Mate choice in white and common Threespine Sticklebacks

(*Gasterosteus aculeatus*)

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ABSTRACT

During speciation, mate choice can reproductively isolate one group from another through selection on divergent traits and behaviours such as colouration, courtship behaviour, and/or body size. Reinforcement can also limit interbreeding between ecotypes in sympatric populations through these sexually selected traits. The primary goal of this research was to investigate male mate choice in common and white Threespine Sticklebacks *(Gasterosteus aculeatus)* that are known to mate assortatively in sympatric populations and who differ markedly in their propensity for paternal care. I observed mate choice of both male and female sticklebacks from sympatric and allopatric populations. Neither common nor white male stickleback courted or mated assortatively. Additionally, female stickleback did not differ in their responses to males of either ecotype. Our research suggests that there is no ecotype-based preference for white and common Threespine Sticklebacks and that mechanisms other than reinforcement may be responsible for the maintenance of common and white Threespine Stickleback ecotypes.

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1. BACKGROUND

1.1 Sexual selection and speciation

Sexual selection is one of the mechanisms that can lead to speciation as it can act as a barrier to reproduction for sexually reproducing organisms (Fisher 1930; 1958; Lande 1981; 1982). Biological speciation occurs when reproductive isolation between two closely related organisms exists, creating genealogically distinct individuals that do not produce viable offspring (Coyne and Orr 2004). This isolation may arise from sexual selection acting on traits associated with mating success (Darwin, 1871; Hosken and House 2011), which occurs via two primary mechanisms: intra-sexual competition and mate choice (Andersson 1994; Maan and Seehausen 2011). Both mechanisms can occur when members of the non-limiting sex vary in their ability to acquire mates, which can influence the variation in reproductive success and alter the intensity of sexual selection for favourable traits. The intensity of sexual selection and variance in reproductive success are expected to be higher in situations where mates or resources are spatially clumped and/or asynchronous and can be monopolized by a few individuals (Emlen and Oring 1977). The distribution of mates in space and time directly affects the operational sex ratio (OSR: amount of sexually active males to receptive females in a population, Emlen 1976), which can be used to predict the direction and intensity of sexual selection. Similarly, the potential reproductive rate (PRR) can influence the availability of males and females to mate, as 'time-outs' occur when gametes are replenished or if parental care is required (Clutton-Brock and Vincent 1991; Clutton-Brock and Parker 1992). In natural populations, males are typically the non-limiting sex; however the inverse mechanisms do certainly occur (see Hosken and House 2011 for a review).

When the sex ratio is skewed toward females, males become the limiting sex if they are the only parent to invest in the care of their offspring (i.e. uniparental male care, Kraak and Bakker 1998; Emlen and Wrege 2004). Additionally, when a male is the sole provider of parental care, males may be choosy and, in some situations, females may compete for mates (Emlen and Wrege 2004; Myhre et al. 2012). As a result, sexual selection may be acting on female traits that would provide males with increased reproductive benefits. Males may base their selection preferences on factors such as female size or attractiveness, thus providing males with higher direct or indirect fitness. However, there are situations where the OSR can shift from a male to a female bias, or vice versa, over the course of the breeding season and sex-roles can alternate between conventional (e.g., female-choice) and reversed (e.g., male-choice). This shift can occur when both sexes become limiting at varying times throughout a breeding cycle due to their reproductive investment through parental care and gamete production (Clutton-Brock and Vincent 1991; Clutton-Brock and Parker 1992; Kokko and Jennions 2008). This shift will result in instances in which both males and females in a population may become choosy and competitive over mates. For example, this shift between OSR and sex-roles has been shown in populations of Two-spotted Gobies (*Gobiusculus flavescens*), where males are more abundant at the beginning of the breeding season (male-biased OSR) and females are plentiful at the end of the season (female-biased OSR; Forsgren et al. 2004). This variation between the availability of males and females in the population results in both sexes being choosy and competitive during mating (Forsgren et al. 2004; Myhre et al. 2012). Thus, when female choice for high-quality males is paired with male choice for high-quality females, a mutual mate choice system may arise. As a result, assortative mating could occur as only the favoured (i.e., high-quality) individuals can sustain a choosy lifestyle; thus, high-quality males and females will mate with one another and low-quality individuals will mate with each other (Kraak and Bakker 1998). Therefore, if both males and females can differentiate between low- and high-quality mates, they may receive the greatest benefits from their selected partner and attain overall higher reproductive success.

1.2 Reinforcement

For individuals to select high quality mates they must first be able to recognize individuals of their species or ecotype. This pre-zygotic differentiation typically occurs in sympatric populations, where two closely related groups live in the same area but do not interbreed. Avoidance of interbreeding among sympatric populations that have the potential to hybridize may be a result of strong prezygotic barriers that have evolved to select against interspecific matings (Dobzhansky 1940; Servedio and Noor 2003). This process, known as reinforcement, ultimately selects against the production of hybrids (Dobzhansky 1940; Servedio and Noor 2003); this may provide a possible explanation for a lack of crossbreeding between closely related sympatric populations. Reinforcement has been studied in various organisms that maintain both sympatric and allopatric populations by comparing traits that aid in species recognition such as mating calls (Pfennig and Rice 2014) and morphology (Hołyńska 2000). The importance of studying species that maintain both types of populations is to understand how individuals recognize one another and how these traits function in selection to avoid unstable hybridization. These traits may help determine the outcome of contact between allopatric populations by demonstrating whether allopatric populations can differentiate conspecifics from heterospecifics using traits that sympatric populations use for recognition and mate selection. Where secondary contact might arise, extinction of one of the populations, hybridization, or reinforcement due to incomplete reproductive barriers may occur (Servedio and Noor 2003; Liou and Price 1994 and references therein). In cases where incomplete barriers are present, selection against hybrids will sustain the separation of the populations, though selection for isolation prior to matings between the populations will also occur to reduce the production of low fitness hybrids. This selection can be strengthened through sexually selected mating signals if they were present prior to secondary contact (Servedio and Noor 2003). Thus, sexually selected traits will not only be useful for choosing high-quality mates, but can aid in differentiating individuals of the same species when introduced to and/or interacting with closely related populations.

1.3 Mate characteristics

Nuptial colouration, or colouration used during the breeding season, is one characteristic that may have evolved because of mate choice (Hunt et al. 2009). Nuptial colouration has been associated with increased benefits or indicators of quality in both males and females for the choosing sex. A positive relationship exists between colour intensity and benefits such as enhanced foraging ability, sperm quality, and health, as seen in various species of fish (Maan et al. 2006; Locatello et al. 2006; Takahashi 2018), birds (Hill 1991), lizards (Bajer et al. 2010), and turtles (Polo-Cavia et al. 2013). Female nuptial colouration is also indicative of their quality through traits such as fecundity, readiness to spawn, and maternal quality (Berglund et al. 1986; Amundsen et al. 1997; Amundsen and Forsgren 2001; Baldauf et al. 2011). The information provided about the quality of an individual through nuptial colouration can be enhanced through the use of other breeding-related traits, such as behaviour.

The energy expenditure associated with courtship displays can also be a reliable indicator of mate quality, such that increased frequency, or duration, can signal male vigour. For example, in Fifteen-spine Sticklebacks (*Spinachia spinachia*), males that perform a relatively high frequency of body shakes are more attractive to females; the number of body shakes has been positively correlated with higher offspring hatching rates (Östlund and Ahnesjö 1998). Male vigor can also be demonstrated vocally as well as behaviourally. For male Sage Grouse (*Centrocercus urophasianus*), the interval between vocalizations and display rate with their air sacs is considered attractive to females (Gibson 1996). The attractiveness arises from the greater energetic costs associated with short intervals between vocalizations and rapid display rates which may be connected to male health (Vehrencamp, 1989; Gibson, 1990), male reproductive quality (e.g., testosterone levels in domesticated goats [*Capra hircus*]; Longpre et al. 2011), or gamete viability (e.g., sperm count and motility in the Houbara Bustard [*Chlamydotis undulata undulata*]; Chargé et al. 2010).

Additionally, parental care can be used to assess the quality of an individual as a potential mate. Parental care is a form of investment that enhances offspring fitness (Gross and Sargent 1985; Clutton-Brock 1991). The main benefit of parental care is the increase in offspring survival, which is likely the primary reason parental care has evolved. However, traits related to the quality of parental care may also influence the chance of a particular individual to procure a mate by attracting individuals through these caring behaviours. To elaborate, previous studies have shown that females prefer males who court (Knapp and Kovach 1991; Östlund and Ahnesjö 1998), build nests (Soler et al., 1998; Jones and Reynolds, 1999; Clotfelter, Curren, and Murphy 2006), and possess eggs already in their nest as signs of their caring abilities (Marconato and Bisazza 1986; Clutton-Brock 1991). For example, Tallamy (2000), noted that across all arthropod taxa with exclusive paternal care, males who built nests and were already guarding eggs were preferred by a larger number of gravid females as these behaviours were considered signals of their paternal quality. Despite the benefits, there are multiple costs associated with parental care. These costs include a reduction in matings, survivorship of the caring parent, and lowered future fertility (Gross and Sargent 1985; Smith and Wootton 1995; Reynolds, Goodwin and Freckleton 2002; Clutton-Brock 1991). However, the loss of potential matings may not be a significant as previously thought and may be insignificant when the male becomes territorial (Blumer 1979; Gross and Sargent 1985).

Territoriality is one of the least debated hypotheses behind why parental care has evolved, after the main benefit of offspring survival. Barlow (1962), proposed that early stages of paternal care involved multiple matings and male territoriality ((Barlow 1962): Baylis 1981). Territorially is assumed to have been a pre-cursor to the evolution of male parental care and paternal care is more likely to evolve in the species where males are territorial (Ah-King et al. 2005). The association between territoriality and male parental care may explain why paternal care represents a phylogenetically advanced state (Gross and Sargent 1985; Reynolds et al. 2002), as some costs may be reduced if the male is territorial. If a male is territorial, he is already on “site” defending that area, therefore, protecting his eggs would not be more energetically expensive (Gross and Sargent 1985; Williams 1975). There is a potential for him to gain benefits towards an increased current reproductive effort and a decrease in future reproduction costs, as the costs associated with remaining on site are outweighed by the increased number of matings (Gross and

Sargent 1985; Blumer 1979; Barlow 1981). Current mating costs may also be reduced due to multiple females being able to spawn with the male on his territory (Blumer 1979; Clutton-Brock 1991; Williams 1975). However, there are certain cases where the caring male parent may be more vulnerable to predation such as when they are brightly coloured to attract females (Semler 1971; Blumer 1979). Despite the possible reduction in costs associated with a territorial male conducting parental care, there is still an energetic cost. Thus, the amount of energy and time a male invests into caring for his offspring alone may provide him an opportunity to select who he mates with, as this would influence which sex is limiting in a population. When a male is favoured by multiple females, that male may select the higher-quality female (Kraak and Bakker 1998). For example, by using traits such as female body size or colouration, males may be able to assess female fecundity and thus their quality (Berglund et al. 1986; Baldauf et al. 2011). This connection between colouration, behaviour, body size, and the association with mate choice is demonstrated exceptionally well in a model fish species – the Threespine Stickleback (*Gasterosteus aculeatus*).

1.4 Threespine Sticklebacks

The Threespine Stickleback is a very diverse species of fish. Seven ecotypes comprise the Threespine Stickleback species complex that inhabit a range of salinities and temperatures as a result of the last glaciation (McKinnon and Rundle 2002). *Gasterosteus aculeatus* is a small, euryhaline, ray-finned fish (Jordan and Garside 1972; Wootton 1984). I will be focusing on two marine forms of the seven ecotypes: the "white" and "common" ecotypes (Fig. 1.4.1). The common Threespine Stickleback can be found anywhere within its range of the Northern Hemisphere, whereas the white stickleback is endemic to Nova Scotia, Canada (Blouw and Hagen 1990). The two ecotypes typically occur sympatrically (Blouw and Hagen 1990), although allopatric populations comprising of only white or common sticklebacks do exist (personal observations). Despite the overlap in breeding habitat, white and common ecotypes are genetically distinct, though closely related enough that there is still some gene flow (Samuk 2016). However, behavioural studies have noted that in the field and laboratory the two ecotypes mate assortatively (Haglund et al. 1990; Blouw and Hagen 1990). Additionally, current research has found that artificially crossed hybrids do not produce viable offspring because the eggs are not adequately cared for by the father, though they survive if artificial aeration is provided (C. Behrens, personal communication).

Behavioural, morphological, and physiological variation between and within the common and white ecotypes allows for easy differentiation. During the breeding season, common Threespine Stickleback males provide parental care for their offspring, whereas the white males do not (van Iersel 1953; Blouw 1996; Jamieson, Colgan, and Blouw 1992b). Both common and white Threespine Stickleback males acquire and defend a nesting territory on which they build a nest using either mud/sand (common males) or algae (white males; van Iersel 1953; Jamieson, Colgan, and Blouw 1992; Haglund, Buth, and Blouw 1990). Once their nest is built, common and white males will begin to court females. Both ecotypes will conduct various courtship behaviours, such as moving quickly in a zig-zag pattern (zig-zag dance) and displaying his nest to the female by swimming towards it then back to her (leading/pointing). However, white males do not prick the female with their dorsal spines (dorsal pricking) or circle the female (circling) as commons do (Wootton, 1976; Tinbergen 1952; van Iersel 1953; Mclennan and McPhail

1989; Blouw and Hagen 1990; Jamieson, Colgan, and Blouw 1992; Haglund, Buth, and Blouw 1990). Additionally, both types of males conduct nest-oriented behaviours during the courtship encounter which include fanning, gluing, and boring through the nest (Wootton 1984; see Table 2.3.2 for behaviour descriptions).

While maintaining their nests and courting, males will also display their nuptial colouration to attract females. Both ecotypes have light blue eyes with a pink to red throat colouration, which in common males is indicative of their quality and availability (Ostlund-Nilsson, Mayer, and Huntingford 2007; Hagen, Moodie, and Moodie 1980; Frischknecht 1993). However, there is a striking difference in their dorsal colouration; common male dorsi will turn a shade of blue/brown, whereas white males turn a pearlescent white during the breeding season (Tinbergen 1952; van Iersel 1953; Blouw and Hagen 1990).

An important aspect that must also be considered, along with mating cues and signals, while studying the mating systems of Threespine Sticklebacks is the reproductive availability of both sexes. In general, the sex that is available for a shorter reproductive period and who invests more in their offspring becomes limiting, which will shift the instantaneous sex ratio towards the opposite sex (Clutton-Brock and Vincent 1991; Clutton-Brock and Parker 1992; Kokko and Jennions 2008). In Threespine Sticklebacks, there is variation in the amount an individual invests in reproduction between both sexes and ecotypes. Females of both ecotypes must produce eggs and will take three or more days to do so depending on food availability (reviewed in Wootton et al. 1995). During this time period, females are the limiting sex as there is a fluctuation in the amount of reproductively available females, with the sex ratio shifting towards being male biased.

However, male reproductive availability must also be considered, because common male Threespine Sticklebacks provide parental care (van Iersel 1953; Blouw 1996; Jamieson, Colgan, and Blouw 1992b). During the paternal care period common males are not available to mate for seven or more days (reviewed in Wootton et al. 1995). A male can mate with up to 30 females that can produce egg clutches that contains ~50-200 eggs (see Wootton 1976; 1984; Kraak et al. 1999 and references therein) that are all laid in one nest that the male guards during this parental care period until the fry become independent, thus, they can also become the limiting sex if these "time-outs" shift the sex ratio of available mates such that it becomes female-biased. This back-and-forth between the reproductive availability of male and female Threespine Sticklebacks generates a mutual mate choice system (Rowland 1982; Blouw and Hagen 1990; Kraak and Bakker 1998). In contrast with common males, white male Threespine Sticklebacks do not provide parental care, but rather disperse eggs from their nest after fertilization (Jamieson et al. 1992b; Blouw 1996) and thus avoid or markedly shorten any reproductive "time-outs". The white Threespine Stickleback's sex ratio will thus be male-biased throughout the mating season. As a result, male mate choice may not occur in this ecotype, or it may be dampened compared to the mating system of common Threespine Sticklebacks. Thus far, no studies have examined male mate choice preferences among the white Threespine Sticklebacks. While there is some speculation that white males do not actively choose mates (Blouw and Hagen 1990), that study only considered consecutive and not simultaneous female interactions with the white males, so it is currently unknown whether males are choosy in this ecotype.

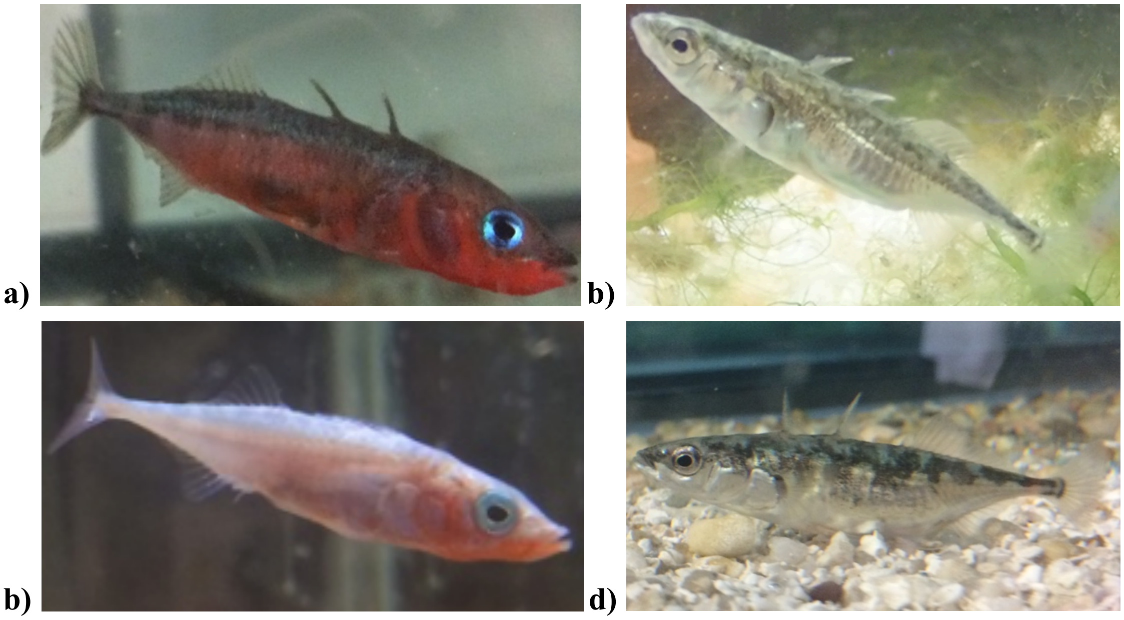

Figure 1.4.1. The **(a)** male and **(b)** female common marine ecotype and **(c)** male and **(d)** female white marine ecotype of the Threespine Stickleback. Both males are in breeding colouration.

Additionally, previous studies such as that by Blouw and Hagen (1990) mainly focused on either same ecotype mate choice (common male and female; van Iersel 1953; Rowland 1982; Kraak and Bakker 1998) or sympatric populations of common and white sticklebacks (Jamieson et al. 1992a; Blouw 1996). Therefore, this ecotype pair (i.e. common and white Threespine Sticklebacks) is a good model for examining reinforcement in the context of sexual selection as allopatric and sympatric populations exist and crossbreeding is not known to occur in sympatric populations (Blouw and Hagen 1990). Thus, due to the considerable variation within this species with reference to their behaviour, colouration, and size, the ability to study them both *in situ* and in the laboratory (Foster and Bell 1994), and the lack of information surrounding reinforcement and mate choice in white and common sticklebacks, the Threespine Stickleback is an exceptional fish to study.

1.5 Research objective and predictions

While studies have examined female mate choice in populations of both common and white Threespine Sticklebacks (Blouw and Hagen 1990; Milinski and Bakker 1990), less research has been conducted on male mate choice in the common stickleback (Rowland 1982; 1989; Bakker and Rowland 1995; Kraak and Bakker 1998), and none of that research has been conducted on the white stickleback. Moreover, few studies have investigated the influence of mate choice on the maintenance of barriers between white and common Threespine Sticklebacks. Sympatric populations of white and common sticklebacks mate assortatively (Haglund et al. 1990; Blouw and Hagen 1990), which may have evolved because of hybrid breakdown (F1 males are poor fathers, leading to egg death; Behrens, personal communication). Through evolutionary time, sympatric populations of white and common sticklebacks should evolve strong mate selection preferences to avoid the fitness costs associated with producing unfit hybrids, thus reinforcing their separation. However, allopatric populations have not experienced this selection pressure as they have not experienced the fitness cost (hybrid breakdown) associated with interbreeding. Therefore, I hope to understand how this selection pressure, which is enhanced through reinforcement, has contributed to the divergence of the common and white Threespine Sticklebacks by comparing the two types of populations.

In their work with sympatric white and common threespines, Blouw and Hagen (1990) observed that female white sticklebacks initiated the receptive "head-up" display (willingness to spawn) more to white male sticklebacks. They also found that, although common females initially showed a "head-up" display to white males, they ultimately chose to spawn with common males. This initial response by the common females may be explained by the vigorous courting and conspicuous colouration of the white males (Jamieson et al. 1992a; Haley 2018). Blouw and Hagen's (1990) study was not specifically designed to assess male mate choice, but they did note that when males of both ecotypes were presented with either common or white females, common male sticklebacks courted their own ecotype more and the whites courted the two equally. My research expands on Blouw and Hagen's work by investigating mating preferences in both sexes, ecotypes and population types. Given what we know about mate choice in *Gasterosteus aculeatus* and the costs associated with reproduction (e.g., gamete production), I predict that:

(i) Female mate choice will occur in both populations as both female ecotypes invest in gamete production.

(ii) Male mate choice will be more important for common, compared to white, male sticklebacks as only the common males provide paternal care. Because common males are conducting a costly behaviour, selection is expected to favour males who choose the best quality and quantity of eggs to fertilize, therefore they may be more choosy about the individual with whom they will mate.

(iii) Males and females in the allopatric populations of common and white Threespine Sticklebacks will have weaker mate preferences, measured as courtship and response frequency by males and females respectively, for their own ecotype than their sympatric counterparts, as they have not experienced the potential fitness costs associated with interbreeding. Based on previous findings about sympatric mate preferences of common and white Threespine Sticklebacks (see Blouw and Hagen, 1990), I predict that allopatric males will court, and females will respond, to both their own ecotype as well as the other ecotype.

Through conducting this mate choice experiment on sympatric and allopatric populations of the Threespine Stickleback, I will gain insight into the potential drivers of mutual mate choice through sexual selection. Exploration of mutual choice will yield a more comprehensive understanding of mating systems and provide additional information about the possible mechanisms that drive speciation.

