible genetic link between mate-preferences and expression of the trait. Recent studies are increasingly finding such links located on sex chromosomes (Pryke, 2009). Linkage of genes controlling expression and preference for a trait on sex chromosomes reduce recombination and presents one explanation to the persistence of assortative mate-preferences through sexual selection in the face of frequent gene flow, suggesting genes on sex chromosomes to be of particular importance in evolution of assortative mating (Pryke, 2009). Reduction of threespine stickleback pelvic complex is controlled by regulative changes in the expression of the pitx1-gene (Shapiro et al., 2004), yet these changes are not linked to the linkage group corresponding to sex-chromosomes in the threespine stickleback (Peichel et al., 2004; Shapiro et al., 2004), thus divergence of pelvic morphs seems highly unlikely to arise through strong sexual selection on the trait. Kondrashov and Shpak (1998) through modeling show that assortative mating might arise and become fixed without any sort of disruptive selection, however they further stress that this requires strictly defined circumstances which rarely applies in nature. Thus if the two studied populations are to have assortative mating preferences based on pelvic complex itself, it should likely arise from ecological similar trait-preferences, a process of divergence that might proceed at very slow rates initially (Dieckmann & Doebeli, 1999; Kondrashov & Shpak, 1998). Giles (1983) hypothesize limiting calcium concentrations to explain differences in expression of pelvic complex, however, calcium concentrations between Lake Vigdarvann and Lake Nesavann are both low and barely differ, as such unable to explain differences seen in these two populations' morphology. Extreme differences were found in parasite infections from the two pools of sampled individuals from Lake Vigdarvann and Lake Nesavann, and if this reflects a real difference in parasite pressures experienced by the two populations, differences in parasite pressure should favor allocation of more energy towards immunological defence in the Lake Nesa population (Tschirren & Richner, 2006), potentially on the expense of pelvic complex development. A recent study of parasite resistance found no evidence of a difference between the two populations, yet further investigations are required (Ole J. Fosså̊s, Master Thesis, submission June, 2013). An increase in resource allocation toward immunological defence expectedly should affect allocation of carotenoids for sexual display (Wedekind, Meyer, Frischknecht, Niggli, & Pfander, 1998). Sampled Lake Vigdar males were in general more sexually ornamented with respect to overall body coloration, nuptial red coloration and blue eyes (Torben Lode, 2012, personal observation); dietary differences do not explain differences as both populations were fed the same. Towards the end of the study, Lake Vigdar males started to die off at a much greater rate than Lake Nesa males, and fish from both populations brought in from a late-summer sampling and kept in a separate pair of tanks showed the same difference in mortality, ruling out the possibility of a local disease outbreak in one storage tank explaining mortality trends. If seen differences in parasite load on sampled populations reflect a difference experienced in nature, Lake Vigdar males could be selected to allocate more resources for intensive sexual ornamentation (Boughman, 2007; Wedekind et al., 1998); which could potentially lead on to assortative mating (Kondrashov & Shpak, 1998). Predation is another factor to consider. If differences in predation pressure experienced exert high selective pressure on different morphological anti-predator traits, this could effectively wipe out all fish migrating downwards into Lake Vigdarvann (Reimchen, 1980). Using a fish-mimicking predator model, Lake Nesa fish are found to be bolder, suggesting a difference in predator pressures experienced in the two lakes (Lars E. Torgilstveit, Master Thesis, submission June, 2013). Differences in fish and invertebrate predator regimes combined should mediate selection for different anti-predator traits (Reimchen, 1980). Studies on invertebrate predation on sticklebacks have found predation by *Notonecta* sp. to have a greater effect than predation by *Aeshna* sp. (Marchinko, 2009; Mobley et al., 2013; Vamosi, 2002; Zeller et al., 2012), and investigation of invertebrate faunas in these two lakes combined with an experimental test of effects would be interesting. Differences in parasite and predation pressures coupled might explain evolution and maintenance of the two distinct stickleback morphs studied. A pond-experiment aimed at testing correlational effects of trout predation and infection by *S. solidus* both individually and coupled was started in collaboration with 2 other students and supervisors for this study. However, practical challenges with infections delayed progress and lead to the onset of winter, which further complicated matters; thus this was never completed. Ziuganov and Zotin (1995) do a similar experiment, only without parasites and using both fish and insect piscivorous predators. Testing ninespine sticklebacks with fully expressed or absent pelvic complex, Ziuganov and Zotin (1995) find clear effects of predation on survival of ninespine sticklebacks differing in pelvic expression, in support of Reimchens'

(1980) predation hypothesis. Furthermore, if different mortality pressures are experienced by Lake Nesa and Lake Vigdar populations this could impact on life histories and rates of sexual maturation (Begon, Townsend, & Harper, 2006), and potentially result in interval-based assortative mating (Kondrashov & Shpak, 1998).

