Nuptial colouration and breeding behaviour in the white Threespine Stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

The white Threespine Stickleback (*Gasterosteus aculeatus*) is an ecotype endemic to brackish environments in mainland Nova Scotia and the Bras d'Or Lake. During the breeding season, 'white' males are bright white and provide little parental care. By contrast, 'common' males have blue/brown dorsal nuptial colouration and show extensive parental care. My main goal was to quantify breeding behaviour and nuptial colouration in the two ecotypes and compare the mainland and Bras d'Or populations. To address this goal, I conducted field observations of behaviour and colouration, and investigated the cellular basis for colour differences. My findings indicate that: quantitative behavioural traits match earlier observations that white males court at a higher intensity than common males; the cellular basis for male brightness is associated with a reduced number or size of melanophores in both mainland and Bras d'Or white stickleback males; and white colouration becomes brighter in association with increased courtship.

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To Papa

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1. INTRODUCTION

1.1 Selection and speciation

Many species concepts have been put forth to understand and organize biological diversity (Coyne and Orr 1998). Of these species concepts, the most common is Ernst Mayr's biological species concept used to delineate sexually reproducing species (Mayr 1969). The biological species concept defines a species as a group of interbreeding populations that are reproductively isolated from other groups (Mayr 1969), and who do not produce viable offspring (Nosil et al. 2003; Kitano et al. 2007). Therefore, gaining an understanding of reproductive isolation is key to disentangling the mechanisms involved in the process of speciation. Reproductive isolation can occur in allopatry, when populations are geographically separated (e.g., Schluter 2001; Schluter and McPhail 1992; Östlund-Nilsson et al. 2007; Hendry et al. 2009), or in sympatry, when populations inhabit overlapping or identical ranges (e.g., Schluter 2001; Rundell and Price 2009). Both allopatric and sympatric speciation can be driven by random processes such as genetic drift and/or deterministic processes such as natural selection, the latter occurring when groups adapt to different environments (Nagel and Schluter 1998; Hendry et al. 2007).

Natural selection occurs when organisms experience differential survival and reproductive success in the face of different factors (e.g., predation, habitat availability and/or food availability), and the alleles of individuals that survive and reproduce are subsequently passed on to offspring (Nagel and Schluter 1998; Nosil and Crespi 2006). When selective pressures differ among populations with limited gene flow, they can

diverge over evolutionary time to such an extent that they become different species (Schluter 2000, 2001). Similarly, differences in sexual selection can also be an important driver of speciation (Nosil et al. 2003; Kitano et al. 2007; Ritchie 2007). Sexual selection occurs due to differences among individuals in their ability to secure and attract mates resulting in genetic change that may or may not be influenced by environmental factors (Hosken and House 2011). The African crater lakes cichlids are a classic example of speciation in sympatry arising from differential sexual selection (Galis and Metz 1998; Wilson et al. 2000). Assortative mating, based upon polychromatic breeding colouration, may contribute to reproductive isolation among populations more strongly than ecological factors, and can lead to a remarkable diversity of sympatric species (Galis and Metz 1998; Wilson et al. 2000; Allender et al. 2003). However, more often than not it is the interaction of this selective pressure in combination with pressures sustained in an ecological context, such as predation or immune system function, that plays an important role in pre-mating isolation (Ritchie 2007; Scordato et al. 2014). For example, Poeciliid fishes display prominent nuptial colouration for species recognition and to signal male health thereby enhancing female preference and mating success, yet must balance the potential predation pressures associated with conspicuous breeding colouration (Endler 1983).

1.2 Mate choice and nuptial colouration in fishes

The evolution of ornate behavioural and morphological signals to attract potential mates occurs in many fish species when competition for mates is intense (Endler 1983; Seehausen and Van Alphen 1998; Bay et al. 2017). For this reason, fishes are excellent models to study the evolution of mate choice, and how it may play a role in speciation

(Endler 1983; Seehausen and Van Alphen 1998; Bay et al. 2017). In particular, 'nuptial colouration', colour patterns associated with mating, have become increasingly important morphological signals examined in the study of speciation owing to the role they can play during mate competition and choice (Kodric-brown 1998; Price et al. 2008). Nuptial colouration can be an inter- and intrasexual signal to potential mates and competitors and is important to the study of the link between sexual selection and incipient speciation when inter-species colouration differs (Kodric-brown 1998; Seehausen and Van Alphen 1998; Price et al. 2008).

Differences in nuptial coloration are the result of changes in pigment cells, or chromatophores (reviewed by Sköld et al. 2016). Chromatophore cell types are differentiated by the pigments they contain (reflecting and/or absorbing light), their shape, arrangement, and responses to the presence of stimulating hormones (Schartl et al. 2016). There are many types of chromatophores in fishes: e.g. melanophores (dendritic cells containing the brown pigment melanin, or yellow eumelanin), iridophores (reflective platelets that can produce many colours), leucophores (dendritic cells containing reflective pigments that appear white), and xanthophores/erythrophores (dendritic cells containing red or orange pigments). These pigment cells create patterns and colours that are physiologically regulated in short-term responses to stress and excitatory stimuli and can also vary in number, size and pigment density over longer-time scales (Schartl et al. 2016; Sköld et al. 2016).

There are typically three types of colour change associated with nuptial colouration in fishes: permanent, long-term, and rapid colour change (Kodric-brown 1998; Price et al. 2008). Permanent nuptial colouration is often found in tropical fishes

that maintain breeding grounds year-round, such as cichlids and guppies (Galis and Metz 1998; Price et al. 2008; Kottler et al. 2013). By contrast, long-term nuptial colouration is found in fishes with restricted breeding seasons, and has been extensively studied in models such as the Threespine Stickleback (Reimchen 1989; McKinnon 1995; McKinnon and Rundle 2002; Marques et al. 2017). This seasonal colour change, also termed 'morphological' colour change, occurs over longer time-periods through changes in the number of chromatophores, chromatophore morphology, and/or the deposition of pigments acquired from the diet or endogenously synthesized (Bagnara and Matsumoto 2007; Price et al. 2008; Sköld et al. 2016). Lastly, rapid nuptial colour changes are ephemeral changes (seconds to minutes) that serve to enhance colour patterns, and/or signal aggression and courtship behaviour (Kodric-brown 1998; Price et al. 2008; Nilsson Skold et al. 2013). This short-term 'physiological' regulation of colour occurs when pigments and light-reflecting platelets move within pigment cells, such as the observed changes in the iridophores of the paradise whiptail (*Pentapodus paradiseus*), that leads to changes in colour from blue to red in 0.25 seconds (Mathger 2003).

The Threespine Stickleback (*Gasterosteaus aculeatus*) is an excellent model for evolutionary and behavioural studies on breeding behaviour and nuptial colouration because of considerable intraspecific variation in these traits, ease of rearing under laboratory conditions, and the ability to observe breeding behaviour in the field (Kynard 1978; Bell and Foster 1994; Östlund-Nilsson et al. 2007). Because the breeding season is restricted to a few months of the year, males of this species are good candidates for the study of long-term and rapid colour change associated with sexual selection.

