Extent of plasticity in the nuptial body coloration of the white ecotype of the

Threespine Stickleback (*Gasterosteus aculeatus*)

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A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of BSc. Biology with Honours.

March 2019, Halifax, Nova Scotia

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Date: April 17th, 2019

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Abstract

During mating, animals use both behavioural and morphological traits to compete for and attract mates. In some species, one of the sexes may exhibit nuptial colouration, a type of signal that is under sexual selection. This colouration may function in female choice to indicate mate quality, and/or male competition to assert possession of a territory to competitors. During their breeding season, male Threespine Stickleback build and defend a nest from competitors and actively court females. They display red and blue colouration in combination with these mating behaviours. Male white Threespine Stickleback, an ecotype endemic to Nova Scotia, display red and blue colour, and also turn a pearlescent white. However, whether this colouration functions in female choice or male-male competition (or neither) is not well understood. Therefore, the goal of this study was to determine the association between the phase of the breeding cycle and nuptial colouration of the white ecotype. Males were exposed to experimental conditions that mimicked the three main phases of the breeding cycle: nest-building, territory defense, and mate acquisition. The nest-building condition was used as a baseline to quantify the males' colouration before being exposed to a competitor or a potential mate. Male colouration was recorded and quantified to determine the influence of the different experimental conditions. The intensity of red, blue and white colouration was lower in the nest-building phase than the other phases; however, there was little difference between the male competition and female choice phase. These results suggest that the white Threespine Sticklebacks' nuptial colouration may be associated with both male-male competition and female choice.

March 2019

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Acknowledgments

I would like to thank my supervisor Dr. Laura Weir, for all her support and guidance for the past year. I would also like to thank my honours seminar professors Dr. Anne Dalziel and Dr. Tim Fraiser for their helpful feedback. I would like to give thanks to Caila Henderson, who throughout the project has dealt with any and all questions I had. I thank Anne Haley for her advice and pioneering field work. I also cannot forget to thank the honours seminar class of 2019, for making this experience one of learning and pleasure. Lastly, funding was provided by NSERC and is gratefully acknowledged.

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Introduction

1.1 Nuptial body colouration and breeding behaviour in animals

Many animal species use multiple signals, including colouration and behaviour, to attract

mates (Candolin, 2003). Nuptial colouration is a signal used during mate competition that

may have evolved through sexual selection; this can occur via two different mechanisms:

female mate choice and/or male-male competition (Hunt et al., 2009). Nuptial, or

breeding, colouration can be a display of an individual's quality as a mate, as well as their

ability to ward off competitors. For example, females prefer bright orange over drab

orange colouration in male guppies (*Poecilia reticulata*); orange intensity is correlated

with male swimming ability, which females may use to gauge male vigour (Kodric-

Brown, 1989; Nicoletto, 1991; Kottler et al., 2014). Similarly, the red colouration of the

male house finch (*Carpodacus mexicanus*) is correlated with male attentiveness to the

female during breeding, which is indicative of male quality (Hill, 1991). Comparatively,

the red colouration may signal sperm quality through sperm longevity, and therefore male

quality in *Puntius titteya,* a cyprinid fish (Fukuda & Karino, 2014). Red and orange

colour signals have a carotenoid origin and are considered honest signals of quality as

these pigments must be gained from the animal's diet (Hill, 1991; Olson & Owens, 1998).

Colouration can also be used during male-male competition to assert possession of a

territory to competitors as in Northern cardinals *(Cardinalis cardinalis*), whereby red

colouration is used to obtain and defend a territory by signalling male fighting ability

(Wolfenbarger, 1999)

In addition to colouration, animals may also use a variety of behaviours to signal

their quality or availability to a mate. Courtship displays are a reliable indicator of mate

quality because they require energy expenditure, such that increased frequency, or duration, can signal male vigour. For example, male sage grouse (*Centrocercus urophasianus*) form a lek and then attract and mate with females based on the interval between vocalizations and display rate with their air sacs (Gibson, 1996). Short intervals between vocalizations and rapid display rates have a greater energetic cost and therefore may be connected to male health (Vehrencamp, 1989; Gibson, 1990). These types of courtship displays are also used to attract mates in fifteen-spine sticklebacks (*Spinachia spinachia*), whereby females are attracted to males capable of high frequency body shakes, and the number of body shakes has been positively correlated with higher hatching rates in offspring (Östlund & Ahnesjö, 1998).

While males use a variety of behaviours to attract mates, they also often engage in intrasexual competition. Aggressive interactions may occur to defend or gain access to mates or resources. During the breeding season, elephant seal (*Mirounga angustirostris*) males fight to create a hierarchy that determines which individuals gain access to females (Le Boeuf, 1974). Aggressive behaviour can be associated with the territorial defense of a nest or breeding site, and males may attack any individuals that enter their territory (e.g., Noble & Vogt, 1935). In some cases, aggressive behaviours can be used for a combination of reasons, such as the protection of mates, offspring, and territory from potential threats and/or competitors. In Amazonian cichlids (*Pterophyllum scalare*), both females and males increase their aggression level towards individuals of the same sex to defend their mates, to defend their broods against predators, and, in the case of males, to defend their territory (Yamamoto et al., 1999).

Behaviour and colouration often interact to enhance signalling. For instance, in red-winged blackbirds the red and yellow colour patch on the male bird's wings

(epaulets) are used intrasexually to display territory ownership to other males and may act as a warning to intruding males that they are a threat (Smith, 1972). The males expose their epaulets when conducting behaviours such as a "fluttering-flight display", "bill-up boundary", and more (Orians & Christman, 1968, as cited in Smith, 1972). The epaulets are also used as an intimidating factor to the competitor when in a physical encounter (Noble & Vogt, 1935). From an inter-sexual perspective, animals may use colouration and behaviours to attract mates, such as the male frigate bird (*Fregata magnificens*), who only inflate their red coloured gular pouch as part of a courtship display (Madsen et al., 2004). Lastly, animals use their signalling for both inter- and intrasexual purposes, such as the Threespine Sticklebacks (*Gastrosteus aculeatus*), whose red colouration attracts females and wards off other males when used in combination with courting and aggressive behaviours (Wootton, 1976; Milinski & Bakker, 1990; Frischknecht, 1993). The red colouration of the Sticklebacks has a dual purpose as the red indicates the Stickleback's sexual maturity to the females, and displays territorial status to both females and males (Mclennan & McPhail, 1989). With the use of the behaviours and colouration, male display to both females and males is enhanced.

1.2 Mating behaviour and colouration in Threespine Stickleback

Gastrosteus aculeatus, the Threespine Stickleback, is a streamlined, ray-finned fish, approximately 5cm in length. The marine form typically has bony plates on their lateral sides and three spines on their dorsum (Hubbs & Lagler, 1958; Bell & Foster, 1994). *G. aculeatus* consists of a species complex made up of seven ecotypes, which are classified into one of three lineages. The three lineages diverged from one another between one to

two million years ago and consist of the Japan Sea, Pacific, and Atlantic lineages (McKinnon & Rundle, 2002; Samuk, 2016; Toli et al., 2016).

Marine 'common' and 'white' Threespine Sticklebacks are two ecotypes that occur in Nova Scotia, Canada. While the common marine Stickleback breeds in brackish water throughout a large part of its range, the white ecotype has been found only in Nova Scotia (Blouw & Hagen, 1990). Despite the observation that white and common Sticklebacks mate assortativity (Blouw, 1996), molecular studies suggest recent divergence and/or gene flow between the two ecotypes (Haglund, Buth, & Blouw, 1990; Samuk, 2016).

Male common Threespine Sticklebacks have well-characterized and identifiable mating behaviour and breeding colouration (Blouw & Hagen, 1990). Males begin their breeding cycle by searching for and claiming a territory where they build a nest and continuously defend from competitor males (Jamieson et al., 1992a). During breeding they are a greenish-blue colour on their dorsum, a shade of red on their ventral surface, and have bright blue irises (Bell & Foster, 1994; Figure 1.1a). Males actively court females through a series of behaviours including a zig-zag dance, dorsal pricking, and motioning the female towards their nest by pointing (Mclennan & McPhail, 1989; Blouw & Hagen, 1990). Following successful mating with one or more females, males guard and fan the eggs until they hatch, and then guard their hatched fry. The parental care routine of the common Threespine Stickleback ensures a greater survival rate of their embryos than if the common male provided no care at all, as they supply a constant stream of oxygen and protect them from predators (Blouw, 1996). However, not all ecotypes of the Threespine Stickleback share these traits.