2. METHODS

2.1 Sampling sites and fish collection

Fish collection took place during the month of June 2020 at three sites located across Nova Scotia, Canada. All three locations are brackish water environments supplied by the Atlantic Ocean, with a salinity range of ~18 to ~30 ppt and temperature ranging between ~13 to ~21 ºC (personal observations). The locations contained either sympatric (Rainbow Haven Estuary) or allopatric, (Antigonish Landing [common] and Crossing Road [white], populations of white and common Threespine Sticklebacks, Fig. 2.1.1). These sites were selected based on knowledge about the populations from previous sampling years and prior research studies (Blouw and Hagen 1990; Samuk 2016; Haley 2018, personal observation). Antigonish Landing is characterized by its muddy substrate and is relatively bare of large rocks and algae. Crossing Road has a rocky substrate surrounded by patches of filamentous algae, such as *Cladophora* spp.. Rainbow Haven Estuary is intermediate between the other two sites with a combination of a muddy and rocky substrate surrounded by filamentous algae and tall grass. Presence or absence of these environmental factors is important as Threespine Sticklebacks have specific habitat requirements for nesting sites (Blouw and Hagen 1990).

Fish were collected using a combination of unbaited Gee's minnow traps (1/4 inch mesh) that soaked for three to twenty-four hours, and dip-netting by hand. Fish were identified visually in the field for sex and ecotype by observing their size, colouration, and abdominal shape as these traits vary between the sexes and ecotypes (Blouw and Hagen 1990). Females were collected mostly by minnow traps while most males were dip-netted above their nest. Females and males were identified by size as previous studies have shown that common Threespine Sticklebacks are larger than the white ecotype (Blouw and Hagen 1990; Samuk 2016). Male ecotype was determined by breeding colouration and nest location. Common male stickleback were differentiated by their bright blue eyes, red throat, and dark dorsum, and their nests were built in mud; while white male sticklebacks were identified by their conspicuous white dorsum and their nests were built in the filamentous algae (Blouw and Hagen 1990; Jamieson et al. 1992a). If male sticklebacks were caught in the minnow traps and easily identifiable via their colouration, they were retained. Across all three sites a total of 120 fish (Table 2.1.1) were collected and transported to Saint Mary's University's aquatic facility.

2.2 Fish husbandry

Fish were housed at Saint Mary's University's aquatic facility and held in 15-gallon stock tanks with the sexes, ecotypes, and populations housed separately. The stock tanks were maintained at a water temperature of 20-22°C and salinity of 15ppt ± 1ppt supplied by reverse osmosis de-ionized (RODI) source water. The tanks contained gravel, artificial plants, hiding structures, algae collected from Rainbow Haven estuary for nesting material, and a waterfall filter for aeration. The fish were maintained on a light cycle of 16 hours light and 8 hours dark, which is consistent with the summer breeding photoperiod of Nova Scotia (Blouw and Hagen 1990) and were fed a diet of frozen *Mysis* shrimp and Bloodworms once a day. All fish collection and laboratory work were conducted in accordance with Saint Mary's Universtiy animal care protocol 20-09.

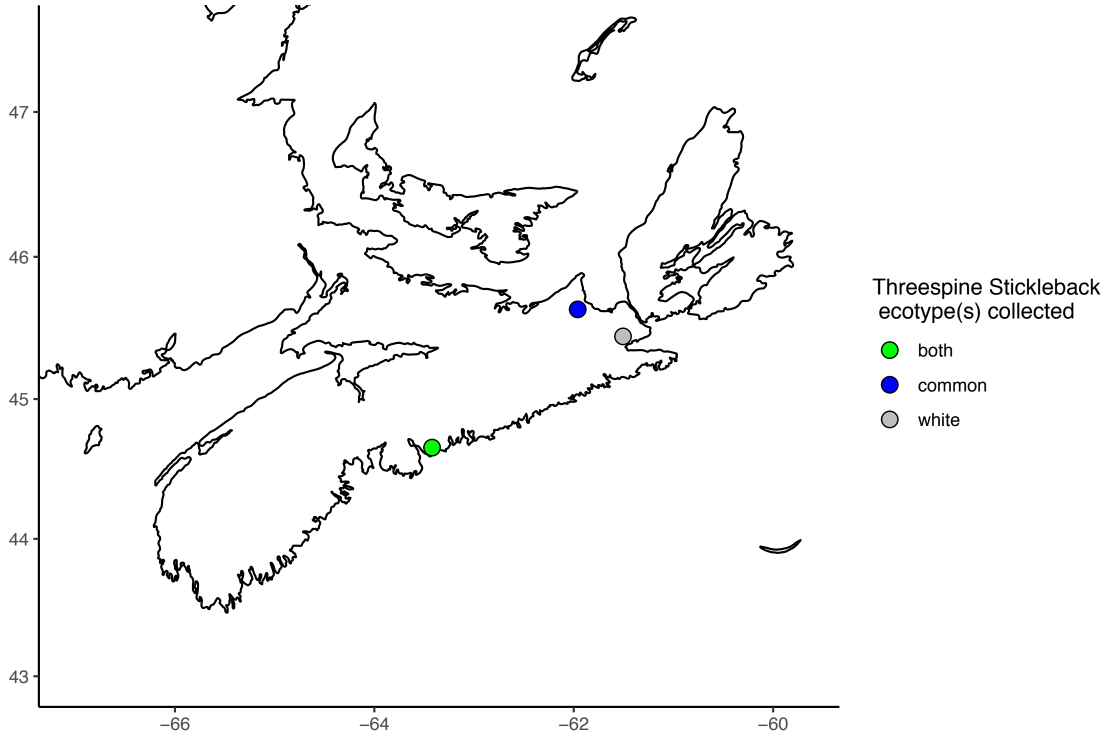

Figure 2.1.1. Map of Nova Scotia Threespine Stickleback sampling locations. Antigonish Landing (blue circle) contained an allopatric population of common Threespine Stickleback. Crossing Road (grey circle) contained an allopatric population of white Threespine Stickleback. Rainbow Haven Estuary (green circle) contained a sympatric population of white and common Threespine Stickleback.

Table 2.1.1. Total number of fish collected by site, ecotype and sex during the month of June 2020, across Nova Scotia.

Site	Fish ecotype and sex			
	Common Male	Common Female	White Male	White Female
Rainbow Haven	12	12	15	15
Antigonish Landing	15	15	NA	NA
Crossing Road	NA	NA	18	18

2.3 Experimental setup and design

2.3.1 Tank set-up and male nest-building

The experimental setup consisted of six 10-gallon focal tanks that housed one male and two females. Tanks were partitioned into three compartments; one large compartment comprising three-quarters of the tank containing the focal male stickleback and nesting material; the other two compartments were evenly divided into the remaining one-quarter of tank space and housed the two females separately (Fig. 2.3.1a). Prior to observations and the addition of female stickleback to the tanks, male stickleback were placed in the focal tanks and provided with two types of nest-building material: sand in a petri dish and algae. Nest material preferences of common and white stickleback have been previously reported: common stickleback prefer sand and white stickleback prefer algae (Blouw and Hagen 1990; Jamieson et al. 1992a), but both ecotypes may build nests using both materials (Blouw and Hagen 1990; Corney 2019). If a male did not build a nest within four days, he was removed and a new male was added to the focal tank. Males were given a minimum of 24 hours to build a nest prior to any observations. Nests were identified as an opening in a constructed mound made of algae and/or sand. One of the two materials always made up the majority of a nest (≥70%), and thus I classified nests as either sand or algae-based. These observations acted as a confirmation of the visual identification of male ecotype in the field.

2.3.2 Experimental design and data collection

The experimental design for the mate choice study followed a procedure similar to that of Kraak and Bakker (1998) with a few modifications. The experiment consisted of four combinations of the layout displayed in figure 2.3.1. Each layout was a combination of one male and two females. Both males and females varied by ecotype (white and/or common) and in geographical distribution (population type: sympatric or allopatric population). For example, an allopatric white male with a common female and a white female would be one of the four possible combinations (Table 2.3.1). There was always a white and common female in each trial. There were eight to ten replicates of each combination for a total of 35 observational trials. During one trial, two choice experiments were conducted sequentially, male preference and no-choice. A trial consisted of a one-hour observational period divided into four 15-minute sections (Fig. 2.3.1c). Two of the four 15-minute sections were for the male preference stage (Fig. 2.3.1a) and the remaining two 15-minute sections were for the male and female no-choice stage, herein referred to as the “interaction” stage (Fig. 2.3.1b). All trials were recorded with an Enviro R jvc camcorder (GZ-R460D model). To investigate mate preference of male and female Threespine Sticklebacks, video recordings were uploaded to JWatcher, a quantitative behavioural analysis program (Blumstein, Daniel, and Evans 2006). JWatcher allows for the observer to record events to assess the frequency and time allocated to specific behaviours conducted by the focal subject.

Table 2.3.1. Number of observations for each combination of male and female ecotype and population type. There was a total of 35 trials with each trial consisting of one male and two females.

Male ecotype and population type	Female ecotype and population type			
	Allopatric common	Sympatric common	Allopatric white	Sympatric white
Allopatric common	4	4	3	5
Sympatric common	5	5	5	5
Allopatric white	3	5	3	5
Sympatric white	4	5	5	4

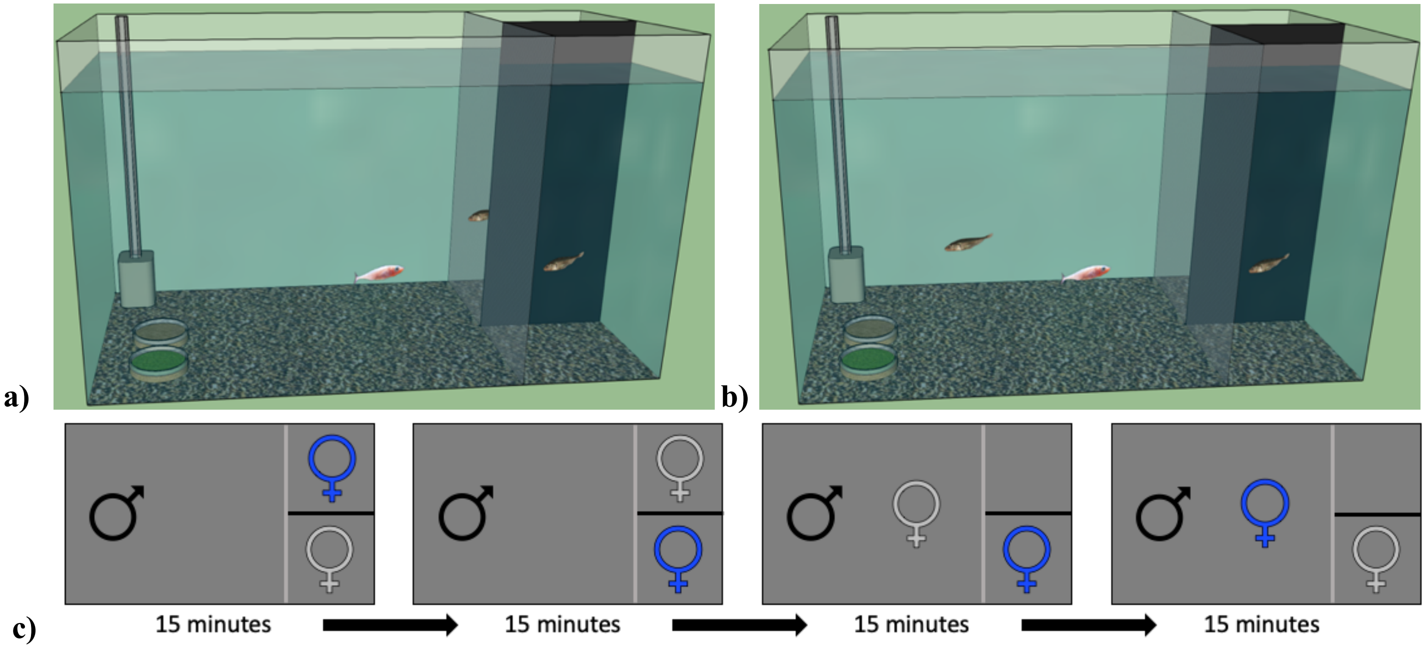

Figure 2.3.1. Experimental setup for the (**a**) male preference and (**b**) male and female no-choice experiments. The larger part of the tank contained the focal male fish and nest-building materials. The two smaller portions of the tank contained one female each for the first part of the male preference trial (**a**). For the second part of the experiment, the male and female no-choice trials, one of the two females was released into the larger portion of the tank with the male (**b**). The order of the observational events, each event consisting of 15-minute intervals totaling a one-hour long experimental trial (**c**). The black partition in figure a and b, or black line in figure c, indicates an opaque partition separating the fish visually from one another, the grey line in figure c and clear plexiglass in figures a and b indicate a clear partition separating the fish physically from one another.

Preference stage

The preference stage was designed to assess male choice based mainly on visual cues and involved a focal male who was physically separated from a female of each ecotype, but could see them (Fig. 2.3.1a). For the two 15-minute periods the male and females were physically separated from one another (Fig. 2.3.1c box 1); after the first 15 minutes elapsed the females' positions were swapped with one another and the second 15 minutes were completed (Fig. 2.3.1c box 2). This was to ensure that the male was interacting with a specific female not simply exhibiting a preference for one side of the tank.

Male preference was determined by observing the amount of time a male spent in proximity to each of the contained females. Proximity was determined when the male was located directly in front of the clear plexiglass barrier of the female's compartment. If the male was located away from the clear plexiglass barrier or directly in the middle of the two female compartments (black line fig. 2.3.1c) then this was considered no preference by the focal male. Time recording was initiated each time the male "poked" the clear plexiglass barrier with his mouth ("glass poking"), and ceased when he stopped the behaviour for more than one second or retreated from the barrier. The male needed to conduct the poking behaviour to ensure that we were recording his potential interest in the contained female and not for other reasons such as resting near the barrier.

Interaction stage

A 30-minute interaction stage was used to assess both visual and behavioural cues of male and female stickleback when the male was able to interact with one of the two females (Fig. 2.3.1b). For the first 15-minute interaction stage, one of the two females was randomly chosen to be released into the larger portion of the tank with the male (Fig.

2.3.1c box 3) while the other remained behind the clear barrier. For the second 15-minute interval, she was then swapped with the other female (Fig. 2.3.1c box 4). During the interaction stage, the male was still able to view the other female while interacting with the released female allowing for a potential additional measure of preference between the two females (Fig. 2.3.1b).

Male courtship effort was divided into two sections; first, by male exertion, which was determined by the total courtship behaviours conducted towards the released female during the interaction stage; and second, by male persistence, determined by the amount of "glass poking" behaviour that a male conducted towards the contained female during the interaction stage. Male courtship behaviours are described in Table 2.3.2.

Interaction stage – female preference

Female preference was measured as a binomial response associated with whether or not she conducted any of the typical "head-up", "follow", and/or "inspect nest" response behaviours that characterize female willingness to mate when released with the male. The pairs were also observed for spawning events. When spawning occurred, the time that the female and male entered and left the nest were recorded along with whether or not the female deposited her eggs, which was determined by a change in size of her abdomen. After spawning took place, the portion of the trial for the female that spawned was terminated and nests were checked for the presence of eggs.

After a trial was complete, the male and the two females were tagged using subcutaneous visual implant elastomer tags (Northwest Marine Technologies) for individual fish identification. Body size was also collected to confirm the ecotype of the fish by taking measurements of standard length to the nearest 0.01mm and a piece of the anal fin was collected for later genetic analysis.

There was a total of 35 observations for male sticklebacks, with each male interacting with a common and white female in random order. As two females were present per trial, there was a total of 70 female observations recorded (Table 2.3.1). Male fish were only used once per mate choice trial, while female fish were used one to four times (mean ≈ 2). Only one female was reused with a male of the same ecotype and population type combination as a previous trial. Female fish were reused due to the necessity of having a gravid female in the choice trails. There was a total of 39 female fish and 35 male fish used during the experiment.

Nest tending

While male and female preferences were being recorded, male nest tending was also documented. Nest tending time and frequency were recorded to determine if additional differences between the nesting habits of white and common stickleback existed (during the preference stage) and whether nest tending might be associated with mate choice, i.e., whether common and/or white male stickleback tend to their nest more depending on the female with whom they can interact (during the interaction stage). Male nest-tending was recorded in a similar manner to male preference, with the amount of time spent nest-tending recorded during the preference stage and the frequency of individual behaviours recorded during the interaction stage.

Table 2.3.2. Description of focal behaviours observed for both male and female Threespine Sticklebacks. Modified from Haley, 2018 and Berhens, 2020.

Sex	**Behavioural category**	**Focal behaviour**	**Definition**
Male	Nest building	Material retrieval	Male brings material to where he is building a nest
		Nest tending	Gluing – male uses spiggin to hold the nest together
			Poke – male bites/pecks at his nest
			Fanning – male moves his pectoral fins back and forth
			Creep-through – male enters and squirms through his nest
	Courtship	Zig-zag	Male swims in a 'Z' configuration quickly
		Dorsal pricking	Male swims against female, often slightly on his side and pokes the gravid stomach with his spines
		Biting	Male bites female's lateral and/or ventral surface
		Lead	Male leads the female back to his nest
		Side fan	Similar to dorsal pricking in commons, male wiggles their body in a horizontal position
		Show nest	Male will poke at the opening to indicate where the female should go
		Glass poking*	Male pokes the plexiglass barrier with his mouth
Female	Courtship response	Head-up	Female holds her head pointing upwards towards the top of the tank (~45 degree angle)
		Inspect nest	Female pokes her head into the nest entrance
		Follow male	Female follows closely behind/or next to the male
Both	Spawning	Enter nest	Female – enters/squirms into the nest and stops inside of it
			Male – enters his nest after a female deposits her eggs to spread sperm onto the eggs
		Quiver	Male quivers against the female when she is in the nest

* This behaviour was given a description specifically for this experiment, it is not an officially documented courtship behaviour of Threespine Sticklebacks.

2.4 Statistical analyses

All analyses were implemented using the statistical software R v4.0.2 (R Core team 2020). Two statistical methods were performed. First, I used a model comparison method utilizing Akaike Information Criteria (AIC) that is described in the following sub-sections; second, I explored my data using a Bayesian approach that is described in Appendix A.

2.4.1 Body size and nest material

Body size, measured as standard length (mm), and nest material were collected as extra measurements to confirm the visual identification of the common and white Threespine Stickleback ecotypes. Body size was analysed using a generalized linear model (GLM), as the data were normally distributed and continuous. The full model was fitted with ecotype (white or common), sex (male or female), and their interaction as the fixed effects; no random variables were included. All possible models containing different combinations of predictors were compared and ranked using Akaike Information Criteria corrected for small sample size (AICc). This was accomplished by running an automated model selection (dredge) using the 'MuMin' package, version 1.43.17 (Bartoñ 2020). Model fit was assessed using quantile-quantile (QQ) plots and pairwise differences between groups were then analysed using post-hoc analyses for the full model.

To confirm the nest material preferences of the white and common Threespine Stickleback ecotypes, regardless of male population type, χ^2-squared tests were performed on the nesting material (sand or algae) of males' nests and their ecotype (common or white). Male population type (allopatric or sympatric) was omitted from the analysis as we do not expect any change in nesting material preference based on the presence or absence of either ecotype from a population. For nesting material, when both options were used to construct a nest only the dominant material was included in the chi-squared analysis to account for the small sample size of mixed material nests. Additional analyses related to differences between white and common nest tending habits can be found in the supplementary information section one.

2.4.2 Male preference

Preference stage

Male preference was examined by assessing the relationship between the proportion of time a male spent with each female and the male's ecotype, population type, and female body size. The proportion of time was calculated by dividing the time the male was in proximity to the contained female, by the total observation time (30 minutes); this was done for both females. Because the response variables were proportions, I used a generalized linear mixed model (GLMM) with a binomial error distribution. Fixed effects included in the model were male ecotype, male population type (allopatric or sympatric), whether the female they were near was the same ecotype as themselves or not (ecotype relationship), and their interactions. The ecotype relationship effect was evaluated as a value of 0 indicating that the male and female were of different ecotypes and a value of 1 denoted that the fish were of the same ecotype. Individual males were included a random effect to account for each male being able to associate with both a white and a common female during this stage. Automated model selection using the dredge function was conducted to determine the best model based on the AICc values, followed by assessing the model fit using QQ plots and calculated pairwise differences.

Interaction stage

Male courtship effort

Male courtship effort was investigated using both male exertion and male persistence which were recorded during the interaction stage. Prior to the analyses, the frequency of courtship behaviours and glass poking were corrected for time. Nine of the 35 trials did not span the full 15-minute observation period as spawning events occurred. Therefore, the behavioural frequencies had to be corrected to account for how many behaviours would have occurred if the trial lasted the full 15 minutes. This was done by dividing the typical observation time (15 minutes) by the duration of the trial and then multiplying by the total combined frequency of all the behaviours (see Table 2.3.2 for descriptions). The final value was rounded to the nearest integer to approximate count data (see supplementary information section two for analyses using each courtship behaviour separately). Following the correction for time, two negative binomial generalized linear mixed effect models were run: one for male exertion, the second for male persistence.

For male exertion, the adjusted total courtship frequency was the response variable and male ecotype, male population type, the ecotype relationship, and all possible interactions were the fixed effects. Individual male was the only random effect to control for the effect of repeated observations of the same male. The model was run using a negative binomial error distribution from the glmmTMB package, version 1.0.2.1 (Magnusson et al 2020) as the data were over-dispersed counts. Again, significance was determined by the model with the lowest AICc followed by a QQ plot to assess the fit of the model to the data. The models were then followed by post-hoc testing.

To assess male persistence, the glass-poking frequency conducted by male stickleback was the response variable in a new GLMM. Predictors and random effects were the same as above for male exertion. Automated model selection was performed to determine model significance, and the full model's fit was assessed using QQ plots, followed by calculating any pairwise differences.

Male courtship effort and female body size

Two separate models were run for assessing the impact of female body size on male exertion. The first model consisted of female body size, female ecotype, male ecotype and their interactions as the predictors and the total courtship frequency as the response variable, with individual male and female fish as the random effects. The second model consisted of female body size, male population type, the ecotype relationship, and their interactions as the predictors; the response variable and random effects remained the same. Both models were run using a Poisson error distribution, as the data were counts that were not overdispersed, with the female size variable scaled and centered using the R Base Package (version 4.0.2). As with all other models, the best model was determined by the lowest AICc value and the fit was evaluated with a QQ plot. Both models were created to assess both a male's preference for female body size based on ecotype, while the second model was aimed at determining whether there was a pattern for males from different geographic backgrounds having a preference for female body size (e.g. larger females), and whether she was the same ecotype as himself.

2.4.3 Female preference

Interaction stage

Female preference was recorded as a binary response with a value of 1, indicating the female responded to a male by following him, inspecting his nest, or conducting the typical "heads up" display, and a value of 0 indicating she did not respond during the interaction stage. A GLMM with a binomial error distribution was run with female response as the binary response variable to determine the effect of male ecotype, female population type, whether the male and female were of the same ecotype or not, and their interactions on a female's response. Individual females were a random effect to account for the re-use of 20 of 39 females that were used more than once in the trials. Model selection and fit were determined in the same manner as the previous GLMMs using the dredge function on the model to determine the AICc values and assessing the model fits with QQ plots.