1.3 The Threespine Stickleback (*Gasterosteus aculeatus*) as a model species for breeding behaviour and nuptial colouration

The Threespine Stickleback is a small teleost fish that is approximately five centimetres long, has successfully adapted to both marine and freshwater environments (Moodie 1972b; Nagel and Schluter 1998; Matthews et al. 2010), and includes anadromous marine populations (Wootton 1976; Bell and Foster 1994; Östlund-Nilsson et al. 2007). Freshwater and anadromous/marine populations differ in many physiological, morphological and behavioural traits influenced by environmental and genetic variation (e.g. Bentzen and McPhail 1984; Schluter 2001; Kume et al. 2010). As such, this species is an ideal model for behavioural ecologists and evolutionary biologists studying speciation and local adaption (Lee 1976; Orti et al. 1994; Östlund-Nilsson et al. 2007).

While considerable work indicates that natural selection may drive speciation in some populations of Threespine Stickleback (Nagel and Schluter 1998; Barrett et al. 2008; Conte and Schluter 2013), differential sexual selection on breeding colouration, mating strategies, and parental care strategies can also contribute to positive assortative mating and limit gene flow among populations (Olafsdóttir et al. 2006; Marques et al. 2016). Typically, marine Threespine Stickleback males, hereafter the 'common' ecotype of the Threespine Stickleback, develop red throat pigmentation, blue eyes, and a blue/brown dorsal colour during the breeding season, and aggressively defend a territory in which they build a nest (Tinbergen 1952; van Iersel 1953). Males seek out materials appropriate for nest construction (e.g., bits of algae or sand) and secrete glue from the kidneys to keep nesting material in place (De Ruiter and Wendelaar Bonga 1985). Nests are constructed in muddy or sandy substrate, and females are attracted to these nests with a series of stereotypical male courtship behaviours including a zigzag 'dance', dorsal

pricking, and circling, followed by leading females to the nest (Tinbergen 1952; van Iersel 1953). Females lay eggs in these tunnel-shaped nests and are then followed by males that fertilize these eggs. After one or several females has laid eggs in the nest, males will defend the nest from predators and exhibit a fanning behaviour to oxygenate developing eggs and offspring (van Iersel 1953; Wootton 1976).

Inter-population variation is observed in nuptial colouration, nest-building, courtship, and male parental care behaviour (Östlund-Nilsson et al. 2007; reviewed by Kitano et al. 2017). For example, two isolated Threespine Stickleback populations on the Sechelt Peninsula in British Columbia have lost the typical red and blue nuptial colouration and aggressive behaviour usually seen in breeding males (Pressley 1981). In addition, on the Pacific coast of North America, some populations display black (as opposed to typical red) nuptial colouration along the throat (Moodie 1972a; Kynard 1978). Furthermore, these males have the ability to rapidly lighten their dark colouration by decreasing pigment dispersion and increasing light reflectance within their chromatophores (Kynard 1978) A unique population in Southeast Sweden lacks red nuptial colouration along the throat, displays a conspicuous blue nuptial colouration along the dorsum, and engages in frequent courtship while lacking typical nesting material (Borg 1985). These males bear resemblance to a marine Threespine Stickleback ecotype on the Atlantic coast of Canada that displays white dorsal and lateral nuptial colouration and does not provide male parental care (Blouw and Hagen 1990; Haglund et al. 1990; Blouw 1996). This ‘white’ ecotype, along with the previously described common ecotype that displays typical breeding traits and parental care, is the focus of this dissertation.

Blouw and Hagen (1984, 1990) first characterized the ‘white’ Threespine Stickleback ecotype along the coasts of Nova Scotia. White males develop a conspicuous bright white dorsal and lateral colour, a pale red coloured throat, and a white-blue iris (Blouw and Hagen 1990). Outside of the breeding season, both sexes of the white and common Threespine Stickleback have similar colouration ranging from subtle blue and brown dorsal colour, and silver ventral colouring (Hagen and Gilbertson 1972; Blouw and Hagen 1990; Jamieson et al. 1992a). However, both males and females of the white ecotype are smaller than their common ecotype counterparts, with shorter and narrower bodies (Blouw and Hagen 1990; Samuk 2016). The breeding territories of white and common males occur in different habitats within estuaries, with the white ecotype making use of algae and the common male nesting in muddy substrate (Jamieson et al. 1992a). Although males of the white ecotype nest above the substrate, the filamentous algae present in all white Threespine Stickleback breeding grounds provides adequate cover and is found at greater depths than the common Threespine Stickleback nests of the same area (Blouw and Hagen 1990; Jamieson et al. 1992a).

Differences in courtship behaviour occur between ecotypes; common Threespine Stickleback males perform zig-zag, dorsal pricking and circling rituals, but white males perform only the zigzag dance (Wootton 1976; Blouw and Hagen 1990; Jamieson et al. 1992a). Both types of males attempt to lead the female to the nest during courtship and highlight nest location, however, white-coloured males remove embryos from their nests after breeding to scatter them in filamentous algae and return to their nest to breed, upon which courting and spawning is resumed (Blouw and Hagen 1990; Jamieson et al. 1992a). By contrast, after a set of successful spawnings, ‘common’ males cease courtship

and remain at the nest to fan and guard the eggs and do not begin courting again until offspring have vacated the nest (van Iersel 1953; Wootton 1976). Because male white Threespine Stickleback disperse their eggs after fertilization and provide little to no parental care, Blouw and Hagen (1996) suggested that the filamentous algae in which the embryos are deposited must be an adequate substitute for parental care in terms of protection from predation and oxygenation of eggs in tidal waters. As a result, white males have more numerous and longer mating bouts, and likely an increase in the intensity of sexual selection compared to common males (Jamieson et al. 1992a,b). An increase in the intensity of sexual selection is further supported by evidence in the field when the territories of both white and common males are adjacent. Some common females are initially attracted to the energetic courtship displays and bright colouration of the white males, but will not follow through with spawning upon arrival at the white male's nest (Jamieson et al. 1992a). Positive assortative mating, driven by female choice, occurs between white and common ecotypes (Blouw and Hagen 1990).

The white ecotype likely evolved from an ancestor similar to the 'common' marine Threespine Stickleback, and the ecotypes currently breed in sympatry in brackish waters (Blouw and Hagen 1990). These two ecotypes are genetically differentiated; recent population genomic studies have found that white Threespine Stickleback in mainland Nova Scotia can be genetically distinguishable from common Threespine Stickleback populations (Samuk et al. 2014; Samuk 2016), and laboratory breeding studies have found that differences in breeding colouration and behaviour between white and common ecotypes have a genetic basis (Jamieson et al. 1992a; Blouw 1996). However, the overall level of genetic differentiation between the two ecotypes is relatively low (Haglund et al.

1990; Samuk et al. 2014; Samuk 2016) and laboratory crosses between white and common fish produce viable offspring (Blouw 1996). The persistence of the two ecotypes suggests some degree of reproductive isolation, but the low genetic differentiation suggests ongoing gene flow and/or recent divergence (Samuk et al. 2014; Samuk 2016). Thus, like other ecotypes of the Threespine Stickleback complex, the white Threespine Stickleback occupies a "grey zone" of speciation that challenges the biological species concept (Roux et al. 2016). This makes the 'white' and 'common' marine ecotypes in Nova Scotia important example systems for comparative studies of local adaptation and sexual selection in populations at the early end of the speciation continuum (Blouw and Hagen 1990; Jamieson et al. 1992a; Blouw 1996; Samuk 2016), and the white Threespine Stickleback an especially useful model to study the evolution of male nuptial colouration, breeding behaviour, and parental care.