The white ecotype of the Threespine Stickleback shares some traits with, but also differs from the common ecotype. White males are similar to common males in that the males claim and defend a territory where they build a nest, use zig-zag dances, and lead females to their nest during courtship. The white males also have blue irises during courtship; however, instead of turning a greenish-blue on their dorsum and red ventrally, the white Sticklebacks turn a shimmering white on their dorsum and a shade of pink ventrally ((Jamieson et al., 1992a; Blouw, 1996); Figure 1.1b). In addition, they do not use dorsal pricking during courtship, and they build nests using filamentous algae instead of building in the muddy or sandy substrate (Blouw & Hagen, 1990; Macdonald et al., 1995). Haglund et al. (1990) suggest that the difference in nesting substrate is due to differences in nuptial colouration, as the white colouration would be too conspicuous in barren territory. In addition to differences in colouration, courtship, and nest site selection, white males differ from common males in that they do not provide any parental care to their offspring (Blouw, 1996; Samuk et al., 2014). Instead, once the female has laid her eggs and the male fertilizes them, he collects them in his mouth and transports them from his nest to be spread into open algae (Jamieson et al., 1992b). In the laboratory environment, embryos of the white Sticklebacks survive and mature equally as well as the common embryos who receive parental care (Blouw, 1996).
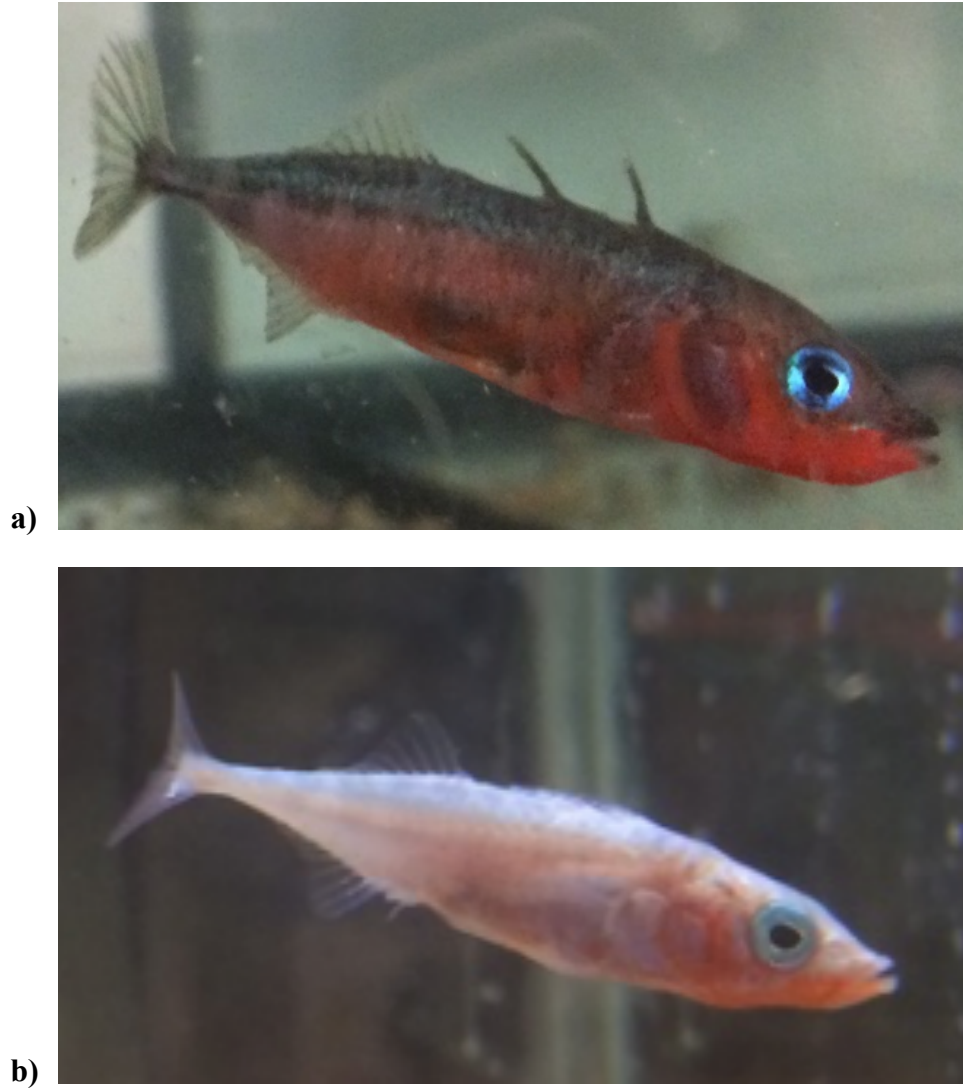
Figure 1.2.1: The marine (a) and white (b) ecotype of the Threespine Stickleback in breeding colours.

1.3 Colouration of the Threespine Stickleback

Males of both ecotypes of *G. aculeatus* use a combination of behaviour and colouration to attract a mate. The use of multiple cues in Sticklebacks is important as it allows for mate assessment by combining one trait such as colouration with the use of another such as behaviour (Mclennan & McPhail, 1989). Male Sticklebacks use multiple-component signals that combine their courtship dance and their bright colouration to intensify their signalling to the female. This is also known as amplification (Mclennan & McPhail, 1989; Candolin, 2003). The colouration in fish is produced by pigmented cells known as chromatophores (Fujii, 2000; Cal et al., 2017). Males portray their colouration and can alter their display when encountered with various environments and conditions, and can change in as little as 90 seconds (Blouw & Hagen, 1990; Cal et al., 2017).

The red and blue breeding colouration of male common Threespine Sticklebacks have been linked to territoriality and aggressive behaviour, as well as male quality and courtship behaviours (Hagen et al., 1980; Milinski & Bakker, 1990; Frischknecht, 1993). Females use the intensity of the male's blue eyes and red throat as an indicator of their quality (Frischknecht, 1993). The blue eyes signal a male's overall health, through long-term vigour and growth (Hagen et al., 1980; Frischknecht 1993). In addition, a male with an intense red throat portrays to the female that he is good at finding resources and that he is not hindered by parasites (Milinski & Bakker, 1990; Frischknecht 1993). The red throat can also be used to display to other males that they possess a territory, as only the males with a territory turn red (Mclennan & McPhail, 1989). In addition, for common males the red and blue colouration is associated more with courtship and aggression, and only faintly with nest-building. (Mclennan & McPhail, 1989). For the white ecotype of the Threespine Stickleback, there is an added factor of their white dorsum; a field study has

shown that the degree of dorsum white colour intensity is positively correlated with the frequency of courtship behaviours to attract females (Haley, 2018).

1.4 Objective and predictions:

The extent of plasticity of male white Threespine Stickleback nuptial colouration and its association with different phases of the breeding cycle is not well understood. Therefore, the objective of this study is to quantify the association between nuptial colouration and different breeding behaviours of the white ecotype. By linking breeding behaviour and colouration, we can begin to understand the mechanism behind the white colouration in males, and assess whether it is primarily associated with female mate choice or male-male competition. I predict that the white male's breeding colours (white, red and blue) will change in intensity depending on whether they are in the presence of competitor males or gravid females. Specifically, I predict that the red and blue colouration will be of higher intensity when a female or a competitor male is present and that the white dorsal colouration will be of highest intensity when a female is present.