2.4.4 Spawning

Spawning events were recorded during the interaction stage of the experiment. For each spawning event, the ecotype and population type of the male and female fish were recorded. Assortative spawning was determined based on whether the male and female were of the same (pure cross) or different (hybrid cross) ecotype and whether the fish were from the same (sympatric), different (allopatric), or a combination of population types.

In addition to the model selection approach used above, I also did all analyses using a Bayesian framework. Results from the Bayesian analyses did not differ from those retained from the model comparison analyses and can be found in appendix A.

3. RESULTS

3.1 Ecotype confirmation

Ecotype identity of male and female Threespine Sticklebacks were visually categorized *in situ* and identification was confirmed in the laboratory by measuring the standard length, to the nearest millimetre, of both sexes. Body size of the Threespine Stickleback was best explained by the model that included the effects of ecotype, sex, and their interaction (Table 3.1.1). Common stickleback of both sexes were larger than their white counterparts (Fig. 3.1.1). Additionally, common female stickleback were larger than common male stickleback, while there were no differences between the sexes for the white stickleback (Fig. 3.1.1).

I also compared nest material (sand or algae) preferences of common and white male stickleback to confirm male ecotype. As per Blouw and Hagen (1990), in my study, common Threespine Stickleback males more frequently constructed a nest using predominately sand, while the white ecotype constructed nests out of filamentous algae ($\chi^2 = 16.56$, $p < 0.0001$, Table 3.1.2).

Table 3.1.1. Linear model selection for the effects of ecotype and sex on the body size of Threespine Sticklebacks. Shown below are the degrees of freedom (df) for the predictors,

Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 5). The bolded models are those with the lowest AICc values by a difference of two or more.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
body size	**ecotype + sex + ecotype x sex**	**5**	**395.2**	**0.00**	**0.968**
	ecotype + sex	4	403.2	7.84	0.019
	ecotype	3	403.9	8.67	0.013
	(intercept)	2	474.6	79.41	0.000
	sex	3	476.5	81.25	0.000

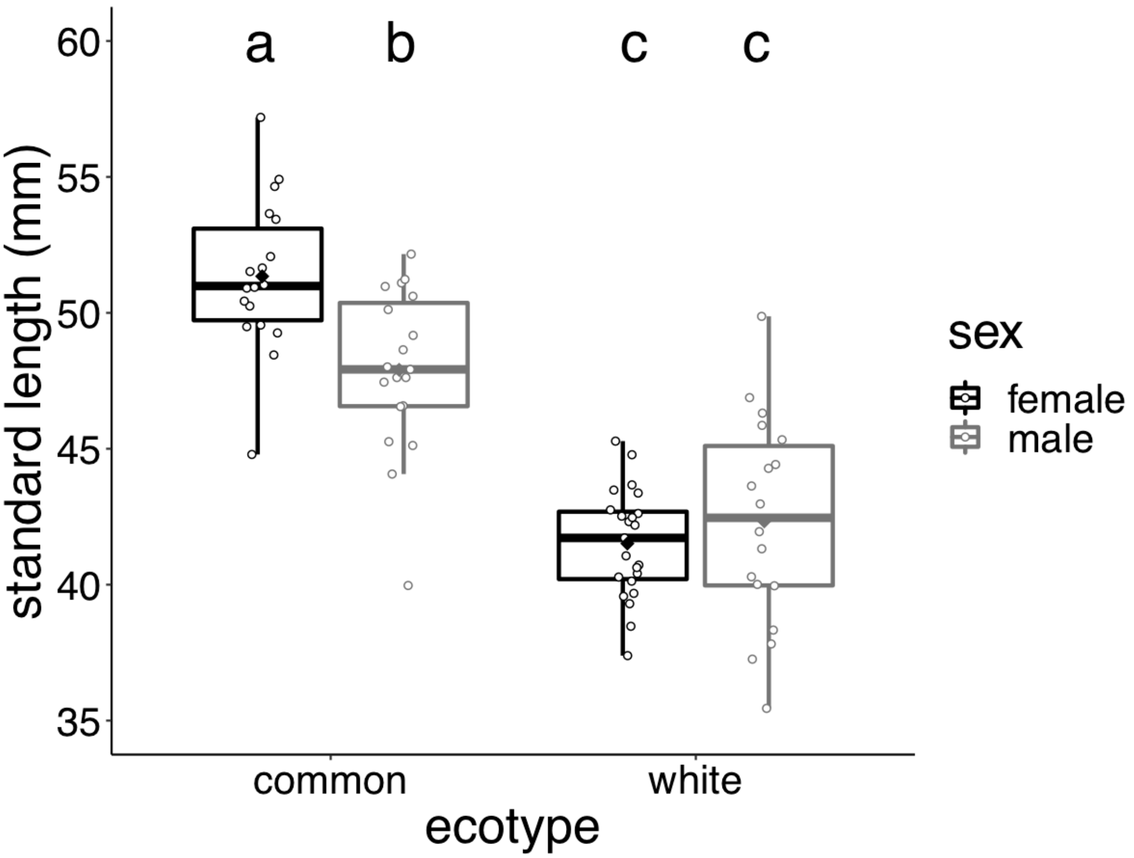

Figure 3.1.1. Standard length of male (grey) and female (black) common and white Threespine Sticklebacks (n = 78). Black and grey horizontal lines indicate the median, the black and grey diamonds are the sample means, and the coloured dots are the body size (standard length [mm]) for individual fish. Letters above the data represent post-hoc test groupings.

Table 3.1.2. Number of nests built by common and white Threespine Stickleback males using either algae or sand as their dominant material (n = 35).

Nest material	**Male ecotype**	
	Common	**White**
Algae	5	17
Sand	13	0

3.2 Mate preference

3.2.1 Male courtship trends

I was interested in four questions related to male Threespine Stickleback mate choice: first, whether one male stickleback ecotype courted females more than the other ecotype; second, whether sympatric or allopatric male stickleback courted females more than the other population type; whether males of different ecotype or population type preferred a female that was the same or different ecotype than themselves; lastly, whether female body size influenced a males' preference. I answered these questions by running various generalized linear mixed effect models with the respective variables for the question posed, with males as the random effect.

Preference stage

During the preference stage, I assessed the proportion of time males spent in proximity to the contained females of two different ecotypes. There was a degree of uncertainty as to which model best predicted the proportion of time male stickleback spent near a female, as there were seven models within two AICc values of one another (Table 3.2.1), indicating that all seven models influenced the proportion of time. There was a broader range of times that white and common males spent in proximity to common females compared to the range of times white and common males spent in proximity to white females (Fig. 3.2.1). Additionally, sympatric males spent slightly more time near a female regardless of his or her ecotype, with the exception of sympatric common males with white females (Fig. 3.2.1). However, overall males of either ecotype and population type spent relatively the same amount of time near each female (Fig. 3.2.1 and Table 3.2.1).

Table 3.2.1. Generalized linear model selection for the effects of male ecotype, male population type (allopatric or sympatric), and whether the female was the same ecotype as the male (ecotype relationship) on the proportion of time male Threespine Sticklebacks spent in proximity to the contained females during the preference phase of the experiment. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19). The bolded models are those with the lowest AICc values by a difference of two or more. Only the top models are shown below.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
proportion of time spent in proximity to a female	**(intercept)**	**2**	**14.7**	**0.00**	**0.188**
	male ecotype	**3**	**15.4**	**0.72**	**0.131**
	ecotype relationship	**3**	**15.4**	**0.78**	**0.127**

	male population type	**3**	**15.6**	**0.95**	**0.117**
	ecotype relationship + male ecotype	**4**	**16.2**	**1.56**	**0.086**
	male ecotype + male population type	**4**	**16.3**	**1.68**	**0.081**
	ecotype relationship + male population type	**4**	**16.5**	**1.79**	**0.077**

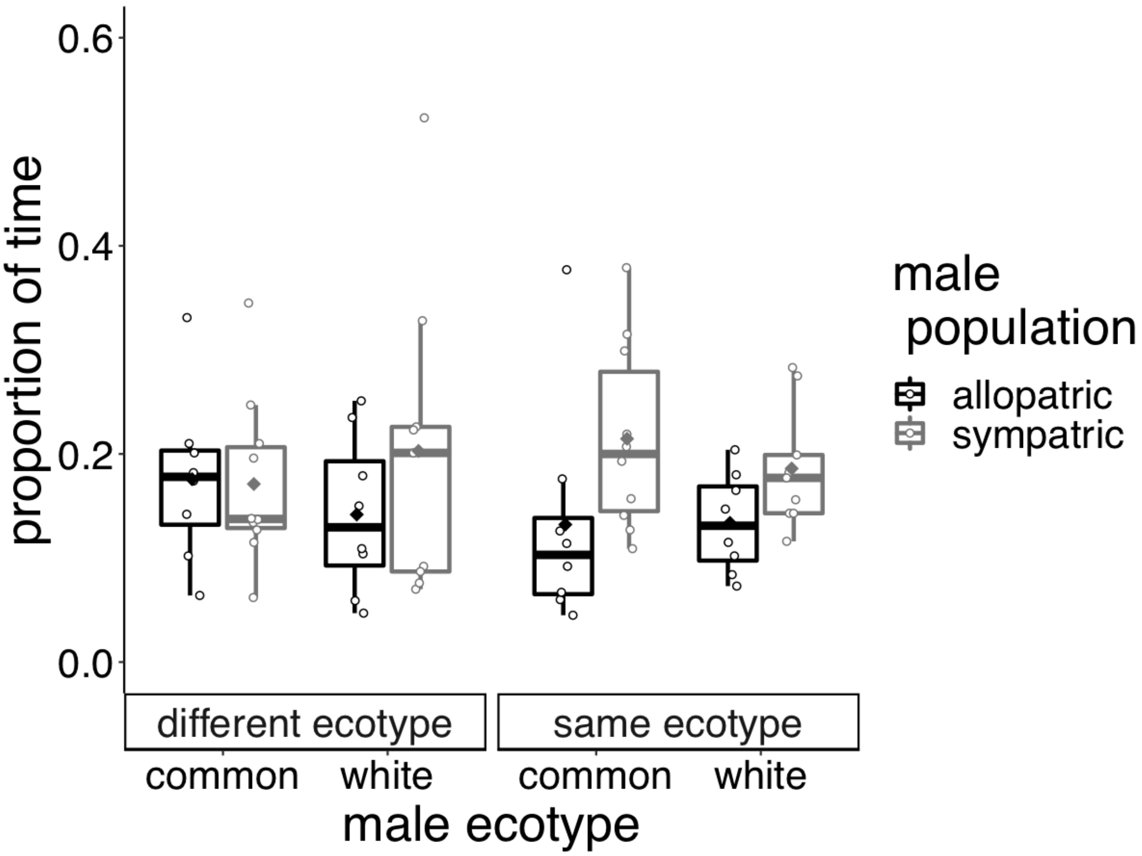

Figure 3.2.1. Proportion of time allopatric (black) and sympatric (grey) common and white male Threespine Sticklebacks spent in proximity to females of a different (left) or

similar (right) ecotype to themselves during the 30-minute male preference stage of the experiment (n = 35). Black and grey horizontal lines indicate the median, the black and grey diamonds are the sample means, and the coloured dots are the proportion of time for individual males. Individual males are counted twice as each male had an observation recorded for both a common and white female stickleback.

Interaction stage

Male courtship effort

During the interaction stage, I determined male exertion and persistence to gain access to the two females. Male exertion was assessed by analysing the total courtship behaviours performed towards the released female. Male exertion was explained by three models that were retained from this analysis; male population type, and an additive and an interactive effect between male population type and ecotype relationship (Table 3.2.2.). Males from sympatric populations courted females at a higher frequency than allopatric males and males courted females of a different ecotype than themselves at a slightly higher rate with the exception of white males courting common females (Fig. 3.2.2). Specifically, sympatric white male stickleback courted common females significantly more frequently than allopatric common male stickleback courted common females (Fig. 3.2.2).

Male persistence was analysed using the amount of “glass poking” male stickleback performed on the clear barrier separating the male from the contained female.

Although there is variation in the frequency of “glass poking” (Fig. 3.2.3), no predictors were retained in the best model (Table 3.2.2).

Table 3.2.2. Generalized linear model selection for the effects of male ecotype, male population type (allopatric or sympatric), and whether the female was the same ecotype as the male (ecotype relationship) on the total courtship behaviour frequency conducted toward the released female stickleback (effort) and the frequency of “glass poking” (persistence) toward the contained female stickleback by male Threespine Sticklebacks. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19). The bolded models are those with the lowest AICc values by a difference of two or more. Only the top five models are shown below.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
released female	**ecotype relationship + male population type**	**5**	**827.6**	**0.00**	**0.275**
	male population type	**4**	**828.0**	**0.45**	**0.219**

	ecotype relationship + male population type + ecotype relationship x male population type	**6**	**828.8**	**1.23**	**0.149**
	ecotype relationship + male population type + male ecotype	6	829.8	2.24	0.090
	male population type + male ecotype	5	830.2	2.57	0.076
contained female	**(intercept)**	**3**	**783.6**	**0.00**	**0.387**
	male ecotype	4	785.6	2.06	0.139
	male population type	4	785.6	2.06	0.138
	ecotype relationship	4	785.8	2.23	0.127
	male population type + male ecotype	5	787.8	4.19	0.048

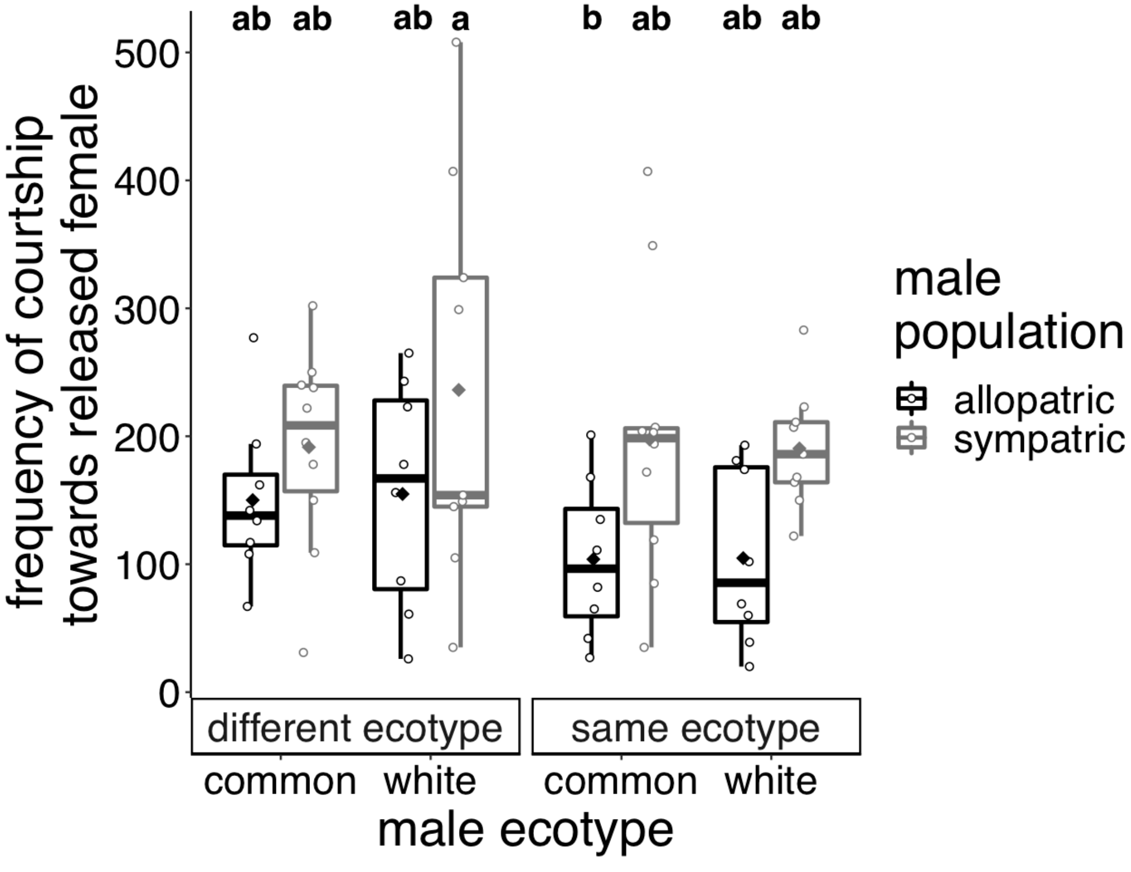

Figure 3.2.2. Frequency of total courtship behaviours allopatric (black) and sympatric (grey) common and white male Threespine Sticklebacks displayed towards a female of a

different (left) or similar (right) ecotype as himself that was released in the tank with him during the male interaction stage of the experiment (n = 35). Black and grey horizontal lines indicate the median, the black and grey diamonds are the sample mean, and the coloured dots are the frequency for individual males. Individual males are counted twice as each male had an observation recorded for both a common and white female stickleback. Letters above the data represent post-hoc test groupings.

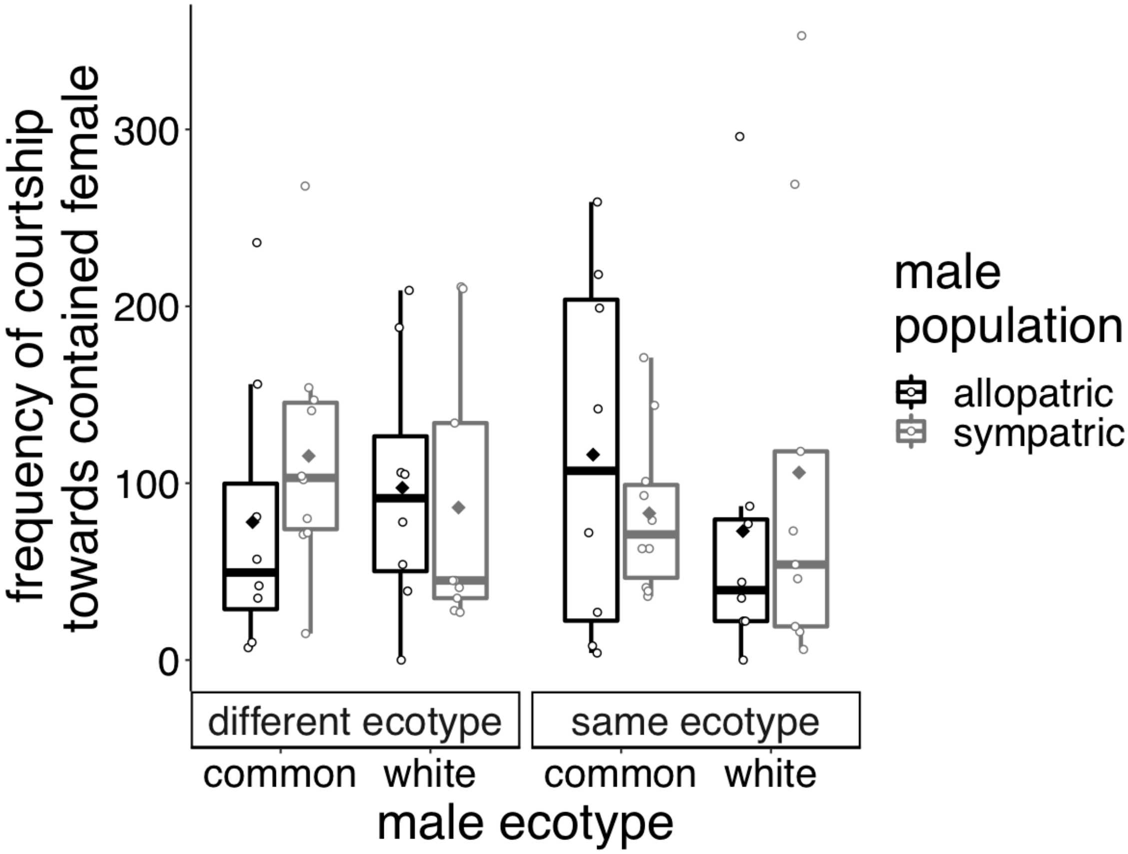

Figure 3.2.3. Frequency of "glass poking" allopatric (black) and sympatric (grey) common and white male Threespine Sticklebacks performed towards a female of a

different (left) or similar (right) ecotype to themselves that was contained behind the clear plexiglass barrier during the male interaction stage of the experiment (n = 35). Black and grey horizontal lines indicate the median, the black and grey diamonds are the sample mean, and the coloured dots are the frequency for individual males. Individual males are counted twice as each male had an observation recorded for both a common and white female stickleback.

3.2.2 Male courtship effort and female body size

Male ecotype

Body size preference of male stickleback was determined by analysing the frequency of courtship behaviours conducted towards females of different sizes during the interaction stage. The two models that best explained the frequency of courtship toward females of different sizes both contained the interactive effects of female ecotype and male ecotype, and female size and male ecotype (Table 3.2.3). Interestingly, only white males increased their courtship toward the largest females regardless of ecotype (Fig. 3.2.4). Meanwhile, common male stickleback courted larger females with a higher frequency than smaller females, but did not show a continuous preference with body size (Fig. 3.2.4). Because of the interactive effects, I also examined patterns within subsets of my data. When only common females were included in the analysis, both female size and male ecotype weakly influenced courtship frequency (Table 3.2.4). Common and white male stickleback males courted larger common females more than the smaller common females

(Fig. 3.2.4). By contrast, when a subset of the data containing only white females was examined, there remained an interaction between female size and male ecotype (Table 3.2.4). Common male stickleback courted larger white females more, while white male stickleback courtship frequency remained fairly constant regardless of white female body size (Fig. 3.2.4).