As previously mentioned, Blouw and colleagues have detected behavioural differences in wild common and white populations (Blouw and Hagen 1990; Jamieson et al. 1992a) and quantified breeding and parental care behaviour in the laboratory (Jamieson et al. 1992b; Blouw 1996), but treated Bras d'Or lake and mainland Nova Scotian stickleback as members of the same population. However, populations of white and common ecotypes that inhabit the mainland and Bras d'Or Lake may have limited gene flow (Samuk 2016) and white populations from the mainland and Bras d'Or may show differences in these traits. Recently, Samuk (2016) found that Bras d'Or common marine stickleback are genetically distinct from mainland white and common marine ecotypes of Threespine Stickleback. Thus, it is possible that the Bras d'Or Threespine white ecotype evolved from the Bras d'Or commons, independently from mainland white

populations. Alternatively, the white colouration in Bras d'Or and mainland Nova Scotia stickleback populations may have a shared genetic basis if gene flow between mainland Nova Scotia and the Bras d'Or Lake white ecotypes is the source of the opposite location's 'white alleles'. Because recent population genetic analyses did not include the Bras d'Or white form (Samuk 2016), these predictions have not yet been directly tested. Similarly, behavioural and nuptial colouration differences among White Threespine Stickleback populations found on mainland Nova Scotia and in the Bras d'Or Lake have not yet been directly compared to determine if male nuptial body colour and breeding behaviours are similar in these two potentially unique populations. Therefore, the main goal of my thesis is to determine whether white stickleback breeding behaviours differ between mainland and Bras d'Or populations, and to study skin colouration divergence among these white populations and among ecotypes (white versus common).

1.4 Study Objectives

This study was designed to further investigate behavioural differences between white and common males that may provide insight into differences in the intensity of sexual selection between the two ecotypes. I also compare behaviours and long-term colour change between both mainland and Bras d'Or populations to study the proximate signals leading to rapid colour change in the field. To this end, I address three questions in this thesis: (1) How does breeding behaviour differ between white and common ecotypes, and do mainland and Bras d'Or populations differ? To answer this question, I quantified and compared courtship and nestbuilding in wild populations of white and common stickleback to test predictions from earlier studies, as well as provide new information on aggression. (2) How do the mechanisms leading to nuptial colouration

differ between white and common ecotypes in both locations? To address this question, I quantified and compared pigmentation density predicted to lead to white skin colouration in these two ecotypes from both locations. (3) Can 'whiteness' be correlated with specific breeding behaviours? I analyzed behavioural correlates and the degree of brightness, which was measured using two separate methods, to identify which breeding behaviours are related to white colouration.

1.4.1 Goal 1: Quantify and compare breeding behaviour of mainland and Bras d'Or white stickleback

Blouw and Hagen (1990) noted that white males courted at higher intensities than common males, which was further supported by field observations quantifying courtship and nestbuilding activity in Bras d'Or Lake sticklebacks (Jamieson et al. 1992a). Positive assortative mating between white and common Threespine Stickleback ecotypes from mainland populations, as well as Cape Breton populations (but not in Bras d'Or), was also confirmed (Blouw and Hagen 1990). In this study, I directly compared behavioural differences between white and common ecotypes from the mainland and Bras d'Or lake populations in the field. Focal observations characterized courtship, nestbuilding, and aggression. I predicted that male white Threespine Stickleback would show increased intensity of all types of breeding behaviour in comparison with the common ecotype as qualitatively noted by Blouw and Hagen (1990) and Jamieson et al. (1992a).

1.4.2 Goal 2: Compare melanophore density between mainland and Bras d'Or white and common ecotypes

A determination of the chromatophore combinations that lead to the white colouration in the Threespine Stickleback can provide insight to the physiological and genetic basis for differences in colouration among ecotypes and the factors controlling

rapid colour change. The white colouration of the Nova Scotia ecotype may be related to a decrease in the number of melanophores responsible for dark colouration, an increase in pigment cells responsible for their white colouration (e.g. iridophores), or a combination of these factors. In this study, skin samples of wild fish from both ecotypes were compared to assess melanophore content and dorsal, lateral, and ventral melanophore coverage. I predicted that white Threespine Stickleback males would have a lower density of melanophores in comparison with common Threespine Stickleback males, and that Bras d'Or and mainland populations would achieve nuptial colouration in a similar fashion because they are morphologically similar.

1.4.3 Goal 3: Test for an association between brightness and behaviour in white ecotypes

The ability to increase or decrease brightness is a feature of the male white ecotype's nuptial colouration; males can rapidly change from bright white to dull grey (Blouw and Hagen 1990). Blouw and Hagen (1990) also noted that the intensity of brightness appeared to be correlated with an increase in courtship, nestbuilding and aggressive behaviours, but these changes were not quantified, nor was it clear which activity, if any, was more strongly associated with white colouration. To investigate this, colour in the white ecotype was assessed *in situ* and correlated with behavioural observations. I predicted that both courtship and aggression would be positively correlated with the intensity of brightness because bright nuptial colouration serves to enhance courtship and aggressive displays in populations of Threespine Stickleback that display typical colouration. I also predicted that the behavioural correlates with brightness would not differ between Bras d'Or and mainland populations because, if variables are

similar in both locations, conspicuous nuptial colouration is likely an inter- or intra-sexual signal useful to males of both populations.

2. MATERIALS AND METHODS

2.1 Sampling sites

I collected and observed white and common Threespine Sticklebacks from a variety of locations throughout Nova Scotia (Table 1). I chose these sites based on previous behavioural (Blouw and Hagen 1990; Jamieson et al. 1992a) and genetic studies (Samuk et al. 2014; Samuk 2016). New field sites were also discovered throughout the course of this study and were selected based on ease of access and presence of algae and substrate typical of nest material for breeding white and common males.

Breeding white and common Threespine Stickleback in Nova Scotia were found in brackish tidal basins separated from the ocean by raised highway beds or gravel roads. During the breeding season the tidal cycle was a regular twelve hours, which caused the temperature and salinity to fluctuate at these sites throughout the day. Filamentous algae, likely *Cladophora* sp., was present in all sites which matches the observations of Blouw and Hagen (1990). Algal density varied throughout the breeding season, with more algae blooming as the ambient temperature and daylight increased. Boulders and large rocks were present on the shoreline of all sites except for two: Rainbow Haven, which was bordered by marshy grass, and Middle River, which was a riverbank. The substrate at all sites was characterized by muddy detritus with fallen leaves and pieces of grass. On the seaward side of each inlet, wave action was stronger, the density of filamentous algae decreased, and breeding males were not observed to have nest-sites. Water turbidity appeared similar at all sites; the water had a tea-stained appearance which seemed to be exacerbated by heavy rain or high tides.

In June – July of 2017 and 2018, I conducted behavioural observations (Section 2.2) in Canal Lake and Rainbow Haven on the mainland of Nova Scotia, and in Baddeck, in the Bras d'Or Lake, which is a saltwater lake in Cape Breton island, Nova Scotia, that is largely isolated from the Atlantic Ocean (Table 2.1.1, Fig. 2.1.1). Within that timeframe individuals were also collected for chromatophore analysis (Section 2.3) from 6 sites on the mainland of Nova Scotia, Bras d'Or Lake, and from 1 site in Corner Brook, Newfoundland (Table 2.1.2, Fig. 2.1.2).

Table 2.1.1 Behavioural observation sites in Nova Scotia. Ecotypes were found in different locations on the Nova Scotian mainland, and Bras d'Or Lake, Cape Breton. All fish collected were males.