4.5. Experimental Setup: Suggestions for Improvement

The by far most limiting factor in this study is the low amount of successfully run parallels, to a great extent resulting from problems establishing males and male mortality. By using separate tanks for male establishment, and when ready transfer males alongside their nests to test compartments (Milinski & Bakker, 1992; Östlund-Nilsson & Nilsson, 2000), one may create a pool of males ready to use. Thus, parallels can be started immediately after a short acclimatization phase for males, reducing the total time it takes to fulfill a satisfyingly amount of parallels. Saving of time clearly can be of crucial importance as challenges with mortality and establishment failure increase towards the end of the season. Several studies use behavioral cues to assess males and females readiness to court/ spawn (Milinski & Bakker, 1990; Ólafsdóttir et al., 2006; Östlund-Nilsson & Nilsson, 2000), and adding usage of such cues would decrease likelihood of using *S. solidus* infected females and likely lower mortality amongst males used. Furthermore, using a maximum time limit of a few hours for trials to run would likely present little problem (Luttbeg et al., 2001; Scott, 2004); saving time thus facilitating a greater amount of successfully run parallels.

The open design allowing females to freely interact with both males is necessary when testing for assortative mating preferences (Luttbeg et al., 2001; McLennan & McPhail, 1990; Östlund-Nilsson & Nilsson, 2000), yet at the same time presents confounding male-male interactions (Bakker & Sevenster, 1983). Although video analysis is used here to correct for male-male dominance relationships, it is preferable to limit the extent of male-male interactions firsthand, e.g. by leashing males (Östlund-Nilsson & Nilsson, 2000) or increasing length between males (Luttbeg et al., 2001). Alternatively, inserting enclosed females in bottles during trials into male nesting areas, in the same manner as used for nest-building stimulation, might lower male-male interactions. Focusing on selecting males of more similar red nuptial coloration intensity is also likely to reduce problems with male dominance

(Bakker & Sevenster, 1983); however, there could be differences between populations' condition-dependent expression of red coloration (Boughman, 2007), see 4.5. Possible Explanations and Suggestions for Future Research.

To accomplish the initially planned amount of 40 parallels, camera positions were rigged to allow running 4 parallels simultaneously, using 1 camera. However, this comes at the cost of lower detail level, and with the setup used especially at the one side furthest from camera. Camera position was changed at the end of the study (only one successful parallel run with this new position), placed over two test compartments middle; allowing recording of only two parallels simultaneously. This is preferable, as it gave higher levels of details e.g. easier to observe female entering nest, and removes problems related to camera-side and refraction given that it evens out on each side; wide-angle preferably should still be corrected for. This closer camera position also enables more detailed scoring of different sorts of male-female interactions and displays. Using same-length (1.6m) test compartments and depressing male aggression with bottled females would allow such high-detailed recording; see McLennan and McPhail (1990) and Nagel and Schluter (1998) for examples of more detailed behavioral scoring. Nest-box sand contrasting more to sticklebacks would enhance registering of fish and activities; however it should not be so conspicuous that it repels males from establishing nests. Furthermore, the more structural complexity, the harder to track fish; while some studies use plants to provide shelter, this would be highly disfavored when afterwards tracking fish on video. The necessity of partition walls should be reconsidered if males' aggression and intra-sex interactions are staggered elsewise. If sufficiently contrasting background and low compartment complexity is provided, one might even be able to test usage of automatic fish tracking, more information on this in Haverflock guide to image analysis with ImageJ (Zamani et al., 2012).

The importance of including adaption phases in the analysis must be emphasized, especially in parallels having early spawning. What is seen in main phase could be the result of an already established male dominance relationship from adaption phase, e.g. parallels having early spawning and no registered dominance relationship between males in main phase could result from one of the males already being suppressed, hence no male-male interactions seen from analyzed part of main phase. Furthermore, adaption phases are found quite accurate in predicting winner male in cases of total or strong dominance relationships during adaption phase; however, this could be a random artifact resulting from low sample size.