Table 3.2.3. Generalized linear model selection for the effect of male ecotype, female ecotype, and female body size (standard length [mm]) on male Threespine Sticklebacks courtship frequency. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19 for the first model, n = 5 for the other two). The bolded models are those with the lowest AICc values by a difference of two or more.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
total courtship frequency	**female eco. + female size + male eco. + female eco. x male eco. + female size x male eco.**	**8**	**874.2**	**0.00**	**0.619**
	female eco. + female size + male eco. + female eco. x male eco. + female size x male eco. + female eco. x female size	**9**	**875.7**	**1.43**	**0.302**

	female eco. + female size + male eco. + female eco. x male eco. + female size x male eco. + female eco. x female size + female eco. x female size x male eco.	10	878.3	4.11	0.079
	(intercept)	3	921.7	47.52	0.000
common females	**(intercept)**	3	**431.2**	**0.00**	**0.430**
	female size	**4**	**432.3**	**1.11**	**0.247**
	male ecotype	**4**	**432.9**	**1.73**	**0.181**
	female size + male ecotype	**5**	434.6	3.42	0.078
	female size + male ecotype + female size x male ecotype	6	434.9	3.79	0.065

Table 3.2.3 (continued). Generalized linear model selection for the effect of male ecotype, female ecotype, and female body size (standard length [mm]) on male Threespine Sticklebacks courtship frequency. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19 for the first model, n = 5 for the other two). The bolded models are those with the lowest AICc values by a difference of two or more.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
white females	**(intercept)**	*3*	**414.6**	**0.00**	**0.279**
	male ecotype	**4**	**414.9**	**0.33**	**0.237**
	female size + male ecotype + female size x male ecotype	**6**	**415.0**	**0.44**	**0.224**
	female size	**4**	**415.8**	**1.19**	**0.154**
	female size + male ecotype	**5**	**416.5**	**1.94**	**0.106**

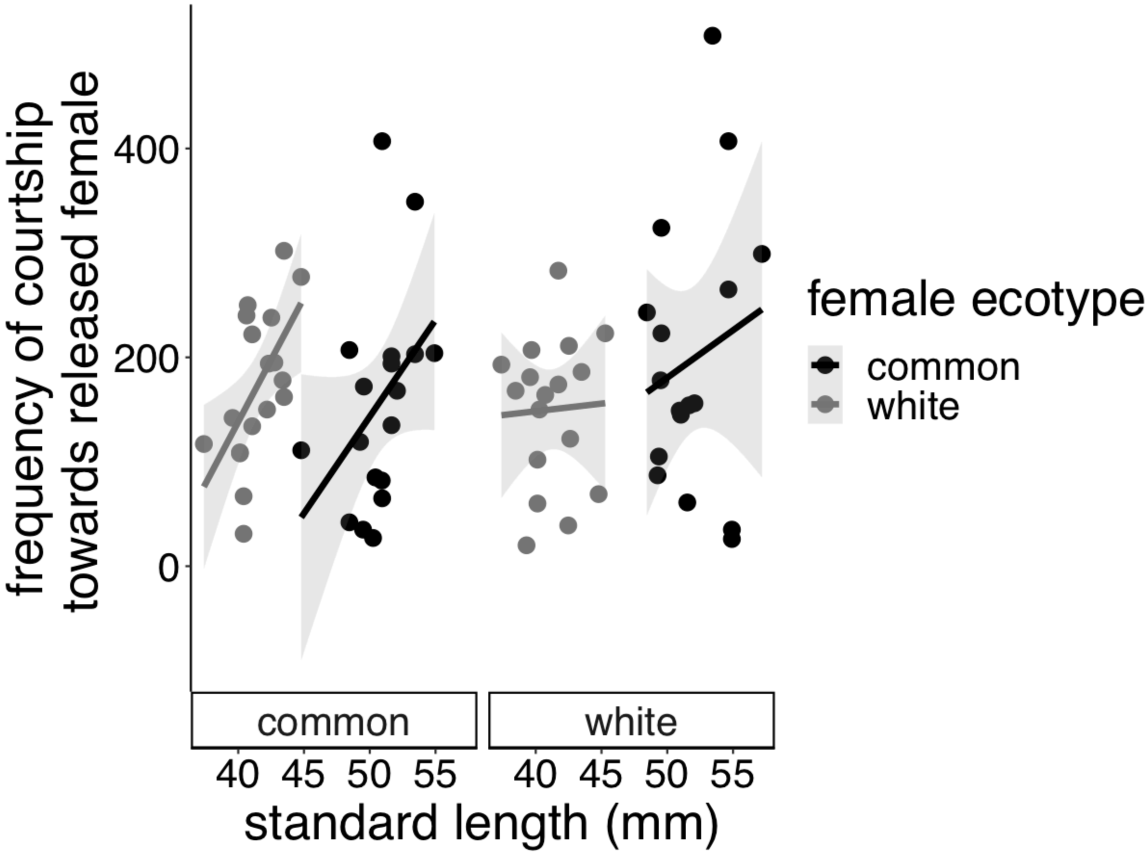

Figure 3.2.4. Total courtship frequency of common (left) and white (right) male Threespine Sticklebacks towards common (black) and white (grey) female sticklebacks based on the standard length (mm) of the females during the 15-minute interaction stage (n = 35). Black and grey circles represent the frequencies for individual males. Individual males are counted twice as each male had an observation recorded for both a common and white female stickleback.

Male population type

Male preference for female body size was also analysed for males from different population types (allopatric or sympatric). The frequency of courtship behaviours conducted towards the released females during the interaction stage was again analysed but with a focus on male population type instead of male ecotype. One model consisting of two interaction terms between the ecotype relationship with both female size and male population type best predicated the courtship behaviours conducted by sympatric and allopatric male stickleback (Table 3.2.4). As female body size increased, sympatric male stickleback increased their courtship frequency showing a higher preference for larger females (Fig. 3.2.5). Allopatric males remained relatively constant in their courtship towards females, regardless of body size, with a very slight increase for allopatric males courting females of a different ecotype (Fig. 3.2.5). Interestingly, both allopatric and sympatric males had a higher frequency of courtship behaviours oriented towards females that were not of the same ecotype as themselves (Fig. 3.2.5). As before, due to the interactive effects, I also examined subsets of my data. When only females that were the same ecotype as the male were included in the analysis, male population type was the only predictor influencing the data (Table 3.2.4). Sympatric males courted females that were the same ecotype as themselves not only at a higher frequency but with an increasing frequency as body size increased (Fig. 3.2.5). Similarly, when only females that were of a different ecotype than the male were included in the analysis, male population type was the best predictor (Table 3.2.4). Both allopatric and sympatric males increased their courtship frequency as female body size increase, albeit very slightly for allopatric whereas sympatric males increased a considerable amount (Fig. 3.2.4).

Table 3.2.4. Generalized linear model selection for the effect of female body size (standard length [mm]), male population type (allopatric or sympatric), and whether the female was the same ecotype as the male (ecotype relationship) on male Threespine Sticklebacks courtship frequency. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19 for the first model, n = 5 for the other two). The bolded models are those with the lowest AICc values by a difference of two or more.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
total courtship frequency	**eco. relation + female size + male pop. + eco. relation x female size + eco. relation x male pop.**	**8**	**902.0**	**0.00**	**0.707**
	eco. relation + female size + male pop. + eco. relation x female size + eco. relation x male pop. + female size x male pop.	9	904.3	2.32	0.222
	eco. relation + female size + male pop. + eco. relation x female size + eco. relation x male pop. + female size x male pop. + eco. relation x female size x male pop.	10	906.9	4.90	0.061
females of the same ecotype	**male population type**	**4**	**411.0**	**0.00**	**0.750**
	female size + male population type	5	413.8	2.73	0.192

	female size + male population type + female size x male population type	6	416.7	5.64	0.045
	(intercept)	3	419.7	8.66	0.010
	female size	4	422.0	10.97	0.003

Table 3.2.4 (continued). Generalized linear model selection for the effect of female body size (standard length [mm]), male population type (allopatric or sympatric), and whether the female was the same ecotype as the male (ecotype relationship) on male Threespine Sticklebacks courtship frequency. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19 for the first model, n = 5 for the other two). The bolded models are those with the lowest AICc values by a difference of two or more.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
females of a different ecotype	**male population type**	**4**	**417.0**	**0.00**	**0.514**
	(intercept)	**3**	**418.7**	**1.68**	**0.222**
	female size + male population type	5	419.7	2.68	0.134
	female size	4	421.1	4.12	0.065
	female size + male population type + female size x male population type	6	421.1	4.14	0.065

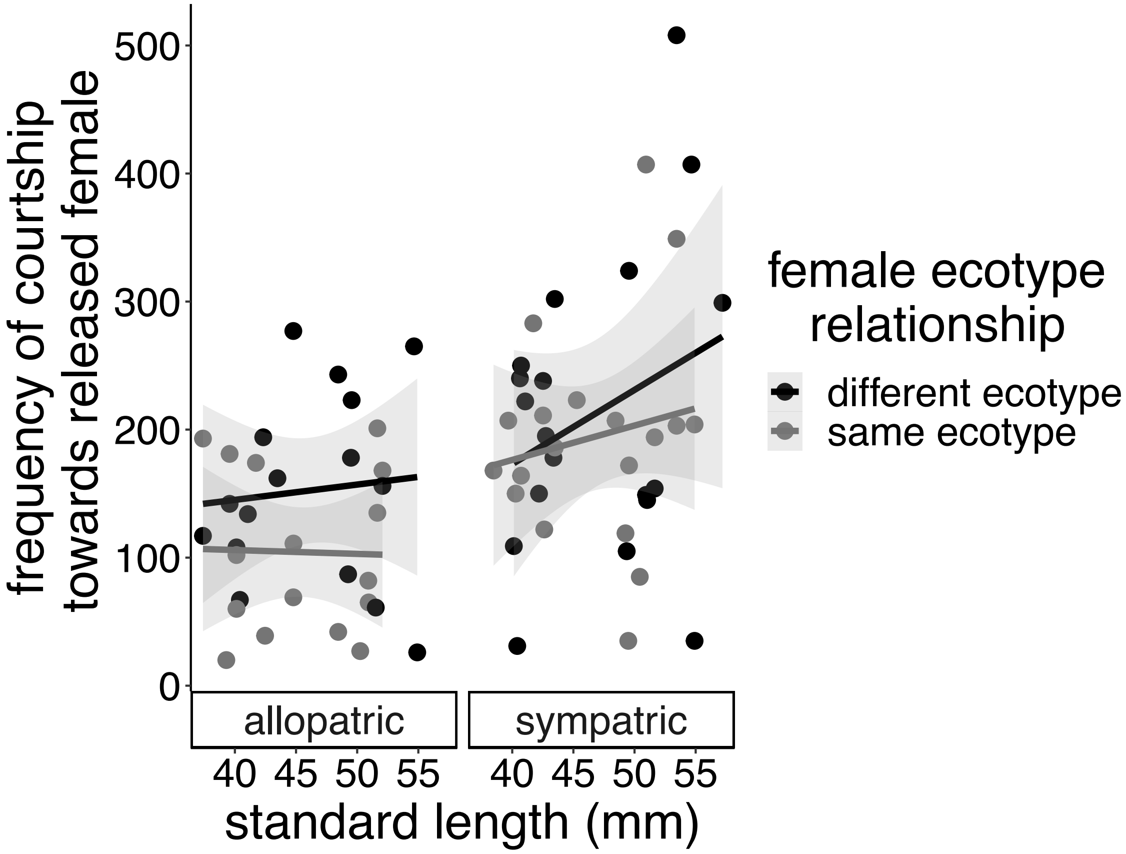

Figure 3.2.5. Total courtship frequency of male Threespine Sticklebacks from allopatric (left) and sympatric (right) populations conducted towards females of the same (grey) or different (black) ecotype than themselves based on standard length (mm) of the females during the 15-minute interaction stage (n = 35). Black and grey circles represent the frequencies for individual males. Individual males are counted twice as each male had an observation recorded for both a common and white female stickleback.

3.2.3 Female preference

Interaction stage

In addition to assessing male preference during the interaction stage of the experiment, I also quantified female preference using female behavioural cues. For the analysis, individual behaviour frequencies were not used, instead, a binomial data set was created where a value of 1 indicated that any of the three behavioural cues (heads-up display, follow, and/or inspect nest) were observed, while a value of 0 denoted that none of the behaviours were observed. I used a GLMM with male ecotype, female population type, whether the male and female were of the same ecotype or not, and their interactions as the fixed effects. Individual female identity was the random effect because some females were used more than once. Three models were retained that contained interactive and additive effects between the three predictors (Table 3.2.5). Sympatric females generally responded more than allopatric females, with the exception of allopatric common females who responded more to white males than their sympatric counterpart. White females also responded more to white males in comparison to the response of common females towards common males and allopatric common females performed no response behaviours towards common males. Moreover, both female ecotypes responded in a similar quantity to males of the opposite ecotype; however, post-hoc tests revealed that these trends were not significantly different from one another (Fig. 3.2.6).

Table 3.2.5. Generalized linear model selection for the effects of male ecotype, female population type (allopatric or sympatric), and whether the male was the same ecotype as the female (ecotype relationship) on whether a female responded to a male or not. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 19). The bolded models are those with the lowest AICc values by a difference of two or more. Only the top five models are shown below.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
female responded	**ecotype relation + male ecotype + ecotype relation x male ecotype**	**5**	**84.5**	**0.00**	**0.379**
	ecotype relation + male ecotype + female population type + ecotype relation x male ecotype	**6**	**86.1**	**1.67**	**0.164**
	ecotype relation + male ecotype + female population type + ecotype relation x male ecotype + male ecotype x female population type	7	87.3	2.87	0.090
	male ecotype	3	87.7	3.27	0.074
	(intercept)	2	88.2	3.78	0.057

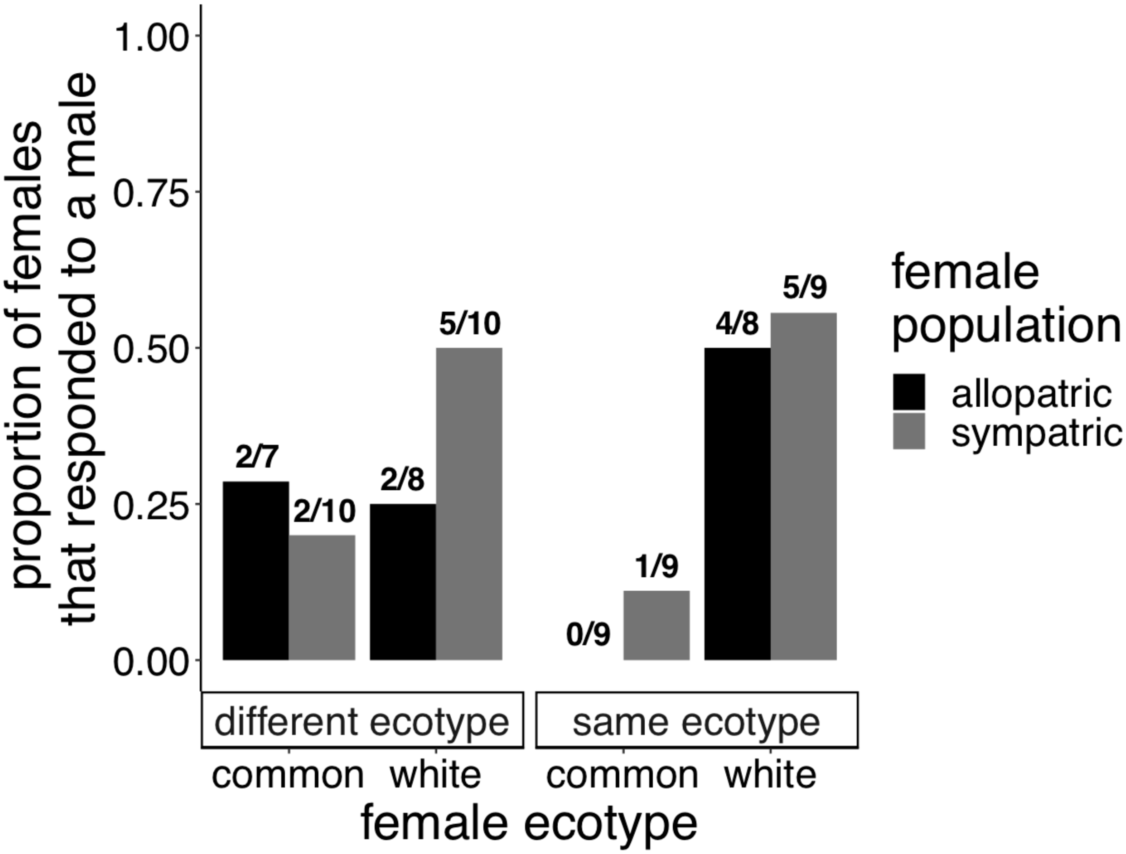

Figure 3.2.6. Proportion of common and white Threespine Stickleback females that originated from an allopatric (black) or sympatric (grey) population and responded to a male of a different (left) or similar (right) ecotype as themselves, during the interaction stage of the experiment. A response indicates that females performed at least one of the three response behaviours ("heads-up" display, following the male to his nest, and/or inspecting the male's nest). Numbers above each bar represent the number of trials where a female responded at least once out of the total number of replicates of that female ecotype, male ecotype, and female population type. Of the 70 trials 21 had at least one response behaviour performed by a female.

3.3 Threespine Stickleback spawning events

Ten spawning events occurred during the interaction phase of the 35 mate choice trials (28.6%). Five of the ten spawnings were hybrid matings; four of these were between common males and white females (Table 3.3.1). For four of the five hybrid crosses, at least one of the fish originated from the area containing both common and white stickleback (sympatric populations). The fish for the remaining hybrid cross were both from allopatric populations. Of the five pure crosses, one was a common male and female cross, while four were between a white male and female (Table 3.3.1).

Table 3.3.1. Number of spawning events for each combination of male and female Threespine Stickleback of both ecotypes and population types.

Male Population and Ecotype	**Female Population and Ecotype**			
	Antigonish Landing Common	Rainbow Haven Common	Crossing Road White	Rainbow Haven White
Antigonish Landing Common	0	0	1	1
Rainbow Haven Common	0	1	1	1
Crossing Road White	0	0	2	1
Rainbow Haven White	0	1	1	0

4. DISCUSSION

The Threespine Stickleback species complex provides a unique opportunity to study how these fish have evolved and diverged in various environments. The common and white morphs in Nova Scotia, Canada are an excellent study species for understanding how sympatric speciation may occur (Blouw and Hagen 1990; Samuk 2016). The two ecotypes are genetically distinct (Samuk 2016), though there is still evidence of gene flow between morphs (Samuk 2016), and allopatric and sympatric populations have shifted over the past 30 years with previously undocumented populations arising and known populations disappearing (Blouw and Hagen 1990, personal observations). However, hybrids have not been found in the wild, as all observed spawnings prior to this work have been positive assortative (Blouw and Hagen 1990; Jamieson et al. 1992a), and genetic evidence suggests that common and white stickleback have not collapsed into a single morph (Samuk 2016). To further understand the mechanisms that maintain the two Threespine Stickleback ecotypes, I examined the mate preference of males and females using behavioural and visual cues as possible pre-mating isolation mechanisms. Despite previous research that found that the ecotypes mate assortatively (Haglund et al. 1990; Blouw and Hagen 1990; Jamieson et al. 1992a), my findings indicate that mixed-matings occur as frequently as within-ecotype matings. Additionally, my results indicated that there is no strong mate preference by males or females for either ecotype based on the time males spent in proximity to females and the response behaviours performed by females, nor do the sexes perform these behaviours assortatively based on ecotype. However, when considering male courtship frequency, sympatric white male stickleback courted common females more than allopatric common stickleback males. Moreover, there was evidence that male stickleback of both ecotypes and population types adjusted their courtship frequency based on the body size of females. Common male stickleback preferred to court larger-bodied females of both ecotypes, while white male stickleback showed no body size preference among white females, but increased their courtship for larger-bodied common females. In addition, males from allopatric populations remained relatively constant in their courtship frequency as female body size increased, but sympatric males courted larger females at a higher frequency than they did smaller females.

4.1 Ecotype confirmation

I examined male nesting material preference and body size of the Threespine Sticklebacks collected to ensure proper ecotype identification for the experiment. Nest preference and body size differences between common and white Threespine Sticklebacks were congruent with previous reports that common stickleback males preferred nests made of sand while white stickleback males preferred algae nests, and that common stickleback are larger than white stickleback.

Studies have reported the nest preferences of white and common Threespine Stickleback males and the requirement of sand or mud and/or algae specifically for nest construction (van Iersel 1953; Haglund et al. 1990; Blouw and Hagen 1990; Jamieson et al. 1992a; Blouw 1996). Blouw and Hagen (1990) suggested that the algae preference of the white stickleback may be related to benefits associated with cover and protection because the white stickleback is very conspicuous in colour, and as a potential source of aeration for fertilized eggs. By contrast, common stickleback typically prefer more open areas not obstructed by dense algae, as the covered areas may reduce visibility and the prospects of encountering a female (Candolin and Salesto 2006). My findings are consistent with these previous reports indicating that nesting material preference is an accurate method with which to identify the ecotype of male Threespine Sticklebacks. In addition to nesting material, my results support the use of relative body size as a determinant of ecotype. Common stickleback of both sexes were larger than both sexes of white stickleback which is consistent with the initial findings of Blouw and Hagen (1990) and other, more recent studies (see Samuk, Iritani, and Schluter 2014; Samuk 2016).

4.2 Interbreeding and hybridization in white and common Threespine Sticklebacks

During the experiment ten spawning events occurred resulting in ~29% spawning success. Spawning success of Threespine Stickleback in laboratory experiments varies greatly from 0-100% (see Ridley and Rechten 1981; McLennan and McPhail 1989; Blouw and Hagen 1990; Jamieson and Colgan 1992; Dean, Dunstan, Reddish, and MacColl 2021 for examples). Of these ten spawnings, five were pure crosses, while the other five were hybrid crosses that occurred in both directions and involved fish from both population types (allopatric or sympatric).

The observation that white and common Threespine Sticklebacks will interbreed is not congruent with the findings from previous studies on these ecotypes, where mating was completely assortative both in the laboratory and the field (Blouw and Hagen 1990; Jamieson et al. 1992a). Additionally, white Threespine Sticklebacks are no longer found in some of the locations previously discovered by Blouw and Hagen (1990, e.g. Antigonish Landing) and new populations of allopatric and sympatric white stickleback have been found (personal observations), indicating that there may be spatial and temporal effects influencing the distribution of stickleback ecotypes over the past 30 years. My findings indicate that reinforcement may not be as strong as previously suspected in these stickleback populations.

Reinforcement is related to the prezygotic barriers that aid in selecting against hybrids in sympatric populations (Dobzhansky 1940; Servedio and Noor 2003). It is thought that sympatric populations should evolve stronger mating preferences compared to allopatric populations if hybrids are less viable or have reduced fertility, as selection can act on populations in sympatry to favour conspecifc matings (Dobzhansky 1940; Rundle and Schluter 1998; Servedio and Noor 2003 and references therein). While there is evidence that some sympatric populations of Threespine Stickleback ecotype pairs found in Washington, USA have evolved strong mate choice based on male colouration to avoid inbreeding (McPhail 1969; Scott 2004), there are instances where populations from the same watersource in Washington show little preference for colouration (McKinnon 1995; Tinghitella et al. 2015). Similarly, there are some sympatric populations of stickleback that show preferences for specific traits based on ecotype that may also interbreed under certain conditions (Hagen 1967; Ólafsdóttir et al. 2006; Conte and Schluter 2013). For example, benthic and limnetic Threespine Sticklebacks are known to hybridize in the wild and do so based on size matching. Benthic stickleback are larger in size and heavier in mass than limnetic stickleback (Schluter and Mcphail 1992; Nagel and Schluter 1998) and hybridization may occur when individuals on the extreme end of their ecotype's size distribution overlap with the extreme of the other ecotype (e.g. a small benthic with a large limnetic; Nagel and Schluter 1998; Conte and Schluter 2013). In the Little Campbell River in British Columbia, Canada, pure marine and freshwater stickleback occupy two separate ends of the coastal stream, but there is evidence of hybridization where the two populations connect in the middle (Hagen 1967). Moreover, there are Icelandic sympatric stickleback populations for which instances of inter-ecotype spawning events have been observed, despite the general observation that they mate assortatively (Ólafsdóttir et al. 2006). These Icelandic stickleback, like the common and white stickleback, are genetically distinct but most likely diverged recently (Ólafsdóttir 2004).