Site	**Location (GPS coordinates)**	**Ecotype(s) present**	**Number of fish observed**	**Number of observations**	**Average water temperature (°C)**	**Salinity at depth (ppt)**	**Dates of observations**
Canal Lake *Previously studied by Samuk (2016)	Mainland 44.497627° N, 63.900449° W	White Common	50 white 5 common	96 white 9 common	13.0 – 28.0	10.3 - 22.6	Jun 5 – Jul 17 2017
Rainbow Haven	Mainland 44.654799° N, 63.421140° W	White Common	16 common	25 common	Not collected	Not collected	Jul 4 - 5 2018
Baddeck	Bras d'Or 46.101757° N, 60.745549° W	White Common	19 white 8 common	36 white 8 common	21.4 -24.1	17.8 -18.4	Jul 10 – 11 2017 Jun 11 2018

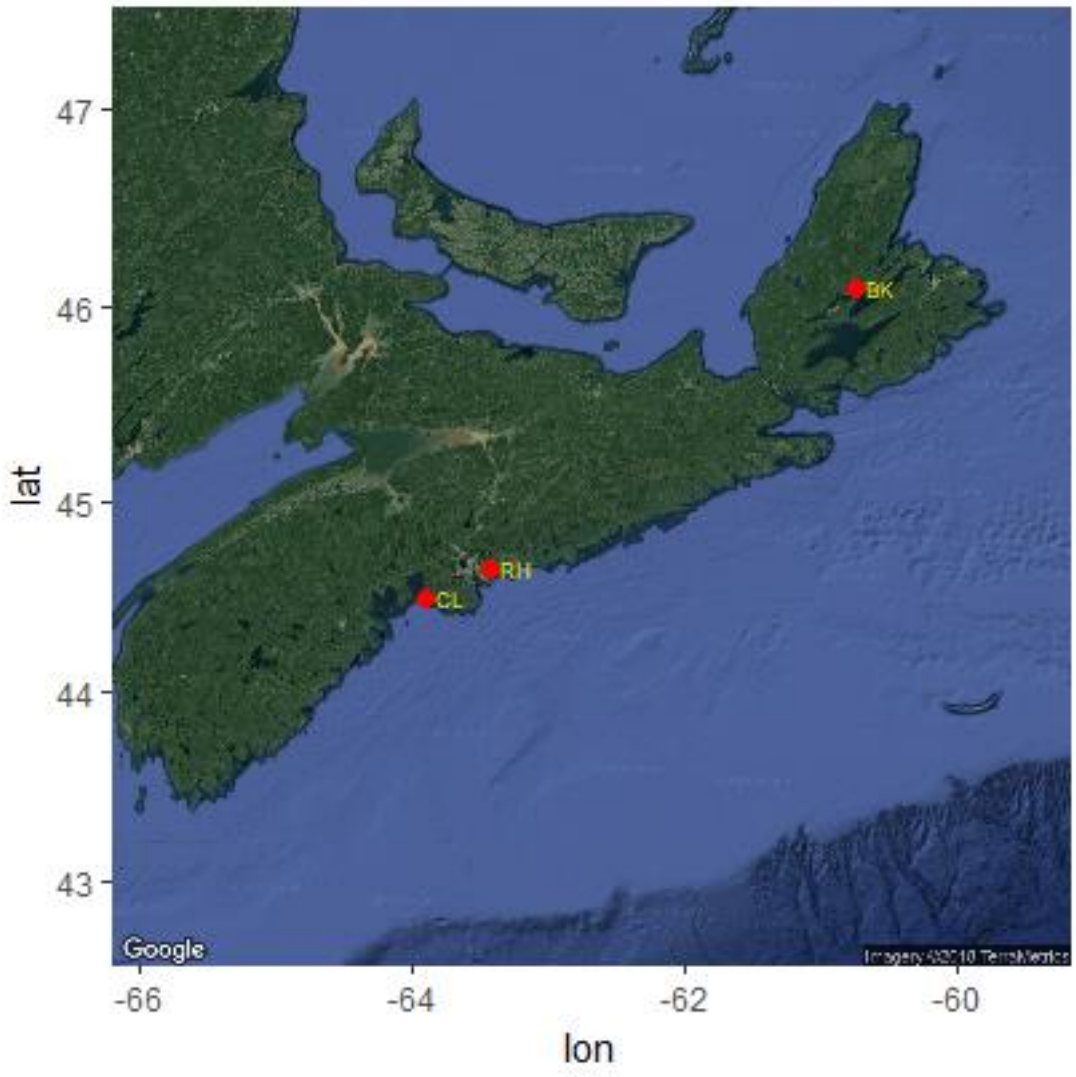

Figure 2.1.1 Map of Nova Scotia outlining behavioural sampling sites on the mainland (CL, RH), and Bras d'Or (BK). CL = Canal Lake, RH = Rainbow Haven, BK = Baddeck.

Table 2.1.1 Chromatophore sampling sites in Nova Scotia and Newfoundland. Ecotypes were found in different locations on the Nova Scotian mainland, one site in Newfoundland, and Bras d'Or Lake, Cape Breton. All fish collected were males.

Site	**Location (GPS coordinates)**	**Ecotype(s) present**	**Number of fish collected**	**Average water temperature (°C)**	**Salinity at depth (ppt)**	**Date of collection**
Canal Lake *Previously studied by Samuk (2016)	Nova Scotia Mainland 44.497627° N, 63.900449° W	White Common	11 (white)	13.0 – 28.0	10.3 - 22.6	Jul 17 2017
Lawrencetown	Nova Scotia Mainland 44.645293° N, 63.325352° W	White	5	Not collected	Not collected	Jul 21 2017
Rainbow Haven	Nova Scotia Mainland 44.654799° N, 63.421140° W	White Common	2 (common)	Not collected	Not collected	Jul 21 2017
Blues Cove	Nova Scotia Bras d'Or 45.899065° N, 61.086559° W	Common	4	22.6 - 24.8	15.9 - 16.6	Jul 12 2017
Middle River	Nova Scotia Bras d'Or 45.993401° N 60.979736° W	Common	7	Not collected	5.0	Jul 25 2017

Table 2.1.1 (continued) Chromatophore sampling sites in Nova Scotia and Newfoundland. Ecotypes were found in different locations on the Nova Scotian mainland, one site in Newfoundland, and Bras d'Or Lake, Cape Breton. All fish collected were males.

Site	**Location (GPS coordinates)**	**Ecotype(s) present**	**Number of fish collected**	**Average water temperature (°C)**	**Salinity at depth (ppt)**	**Date of collection**
Baddeck	Nova Scotia Bras d'Or 46.101757° N, 60.745549° W	White Common	5 (white)	21.4 -24.1	17.8 -18.4	Jul 11, 25 2017
Corner Brook	Newfoundland 48.95001°N, 57.95202° W	Common	9	13	16.4	Jun 23 2017