Methods

2.1 Field Site and Sampling:

Threespine Sticklebacks were collected from Canal Lake, Nova Scotia (44.498N, -63.902W), in May 2018 (Department of Fisheries and Oceans fishing license #343930). Canal Lake is a tidal, brackish water lake that is supplied by the Atlantic Ocean, and freshwater runoff from nearby barrens (Figure 2.1). Fish were collected using Gee's minnow traps that were set and left for 24 hours (in accordance with SMU Animal Care protocol 17-18A). Fish that were found in the traps were sorted and counted based on ecotype (common or white) and sex. In total, 40 white males, 14 common males and 21 females were collected and transported back to the aquarium facilities at Saint Mary's University. Filamentous algae (e.g., *Cladophora* sp.) were also collected at this site for use as nesting material in the laboratory. The algae were kept in a 36-inch diameter circular pool filled three-quarters of the way with water. Water was replenished with used fish tank water to maintain adequate nitrogen levels for algal survival.
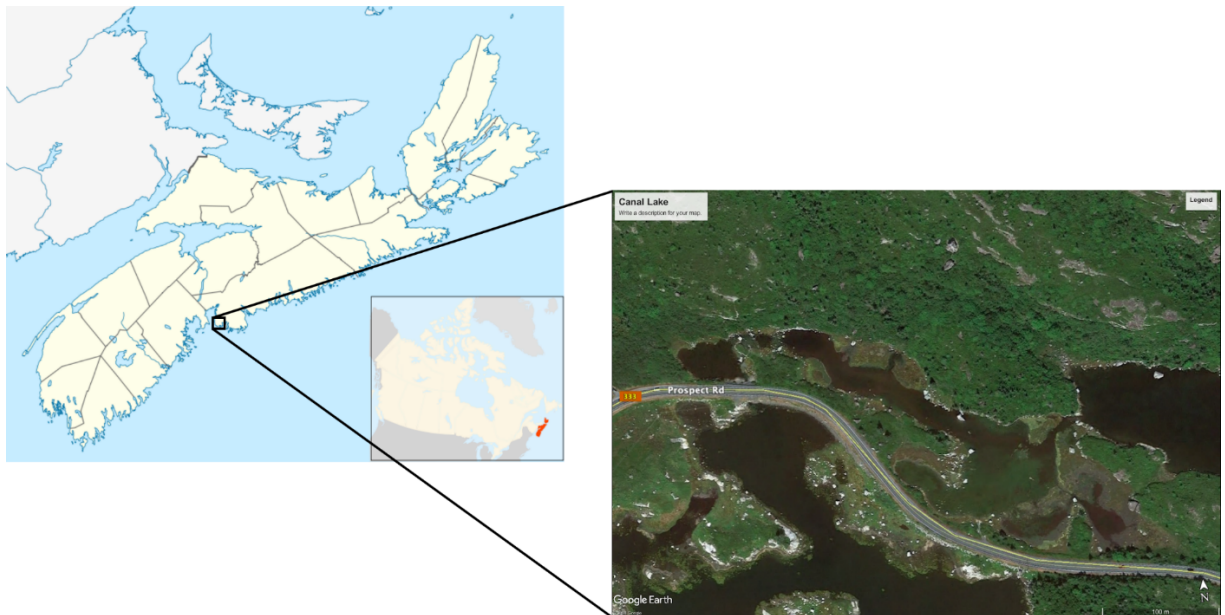
Figure 1.1.1: Canal Lake, Nova Scotia, Canada.

2.2 Laboratory Housing:

Upon arrival at the SMU Aquarium Facility, the collected fish were placed in five 15

gallon holding tanks, with a water temperature range of 20 to 22°C and salinity of 15ppt ±

1ppt. These conditions approximate temperature and salinity in Canal Lake during

breeding season. Females and males were housed separately, but the ecotypes were held

in the same tanks. The photoperiod was set at 16 hours light and 8 hours dark, which is

consistent with the summer breeding photoperiod in Nova Scotia (Blouw & Hagen,

1990). This photoperiod was maintained using over tank Fluval Marine 3.0 LED lights

(Hagen Inc.). The tanks were equipped with aquarium gravel, plastic plants, clay pots and

petri dishes filled with sand for environmental enhancement. Fish were fed to satiation

with a diet of *Artemia nauplii,* frozen blood worms and frozen adult *Mysis* shrimp twice a

day, similar to other studies (McPhail, 1984; Blouw & Hagen, 1990; Clotfelter et al.,

2006). Experiments began on June 11th, 2018, to allow sufficient time (18-26 days) for

the fish to acclimate to the laboratory environment, as required for accurate observations

of typical behaviours (Melvin et al., 2017).

2.3 Experimental Tank Setup:

To begin the study, experimental tanks were set up to house the fish for observations.

Four 15-gallon tanks with the same temperature and salinity as the holding tanks were

used during behavioural experiments (under SMU Animal Care protocol 17-15A). All

tanks had a mesh separator to divide the tank into two equal halves. On one side of the

mesh, nest building material such as gravel, a clay pot, sand in a petri dish, and algae

were available, while the other side contained only gravel. Males were placed on the side

of the divided tank that contained the nest building materials, as well as a homemade

colour checker. The colour checker was fabricated using a laminated collection of six different Canadian Tire paint cards, consisting of three shades of grey, two shades of blue and one shade of red (PR16R08, PR16S08, PR16T08, PR16F30, PR16G32, PR16G04; Haley, 2018). A colour checker was placed in each tank and was left throughout the filming process. This served as a non-changing colour standard for quantification of male colour change (see section 2.5.1 for more information). In total, 14 white males were used in the experiment; common males were not analyzed in this part of the study.

2.3.1 Behavioural observations:

For this study the goal was to observe the white male fish and record their behaviours and colour changes under four different conditions that represented different components of their breeding cycle. These conditions were the presence of nest-building material only, nest-building material and a competitive male who was separated from the focal male by a mesh screen, nest-building material and a gravid female separated by a mesh screen, and lastly nest-building material and a gravid female present with no separation. These different treatments were presented to a male fish once a day, for a trial period of four days to complete all conditions in sequence. To observe and record the males, two thirty-minute behavioural observations were collected on each of the four days of an experimental trial: once in the morning between 9:30am and 11:30am, and once in the afternoon between 1:00pm and 3:00pm.

On the first day of a trial, a white Threespine Stickleback male was placed in one of the four experimental tanks on the side with the nest building materials. I made live observations of behaviour associated with nest-building and recorded the number of times the male retrieved material for his nest, burrowed, fanned or glued his nest (Table 2.1).

On the second day of the trial, I added a competitor male to the other side of the mesh screen, with no nesting materials. The same nest-building behaviours as the first day were recorded, along with the frequency of aggression of the experimental male towards the competitor male. Aggression was defined as "charging" and was quantified as the number of times the focal male rapidly approached the mesh screen to drive away the competitor male (Table 2.1). At the end of the second 30-minute observation period, I removed the competitor male from the tank.

On the third day of a trial, I added a gravid female to the other side of the mesh screen and recorded the number of nest-building behaviours performed by the male, as well as courtship behaviours. These included zig-zag dancing, "chasing" the female when the male approached the mesh screen and pointing to the nest (Table 2.1).

Finally, on the fourth day of the trials, the female that was separated from the male by the mesh was then added to the same side as the male. The male was observed for the same actions as the day before, with an addition of biting. I also recorded whether the pair spawned or not (Table 2.1).

When the observational portion of a trial concluded, the male was removed from the tank, tagged for individual identification, and a small fin clip was taken for later DNA analysis. If spawning had occurred, the female was also tagged. Once the males were tagged, they were placed in a new holding tank, and were not used again as focal males, but could be re-used as a competitor male. Males from previous trials were used as the competitor males in the trials that occurred after their own. The three males from the first week were used as the competitor males in the second week of trials, and the second week males were used in the third week and so on, for a total of ten males re-used. For the first week of trials, competitor males were males that were not used as focal males

yet. When needed, females were re-used if they had not spawned in a previous trial or became gravid once again.

Table 2.3.1. Definition of the focal behaviours observed during the experiment. Modified from Haley, 2018.