While my finding of interbreeding between white and common stickleback was unexpected because there have been no documented common and white stickleback hybrids in the wild and the two ecotypes are considered genetically distinct (Samuk 2016), gene flow can occur between the two ecotypes. Laboratory breeding studies have noted that through artificial crosses common and white ecotypes can interbreed and produce viable offspring (Blouw 1996). The same study also reported that male colouration and whether a male performs parental care duties or not are heritable traits (Blouw 1996). Because the two ecotypes are interfertile, produce viable offspring, and their identifying traits are heritable, it is not illogical to suspect that there may be instances in sympatric populations where hybridization may occur. It is possible that white and common stickleback are at the end of a potential divergence spectrum, whereby they are beginning to collapse back into one ecotype or in the process of diverging into different species. Both hypotheses are supported by the finding that there appears to be no ecotype-based preference for either white or common males or females, nor is there pre-zygotic reinforcement in sympatric populations. However, there is evidence in favour of the maintenance of the two ecotypes, where initial findings reported that the offspring of F1 hybrids are not adequate fathers which results in egg death without artificial aeration

(C. Behrens, personal communication). Additionally, one study has shown that the well-known naturally occurring hybrids of the limnetic and benthic stickleback pair after four generations and across three life history stages showed a decline in hybridity (Gow et al. 2007). Although it appears that reinforcement may not be the main reason for the separation of common and white ecotypes, there could be other mechanisms at play (see section 4.3.1.1) that could explain why we do not find hybrids in the wild but that gene flow still occurs. It has also been suggested by Servedio and Noor (2003) that selection against hybrids may be negligible in other Threespine Stickleback species pairs when compared to other mechanisms such as mating cues (e.g. colouration, Boughman 2001, and body size, Nagel and Schluter 1998) to help in premating isolation (Servedio and Noor 2003). A generational study would be beneficial to fully understand the impacts of mate selection (or lack thereof) within the common and white ecotypes of the Threespine Stickleback.

4.3 Mate choice in Threespine Stickleback

4.3.1 Male preference as an indicator of mate choice

I predicted that common male stickleback would be more likely to choose a female of the same ecotype than white males due to the amount of investment males provide (i.e. parental care vs. no parental care). However, my results indicate that male Threespine Sticklebacks showed no preference for one ecotype or the other as they spent the same amount of time in proximity to either female ecotype during the preference stage.

Because white male stickleback do not perform parental care duties (Blouw and Hagen 1990; Jamieson et al. 1992b; Blouw 1996) they do not experience the reproductive “time-outs” or the energy constraints associated with paternal care as do common male stickleback (Jamieson et al. 1992b). For this reason, appropriate mate selection (i.e., a white female) may not be as important for white males as it would be for common males, because their fitness cost associated with producing hybrid offspring may not be as high.

The allocation of energy reserves is important in common male stickleback since males must distribute their energy between courtship and parental care. A study conducted on common male courtship found that males who courted more vigorously were poor fathers compared to the less vigorous courters as they had less energy to put towards their parental care duties (von Hippel 2000). However, because white males do not have this parental care constraint, they can invest their energy in pre-mating behaviours such as courtship and aggression to optimize mate acquisition and their reproductive success. Previous studies have reported white male stickleback to have higher courtship frequency than their sympatric common counterparts (Blouw and Hagen 1990; Jamieson et al. 1992a; Macdonald et al. 1995; Haley et al. 2019). Even though we expect white males to invest more in mating than parental behaviour compared to common males, aggression levels were similar between the two ecotypes, indicating that white male stickleback may use inter-sexual selection to enhance their mating success (Haley et al. 2019).

It is surprising that common male stickleback showed no preference for the ecotype of the female they were courting, counter to my prediction. Because common male stickleback assume the burden of paternal care and associated energy costs, it is expected that they would be more selective with respect to ecotype. A possible explanation for the interactions between white and common Threespine Sticklebacks is what population type (allopatric or sympatric) the individuals originated from. Previous work that reported positive assortative mating between common and white stickleback only considered sympatric populations (Blouw and Hagen 1990; Jamieson et al. 1992a; Blouw 1996). It is suspected that males from sympatric populations would have stronger mate preferences than males from allopatric populations because sympatric populations may have experienced selection against interbreeding if there are fitness costs to hybridization (egg death when cared for by the father; Behrens, personal communication), while allopatric populations would not have undergone the same evolutionary process and selection for an appropriate mate may not be reinforced. For this study, male population type was included and it was found that during the interaction stage, sympatric white males courted common females at a higher frequency than did allopatric common males but otherwise there were no differences otherwise.

It was not unexpected that allopatric male sticklebacks showed no preference for their female counterpart. Allopatric male stickleback when interacting with a female that is morphologically similar to his ecotype with the exception of body size (Blouw and Hagen 1990; Samuk 2016), may court the only female option over no female at all. Courtship oriented towards the different ecotype may arise as the allopatric males might not have experienced the negative outcome of mating with females of a different ecotype that is reinforced in sympatric populations evolutionarily through selection. Another possibility is poor initial mate recognition. For example, results showed for when allopatric *Calopteryx virgo* Damselfly males were exposed to heterospecific and conspecific females, there was a non-significant trend for males to be able to differentiate between the two female types (Kuitunen et al. 2012). However, when exposed to conspecific females for an extended period of time, allopatric males' discriminating ability was strengthened (Kuitunen et al. 2012). The authors of that study suggest that learning might be a factor in species recognition (Kuitunen et al. 2012). The fact that sympatric white males courted common females with higher frequency than white females, or that sympatric common males courted females of either ecotype seemingly indiscriminately is interesting. If the separation of common and white stickleback in sympatric populations was due to reinforcement, we would have expected male preference to exist for common males who provide care, and for both male ecotypes to avoid related fitness costs as is seen in other species. Peterson and colleagues (2005) have noted that male mating preferences of sympatrically occurring Blue Milkweed beetle (*Chrysochus cobaltinus*) and Dogbane beetle (*Chrysochus auratus*) have evolved with the help of reinforcement (Peterson et al. 2005). Sympatric *Chrysochus cobaltinus* males are more likely to mate with conspecific females than allopatric male *C. cobaltinus* (Peterson et al. 2005). The same study also noted that sympatric *C. auratus* males are less discriminating about their mate compared to the *C. cobaltinus* males (Peterson et al. 2005), which is similar to my observations.

Additional mechanisms that maintain ecotypes

Male preference does not appear to be a strong pre-mating isolation barrier for the white and common ecotypes of Threespine Stickleback, regardless of the population type (allopatric or sympatric). However, there are various additional avenues that may prevent stable hybridization between ecotypes of Threespine Stickleback, such as other isolating mechanisms that visually identify an individual (e.g. body size, colouration, and others, Nagel and Schluter 1998; Boughman 2001; Servedio 2001; Servedio and Noor 2003) or mechanisms that could occur after mating (e.g. differential sperm production and sperm precedence [see Howard 1999 for a review], hybrid sterility, inviability, or fitness [see Wirtz 1999 for examples] and female death and low fertility [Servedio 2001]).

The Threespine Stickleback mating system is typically described as female choice dominated in the common Threespine Stickleback and various other stickleback ecotypes including hybrids (McPhail 1969; Milinski and Bakker 1990; Bakker 1993; Baube et al. 1995; Conte and Schluter 2013; Bay et al. 2017). Because male mate choice (or mutual mate choice) may not be as prominent in the stickleback mating system, though there is evidence (see Rowland 1982; 1989; Bakker and Rowland 1995; Kraak and Bakker 1998), male selection for female identifiable traits may not be as strong. Indicators of female quality focus mainly on body size in relation to fecundity (Hagen 1967; Wootton 1973; Rowland 1989); however, there is little in comparison into identifying other traits of female stickleback. In fact, common and white female stickleback are morphologically very similar (Blouw and Hagen 1990; Samuk 2016). Common and white stickleback have complete lateral plate coverage, similar gill raker numbers, and similar colouration outside of the breeding season (Blouw and Hagen 1990; Samuk 2016). While there is evidence that the two stickleback differ in body depth and size (Blouw and Hagen 1990), when controlling for body size (as depth and other morphological traits scale with size) there are no differences in body shape with the exception of white stickleback having slightly shorter pelvic spines (Samuk 2016). This similarity in morphology could create difficultly when visually identifying one female ecotype from the other. However, there are other instances within the Threespine Stickleback species complex, such as the limnetic and benthic species pair, where even though males are able to distinguish between females of the same or different ecotype as themselves, they courted the two female types differently, they still courted both female types with similar vigor (Kozak et al. 2009). Male preference should be related to not only identifying an appropriate mate but also to distinguishing a good quality mate from a poor-quality mate or else distributing their resources appropriately.

Differentiating sperm production based on the type of female present could be a possible explanation as to why, when looking at courtship behaviours and time spent in proximity to a female, male stickleback appear to have no preference for the female's ecotype. Males may be courting females of a different ecotype than themselves with similar frequency as they would females of the same ecotype while simultaneously manipulating their sperm reserves to properly reflect the different ecotype female. Differential sperm production has been seen in other fish species, for instance, male Sailfin Mollies (*Poecilia lapitinna*) that originated from both allopatric and sympatric populations when in the presence of a conspecific female produced more sperm than when in the presence of a heterospecific female (Aspbury and Gabor 2004b). Through this method the male could still mate without wasting a large amount of genetic resources if a conspecific female becomes available later. Post-zygotic reproductive barriers related to hybrid sterility or inviability may not occur with first generation hybrids because hybrids between the two ecotypes survive and are fertile, though evidence for stickleback hybrids has only been found in laboratory crosses (Blouw 1996, C. Behrens, personal communication). Because the hybrids survive this could result in diminished pre-zygotic barriers (e.g. courtship display and/or colouration), as the parental individuals do not experience the direct fitness cost of eventual egg death of the second generation hybrids (C. Behrens, personal communication). However, the survival of first-generation hybrids indicate that there should be repeated interbreeding among stickleback that results in detection of hybrids in the wild, but wild hybrids are not found (Samuk 2016). If the behavioural difference of the hybrid fathers of the first generation is heritable, as other traits have shown to be (Blouw 1996), then the hybrid lineage will end when the F2 eggs die. Reduced fitness in F1 hybrids is not uncommon in stickleback. Red and Black Threespine Stickleback hybrids experience a 5% reduction in survivability and the fertility of males (McPhail 1969). These hybrids also experience a 50% reduction in viability when backcrossed with parental species (McPhail 1969). Additionally, although the first-generation hybrids are viable, they may experience a reduction in fitness when acquiring a mate as observed in other stickleback hybrids. For example, limnetic and benthic hybrids have lower reproductive success compared to pure individuals as they must attempt to outcompete the pure parental male ecotypes for access to pure parental females (Vamosi and Schluter 1999). Moreover, the same study noted that pure limnetic males courted limnetic females more intensely than hybrid males and that limnetic females preferred pure limnetic males (Vamosi and Schluter 1999). Thus, in common and white Threespine Sticklebacks, reinforcement may not be acting on the “pure” parental species, but rather on the first-generation hybrids who must out-compete their pure rivals, and who appear to not be adequate fathers to produce the next generation. However, as mentioned earlier, common and white stickleback hybrids have only been observed in the laboratory. No hybrids have been documented in the wild, and thus perhaps there are other mechanisms in the natural environment (e.g. temporal differences, if common and white stickleback are reproductively available at different times, or ecological differences, if females are not able to interact with males of the different ecotype due to nesting location/material) that are removing hybrids from the population. *In situ* observations and generational studies of stickleback in their natural habitat could improve our understanding of male mate preferences, ecotype recognition in sympatric and allopatric populations and the prevalence and fitness of hybrids in sympatric populations.

Male courtship effort and female body size

Based on courtship frequency with respect to female body size, common male stickleback preferred larger-bodied females of both ecotypes, while white male stickleback showed only a preference for larger-bodied common females but not larger white females. Common male stickleback increased their courtship for both larger-bodied common and white females, but did not prefer only the largest females.

Female size is positively correlated with fecundity in various fish species (*Nerophis ophidion,* Berglund et al. 1986; *Fundulus diaphanus,* Phillips et al. 2007; *Salvelinus alpinus,* Smalås et al. 2017; review of fishes, Helfman et al. 2009). Threespine Stickleback females fit this pattern, where fecundity varies with female body size (Rowland 1989), with larger females producing more eggs (Hagen 1967; Wootton 1973); however within the white stickleback, similar sized females produce a variety of clutch sizes (Blouw 1996).

Common male stickleback prefer larger-bodied and more distended females (Rowland 1989; Kraak and Bakker 1998), and common males did increase courtship for larger females in this study; as did white males (for common females). Preference for larger females stems from a female's body size being a good proxy for her spawning readiness and egg quantity or size (Rowland 1989; Kraak and Bakker 1998). White males did not, however, increase courtship for white females. White male stickleback may be courting white females of all sizes since there is variation in clutch sizes with similarly sized females producing differing amounts of eggs (Blouw 1996). Mating based on body size is not uncommon in the Threespine Stickleback species complex; limnetic and benthic Threespine Stickleback ecotypes are known to interbreed and in some instances they differentiate between one another by size matching, though when the two ecotypes are within similar sizes to each other, hybridization may occur (Nagel and Schluter 1998; Conte and Schluter 2013). Additionally, populations of anadromous and freshwater Japan Sea and Pacific Ocean ecotypes of Threespine Stickleback also demonstrated some variation in courtship behaviours towards differently sized individuals (Ishikawa and Mori 2000). Moreover, some male fish such as Sailfin Mollies differentially allocate their sperm production, by producing more sperm in the presence of larger-bodied females compared to smaller females (Aspbury and Gabor 2004a). In organisms other than fish, there is evidence of males actively choosing heterospecific females due to their larger size (and fecundity) over conspecifics females (e.g. Groundhoppers, Hochkirch et al. 2007; Pacific Island Geckos, Dame and Petren 2006). It is possible that white and common stickleback may not only consider the size of a potential mate as a measure of her fecundity but also as an indicator of compatibility. The gravidity of females was not measured in this study, and future avenues of work may involve quantifying the relationship between female fecundity and male preference in the white stickleback.

4.3.2 Female response as an indicator of mate choice

Based on previous findings (Blouw and Hagen 1990), I expected that common females would respond to both male ecotypes, but ultimately mate with common males, while white females would show only a preference for white males. I also expected that, as with the male stickleback, sympatric females would be more selective of their mate than allopatric females, due to the fitness costs associated with interbreeding. Preference for common males may arise due to their parental care (van Iersel 1953), where preference for white males may be associated with their vigorous courting and conspicuous colouration (Jamieson et al. 1992a; Haley 2018). By contrast, I found that female stickleback of both ecotypes and population types have no preference for either male ecotype. My findings show that although all predictors (male ecotype, female population type, and ecotype relationship) were considered to have influenced female response, there were no significant differences in the probability of a response from females of both ecotypes towards males of either ecotype, nor did the population type (sympatric or allopatric) of the female influence her behaviour.

The probability of response towards common males by common females was lower than expected. Only sympatric common females responded with stereotypical mating displays directed toward common males, while common females from both population types responded to white males. This finding is similar to what has been reported previously for this ecotype (Blouw and Hagen 1990; Jamieson et al. 1992a), where common females are initially attracted to white males. However, white females from allopatric and sympatric populations also responded to both male ecotypes similarly, not only white males, indicating that my findings for female behaviour are different from the original study published 30 years ago (Blouw and Hagen 1990). A lack of clear preference by common and white females for either male ecotype may arise if both female ecotypes are attracted to the paternal care provided by common males (van Iersel 1953), while also being interested in the more energic courtship attempts and bright colouration of white male sticklebacks (Jamieson et al. 1992a; Haley et al. 2019).

Because female stickleback do not provide maternal care, the benefits associated with the male providing parental care are high. Selecting a mate who appears to be a good father would be beneficial to the female to ensure her eggs will thrive. Females can assess a male's parental ability using his red nuptial colouration (Candolin 2000). Furthermore, when in the presence of multiple males, a female can determine the best care-giver as red colouration more accurately represents male parental ability during competition (Candolin 2000). In addition to preferring the most red male, common females prefer to mate with males who already have eggs in their possession, as this is an indicator of a high quality mate (Ridley and Rechten 1981). However, other studies that examined female preference noted that instead of egg presence in a male's nest, his readiness to court may serve as a better indicator (Jamieson and Colgan 1992 and references therein). Because male stickleback participate in egg parasitism, the eggs present in the courting male's nest may not accurately represent his quality if he stole the eggs from another male (Jamieson and Colgan 1989; Jamieson et al. 1992b). Because egg presence may not be as influential on female response as the studies above hinted, female stickleback, when in the presence of a male who possesses no eggs, as in my study, may have a more difficult time choosing a mate and may respond to a potential caring common male as much as she would toward an energetic courting white male, as was seen in my study.

Although white stickleback may not possess eggs in their nest, their energetic courtship might be initially appealing to females over egg presence. Additionally, egg presence indicates that a male already mated with one or more females, which could indicate sperm depletion. Females may avoid mating with potential sperm-depleted males in favour of an individual who could fertilize all of her eggs (Nakatsuru and Kramer

1982; Harris and Moore 2005). White males may show no eggs in his nest, which could act as an indication that he is not sperm-depleted. While nothing is known about sperm regeneration in white males, common male stickleback are limited by their sperm supply. Spermatogenesis in Threespine Sticklebacks is physiologically inhibited by androgens until breeding ceases at the end of the season (Borg 1981; Borg and Mayer 1995). Post-breeding, spermatogenesis commences once more to produce more spermatozoa for the following breeding season (Borg 1981; 1982). As a result, male stickleback who mate multiple times have significantly reduced sperm counts and smaller ejaculate size compared to virgin males (Zbinden et al. 2001).

Additionally, larger males invest more sperm into a spawning event compared to smaller males (Zbinden et al. 2001), which could allow male size to be an indicator of quality to selecting females as it would ensure all of her eggs get fertilized. Trends for male size as an indicator for females has been observed in *Poecilia reticulata* which suggested females preference for larger males may result from indirect genetic benefits (Reynolds and Gross 1992) and in some stickleback populations, such as the limnetic and benthic and Icelandic species pairs (Boughman et al. 2005; Ólafsdóttir et al. 2006). As well, in common Threespine Stickleback, larger males who defended a larger and higher quality territory encountered more females than other males, suggesting that these males were of higher genetic and/or phenotypic quality (Candolin and Voigt 2001). Although male body size was not analysed in this study, it could be a measure for females to assess males as potential mates.

Although only male courtship behaviours were analysed in this study, females of one or both ecotypes could also be assessing multiple physical characteristics of males, such as their body size, and other attributes that would represent a male's quality and/or identity that could promote (or hinder) isolation between ecotypes. Within the limnetic and benthic species pairs, female preference for male colouration was determined to be strong for limnetic females and less so for benthic females, and that limnetic males have more exaggerated traits compared to benthic males (e.g. colour; Boughman 2001; Boughman et al. 2005). These differences in traits and preferences creates an environment for strong reproductive isolation as limnetic females will be more selective of their mates compared to benthic females (Boughman 2001; Boughman et al. 2005). Moreover, a study conducted on allopatric populations of red and black ecotypes of Threespine Stickleback that are known to mate assortatively showed that when females could not choose among potential mates, they would mate with the ecotype different from their own (McPhail 1969). Similarly, Threespine Stickleback females once ovulated, their eggs' lifespan shortens as time passes therefore, females become less discriminatory towards the male they respond to in order to ensure her eggs have a chance to get fertilised (Bakker and Milinski 1991; Wirtz 1999). Although, focused on female stickleback selecting common male stickleback of varying brightness, the idea could still apply to common and white females who need to spawn with any male or risk her eggs dying. Within my study, it is possible that many of these situations arose within and between female and male Threespine Sticklebacks, such as no strong response for either male ecotype by females influencing the choice of mating partners in female stickleback.

In addition to colouration and behaviour, several other factors can be used by females to assess males. Common and other female stickleback ecotypes assess male sticklebacks' MHC genetic make-up, as it is directly associated with many beneficial characteristics including parasite and infection resistance, better quality nests, and higher reproductive success (Reusch et al. 2001; Aeschlimann et al. 2003; Milinski 2003; Jager et al. 2007; Eizaguirre et al. 2009; Stutz and Bolnick 2017). It is possible that white female stickleback also assess male MHC genes, as the two ecotypes are so closely related. Another avenue of female choice could be mate copying. Mate choice copying is related to social learning, where the mate selection of a female is related to the choice of another female (i.e. whether she mated with or rejected the male in question; Varela et al. 2018 and references therein). A theoretical study has suggested that mate copying between species that have had secondary contact may promote hybridization via mate preferences of heterospecifics converging (Varela et al. 2018). This study also considered how mate choice copying may influence speciation in different ways such as preventing or reinforcing speciation via mate preferences of conspecifics converging or diverging (Varela et al. 2018). My experimental setup allowed for the contained female to see the first released female and the focal male interacting with each other. Because of this, one can predict that if the first female chose not to mate with the male, this could have influenced the choice of the contained female when she was released. However, female response was only directly correlated in two of the 35 trials when both females respond to the focal male; all other female responses (19 of 35) were singular instances (i.e., only one of the two females responded). The two trials mentioned were instances where the first female that was released spawned with the male, however, a second spawning event did not occur with the second female. To properly understand the relationship between MHC genes and white stickleback and the possibility of the presence of mate copying further research is required. To disentangle the effect of MHC genes in the white stickleback, experiments focusing on a white male's or female's preference for a mate based on the MHC genes of the potential mate through the use of smell (for an example see Milinski 2003) or genetic analyses of white stickleback MHC genes can be conducted. Future studies can also provide females opportunities to interact with multiple males while other females observe and record their mate selection to determine the presence or absence of mate copying.

5. CONCLUSION

Selection can be one of the pathways that leads to the divergence and maintenance of sexually reproducing organisms (Fisher 1930; 1958; Lande 1981; 1982). Reproductive isolation can occur both before and after mating between two different ecotypes or species through pre- or postzygotic reproductive barriers. In sympatric populations, strong prezygotic barriers, including reinforcement, can be a reason for the separation or maintenance of distinct groups of sexual reproducing organisms; this is strengthened through selection over evolutionary time (Dobzhansky 1940; Servedio and Noor 2003). Selection against interspecific matings that do not experience associated fitness costs of producing unfit hybrids could be an explanation for the maintenance of white and common Threespine Stickleback ecotypes that live sympatrically.

My research suggests that there is no ecotype-based preference for white and common Threespine Sticklebacks. Inter-ecotype matings were observed as frequently as pure crossed matings. Moreover, common and white males spent relatively the same amount of time with and courted both female ecotypes with similar frequency. Similarly, female stickleback responded equally to both male ecotypes. This finding differs from a former study that looked at mate choice in white and common Threespine Stickleback and found that both common and white females and common male sticklebacks have a preference for their own ecotype (Blouw and Hagen 1990). However, I did corroborate previous reports that common stickleback are larger in size in comparison to their white counterparts (Blouw and Hagen 1990; Samuk 2016) with an additional finding that both male ecotypes spend more time and energy when in the presence of relatively larger females. Common male stickleback courted more intensely the larger-bodied common and white females within their respective ecotype size range, while white males courted the largest female overall (common females) more intensely.