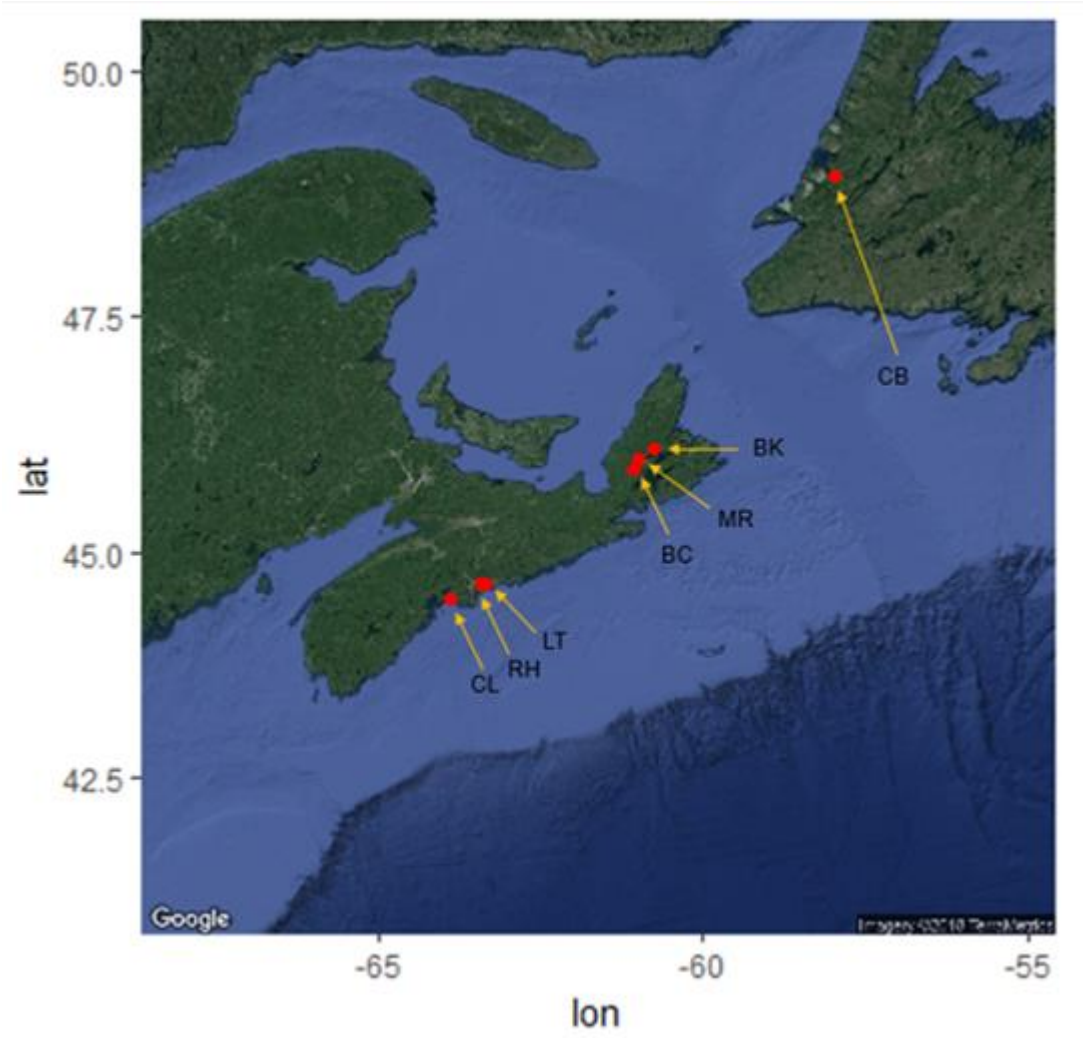

Figure 2.1.2 Map of Nova Scotia outlining chromatophore sampling sites on the mainland (CL, RH, LT), Newfoundland (CB), and Bras d'Or (BC, MR, BK). CL = Canal Lake, RH = Rainbow Haven, LT = Lawrencetown, BC = Blues Cove, MR = Middle River, BK = Baddeck, CB = Corner Brook.

2.2 White and common male breeding behaviour

2.2.1 Behavioural data collection

From mid-June to mid-July of 2017 and 2018, I observed mating behaviour of Threespine Stickleback from Canal Lake and Rainbow Haven (mainland) and Baddeck (Bras d'Or Lake), Nova Scotia. Behavioural observations occurred in Canal Lake from June 16th to 25th, and from July 4th to 7th and July 14th in 2017, and in Rainbow Haven on July 4th and 5th of 2018. Observations in Baddeck occurred on July 12th and 13 in 2017 and on June 11th in 2018.

Prior to observations in Canal Lake, I captured and tagged male Threespine Stickleback using subcutaneous visual implant elastomer tags (Northwest Marine Technologies) to identify individuals occupying nests in the field. I recorded fish length using Vernier calipers, photographed the fish next to a colour standard, and took anal fin clips that were stored in ethanol for later genetic work. Following tagging, I returned individuals to their territories and did not resume behavioural observations until the following day. This gave the male time to return and re-acclimate to his nest site. Upon completion of field observations in July 2017, I recaptured remaining male white Threespine Stickleback from Canal Lake using a handheld dip net.

I chose nine focal behaviours to quantify in the field (Table 2.2.1), emphasizing courtship, aggression, and nest-building based on previous work (van Iersel 1953; Blouw and Hagen 1990; Jamieson et al. 1992a; Wootton and Fletcher 2009).

Table 2.2.1 Description of each focal behaviour quantified in the field to determine courtship, nest-building, and aggressive behaviour for behavioural analysis.

Behavioural category	Focal behaviours	Description
Courtship	Zig-zag dance	Male swims rapidly back and forth in a 'Z'- shaped pattern.
	Dorsal pricking	Male pricks female in the abdomen using dorsal spines.
	Circling	Male swims around female in a circular pattern.
	Leads to nest	Male turns with flank facing the surface of the water and swims in the direction of his nest as female follows.
Aggression	Chasing common Threespine Stickleback	Male swims rapidly towards intruder to defend nest and territory as intruder flees.
	Chasing white Threespine Stickleback	
	Biting	Male charges, then bites intruders. Observed when chasing is not successful (intruder does not flee).
Nest-building	Material retrieval	Male picks up nest-building material (e.g. algae) with mouth and returns to nest.
	Nest tending	Male uses spiggin (glue produced by kidneys) to maintain nest integrity. Male swims through nest to maintain tunnel shape.

I conducted five-minute visual observations of male mating behaviour adapted from the procedure outlined by Macdonald et al. (1995). Because white males are frequently active, five minutes was ample time to observe focal behaviours. Focal observations of individual male fish behaviour occurred at least once a day, and if visibility and nest attendance allowed, I observed an individual a second time during a different tidal cycle (e.g., low and high tide) to account for potential behavioural variability caused by tidal influence. Each instance of behaviour was recorded once the male switched to a different action during the observation period. If the same behaviour was clearly interrupted by more than a one second pause, it was recorded as two separate instances. Each zig-zag to a female was recorded as a separate occurrence, even if the male spent the entire observation period in courtship. When males were behaving aggressively, chasing was recorded as two instances if males returned to their territory after charging at the intruder, and then proceeded to leave its nest-site to charge at the intruder again. Instances of nestbuilding behaviour were recorded when males picked up algae or pieces of substrate in its mouth, demonstrated a gluing behaviour characterized by a circular motion around the nest, and when males used his mouth or performed a fanning motion (different than the fanning observed in the parental care phase) to re-arrange his nest. Because white males do not enter the parental care phase and do not fan embryos, fanning behaviour outside of the nestbuilding and sexual phase could not be compared between ecotypes and was not included in my analyses. Fanning behaviour for nest-building was recorded as a nest-tending focal behaviour. If a male was present on the nest but did not perform a focal behaviour, the behavioural frequency was recorded as a zero. I recorded the behaviour of all individuals possessing territories at the site in a randomized order each day. In addition to the nine focal behaviours, I recorded any

successful spawning events. Additionally, I noted site fidelity by recording which males occupied each nest site using information from elastomer tags, noted egg dispersal events, as well as factors reducing offspring survival, such as nest-raiding and egg predation, following the methods of Jamieson et al. (1992a).