Behavioural Category	Focal Behaviours	Definition
Nest-building	Material retrieval	Using his mouth, the male obtains nest-building material and brings it to where he is building a nest.
	Nest maintenance	Gluing – Male glues nest with the use of spiggin (Jakobsson et al., 1999), to hold the nest together.
		Burrowing – The act of swimming through the tunnel of the nest to keep its form.
		Fanning – The male is stationed in front of the nest and moves his pectoral fins back and forth "fanning" the nest, while remaining in place.
Aggression	Charging	The focal male approaches the mesh screen and drives away the competitor male.
Courtship	Zig-zag dance	Male swims in a 'Z' configuration quickly.
	Pointing	Male faces the female and then rapidly swims back to nest, then returns to the female. When female is close to the nest entrance the male turns on his side and again swims towards the nest.
	Chasing	Male approaches the mesh screen to where the female was.
	Biting	Male approaches the female and bites her lateral and/or her ventral surface.

2.3.2 Colour quantification:

An Olympus Tough TG-5 camera mounted on a tripod was used to record each observation period for later analysis of male colouration. To assess the extent of colour change of each focal male throughout the four parts of a trial, ten photos were extracted from the video recordings for each half hour observation period. All ten pictures were of the fish's left side, five of which were from when the fish was in the front of the tank, and five when the fish was at the back of the tank. Photos of only the left side accounted for any variation associated with asymmetry, and photos from the front and back of the tank accounted for any differences caused by the reflection and capture of light at different depths. The ten photos that were taken for each half hour were analyzed for the degree of dorsal white, red throat and blue eye coloration. In total, 909 photos were analyzed, and the averages for each male in each condition was used in the analysis.

The photos were analyzed using the RGB measure in ImageJ32 (version 1.50i). This plugin functions by measuring the value of red, green, and blue present in a photo. Each photo was saved as a .PNG file and was analyzed in this format because this file type does not distort the colours (Figure 2.2a). For every photo, the colour checker was analyzed along with the fish. All of the measurements were also standardized with the value recorded from the colour checker; the gray for the dorsum, the red for the throat and the dark blue for the eyes. To analyze the colour of the fish, I followed the procedure outlined below.

First, I quantified changes in white colouration in the dorsal region of the fish. This was standardized across animals by drawing a horizontal line from the top of the fish's eye to the top of the caudal peduncle. The area to be analyzed was then chosen from the beginning of the first spine, to the end of the second and extended down to the

horizontal line (Figure 2.2b). Second, I measured red colouration on the fish's throat. This measurement was taken by drawing a horizontal line below the eye of the fish that was the same width of the fish's eye. The analyzed section was from the horizontal line to the bottom of the fish's throat (Figure 2.2c). Finally, I quantified the blue colouration in the fish's eye. This was done by drawing a rectangle on the right side of the pupil with the same height as the pupil, and extended to edge of the eye (Figure 2.2d).

A control was conducted to assess whether the fish's position in the tank would influence my results by measuring any distortion of the colouration of the fish due to the water or light. This was done by recording the colour checker in the back and front, top and bottom, and left and right side of the tank and comparing the colour values of the standard colour checker in the different locations.
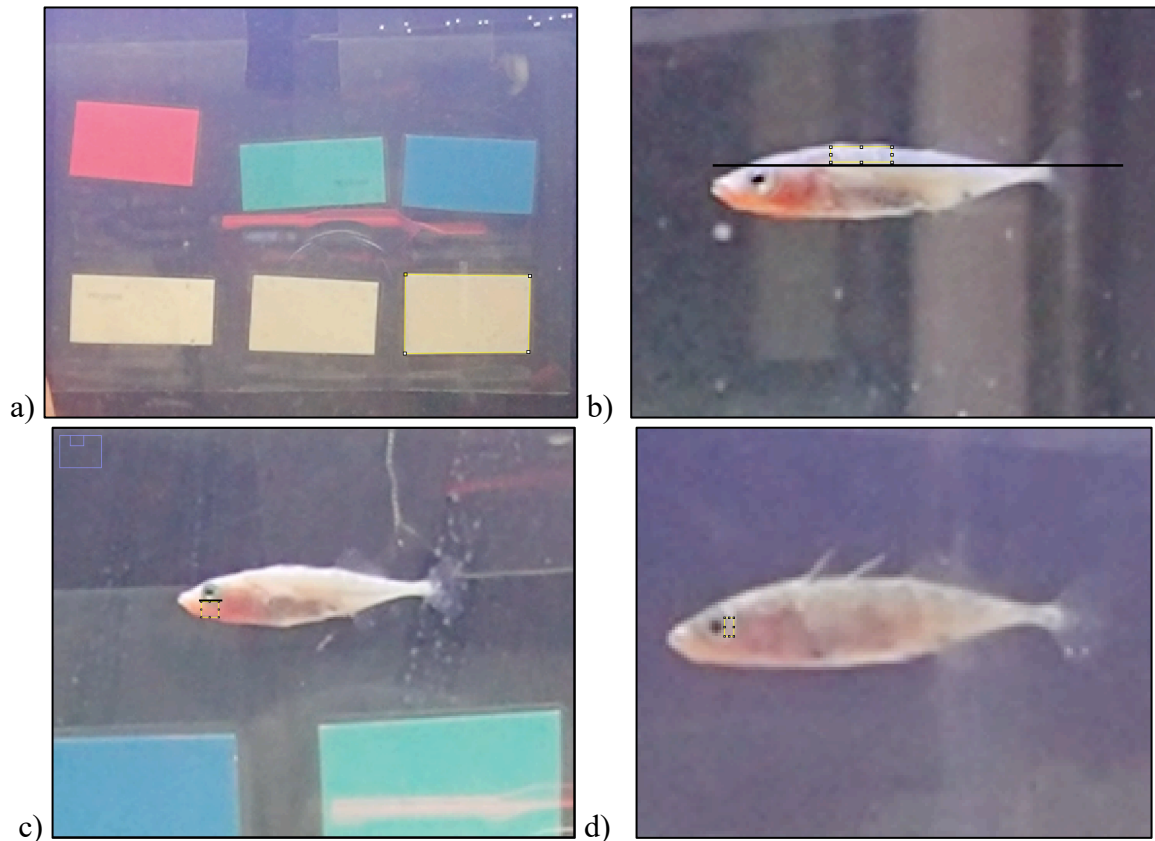
Figure 2.3.1: Examples of how the colour checker (a), the dorsum (b), the throat (c), and the eye (d) of the Sticklebacks were selected to be analyzed.

2.4 Data Analysis:

2.4.1 Standardizing colour values:

To standardize the results from each picture, the colour checker from each tank was photographed in eight different positions as stated above (see section 2.3.2). Colour in these photos was quantified in ImageJ32 (version 1.50i) for the red, white, and blue values. Using R (version 3.4.3), I constructed a generalized linear model (GLM) to assess differences in the placement of the colour checker, with "colour" as the dependent variable and "tank" and "position" as the predictor variables. The colour checker values were analyzed to determine whether different values were obtained for the different

positions in the four tanks (top versus bottom of the tank, back versus front, and left versus right) for all three colours. This was done by running a GLM for the four experimental tanks against the three positions for the white, red and blue colours. My analysis indicated that only the back versus front positioning influenced the colour values, and I used these data to correct for the position of the fish in subsequent analyses. Because the back of the tank was consistently darker than the front, a correction was applied to making the back "lighter" to match the values of the front of the tank. This correction was made by first obtaining an estimate of the relative colour at the back versus front of the tank by dividing the averages of the values for the front of the tank by the values for the back for each tank and each colour. I then multiplied this value by the original recorded colour value of the front. If the corrected colour checker value exceeded 255, then 255 was chosen as the maximum, as this is the highest colour value possible in ImageJ32 (version 1.50i). After I corrected for the position in the tank, the fish colouration value was then standardized. This was done by dividing the colour value of the fish by the colour value of the colour checker. This correction was made for all three colours to get the proportional value for the fish relative to the standard. These values were then multiplied by 255, to get an estimate of the colour value of the fish in the tank. If the final value exceeded 255, then 255 was again chosen as the maximum value. These values were then used in the colour analysis.