Due to time and space restraints along with the Covid-19 global pandemic co-occurring, the full extent of this study, both experimental design and sample size, could not be achieved. Females in this study were subject to a no-choice trial design which may hinder mate choice. However, when presented with only one stimulus (one male stickleback) female response may represent her absolute preference for a mating partner (Wagner 1998; Dougherty and Shuker 2015), if a female rejects a male this may indicate a stronger preference for a different male type since there is the potential that the female would not encounter another male (Dougherty and Shuker 2015 and references therein). Additionally, males were limited to only two females (one of each ecotype) which greatly reduces mate options and males may opt to court any female over no/an inappropriate female. Future studies should consider an experimental design that represents a more natural mating environment with multiple male and female encounters to fully understand the mating preferences of the two stickleback ecotypes. Moreover, this study focused on visual and behavioural mating signals of common and white Threespine Stickleback. While many studies have focused on these characteristics, comparatively fewer have investigated other stimulants, such as olfactory cues and its associated traits. For example, the role of major histocompatibility complex (MHC genes) on mate selection in Threespine Stickleback. Previous reports suggest that common Threespine Stickleback males who possess an optimal number of MHC genes not only gain benefits in terms of health but are more attractive to selecting females (Jager et al. 2007; Eizaguirre et al. 2009; Stutz and Bolnick 2017). However, the influence of MHC genes specifically within the white Threespine Stickleback ecotype and between the common and white ecotypes has yet to be investigated and could potentially be a mechanism separating the two sticklebacks.

My findings associated with male preference for larger-bodied females within an ecotype by common males, and for the largest female overall regardless of ecotype for white males, merit further research to delve into the relationship between preference and size, both from the male and female point of view. Similar outcomes have been found in limnetic and benthic Threespine Sticklebacks that are known to hybridize based on preference for similarly sized individuals (Nagel and Schluter 1998; Conte and Schluter 2013).

Although my results did not support my predictions that mate preference would be present in both female ecotypes and common male sticklebacks due to the amount of investment provided during the breeding season, my research, especially the difference in preference for female body size between common and white males, provides one of many stepping stones on the pathway to discovering what mechanisms are maintaining the separation of common and white Threespine Sticklebacks in the wild.

6. LITERATURE CITED

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SUPPLEMENTARY INFORMATION

List of tables and figures

SI1 Threespine Stickleback nest building trends

I investigated the proportion of time a male spent tending to his nest and the frequency at which he conducted nest tending behaviours to determine if other dissimilarities between common and white stickleback nesting habits were present. These analyses were conducted in a similar fashion to male choice where the during the preference stage nest tending was timed while during the interaction stage nest tending behaviour frequencies (Table 2.3.2) were recorded. A generalized linear model (GLM) with binomial distribution was run to determine if the proportion of time males spent tending their nest differed. The proportion of time was calculated using the time a male spent tending to his nest during the 30 minute preference stage divided by the total observational time. Male ecotype, male population type, and their interaction were included in the model as the fixed effects, there were no random effects included.

The average of the all the nest tending behaviours added together (herein "total nest-tending frequency") was analysed using a generalized linear mixed effect model (GLMM) for each 15 minute interval during the interaction stage. However, prior to analysis, the values for the individual nest-tending behaviours were corrected for time as was done with the total courtship and glass poking frequencies, then summed to create the total nest-tending frequency. Because the data generated from the observations were counts that were overdispersed, the GLMM was run with a negative binomial error distribution. As with the other models, individual males were included as a random effect as each male was observed with a white and a common female. Fixed effects included male ecotype and male population type. The predictive models produced for both the proportion of time and nest tending frequency were then compared and ranked as was done for the body size analysis using AICc and assessed with QQ plots and post-hoc analysis.

Results indicated that males of either ecotype and population type spent the same amount of time tending to their nest along with a similar behaviour frequency. Although, there was a weak additive effect of ecotype and population type for nest tending frequency (Fig. SI1.1, SI1.2 and Table SI1.1).

Male stickleback of both ecotypes and population types did not differ in the amount of time they spent or the frequency of nest tending behaviours they conducted towards building and maintaining their nests. Generally, white male stickleback spent more time maintaining their nest while common male stickleback performed more behaviours.

Table SI1.1. Generalized linear model selection for the effects of male ecotype and male population type (allopatric or sympatric) on the proportion of time and behaviour frequency male Threespine Sticklebacks spent tending to their nest. Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for small sample sizes (AICc), the difference between the model with the lowest AICc value compared to all other predictors (ΔAICc), and ω_i is the weight of a model relative to the complete model set (n = 5). The bolded models are those with the lowest AICc values by a difference of two or more.

response variable	predictor	*df*	AICc	ΔAICc	ω_i
proportion of time spent nest tending	**(intercept)**	**1**	**6.2**	**0.00**	**0.567**
	male population type	2	8.5	2.26	0.183
	male ecotype	2	8.5	2.28	0.181
	male ecotype + male population type	3	10.9	4.69	0.054
	male ecotype + male population type + male ecotype x male population type	4	13.4	7.25	0.015
nest tending frequency	**(intercept)**	**3**	**603.0**	**0.00**	**0.353**
	male ecotype	**4**	**603.5**	**0.48**	**0.278**
	male population type	**4**	**604.4**	**1.39**	**0.176**
	male ecotype + male population type	**5**	**604.9**	**1.91**	**0.136**
	male ecotype + male population type + male ecotype x male population type	6	606.7	3.67	0.056

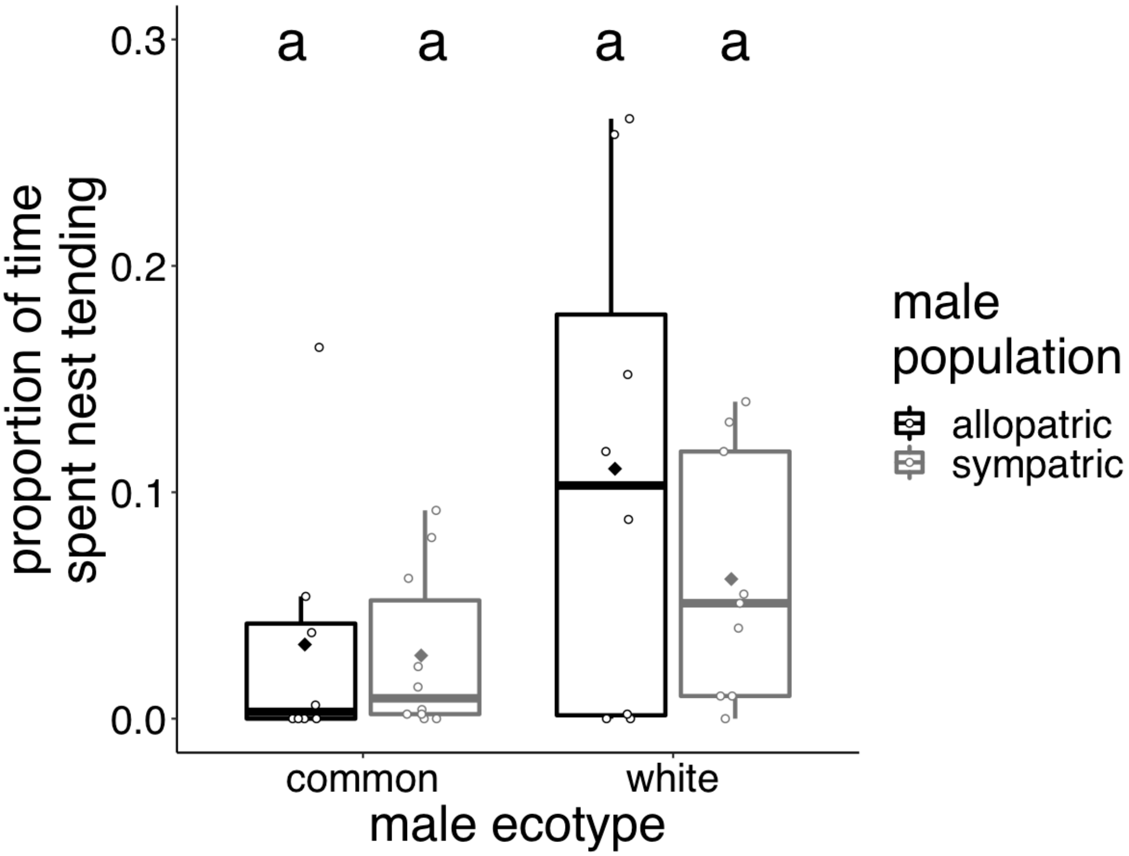

Figure SI1.1. Proportion of time common and white male stickleback spent performing nest oriented behaviours from allopatric (black) and sympatric (grey) populations during the male preference stage (n = 35). Black and grey horizontal lines indicate the median, the black and grey diamonds are the sample means, and the coloured dots are the time for individual males. Letters above the data represent post-hoc test groupings.

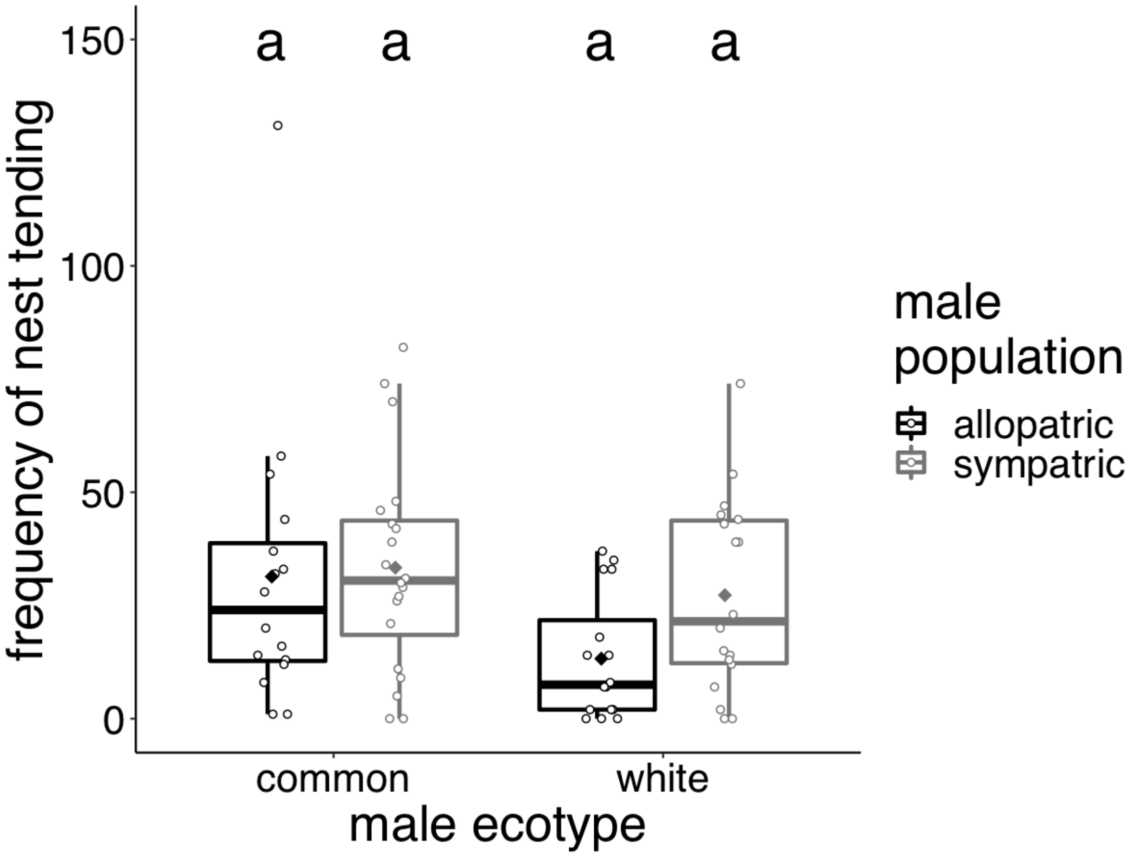

Figure SI1.2. The frequency of total nest oriented behaviours common and white Threespine Stickleback males from allopatric (black) and sympatric (grey) populations conducted during the two 15-minute observation periods from the male exertion stage (n = 35). Black and grey horizontal lines indicate the median, the black and grey diamonds are the sample means, and the coloured dots are the frequencies for individual males. Letters above the data represent post-hoc groupings.

SI2 Courtship behaviours of Threespine Sticklebacks

I was also interested in whether there existed courtship behaviours conducted exclusively by common or white male stickleback. To answer this, I looked at the average frequency of the individual courtship behaviours performed by males of both ecotypes (Table 2.3.2) and compared them between the ecotypes.

Four of the six were performed with relatively similar frequencies between the two ecotypes (Table SI2.1), while side fanning behaviour was conducted exclusively by white male stickleback and dorsal pricking was performed solely by common male stickleback (Table SI2.1).

Jamieson et al. (1992a) found a similar outcome in their field studies noting that there were no behaviour frequency differences between common and white stickleback, though they did note that white males did not perform dorsal pricking. There are other populations of stickleback that also differ in their courtship behaviours, such as the Pacific Ocean ecotype and Japan Sea ecotypes. The Pacific Ocean ecotype conducts a zig-zag courtship display, whereas the Japan Sea ecotype replaced zig-zagging with a lateral display (Ishikawa and Mori 2000). This lateral display may be similar in nature to the change from the dorsal pricking display of the common males to the side-fanning display of the white males.

Table SI2.1. Average frequency of focal behaviours conducted by common and white Threespine Stickleback males towards the released female during the male interaction stage of the experiment (n = 35).

Focal behaviour	male ecotype	
	common (mean ± std. dev.)	white (mean ± std. dev.)
biting	102 ± 82	110 ± 110
leading	11 ± 18	12 ± 12
zig-zagging	45 ± 59	42 ± 48
nest showing	1 ± 3	4 ± 15
dorsal pricking	5 ± 15	0 ± 0
side fanning	0 ± 0	5 ± 10

APPENDIX A

List of figures

A1 Bayesian statistical analyses – methods

In addition to the previous model comparison analysis, a Bayesian statistical approach was also taken to analyse the experimental data. This was to provide another statistical means of analysis since Bayesian statistics allows for the interpretation of the hypothesis given the data with the additional input of prior information about the data. For the Bayesian analyses the statistical software R v4.0.2 (R Core Team 2020) with the additional package of rstan v2.21.2 (Stan Development Team 2020) were used.

A1.1 Statistical model selection

Nest material and body size

Male stickleback nesting material preference was recorded as a binary response with a value of 0 indicating that the dominant nesting material (>70%) used was algae and a value of 1 representing the dominant nesting material as sand/mud. A Bernoulli logistic regression model was run using Bayesian methodology to properly determine male nest material preference, because preference was recorded as binary data. The predicted (response) variable was the dominant nesting material data. The Bernoulli model structure contains only one parameter, μ (mu). Male ecotype was the sole categorical predictor (explanatory) variable that constituted the Bernoulli parameter, as I was only interested in confirming the material preferences of common and white male stickleback. Because Bayesian analysis takes advantage of using prior probabilities to better inform the model, I used a normal distribution with a mean of zero and a standard deviation (S.D.) of one to determine the probability of which male ecotype would prefer what nesting material. An assumption with these analyses is that there is no relationship between the predicted (nest material preference) and predictor variables (in this case just male ecotype), therefore, a prior with a normal distribution with a mean of zero will account for this assumption while also allowing for the estimation of the mean effect of the predictor variables on the predicted variable on a standardized scale. A standard deviation of one was selected as I do not expect any values to fall outside this range since the data were recorded as binary.

To confirm body size differences between common and white stickleback, standard length (mm) measurements were recorded and standardized. Standardized body length was calculated by subtracting mean body size from the size of the individual (for each individual) then divided by the standard deviation of the body size measurements. Because the data for the predicted variable were standardized and continuous numbers, a Bayesian analysis with a normal distribution was executed. The ecotype and sex of each fish and their interaction were used as the categorical predictor variables along with an intercept (mean predicted value for body size when predictor values are zero). The two predictor variables, their interaction, and the intercept were summed to formulate one of the two normal distribution parameters known as μ (mu). The second parameter of the normal distribution was sigma which was derived from a prior of an exponential distribution with a rate of 0.5 to account for the variation around the mean. The exponential distribution was chosen as sigma must be positive, as you cannot have a negative difference between two numbers since the smallest possible difference is zero, and had the highest likelihood for sigma between the values of zero and one. I expect sigma to fall somewhere between zero and one because the body size data was standardized with a standard deviation of one and again the smallest possible difference is zero. The intercept, ecotype, sex, and the interaction terms all derived from normally distributed priors with a mean of zero and a standard deviation of one. Similar to before, the normal distribution with these parameters was chosen as it accounts for the mean effect of each variable while also allowing for some variation (S.D. = 1).

A1.2 Male choice

Preference stage

Male preference was determined by assessing the proportion of time male stickleback spent in proximity to each of the contained females during the preference stage. The amount of time a male spent in proximity to the contained female (for each female) divided by the total observation time (30 minutes) formulated the proportion of time variable. The data was then standardized by subtracting the mean from individual proportion of time data points and then dividing by the standard deviation. Male preference was analysed using a normal distribution because the data were standardised and continuous. Standardized proportion of time was used as the predicted variable in the model. Male ecotype, male population type, female ecotype and their interactions were used as the predictor variables. These variables are similar to the non-Bayesian model comparison analysis however, in the previous model I did not have enough power to run the analysis with female ecotype and ecotype relationship was used instead. With the Bayesian analysis I could use female ecotype directly as intended; therefore, this model differs slightly in that ecotype relationship is replaced with either white or common ecotype for the female. Individual males were also included in the model as a hierarchical variable to account for each male being recorded twice, once for the proportion of time spent he with a common female and once with a white female. Lastly, an intercept was also included in the model (mean predicted value when predictor values are zero). As with the body size analysis, the predictor variables, their interactions, the intercept, and the hierarchical variable were summed to formulate the normal distribution parameter μ, and sigma was calculated from an exponential prior distribution with a rate of 0.5. Prior probability distributions for all variables aside from the hierarchical variable were normally distributed with a mean of zero and a standard deviation of one since the data had been standardized. The individual male hierarchical variable was derived from a normally distributed prior with a mean of zero as the mean was previously defined for the intercept, and a standard deviation deriving from a hyperprior with an exponential distribution with a rate of 0.5 to account for variation around the mean.

Interaction stage

Male courtship effort

Male courtship effort was examined by analysing male exertion and persistence with a Normal distribution likelihood Bayesian model. Male exertion was recorded as the number of courtship behaviours a male conducted towards a released female, while male persistence was defined as the frequency of "glass poking" conducted towards the contained female. The data for both of these variables were standardized before the model was run. As described previously, data were standardized by subtracting the mean from the value for each individual and then divided by the standard deviation. For male exertion, standardized total courtship behaviour was used as the predicted variable. Male ecotype, male population type, female ecotype and their interactions were used as the categorical predictor variables along with an intercept (mean predicted value when predictor values are zero). Individual males were also included in the model as a hierarchical variable to account for the two observations per male. The intercept and the three predictor variables were all derived from normally distributed priors with a mean of zero and standard deviation of one as described in the previous models. The individual male hierarchical variable was derived from a normally distributed prior with a mean of zero, as the mean was previously defined for the intercept, and a standard deviation deriving from a hyperprior of an exponential distribution with a rate of 0.5. All variables were summed and analysed to form the parameter μ. Lastly, the other normal parameter sigma was derived from a prior exponential distribution, it too with a rate of 0.5.

Male persistence was calculated in an identical manner to male exertion with the predicted variable being changed from the standardized total courtship behaviours conducted towards the released female to the standardized amount of "glass poking" a male conducted towards the contained female.

Male courtship effort and female body size

Two separate models were run to assess whether males preferred females of a particular size (i.e., whether they preferred larger females). The two models were identical in structure with the exception of one predictor variable. The first model considered the impact of female body size on the preference of males of different ecotypes (male ecotype model), while the second model focused on the impact of female body size on the preference of males from different population types (male population model). Both models used a Normal distribution likelihood, with standardized total courtship behaviours conducted towards the released female as the predicted variable. Standardized courtship behaviour was calculated by subtracting mean courtship behaviours of all males from the courtship frequency of the individual male (for each male) then divided by the standard deviation of the total courtship behaviours for all males. Each model contained an intercept, female ecotype, and standardized female body size as the predictor variables, and individual males as a hierarchical variable. Standardized body size was calculated the same way as in the body size analysis. The male ecotype model had the additional predictor variable of male ecotype while the male population model contained male population type as a predictor variable. All interactions between the categorical predictor variables were included. These components were then summed to create the normal parameter μ. As before, sigma was derived from an exponential prior distribution with a rate of 0.5. Similar to the previous analyses, all the predictor variables were derived from normally distributed priors with a mean of 0 and a standard deviation of 1, with the exception of the hierarchical variable was derived from a normally distributed prior with a mean of 0 as the mean was previously defined for the intercept, and a standard deviation deriving from a hyperprior with an exponential distribution with a rate of 0.5 to account for variation around the mean.

A1.3 Female preference

Interaction stage

Female preference was recorded as a binary response with a value of 1 indicating the female responded to a male by following him, inspecting his nest, or conducting the typical “heads up” display during the interaction stage, and a value of 0 indicating she did not respond during the interaction stage. A Bayesian analysis was performed with a Bernoulli logistic regression model to properly analyse the binary data. The predicted variable was the binary female response variable. Male ecotype, female population type, ecotype relation (same or different ecotype) and their interactions were the categorical predictor variables. I also added an effect of individual female which was classified as a hierarchical effect within the model to account for individual effects on the data if a female was observed more than once during the experiment (20 of 39 females observed). The sum of the three predictor variables, the hierarchical variable, along with the intercept (mean predicted value when predictor values are zero) composed the single Bernoulli parameter μ. The intercept and the three predictor variables all derived from normally distributed priors with a mean of 0 and a standard deviation of 1 as before. For the individual females hierarchical variable, the prior consisted of a normal distribution with a mean of 0, as the mean was previously defined, and a standard deviation deriving from a hyperprior of an exponential distribution with a rate of 0.5.

A1.4 Checking statistical model performance

To ensure each model performed well and ran appropriately, various checks were executed. Model performance was assessed through the use of traceplots, Rhat values and effective chain length (n_eff) values for each predictor variable. These three methods determine how well the Markov chains performed and explored the sample space. To confirm that each model had appropriate prior distributions selected, the response (predicted) variable was predicted based on the predictor variables values (or category) and their prior distribution; if the predicted values completely overlap the observed/actual data then the priors were considered to be good. As a final check that the statistical model ran appropriately, simulated models were run for each experimental question to check their posterior distributions. Data were randomly selected from the existing observed data set, for each of these data points their corresponding predicted and predictor values were selected. New predicted variable values were estimated, similarly to the prior distribution check, by using the values (or category) of the predictor variables and the calculated posterior values from the model. The newly generated posterior values were then compared to the original observed data; if the simulated data were able to retrieve the true values (i.e., properly predict the observed data value) the model was considered successful.