2.2.2 Statistical analysis of behavioural observations

Prior to analysis, I summed all focal behaviour that occurred in one of the three main categories outlined in Table 2.2.1 (i.e., courtship, nestbuilding and aggression) and looked at separate focal behaviours including leads to the nest, zig-zag frequency, material retrieval, and nest-tending. Analyses were carried out in R v.3.6 (R Core team 2013) using the 'lme4' package for mixed models. Because the data generated from the behavioural observations are counts, generalized linear mixed models (GLMM) with Poisson error distributions were used to test the fixed effects of ecotype (white, common) and location (Mainland, Baddeck) on the frequencies of courtship, nest-building, and aggressive behaviours. Each fish was entered as a random factor to account for unknown differences among individuals and the repeated observations that were made in the experiment. I used Akaike Information Criterion corrected for sample size (AICc) values to compare models and chose the model with the lowest AICc values as the best fit to the data, provided that the AICc difference between the best model and other models was greater than two.

2.3 Long term colour change assessed by chromatophore density

2.3.1 Fish collection and husbandry

From June to July of 2017 I collected white and common males from 7 sites on the mainland of Nova Scotia and Bras d'Or Lake, Nova Scotia, as well as Corner Brook,

Newfoundland (Table 2.1.2). Because I had difficulty finding common ecotype only habitats in Nova Scotia field sites, Threespine Stickleback commons from Corner Brook were analyzed with mainland commons. Fish from Corner Brook are genetically similar to mainland Nova Scotian marine populations and thus were considered to be similar for the purpose of chromatophore work (Antoine Paccard, McGill University, personal communication). Data from white and common ecotypes, mainland and Newfoundland (hereafter Atlantic) and Bras d'Or locations, and breeding and non-breeding condition were used.

Fish were captured from the wild using either minnow traps or a handheld dipnet and transported to the aquatic facilities at Saint Mary's University. Upon arrival at Saint Mary's University, fish were transferred to 15-gallon aquaria at a salinity of 10 ppt equipped with a waterfall filter. Ten to fifteen conspecifics from the same population were housed in each tank and acclimated for at least three weeks prior to chromatophore sampling. Light conditions were similar to the natural environment during the mating season, with a photoperiod of 10 h dark and 14 h light, and water temperatures ranged from 19-21°C. Fish were fed a combination of brine shrimp nauplii, frozen bloodworms, and mysis shrimp twice a day. A YSI probe was used to monitor the salinity, temperature and dissolved oxygen present in the water. Ammonia, pH, nitrite and nitrate levels were also monitored weekly with Hagen water quality test kits, and 20 % water changes occurred on a weekly basis or when nitrogenous waste levels reached levels higher than recommended.

2.3.2 Sample preparation for melanophore analysis

For this study, I examined pigment cells responsible for dark colouration (melanophores) following the methods of Greenwood et al. (2011). I sampled fish in the laboratory from August – September of 2017 after wild specimens had acclimated in aquaria either during the breeding season or following its completion. I compared skin samples of adult males from mainland Nova Scotia and Newfoundland, (Atlantic samples), and samples of adult males from Bras d'Or Lake, to assess melanophore number and density.

Breeding condition of white males was scored prior to removal from aquaria. I gave the fish a score on a scale of one to five to determine the presence, or lack thereof, of nuptial colouration. A score of 1 was recorded when the individual displayed typical non-breeding colouration which includes a silver lateral and ventral colour, dull brown along the dorsum and a complete lack of red and iridescent white colouration. A score of 2 was assigned when the individual showed slight traces of red along the throat. Individuals scored as 3 were a dull white and displayed some traces of red along the throat. A score of 4 was recorded when the fish was fully white, but not as bright and iridescent as a fish scored as a 5. Because males were not collected outside of the breeding season, adult males were housed in aquaria until nuptial colouration and breeding behaviour subsided. White males with a score of 3 and above were categorized as breeding individuals and displayed variable bright white colouration, light blue eyes, and red along the throat.

Common males were not scored, but breeding condition was determined by the presence or absence of typical breeding colouration such as a red throat, blue eyes, and blue/brown dorsal colouration. Fish were then removed from their tank and euthanized with a lethal concentration of buffered tricaine methanesulfonate (MS-222). I removed each fish from the anesthetic, rinsed it with water, and incubated it in physiological saline solution before I took a photo next to a colour checker. Once photographs were completed, I preserved fish in formalin in a 50-ml falcon tube where they incubated for two weeks to dissolve iridophore pigments and facilitate melanophore counts (Greenwood et al. 2011, 2012). I then performed a lateral dissection using scissors and a scalpel and removed the internal organs to eliminate potential colour bias in the photograph. The left lateral section was placed on the dissecting scope stage with a ruler. I photographed the whole section and the area adjacent to the second dorsal spine at 4× magnification using an Olympus dissecting microscope. I analyzed melanophore densities in the ventral, lateral, and dorsal areas directly below and adjacent to the second dorsal spine. I chose the second spine as a landmark due to its central location on the fish. I used trans-illumination to highlight the melanophores through the skin, and all photographs were saved for future melanophore counts and melanophore density analysis. Sample size per treatment (breeding or non-breeding condition included) are outlined in Figure 2.3.1.

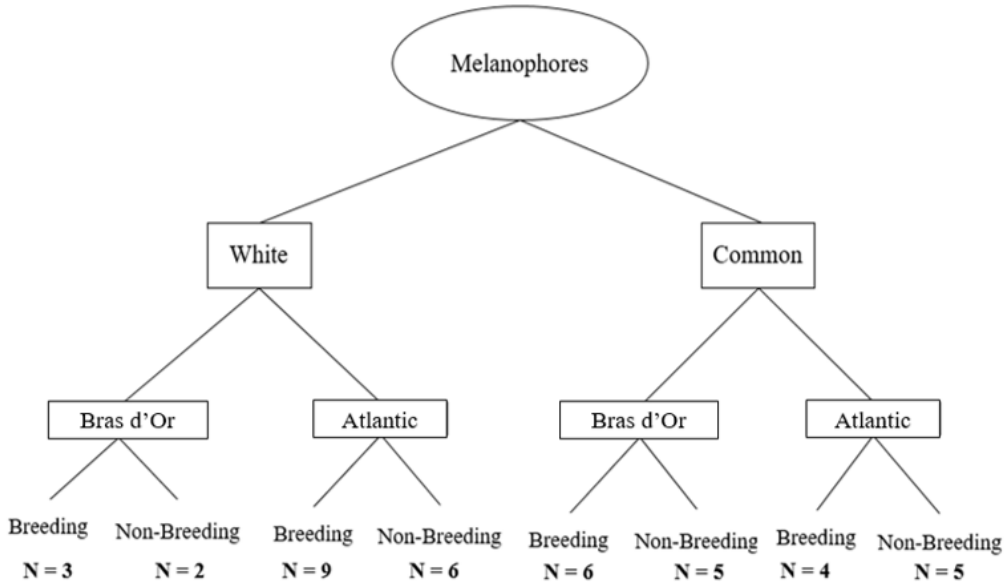

Figure 2.3.1 Sample sizes of ecotype, location and breeding condition used for melanophore counts and coverage analysis.