2.4.2 Colour and behaviour analysis:

I first examined the frequency of the different breeding behaviours (nest-building, aggression, courtship), condition (nest-building, competitive male, female present with and without mesh), and the period (morning or afternoon) for all three colours using the

'lme4' package (version 1.1-18-1) in R (version 3.4.3). This approach was used to generate linear mixed-effect models (LMER) to analyze the relationship between the fixed effects (condition and period) on behavior, while taking into account the random effect (the individual male). Because individual males were used for all conditions, the random effect was included so to avoid pseudo-replication. Following the LMER an automated model selection (dredge) using the 'MuMin' package (version 1.42.1) was run. The dredge test was done to compare all possible models that could be fit to the data. Post-hoc tests were run on to determine pairwise differences between groups.

Standardized colour values were compared against the condition (nest-building, competitive male, female present with and without mesh) and the period (morning or afternoon) for all three colours using same mixed modelling approach described above. Post-hoc tests were run on to determine pairwise differences between groups.

I also investigated whether there were changes in colour intensity throughout the four-day period to account for any temporal changes and to ensure that the males were "resetting" their colouration after each condition and not gradually increasing in colour intensity throughout the trial. This was done by comparing the first photo taken within the first five minutes of the first observational period in a day across the four days of the trials using the LMER approach described above.

To examine whether there was an association between the fishes' behaviours and their colouration, I correlated colour intensity and the frequency of the three breeding behaviours for both observational periods. The nest-building behaviour was recorded across all four days of the trial, therefore all days were included in the analysis. However, because aggression and courtship were only recorded on a fraction of the days in the trial

(aggression day 2, courtship days 3 and 4), a subset of the total data was created to only

include the specific days those two behaviours were recorded.

Results

3.1 Breeding behaviour analysis

I compared the frequencies for each behavioural category (nest-building, aggression, courtship) across conditions (nest-building, competitive male, female present with mesh, or female present without mesh) and observation period (morning or afternoon observations). Both factors influenced the breeding behaviours for all categories. Nest-building was affected by three predictors: condition alone, the intercept only (suggesting a non-zero value for the behaviour, but with no influence of condition or period), and the additive effect of condition and period together, with all three models being roughly similar in weight (Table 3.1.1, Fig 3.1.1 a). The equivocal nature of these three models indicates that nest-building behavior may be influenced by either the condition they were in, a combination of the condition and time of day, or neither. The predictor best describing the effect of aggressive breeding behaviours was an interactive effect of the condition and period (Table 3.1.1, Fig. 3.1.1 b). This interactive effect is driven by a lower level of aggression in the afternoon than the morning observation period when a competitive male was present, compared to no aggression when there was no competitive male (Fig. 3.1.1b). Lastly, courtship frequency was affected mostly by the condition, with a smaller interactive effect of the condition and period (Table 3.1.1, Fig. 3.1.1 c). The interactive effect is caused by the difference in frequency of courtship behaviours occurring when a female was present in the morning and afternoon observational periods compared to no courtship behaviours when no female was present.

Table 3.1.1 Model selection of the effects of condition, period, their interaction and the intercept on the breeding behaviour frequencies (nest-building, aggression, courtship). Shown below are the degrees of freedom (df) for the predictors, Akaike Information Criterion corrected for my small sample sizes (AICc), the variance between the lowest AICc value predictor compared to all other predictors (ΔAICc), and the weight of the model. Condition can be nest-building, competitive male, female present with mesh, or female present without mesh. Period can be either the morning or afternoon observational period. The bolded models are those with the lowest AICc values by a difference of two or more.

Behaviour	Predictor	*df*	AICc	ΔAICc	Weight
Nest-building	**Condition**	**6**	**1124.2**	**0.00**	**0.390**
	(Intercept)	**3**	**1125.3**	**1.11**	**0.224**
	Condition + Period	**7**	**1125.8**	**1.57**	**0.178**
	Condition x Period + Condition + Period	10	1126.8	2.60	0.106
	Period	4	1126.8	2.67	0.103
Aggression	**Condition x Period + Condition + Period**	**10**	**862.9**	**0.00**	**0.734**
	Condition + Period	7	865.7	2.86	0.176
	Condition	6	867.0	4.19	0.090
	(Intercept)	3	926.7	63.88	0.000
	Period	4	928.0	65.10	0.000
Courtship	**Condition**	**6**	**1099.5**	**0.00**	**0.532**
	Condition x Period + Condition + Period	**10**	**1100.8**	**1.30**	**0.278**
	Condition + Period	7	1101.6	2.06	0.190
	(Intercept)	3	1151.3	51.74	0.000
	Period	4	1152.7	53.23	0.000
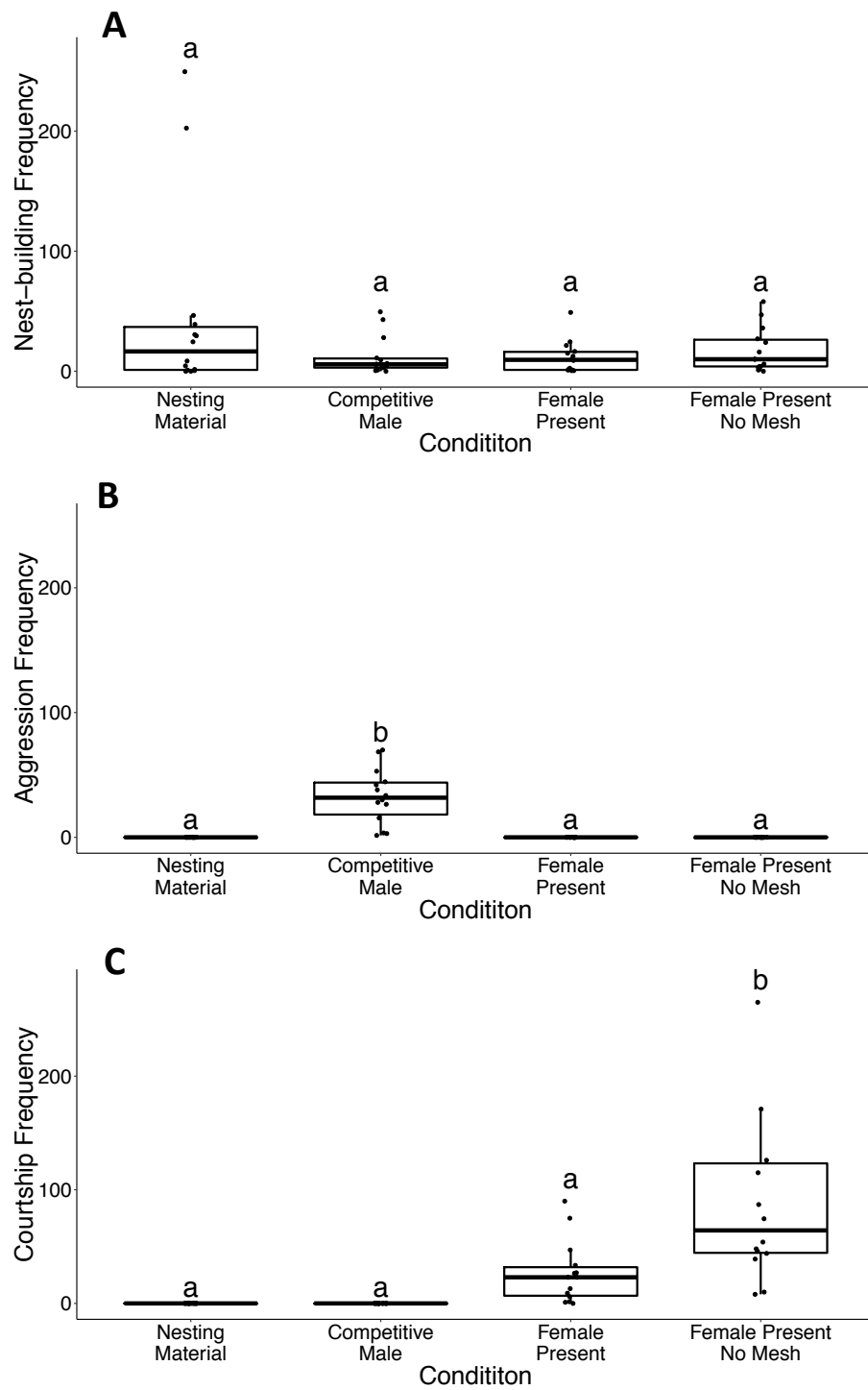
Figure 2.1.1: Frequency of (a) nest-building, (b) aggression, and (c) courtship behaviour of the experimental fish throughout the four conditions during both observational periods of a trial (n=14). Black horizontal line indicates the median, the black dots are frequencies for individual males. Letters above the data represent post-hoc test groupings.