A1.5 Extracting results from the statistical model

Results were extracted from the model by creating a matrix that contained the output values for each step in the Markov chain for all predictor variables in every model including a new matrix for each interactive term. To determine the general trend of each predictor variable and their interaction the mean and 95% highest density interval (HDI) were calculated and compared to the intercept (predicted variable average when all predictor variables are zero). However, to determine the absolute value of each predictor variable and how it differed from the average, the individual effect of each category within the predictor variable was calculated by summing the effect from each time that specific variable occurred with the average (intercept). For example, in the case of the effect of sex on body size, the female variable occurred three times: on its own, an interaction with common females and an interaction with white females. Within each categorical predictor variable, the difference between one category and the other was also calculated by subtracting the effect of one of the categories from the other and then converted back to the original scale. The mean and 95 % HDI of the difference were also calculated. The mean was used to assess how the predicted variable changed depending on the category of the predictor variable and the 95% HDI was used to determine if there were credible nonzero differences from the average effect of being in one of the categories of the predictor variable.

A2 Bayesian statistical analyses – results

A2.1 Ecotype confirmation

Next material preference

Nest material preference was determined by whether the nest was dominantly (>70%) built of algae or sand/mud. Posterior probability plots show no overlap between the nest material preferences of white and common stickleback (Fig. A2.1), indicating that white male stickleback prefer algae as a nesting material and common males prefer sand/mud.

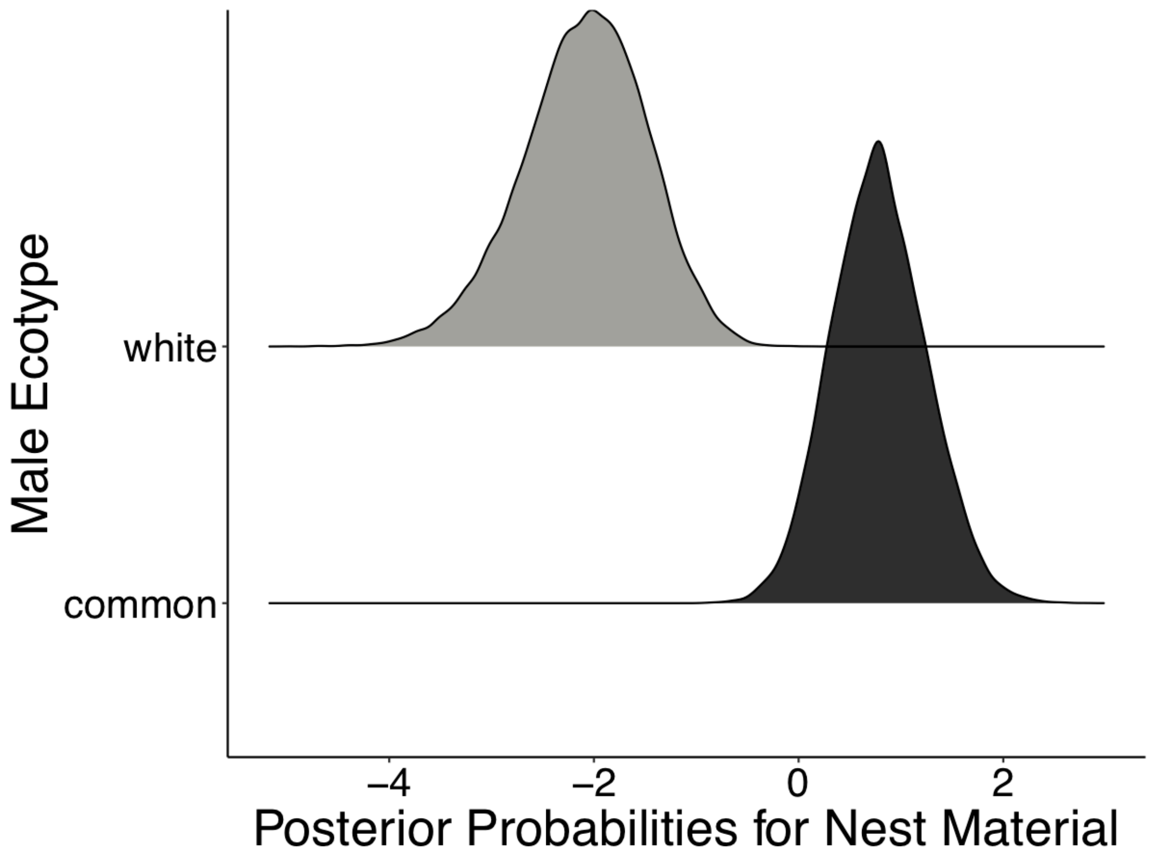

Figure A2.1. Effect of male ecotype (common [black] and white [grey]) on male Threespine Sticklebacks nesting material preference.

Body size

Body size was measured as the standard length of the fish (mm). The estimate for average fish size was 45.55 mm with a 95% highest density interval (HDI) from 37.33 – 53.68 mm. Posterior probability plots show some overlap with the body size of the fish between ecotypes but with common stickleback being larger (Fig. A2.2a), though there is complete overlap in body size between the sexes (Fig. A2.2b). Further, the posterior distributions show that the difference in size between common and white stickleback is different from zero (given that zero does not fall within the 95% HDI). More specifically, these distributions show that common stickleback on average are larger than white stickleback by 10.24 mm (Fig. A2.3a), however, the difference in size between sexes was not considered different from zero (the 95% HDI crosses zero, Fig. A2.3b), indicating that within their respective ecotypes, the sexes do not differ in size.

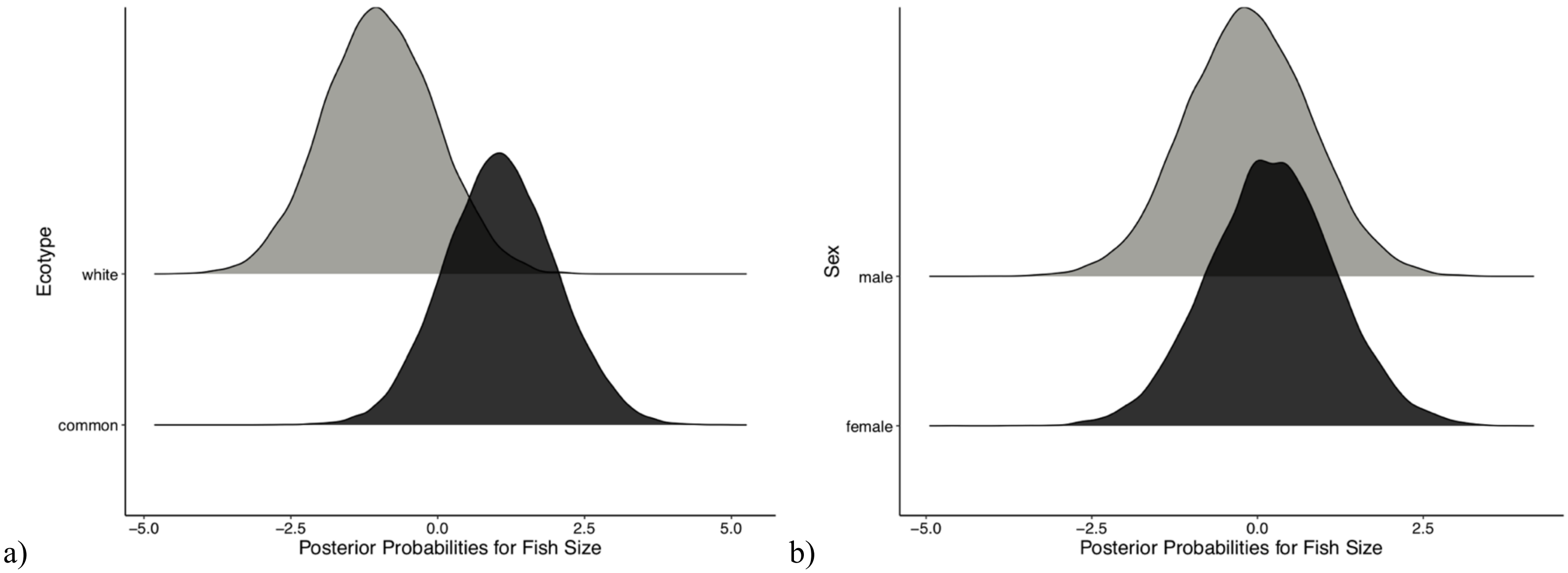

Figure A2.2. Effect of **a)** ecotype (common [black] and white [grey]) and **b)** sex (female [black], male [grey]) on Threespine Stickleback body size (standard length [mm]).

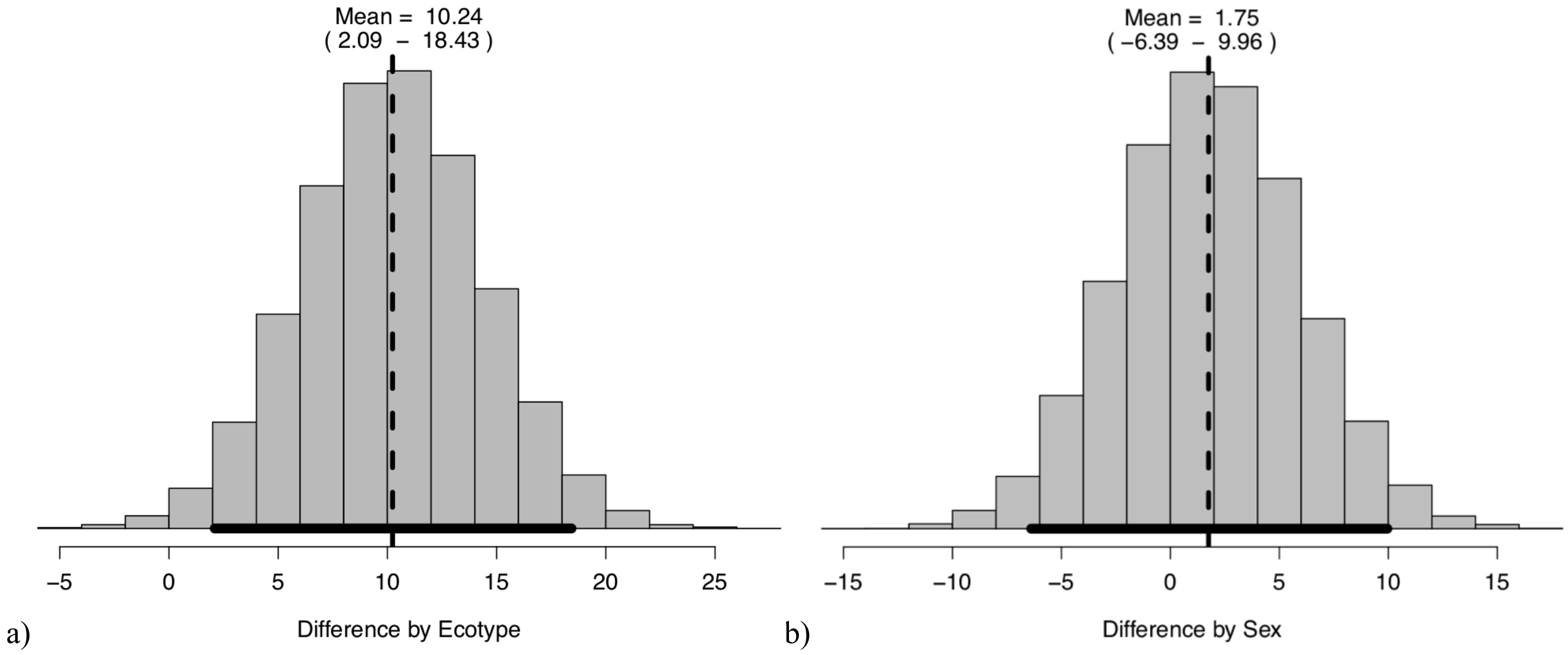

Figure A2.3. The difference between posterior distributions of **a)** ecotype and **b)** sex predictor variables on Threespine Stickleback body size (standard length [mm]). Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences observed when zero does not fall within the 95 % HDI.

A2.2 Mate choice of the Threespine Stickleback

Male courtship trends

Preference stage

Male preference was measured by calculating the proportion of time male Threespine Sticklebacks spent in proximity to the contained females. The estimate for the average proportion of time spent in proximity to a female was 0.17 with a 95% HDI from 0.02 – 0.32. Posterior probability plots show complete overlap for all three predictor variables, female ecotype (common or white; Fig. A2.4a), male ecotype (common or white; Fig. A2.4b), and male population type (sympatric or allopatric; Fig. A2.4c). This overlap is further supported by there being no differences (zero falls within the 95% HDI) between the categories within each predictor variable (Fig. A2.5). These findings indicate that males of either ecotype and from either population had no preference for which female ecotype they were in proximity to.

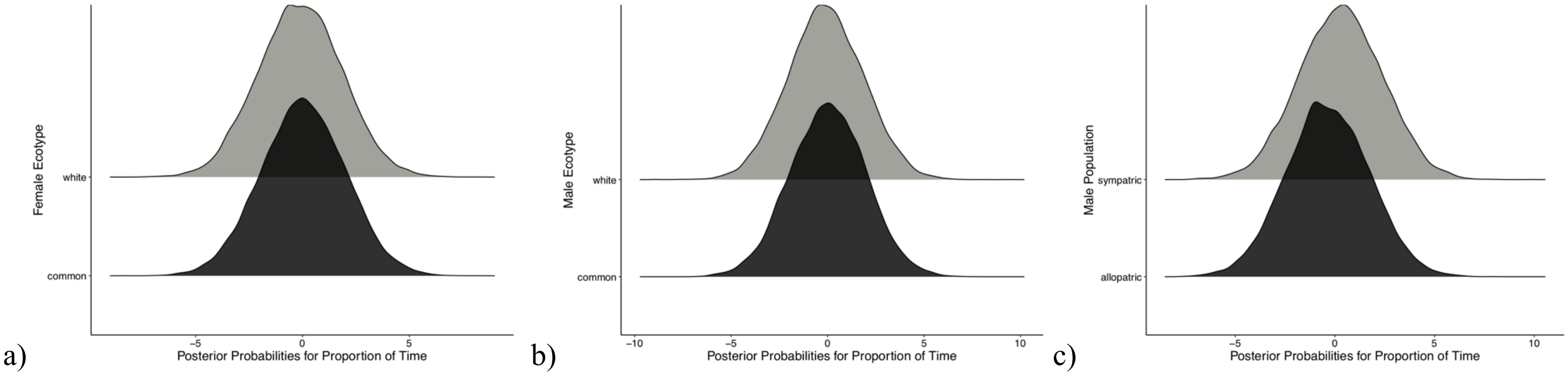

Figure A2.4. Effect of **a)** female ecotype (common [black], white [grey]), **b)** male ecotype (common [black] and white [grey]) and **c)** male population type (allopatric [black], sympatric [grey]) on the proportion of time male Threespine Sticklebacks spent in proximity to the contained females.

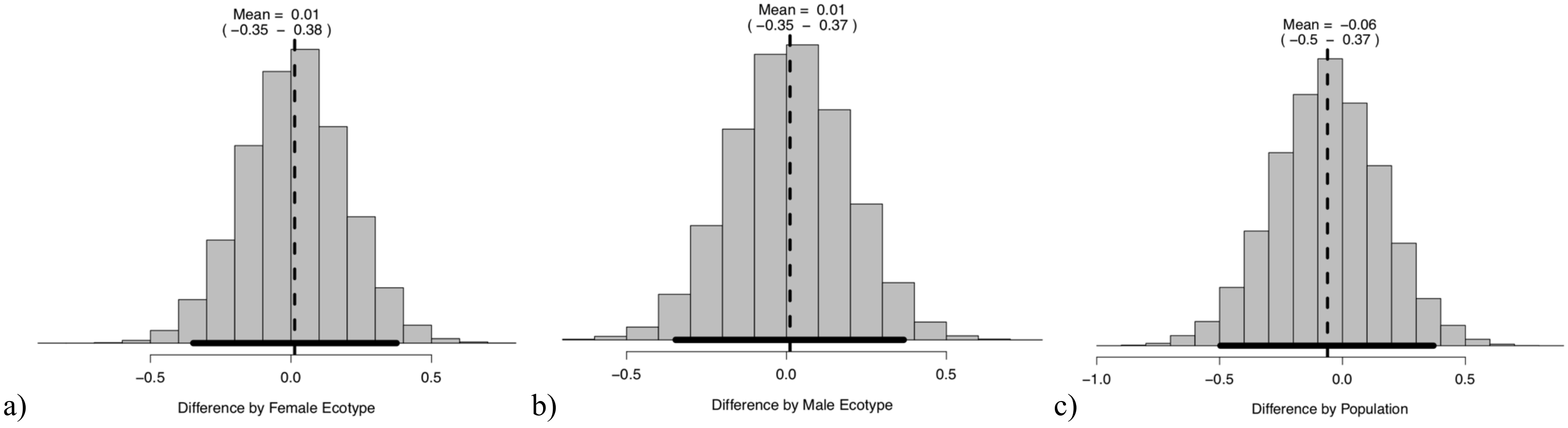

Figure A2.5. The difference between posterior distributions of **a)** female ecotype, **b)** male ecotype, and **c)** male population type predictor variables on the proportion of time male Threespine Sticklebacks spent in proximity to a contained female. Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences are observed when zero does not fall within the 95 % HDI.

Interaction stage

Male exertion

Male exertion was measured by the total amount of courtship behaviours male stickleback performed towards the released female. The estimate for the average courtship behaviour frequency was ~169 instances with a 95% HDI from ~9 – 327 behaviours. Posterior probability plots show complete overlap for all three predictor variables: female ecotype (Fig. A2.6a), male ecotype (Fig. A2.6b), and male population type (Fig. A2.6c). Moreover, no differences (zero falls within the 95% HDI) between the categories within each predictor variable (Fig. A2.7) further explains the overlap seen. These findings indicate that males of either ecotype and from either population type females of either ecotype with similar frequency.

Male persistence

Male persistence was measured by the total amount of "glass poking" male stickleback performed towards the contained female. The estimate for the average glass poking frequency was ~95 instances with a 95% HDI from 0 – ~229 pokes. As with male exertion, posterior probability plots show complete overlap for all three predictor variables: female ecotype (Fig. A2.8a), male ecotype (Fig. A2.8b), and male population type (Fig. A2.8c). There are no differences (zero falls within the 95% HDI) between the categories within each predictor variable (Fig. A2.9) These results show that males of either ecotype and from either population type attempted to gain access to contained females with similar frequency.

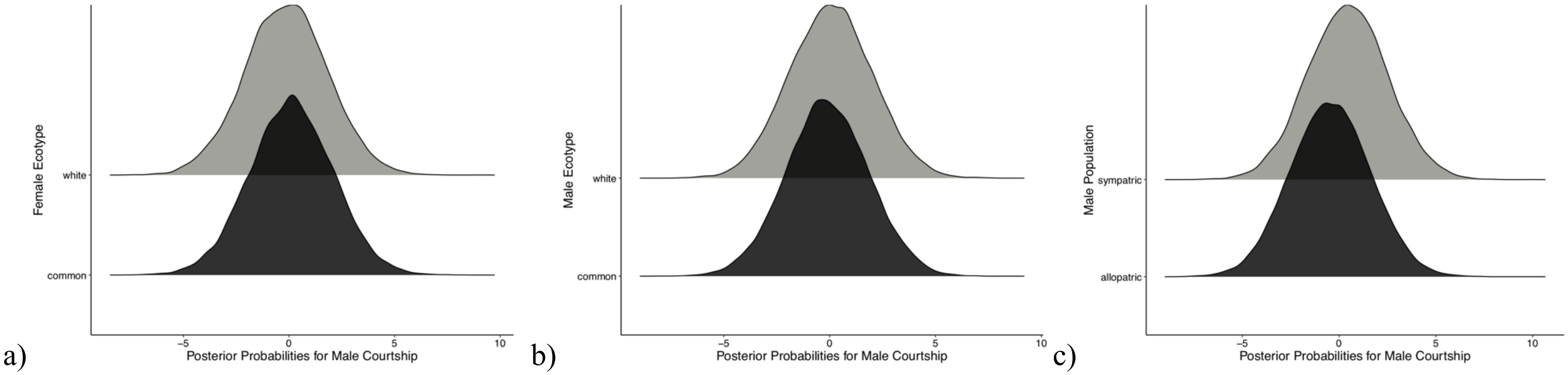

Figure A2.6. Effect of **a)** female ecotype (common [black], white [grey]), **b)** male ecotype (common [black] and white [grey]), and **c)** male population type (allopatric [black], sympatric [grey]) on the total courtship behaviours male Threespine Sticklebacks conducted towards the released females.

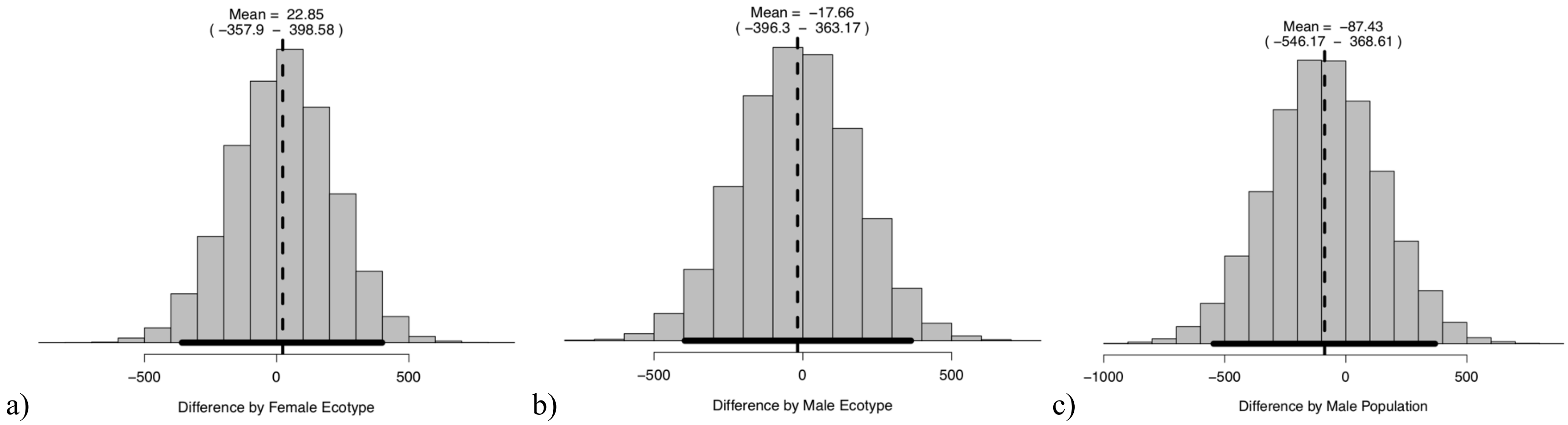

Figure A2.7. The difference between posterior distributions of **a)** female ecotype, **b)** male ecotype, and **c)** male population type predictor variables on the total courtship behaviours male Threespine Sticklebacks conducted towards the released females. Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences observed when zero does not fall within the 95 % HDI.