2.3.3 Counting melanophores

I uploaded photographs of each fish into ImageJ 1.51s. To increase the contrast of dark pigment cells against the light skin, I used the automatic contrast function in ImageJ. I chose this method to avoid user bias with manual contrast settings. I set the scale using the 'Set Scale' function in ImageJ. Photographs were converted to 8-bit greyscale prior to counting.

To quantify melanophore number in lateral and ventral areas, I first divided each fish into lateral and ventral regions (Fig. 2.3.2). I determined the midline of each fish by measuring its depth and dividing it in half. I denoted the midline using the draw function in ImageJ and saved the line in the ROI manager. Using the shape tool, I created two 3 × 1.5 mm rectangles and added these to the ROI manager prior to placement in the ventral and lateral regions. The bottom left-hand corner of the first rectangle was placed on the insertion point of the pelvic spine and was used as the 'ventral' region of interest to be measured. The second rectangle was placed directly above the ventral ROI and just below the midline. This was the 'lateral' region to be measured. I then used the find maxima tool to highlight and count the darkest pigment clusters, or maxima, which represented melanophores in each region.

I determined that ventral and lateral melanophores, which are less dense than dorsal melanophores (personal observation, Fig. 2.3.2), could be individually counted. I tested two functions in ImageJ to count melanophores; the 'Cell Counting' function to individually count the number of cells in each region of interest (ROI), and the automated 'Find Maxima' tool. I counted cells on the left lateral side of each sample. After I tested preliminary protocols using different automated tools, I found that the 'Find Maxima'

tool, which highlights the darkest points in a region of interest, the most accurate at differentiating cells in comparison with the manual cell counting function. I tested this by using both manual cell counting and the automatic maxima method on ten different samples to observe the accuracy with which both tools counted melanophores. Because both methods were almost equal in the ability to pick out separate cells, and the 'find maxima' tool decreased counting time and user bias, I proceeded with this method for all samples. I visually inspected each ROI after maxima were highlighted to account for overlapping melanophores and denoted each individual cell with the 'cell counter plugin' in ImageJ (Roberts et al. 2017).

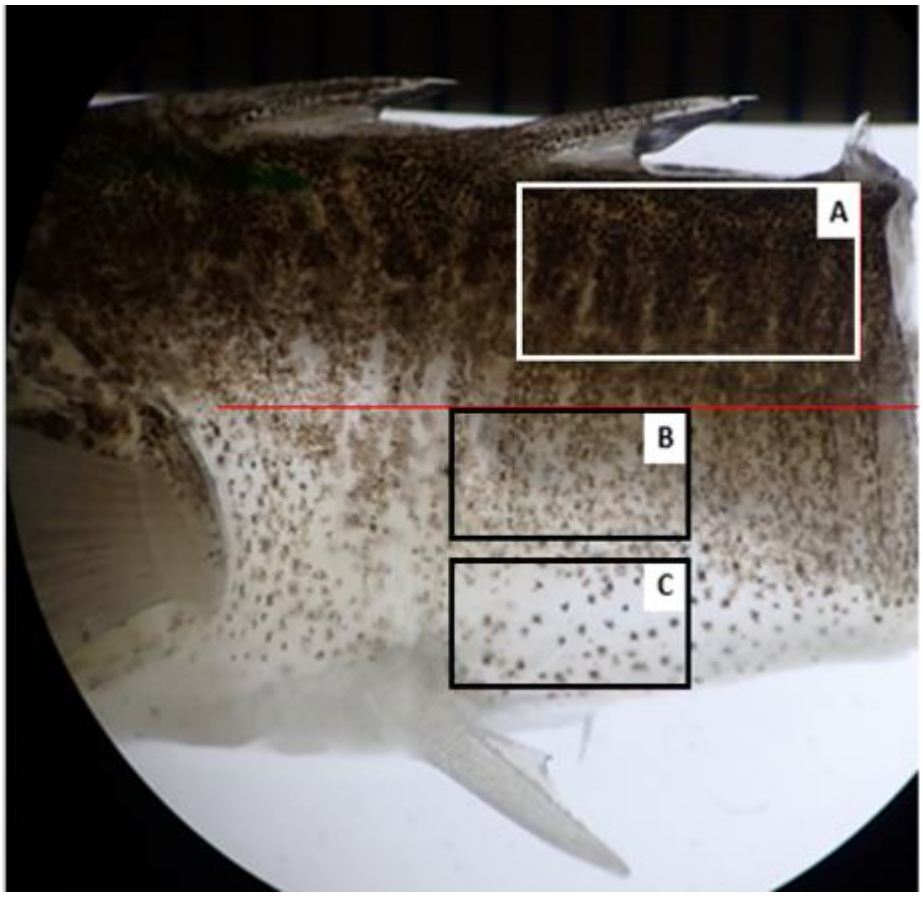

Figure 2.3.2 Lateral left flank of Threespine Stickleback with internal organs removed at 4x magnification under a dissection microscope and trans-illumination. Dorsal (A), lateral (B), and ventral (C) regions of interest are highlighted. Horizontal line indicates the midline.

2.3.4 Percent Melanophore Cover

To measure overall skin darkness of the dorsal, lateral, and ventral regions along the fish's flank, I followed the methods of Rodgers et al. (2010) and Kelley et al. (2016) with some modifications. This method integrates chromatophore number, the amount of pigment deposited in the cells, as well as the dispersion of the cell itself. I chose to measure three areas as opposed to the whole fish to isolate these regions and better understand the deposition of pigment cells in each area and their effect on nuptial colouration of each ecotype. I was particularly interested in isolating the dorsal region because Threespine Stickleback have some capacity to rapidly darken or lighten this area (Östlund-Nilsson et al. 2007; Clarke and Schluter 2011), and this area was the brightest and most iridescent region of the white ecotype when in full breeding colour.

As above, I uploaded photographs into ImageJ, converted each to 8-bit greyscale, and denoted the midline using the draw function. I created rectangles as in section 2.3.3 to measure percent cover in dorsal, lateral and ventral regions of each fish. The dorsal rectangular section was 2.5 mm tall and 4 mm wide, while the ventral and lateral rectangular sections were smaller, both 1.5 mm tall and 3 mm wide (as in the cell counting) to account for the smaller size of these regions in smaller fish. Ventral and lateral rectangular ROIs were oriented using the same landmarks as in the cell counting procedure, and the insertion of the second dorsal spine was used as a landmark for the dorsal ROI; the top left corner of the rectangle was positioned at the insertion point of the dorsal spine, and the rectangle itself was above the lateral ROI and midline.

I followed the binarization and thresholding methods of Rodgers et al. (2010) and Kelley et al. (2016) to determine percent cover. It was especially important to determine

percent cover in the dorsal region because melanophores in this region of some individuals were too dense to count. Thresholding is a useful tool that separates objects from the background by changing colour values to greyscale (Sezgin and Sankur 2004). In this case, the dark pigment cells in the foreground were contrasted against the light background of the skin. Binarization then converts the pixels of a photograph to either black or white, thereby creating a 'binarized' image of black and white (Sezgin and Sankur 2004). Following conversion to its binarized form, the percent cover of dark pixels in the selected ROI was measured using the 'threshold analysis' tool in ImageJ. Binarization can be done manually or with an automatic threshold method. To avoid user bias, I chose an automatic threshold method. After testing both general and local adaptive automatic thresholding methods, I found local adaptive thresholding was the most appropriate method for my samples. Local thresholding methods exploit algorithms that calculate local statistics, such as range, variance and surface-fitting parameters for each pixel in the region of interest (Sezgin and Sankur 2004). This method most accurately recognized and separated the foreground (dark pigment cells) from the background (light skin) in my sample photographs. Finally, after careful comparison of each local automatic method available in ImageJ, I chose the Bernsen automatic local thresholding method (Appendix A) as the most appropriate for this study. The Bernsen method sets the threshold at a midrange value, calculated from the mean of the minimum and maximum gray values in the local area (Sezgin and Sankur 2004).