3.2 Colouration and breeding phases

To account for temporal changes, I analyzed the effect of the condition on colouration within the first five minutes of observation. The intercept factor was also analyze to account for the colouration alone, with no effect of the predictors. This was to ensure that the male white Threespine "reset" his colouration to his standard intensity during a new day in the trial, and was not increasing in intensity throughout the trials. White colouration change was influenced by the condition and the intercept, whereas the red was influenced by the condition alone, and blue by the intercept alone (Table 3.2.1, Fig 3.2.1 a, b, and c).

Following the colouration check, for each time period (morning and afternoon), colour intensity values for white, red, and blue were standardized to quantify their colouration change within the different conditions (nest-building, aggression, and courtship) using the averages from the 909 photos taken throughout the observations. For the white colour variable, the predictor that held the most weight for the colouration change throughout the trial was the effect of condition with a minor influence from the period (Table 3.2.2, Fig. 3.2.2a). For the red and blue colour variables, both best fit models retained the effect of the condition alone (Table 3.2.2, Fig. 3.2.2a and b).

Table 3.2.1 Model selection of the effects of condition, or the intercept on the colour intensity of the fish for all three colours (white, red, or blue) within the first five minutes 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 the weight of the model relative to the complete model set. Condition can be nest-building, competitive male, female present with mesh, or female present without mesh. The bolded models are those with the lowest AICc values by a difference of two or more.

Colour	Predictor	*df*	AICc	ΔAICc	Weight
White	**(Intercept)**	**3**	**514.4**	**0.00**	**0.616**
	Condition	**6**	**515.3**	**0.94**	**0.384**
Red	**Condition**	**6**	**486.3**	**0.00**	**0.963**
	(Intercept)	3	492.9	6.54	0.037
Blue	**(Intercept)**	**3**	**547.6**	**0.00**	**0.881**
	Condition	6	551.6	4.01	0.119

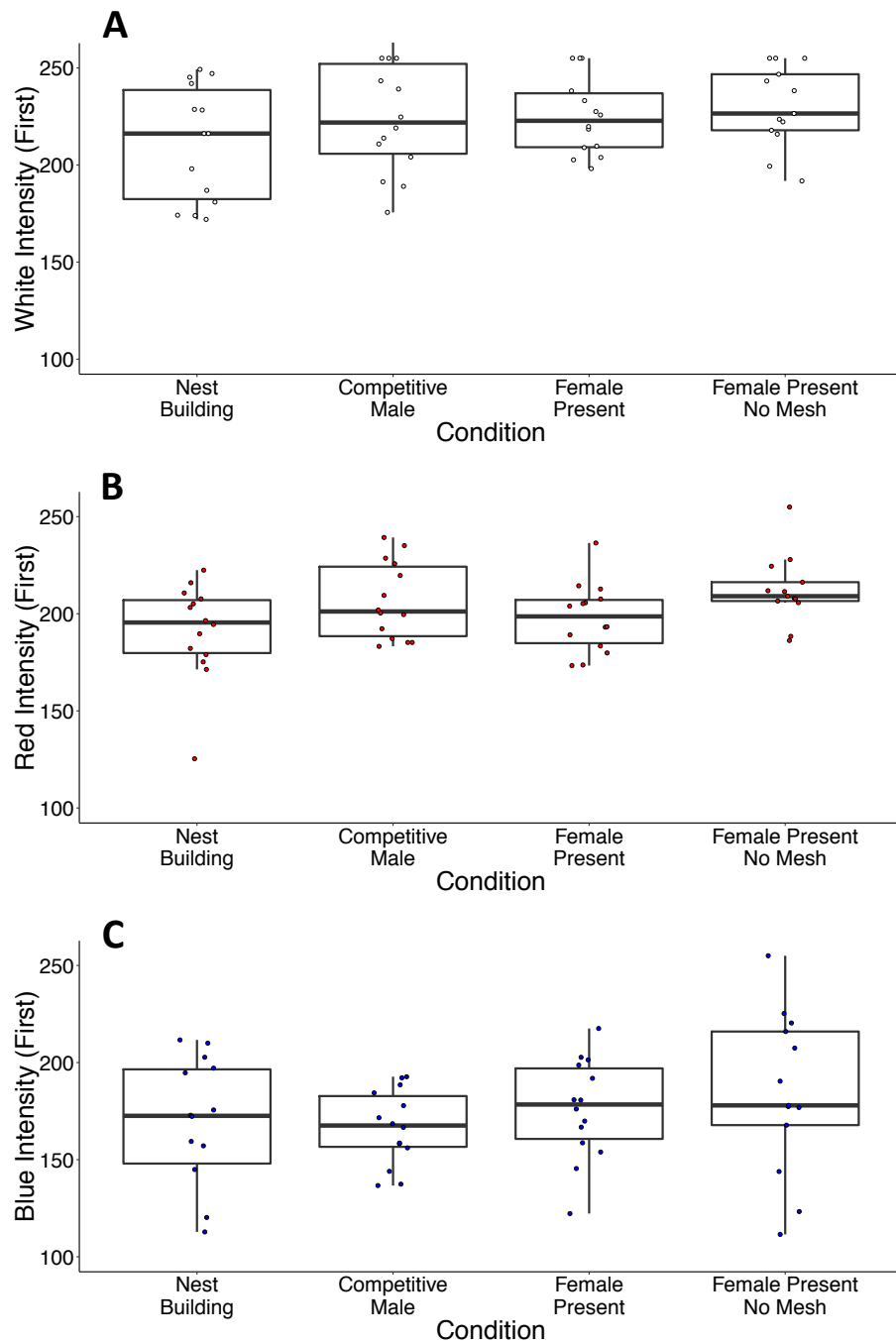**Figure 3.2.1:** Intensity of (a) white, (b) red, and (c) blue colouration of the experimental fish throughout the four conditions during the first five minutes of observations(n=14). Black horizontal line indicates the median, the coloured dots are frequencies for individual males during the first five minutes of observation for a condition. Letters above the data represent post-hoc test groupings

Table 3.2.2 Model selection of the effects of condition, period, their interaction and the intercept on the colour intensity of the fish for all three colours (white, red, or blue). 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 the weight of the model relative to the complete model set. Condition can be nest-building, competitive male, female present with mesh, or female present without mesh. Period is the morning or afternoon observational period. The bolded models are those with the lowest AICc values by a difference of two or more.