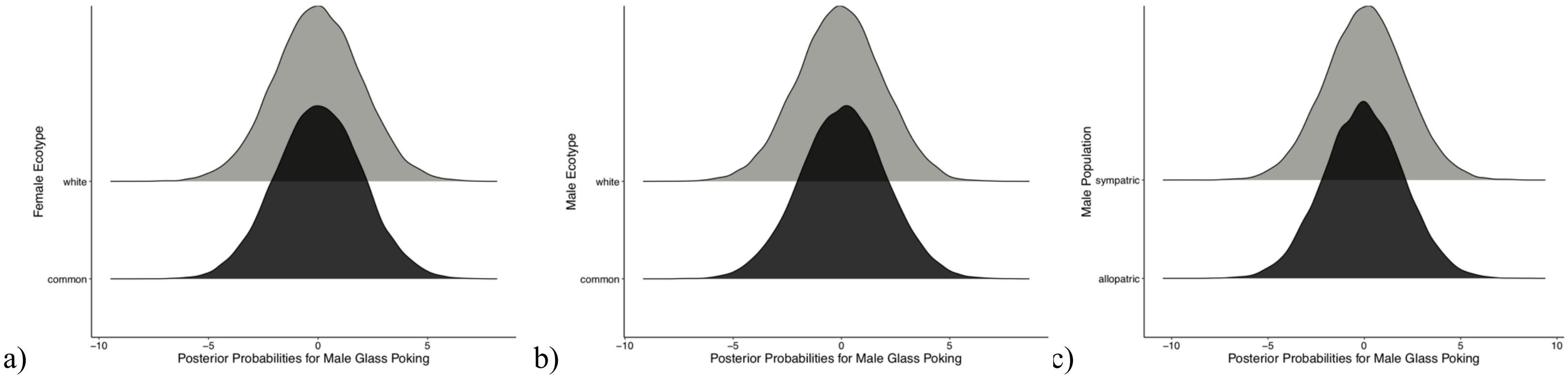

Figure A2.8. Effect of **a)** female ecotype (common [black], white [grey]), **b)** male ecotype (common [black] and white [grey]), and **c)** male population type (allopatric [black], sympatric [grey]) on the frequency of glass poking male Threespine Sticklebacks conducted towards a contained females.

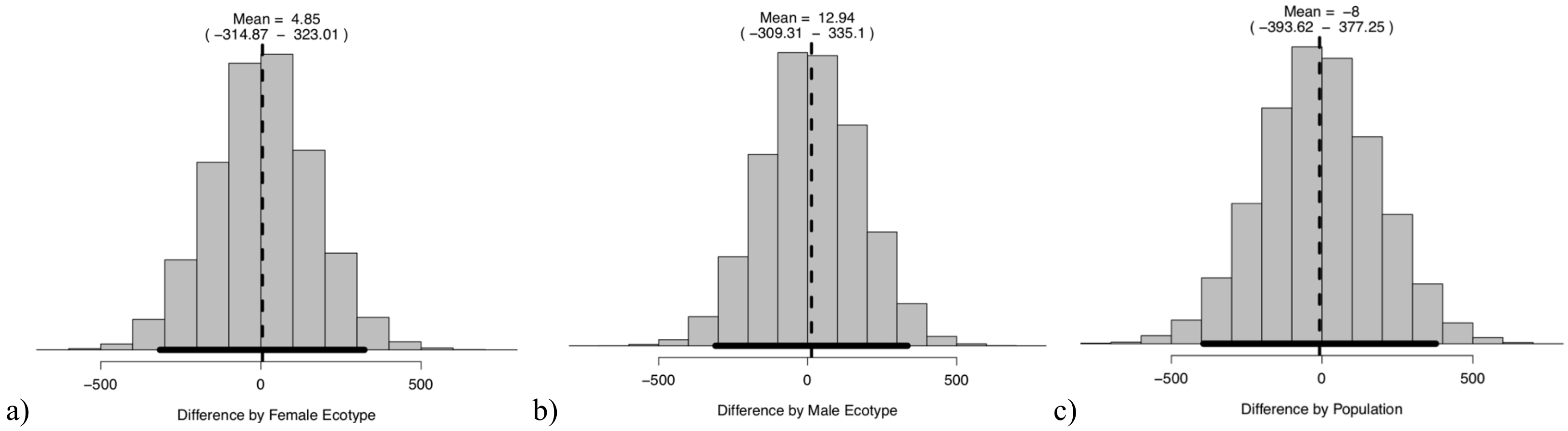

Figure A2.9. The difference between posterior distributions of **a)** female ecotype, **b)** male ecotype, and **c)** male population type predictor variables on the frequency of glass poking male Threespine Sticklebacks conducted towards a contained females. Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences observed when zero does not fall within the 95 % HDI.

Male courtship effort and female body size

Male ecotype

The relationship between male courtship effort towards female body size was measured using the total number of courtship behaviours as the predicted variable, with male ecotype, female size and female ecotype and as the predictor variables. The estimate for the average courtship behaviour frequency with respect to female body size was ~172 instances with a 95% HDI from ~29 – 314 behaviours. Posterior probability plots show complete overlap for the male ecotype (Fig. A2.10a) and female ecotype (Fig. A2.10b) predictor variables. The corresponding posterior plots for the differences between the categories within the two predictor variables indicate no differences between the categories (95% HDI crosses zero, Fig. A2.11a,b), indicating that male stickleback of either ecotype courted females of either ecotype with similar frequency. However, when looking at the effect of female body size alone, the difference between the intercept and the effect of female size is largely different than zero (Fig. A2.11c).

Male population type

This analysis was the same as above, however, the predictor variables were male population type and ecotype relationship. The estimate for the average courtship behaviour frequency with respect to female body size was ~167 instances with a 95% HDI from ~25 – 309 behaviours. Male population type and ecotype relationship posterior probability plots have complete overlap (Fig. A2.13a,b) and non-credible non-zero differences within their categories (Fig. A2.14). This suggests that males from either population have no preference for females of a particular ecotype. However, when examining the effect of female body size alone, as was done in the male ecotype analysis, the difference between the intercept and the effect of female size is largely different than zero (Fig. A2.13c).

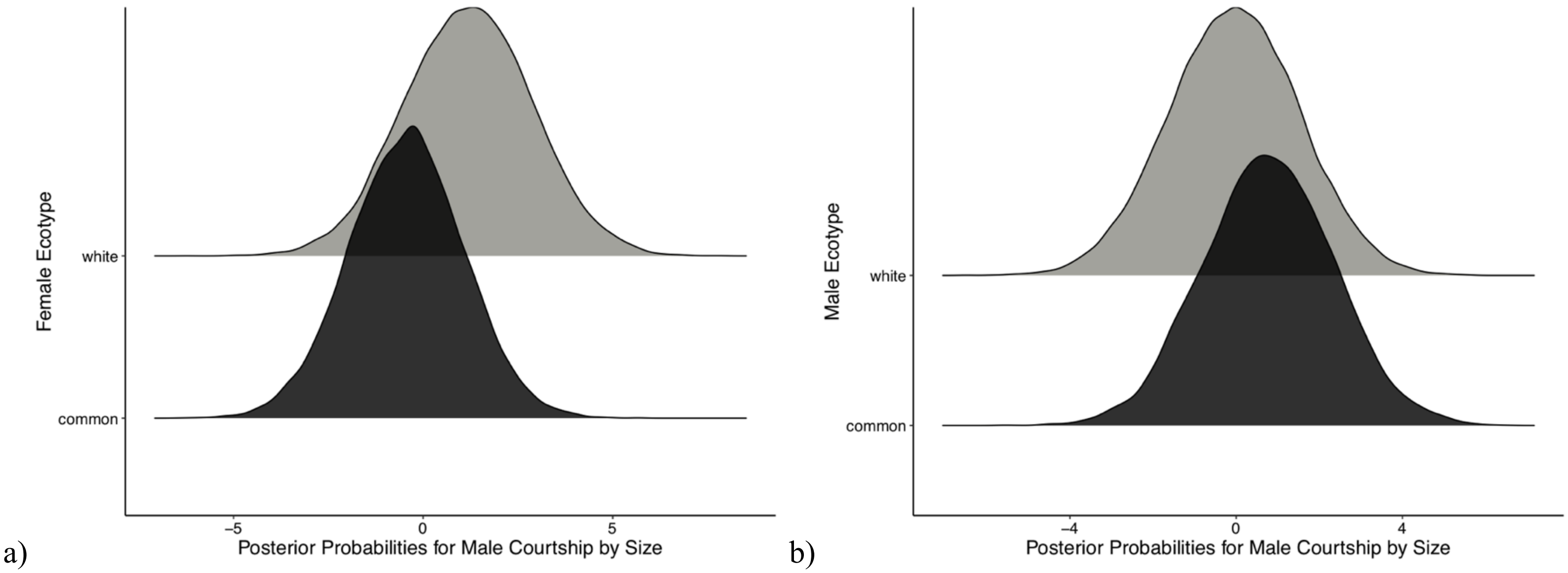

Figure A2.10. Effect of **a)** female ecotype (common [black], white [grey]) and **b)** male ecotype (common [black] and white [grey]) on the frequency of courtship behaviours male Threespine Sticklebacks conducted towards a released female with the additional influence of female body size.

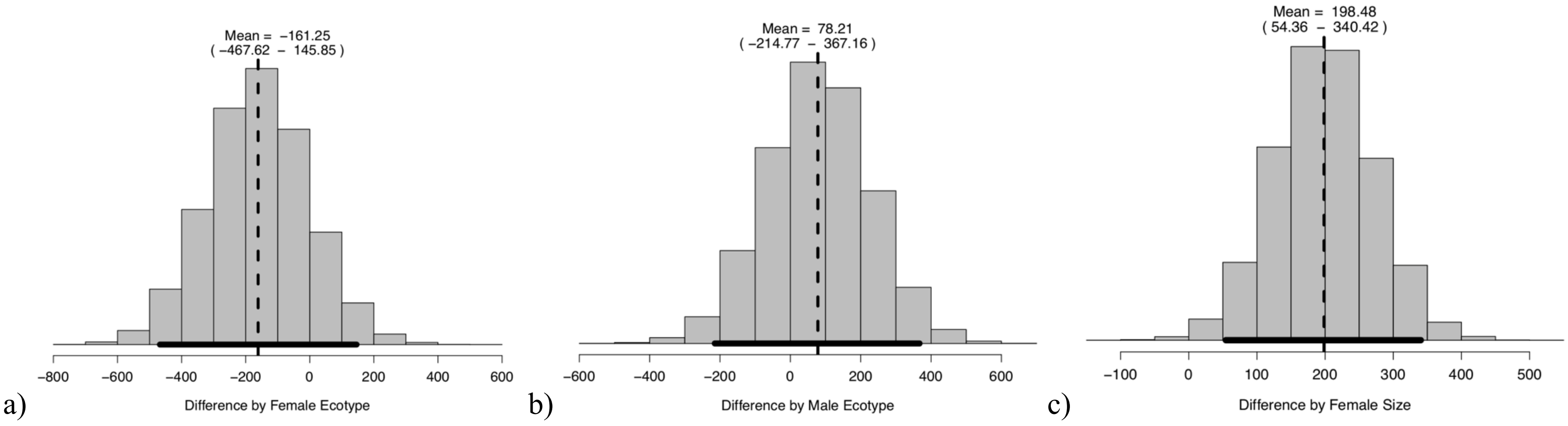

Figure A2.11. The difference between posterior distributions of **a)** female ecotype and **b)** male ecotype predictor variables on the frequency of courtship behaviours male Threespine Sticklebacks conducted towards a released female with the additional influence of female body size. Difference between posterior distributions of **c)** the average courtship behaviours (intercept) and the effect of female body size alone on the frequency of courtship behaviours towards the released females. Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences observed when zero does not fall within the 95 % HDI.

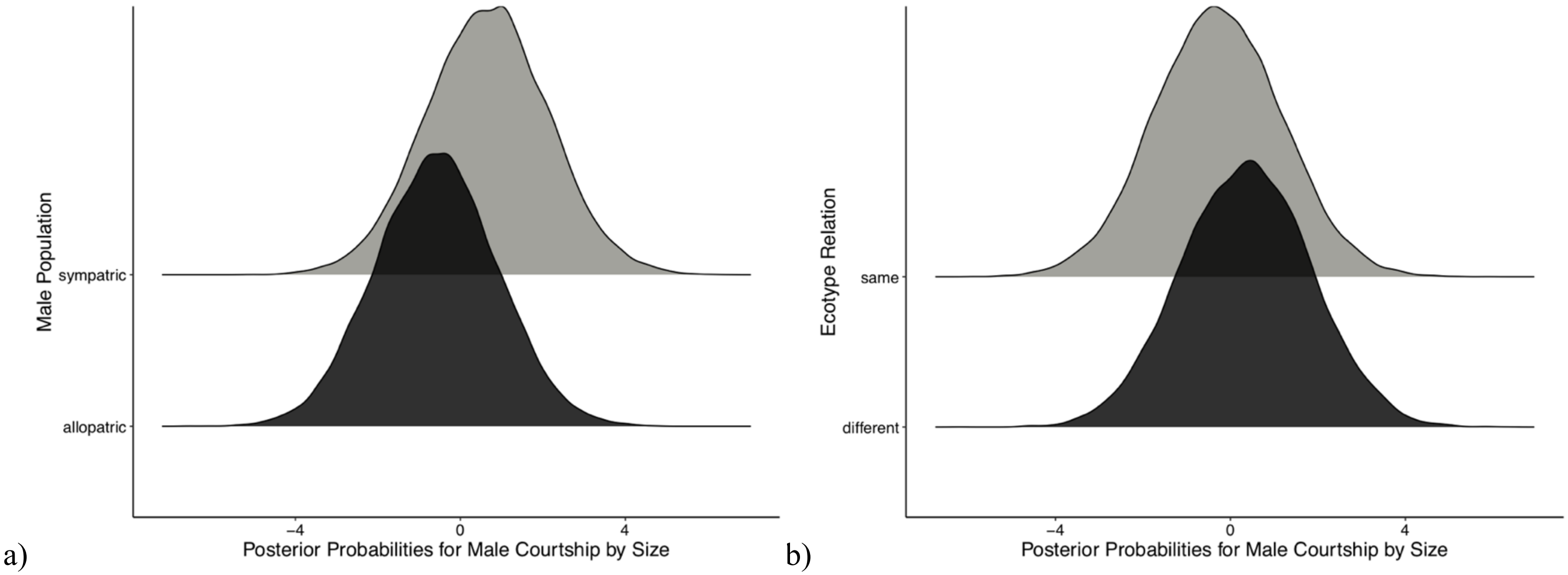

Figure A2.12. Effect of **a)** male population type (allopatric [black], sympatric [grey]) and **b)** ecotype relationship (different ecotype [black], same ecotype [grey]) on the frequency of courtship behaviours male Threespine Sticklebacks conducted towards a released female with the additional influence of female body size.

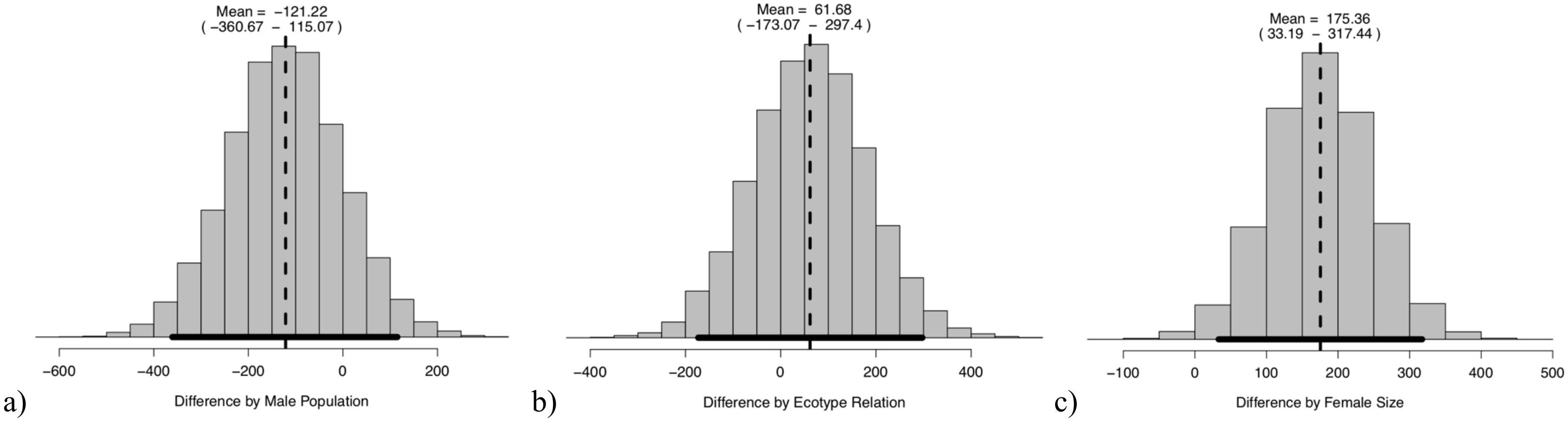

Figure A2.13. The difference between posterior distributions of **a)** female ecotype and **b)** male ecotype predictor on the frequency of courtship behaviours male Threespine Sticklebacks conducted towards a released female with the additional influence of female body size. Difference between posterior distributions of **c)** the average courtship behaviours (intercept) and the effect of female body size alone on the frequency of courtship behaviours towards the released females. Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences observed when zero does not fall within the 95 % HDI.

Female preference

Interaction stage

Female preference was recorded as whether a female responded, or did not respond, to the focal male. The estimate for average female response was 0 with a 95% HDI from 0 – ~1.3. Posterior probability plots show mostly complete overlap for the ecotype relationship (same or different; Fig. A2.14a), male ecotype (Fig. A2.14b), and female population type (Fig. A2.14b) predictor variables. Further, the posterior distributions show that there is no difference among all predictor variables when assessing female preference as they were not considered different from zero (the 95% HDI crosses zero, Fig. A2.15), indicating that females responded similarly from both populations and responded to males of both ecotypes, regardless of if they were of the same ecotype.

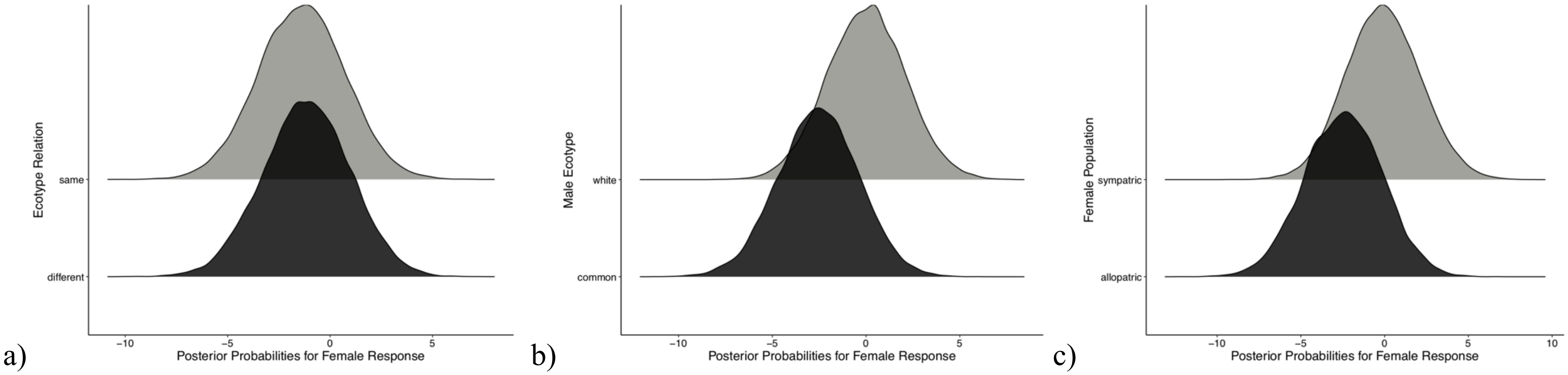

Figure A2.14. Effect of **a)** ecotype relationship (different ecotype [black], same ecotype [grey]), **b)** male ecotype (common [black] and white [grey]) and **c)** female population type (allopatric [black], sympatric [grey]) on whether or not a female responded to the focal male she was released with.

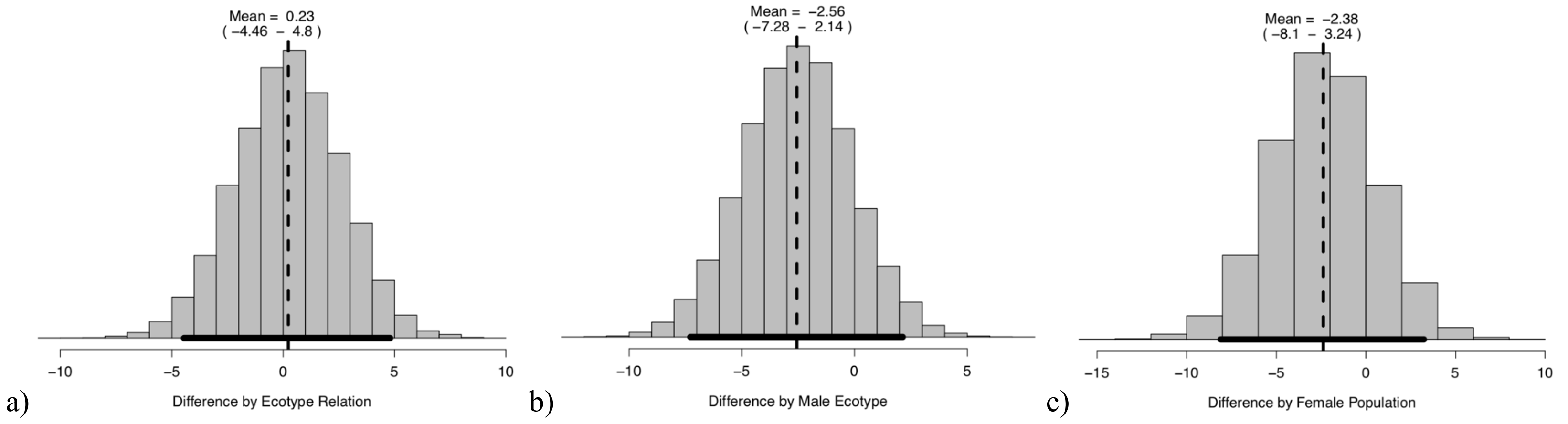

Figure A2.15. The difference between posterior distributions of **a)** female ecotype, **b)** male ecotype, and **c)** female population type predictor variables on whether or not a female responded to the focal male she was released with. Dashed line represents the mean effect of the predictor variable and the bolded line on the x-axis represents the 95% highest density interval (HDI) for the predictor variable. Exact values for the mean and 95% HDI are reported above the plot. Credible nonzero differences observed when zero does not fall within the 95 % HDI.