Finally it must be said that working with behavior and analyzing this, experience goes a long way. When starting this study my experience with threespine sticklebacks and stickleback behavior was at best very limited, and no doubt would I be knowledgeably better equipped if I were to perform a similar series of stickleback mate choice trials and behavioral analyses again.

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Appendix A – Male Location

Gives an overview of male location parameters for adaption phase, parameters measured are males' average distance to female box (fm-box) and males' average distance to own nests (ownnest), both measured in cm. Values are given both before and after camera-side correction; correction factor for camera correction is given in the leftmost column.

	Adaption phase					...>	corrected for camera-side effect			
	Parallel nr	Lake Vigdar Male		Lake Nesa Male			Lake Vigdar Male		Lake Nesa Male	
corr factor		fm-box	ownnest	fm-box	ownnest		fm-box	ownnest	fm-box	ownnest
1,089	1	27,6	53,2	50,5	34,6		30,1	57,9	50,5	34,6
1,086	2	45,4	21,5	37,9	64,4		45,4	21,5	41,2	69,9
1,087	9	33,1	42,6	47,1	23,7		33,1	42,6	51,2	25,8
1,076	11	39,8	30,1	31,6	36,4		42,8	32,4	31,6	36,4
1,138	13	32,1	53,8	55,0	12,1		36,5	61,2	55,0	12,1
1,145	14	27,4	37,5	36,5	20,8		27,4	37,5	41,8	23,8
1,14	16	34,6	33,6	36,6	37,2		39,4	38,3	36,6	37,2
1,113	17	48,8	26,4	27,4	52,3		48,8	26,4	30,5	58,2
1,11	18	48,8	78,5	8,2	57,9		54,2	87,1	8,2	57,9
1,103	19	17,0	50,8	39,0	20,6		17,0	50,8	43,0	22,7
1,105	20	NA	NA	NA	NA		NA	NA	NA	NA
1,106	21	7,7	48,4	38,3	26,4		8,5	53,5	38,3	26,4
1,188	22	NA	NA	NA	NA		NA	NA	NA	NA
cam mid	23	28,6	36,1	39,8	48,9		28,6	36,1	39,8	48,9

Gives an overview of male location parameters for main phase, parameters measured are males' average distance to female box (fm-box) and males' average distance to own nests (ownnest), both measured in cm. Values are given both before and after camera-side correction; correction factor for camera correction is given in the leftmost column.

		Main phase, corrected for spawning				...>	**corrected for camera-side effect**			
	Parallel nr	Lake Vigdar Male		Lake Nesa Male			Lake Vigdar Male		Lake Nesa Male	
corr factor		fm-box	ownnest	fm-box	ownnest		fm-box	own nest	fm-box	ownnest
1,089	1	50,4	24,3	50,5	60,4		54,9	26,5	50,5	60,4
1,086	2	46,7	20,2	49,5	11,2		46,7	20,2	53,8	12,2
1,087	9	50,4	15,7	33,3	30,5		50,4	15,7	36,2	33,2
1,076	11	30,5	45,6	47,9	48,8		32,8	49,1	47,9	48,8
1,138	13	49,3	8,7	42,3	30,5		56,1	9,9	42,3	30,5
1,145	14	38,2	26,6	56,2	5,2		38,2	26,6	64,3	6,0
1,14	16	51,9	6,1	51,6	21,1		59,2	7,0	51,6	21,1
1,113	17	56,2	10,8	31,9	35,8		56,2	10,8	35,5	39,8
1,11	18	50,1	72,8	37,1	67,2		55,6	80,8	37,1	67,2
1,103	19	54,7	11,4	54,8	6,9		54,7	11,4	60,4	7,6
1,105	20	53,2	11,9	30,6	32,5		53,2	11,9	33,8	35,9
1,106	21	32,4	26,0	36,1	29,5		35,8	28,8	36,1	29,5
1,188	22	22,6	37,3	46,6	21,1		26,8	44,3	46,6	21,1
cam mid	23	46,5	46,2	46,9	67,6		46,5	46,2	46,9	67,6