| Colour	Predictor	df	AICc	ΔAICc	Weight
White	**Condition**	**6**	**852.7**	**0.00**	**0.706**
	Condition + Period	**7**	**854.6**	**1.91**	**0.271**
	Condition x Period + Condition + Period	10	859.6	6.86	0.023
	(intercept)	3	888.2	35.45	0.000
	Period	4	889.2	36.47	0.000
Red	**Condition**	**6**	**834.8**	**0.00**	**0.730**
	Condition + Period	7	836.9	2.12	0.253
	Condition x Period + Condition + Period	10	842.8	8.02	0.013
	(intercept)	3	845.7	10.87	0.003
	Period	4	847.8	13.03	0.001
Blue	**Condition**	**6**	**893.3**	**0.00**	**0.604**
	Condition + Period	7	895.6	2.32	0.190
	(intercept)	3	896.1	2.85	0.145
	Period	4	898.2	4.96	0.050
	Condition x Period + Condition + Period	10	901.3	8.00	0.011
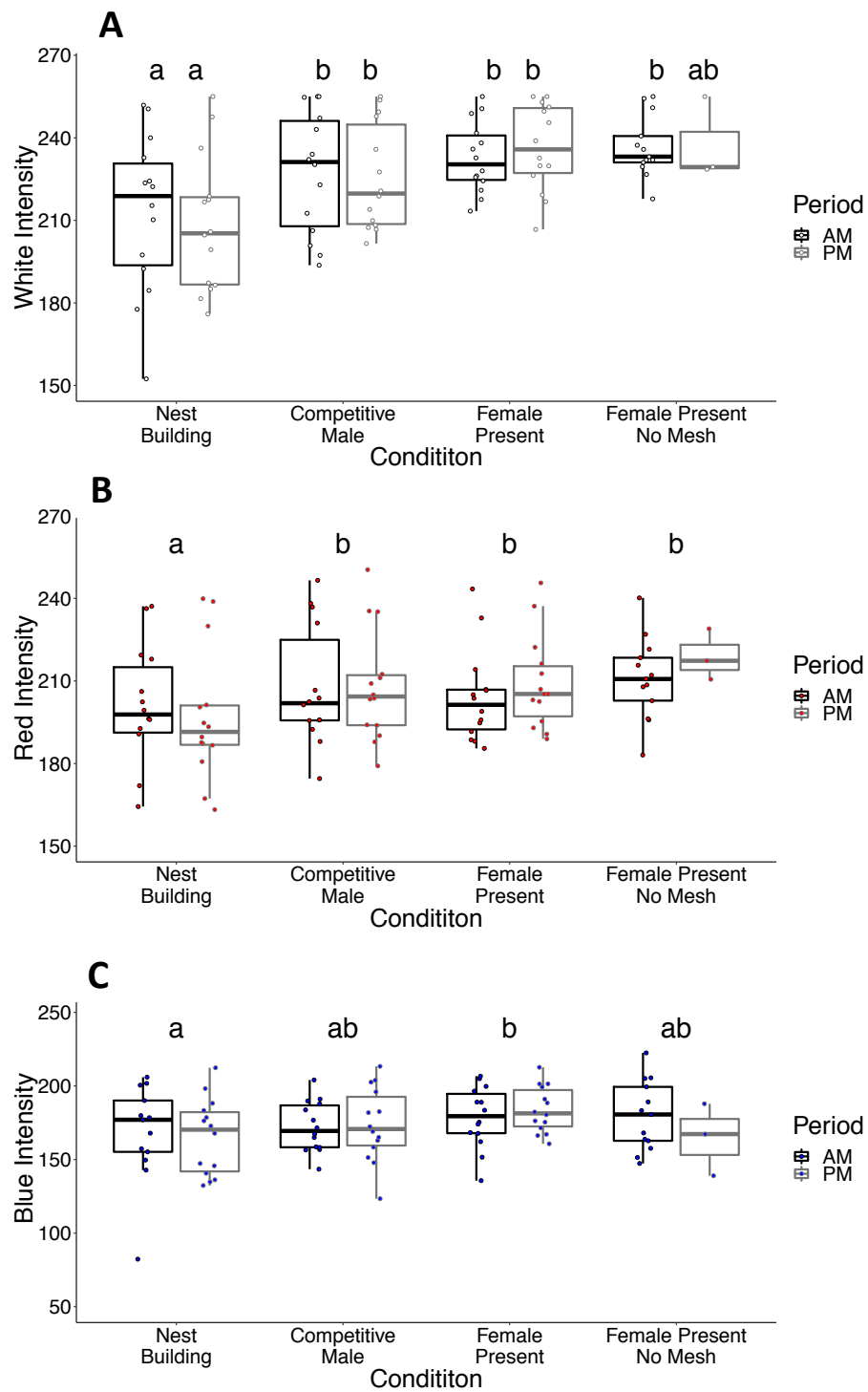
Figure 3.2.2: Intensity of (a) white, (b) red, and (c) blue colouration of the experimental fish throughout the four conditions and both observational periods of a trial (n=14). Black horizontal line indicates the median, the coloured dots are frequencies for individual males. Letters above the data represent post-hoc test groupings.

3.3 Relationship between breeding behaviour and colouration

To further understand the changes in colouration with the components of the breeding phase, I assessed the correlation of white, blue and red intensity of the fish with the frequency of breeding behaviours. A Pearson's product-moment correlation was run between the three behaviour categories for all three colours. Nest-building behaviours were analyzed for all four days of the trials, whereas aggression and courtship behaviours were only analyzed for the days specific to those actions (day 2 = aggression, day 3 and 4 = courtship). For the frequency of nest-building behaviour, all three colour intensities had positive but small correlation values (Table 3.3.1, Fig. 3.3.1a, b, and c). Nest-building behavior frequency went as low as zero when correlated with the white intensity. Similar to nest-building, aggressive behaviour and colouration intensity for all three colours showed a positive but small correlation. However, aggressive behavior frequency was able to be as low as zero when correlated with the red intensity (Table 3.3.1, Fig. 3.3.1d, e, and f). Lastly, for courtship frequencies, the white and red colour intensities again had positive and small correlation values, with courtship behavior frequency possibly reaching zero when correlated with the white intensity. Though, for the blue colouration, it was the only one of the nine correlation tests to display a negative correlation. However, with the standard error the courtship behavior frequency can go higher than zero when correlated with the blue intensity (Table 3.3.1, Fig. 3.3.1g, h, and i).

Table 3.3.1 Pearson's product-moment correlation between behaviour frequency and colouration intensity for all fish. Shown below are the degrees of freedom (df) for the amount of observations analyzed, standard errors (SE), and the correlation value (r).

Behaviour	Colour	*df*	Correlation (r)	SE ±
Nest-building	White	98	0.0917	0.1006
	Red	98	0.1722	0.0995
	Blue	97	0.2751	0.0976
Aggression	White	26	0.2887	0.1878
	Red	26	0.1819	0.1928
	Blue	26	0.3941	0.1802
Courtship	White	42	0.1249	0.1531
	Red	42	0.2098	0.1509
	Blue	42	-0.0384	0.1542
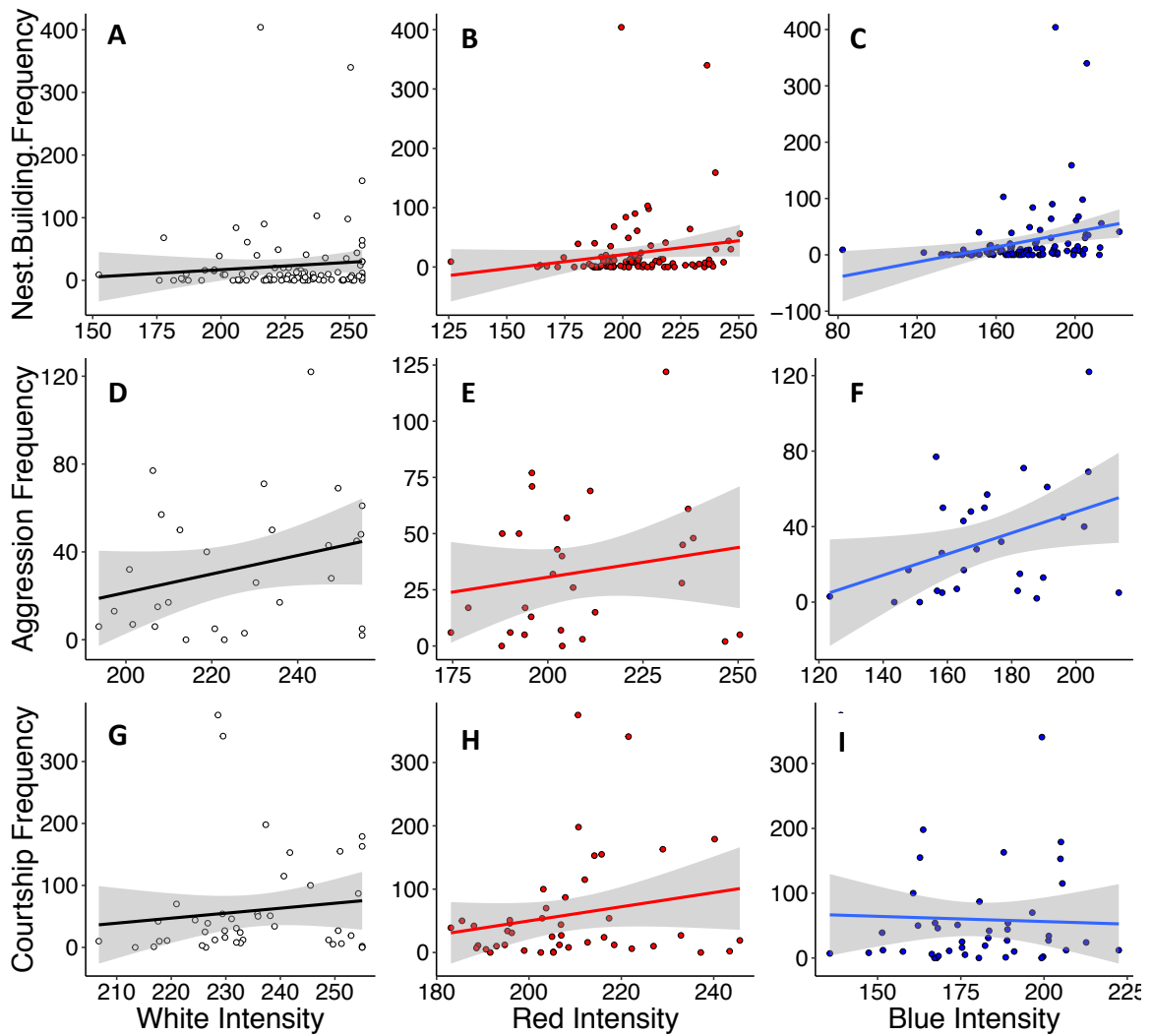
Figure 3.3.1: Frequency of nest-building for white, red and blue colouration intensity (a-c), aggression frequency for white, red, and blue colouration intensity (d-f) and courtship frequency for white, red, and blue colouration intensity (g-i) of the experimental fish throughout the four conditions during both observational periods of a trial (n=14). Nest-building analysis included observations from all four days (n=112), aggression from only day two (n=28) and courtship from only days three and four (n=56).

Discussion

The main objective of this experiment was to quantify the relationship between nuptial colouration and the breeding behaviour of male white Threespine Sticklebacks. This was to understand the use of nuptial colouration as a signal associated with sexual selection through either female mate choice, male-male competition, or both. To do so, I first assessed whether the males behaviours corresponded with the phase of the breeding cycle that they were experiencing; the animals behaved in the way I had predicted by being aggressive only in the presence of competitor males, and by courting only when a female was in the tank. Secondly, I examined whether white male colour intensity changed while performing breeding behaviours and if there was a correlation between the colour intensity and those breeding behaviours. My results revealed that male nuptial colouration did vary among the conditions and that there were small, but mainly positive correlations between the colour intensities and the specific behaviours they were conducting.

4.1 Colouration and Breeding Phases

Throughout all 14 trials, white male nuptial colour intensity changed depending on breeding phase condition (Figure 3.2.1). The results indicate a higher colour intensity for all three breeding-related colours in the presence of a competitor male or a gravid female compared to the baseline colouration during nest-building. This suggests that the colour intensity of the white male is dependent upon the phase of the breeding cycle that they are in. This is similar to past studies on the nuptial colouration of the common Threespine Stickleback, where red and blue colouration were linked to the aggressive and courtship phases of the breeding cycle (Rowland, 1984; Milinski & Bakker, 1990). These data also

corroborates field studies conducted by Haley (2018) that indicated a correlation between courtship frequency and the intensity of white colouration.

In my study, white colour intensity was highest when a gravid female was present, although white colouration was equally intense in the presence of a competitor male. These results support my initial prediction that white male nuptial colouration would change intensity across the different breeding phases. The white male sticklebacks' display of white nuptial colouration is unique to this ecotype (Blouw & Hagen, 1990). My results showed that the white intensity was highest during courtship, suggesting that it is an inter-sexually selected trait. These findings are congruent with other studies that found that the white colouration is possibly associated with sexual selection and female mate choice (Blouw & Hagen, 1990; Jamieson et al., 1992a). However, because there was no difference in colouration between courtship and aggression, colour may also be associated with both female mate choice and male-male competition, as has been proposed by other studies (Blouw & Hagen, 1990).

The results of my experiment support my prediction that the red colouration would be of higher intensity when a competitor male or gravid female were present compared to the nest-building phase. The red colouration of the Threespine Stickleback is of carotenoid origin (Brush & Reisman, 1965). Carotenoid-based pigmentation is costly because the animal cannot produce the pigmentation on their own but must obtain it from their diet (Olson & Owens, 1998). Therefore, some of the energy gained from food must be allocated to colouration (Frischknecht, 1993). The red colouration aids a female in assessing a male as a potential mate; it is used to understand the males current quality by displaying to the female that he is free of parasites, which affect brightness of the red colouration, as well as showing that he is capable of finding resources (Milinski &

Bakker, 1990; Frischknecht, 1993). Furthermore, a common male's red colour intensity may predict how he will react to competitor males impeding on his territory, through an increase in biting behaviour (Rowland, 1984). Therefore, red colouration is also used by competing males.

The results of the blue colouration were congruent with my prediction that the presence of a competitor or a potential mate would result in a higher colour intensity than when the male is nest-building. Threespine Sticklebacks synthesize their own blue colouration through a reduced count of guanine crystals found within guanophores (Titschack, 1922 as quoted in Frischknecht, 1993). Similar to the red colouration, the blue colour can be used by a female to evaluate the male as a possible partner. Blue colouration in males is used by the females to evaluate their long term quality and overall growth (Frischknecht, 1993). Additionally, the intensity of the blue colour of the common male Threespine Stickleback is a predictor of his response of aggressive biting behaviours to intruding competitive males (Rowland, 1984), which is again similar to the red colouration and male competition.

Together, the white, red, and blue colouration is used for attracting mates as well as for competing with other males. In addition, previous studies done on the energy cost of producing the red and blue nuptial colouration of the common Threespine Stickleback indicated that their use of colouration was an honest signal (Frischknecht, 1993). Therefore, I propose that the nuptial colouration of the male white Threespine Stickleback is also an honest signal of quality, and is under sexual selection through female mate choice and male-male competition.

4.2 Colouration and Breeding Behaviours

My results indicated that there were no strong relationships between colour intensity and the frequency of breeding behaviours. When combined with behaviours, the colour signal may be more effective at attracting mates or deterring competitors, than when displayed in the absence of any behaviour. This relationship between nuptial colouration and display behaviours is comparable with a population of guppies (*Poecilia reticulata*) in which female guppies preferred males that displayed carotenoid pigmentation as well as had a high display rate compared to the males who had no pigmentation and a low display rate (Kodric-Brown & Nicoletto, 2001). This study relates to mine in that there was a general trend towards an increase in colour intensity with an increased frequency of breeding behaviour. An alternative explanation for the lack of trends is that regardless of the amount of behaviour conducted, a male may have a maximum colour intensity that they can display. It is possible that the males might have been hindered due to other factors. For example, in the common Threespine Stickleback red colouration is affected by parasitic infection (Milinski & Bakker, 1990). Therefore, if my fish were also infected by parasites there is a chance that their colouration would have been dampened as well. Overall, it appears that the colouration of the white male Threespine Stickleback is associated more so with the condition of the breeding cycle they are currently in (nest-building, competitor male, gravid female), than with the exact frequency at which they are conducting the behaviours associated with these phases.

Conclusion

White Threespine Stickleback males use both morphological and behavioural traits to compete for mates. Males of the white ecotype exhibits white, red, and blue nuptial colouration. Nuptial colouration in previous studies done on common Threespine Sticklebacks showed that their colour acted as a signal that functions in female choice to indicate mate quality and in male-male competition to assert possession of a territory to competitors (Rowland, 1984; Mclennan & McPhail, 1989). Through this experiment I was able to determine that the nuptial colouration of the white ecotype was also associated with both female mate choice and male-male competition. In the future it would be useful to analyze and quantify the rate at which the white male Threespine Stickleback changes in colouration intensity, to add to these analyses. With this study we hope to further understand the plasticity of colouration within and individual and how that relates to their condition and behaviour.

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FIGURES

Figure 2.1 modified from:
https://en.m.wikipedia.org/wiki/File:Canada_Nova_Scotia_location_map_2.svg