2.3.5 Statistical analysis of melanophore counts and percent cover

Analyses were carried out in R v.3.6. Generalized linear models (GLM) with Poisson error distributions to test the effects of ecotype (white, common), location (Bras

d'Or or Atlantic), and breeding condition (breeding or non-breeding) on the number of melanophores present in ventral and lateral areas on the left side of the fish. To test the fixed effects of ecotype, location, and breeding condition on the percent coverage of melanophores in the dorsal, lateral, and ventral regions, GLMs with binomial error distributions were used. As in Section 2.2.2, I used AICc values to determine the model that best fit the data for both melanophore counts and coverage. I used Tukey HSD post-hoc tests to assess differences between groups with the 'multcomp' package in R.

2.4 Colour correlated with breeding behaviour in the white ecotype

2.4.1 Nuptial colouration data collection

Because white males have been documented to rapidly change colour during the breeding season (Blouw and Hagen 1990; Jamieson et al. 1992a), I investigated behavioural correlates of the intensity of white colouration to determine if a specific breeding behaviour is related to colour.

I determined the intensity of brightness in two ways. First, immediately prior to behavioural observations, myself and another observer independently gave the fish a 'brightness score' on a scale of one to five, whereby five was the brightest white. These scores were revealed following the 5-minute behavioural observation period to avoid bias, and the observers agreed upon the ranking prior to recording it. A score of 1 was recorded when the individual was a dull grey colour and barely noticeable. A score of 2 was assigned when the individual was slightly more noticeable than an individual scored as 1. Individuals scored as 3 were intermediates; the fish was white, not grey, but the brightness was dim in comparison to those scored as 4 and 5. A score of 4 was recorded when the fish was white and conspicuous on the nest, but not as bright as a fish scored as

5, which was visible up to a metre from shore. Second, fish were photographed approximately every minute during an individual's five-minute behavioural observation. A total of 50 individual fish were photographed during observations from Canal Lake and Bras d'Or, for a total of 595 photographs for brightness analysis. Photographs were taken from the bank of the lake or rock overlooking the nest site with an Olympus TG4 waterproof camera. I constructed an underwater colour checker from a series of laminated Canadian Tire paint samples, which included shades of red, blue, and grey, for sequential white balance calibration following the methods of Bergman and Jacinta (2008). The colour checker was photographed *in situ* at the approximate depth and location of an individual's nest site prior to behavioural observations to account for water turbidity and luminescence. To avoid alterations to colour that can occur when compressing JPEG files, I saved all photographs in the RAW file format to ensure colour and brightness were accurately depicted.

I exported RAW files as 16-bit TIFF files (Rodgers et al. 2010; Kelley et al. 2016) in Olympus studio, a free photography editing software accessed from the Olympus website, and opened these images in ImageJ (NIH). I measured colour (RGB colour space) by synthesizing the values of Red, Green, and Blue colour channels from each photograph using the RGB measure function. Each set of photographs corresponding to an observation period included a colour checker photograph for calibration purposes. I measured and recorded the RGB values of the light grey square on the colour checker as the white balance reference. Next, I used the free hand draw function in ImageJ to outline the fish in each photograph and recorded the RGB values of each fish. I also recorded the RGB values of the surrounding water in each photograph as a calibration method to

account for rapid changes in cloud cover and light during the 5-minute period. Average RGB values from the colour checker were divided by 255 (the 'true white' value) to calibrate the white value calculated in the observation period. The average RGB value of the fish was then divided by the ratio value corresponding to its colour checker. Lastly, the average RGB value of the surrounding water was subtracted from the calibrated individual fish value to quantify the intensity of brightness in contrast with the surrounding water. This was done to account for brightness reflected in the surrounding water throughout the observation period. In sum, the average RGB value of the surrounding water was subtracted from the average RGB value of the fish, after standardization with a colour checker, to provide a 'contrast value'.

The associations between colouration and different breeding behaviour were based on white males only, and were based on the means of behavioural frequencies, visual colour scores, and contrast value per fish calculated from field photographs over the five-minute observation period described in section 2.2.1. The correlation between visual colour scores and photographic contrast values can be found in Appendix B. The relationship between colour and behaviour was also analyzed as a proportion of total behaviour to account for the effect of brightness on behaviour in individuals that were both active and non-active in certain types of breeding behaviour. These relationships demonstrated a similar pattern to the results shown below, and can be found in Appendix C.

2.4.2 Statistical analysis brightness score and RGB values

As described in section 2.2.2, I used GLMMs with Poisson error distributions to test the effect of brightness on courtship, aggression, and nest-building in white stickleback. I analysed behaviour based on both 'brightness score' (one to five) and the contrast values (ranging from 11.37 to 117), each of which were included in the models as continuous fixed effects. Because observations and colouration were assessed in both Canal Lake and Baddeck, I included location as a fixed effect to test for potential differences due to genetic divergence. As in section 2.2.2, individual identification was included as a random effect and AICc values were used for model selection.

2.5 Animal care protocols

All field and laboratory work was approved by the Saint Mary's University Animal Care Committee (SMU ACC Protocols 17-16, 17-18 & 16-16) and followed the standards for fish care described by the Canadian Council on Animal Care.

3. RESULTS

3.1 Behavioural patterns and analysis

Nine focal behaviours were compiled to quantify courtship (zig-zag dance, dorsal pricking, circling, leads to nest), aggression (chasing and biting conspecifics), and nest-building (material retrieval and nest maintenance). I compared behavioural frequencies (courtship, aggression, and nestbuilding) between white and common males in both mainland and Bras d'Or sites. The model that best explained courtship frequency included the effects of ecotype, location and their interaction (Table 3.1.1, Fig. 3.1.1 A). When common males were removed from the analysis, location did not influence courtship frequency in the white ecotype, and when location was removed, courtship was best explained by ecotype, with white males performing higher frequencies of courtship. There was no difference between the mainland and Bras d'Or ecotypes for nest-building or aggression; however, there was a weak effect of ecotype on nestbuilding frequency and weak effects of location and ecotype on aggression frequency (Table 3.1.1, Fig. 3.1.1 B, C). Within the aggression behavioural category, Bras d'Or and Canal Lake white males displayed similar aggression rates towards conspecifics, yet when potential egg predators, *Fundulus* sp. were included in the analysis, aggression frequency increased in Bras d'Or white males (Appendix D).

I also compared specific focal behaviours including leads to the nest, zig-zag frequency, material retrieval, and nest-tending between white and common males in both mainland and Bras d'Or sites. In the model that explained the number of leads to the nest (males successfully leading females to the nest), ecotype held the most weight with white males leading females more than common males (Table 3.1.2, Fig. 3.1.2 A). However,

the model also retained the interactive effect of ecotype and location. Although white males only perform zig-zag dances during courtship (Blouw and Hagen 1990; Jamieson et al. 1992a), common males perform a combination of zig-zag dances, dorsal pricking, and circling to entice females to their nests (Wootton 1976). In the model, ecotype best explained the frequency of zig-zags, with white males performing more zig-zag dances than common males, as expected (Table 3.1.2, Fig. 3.1.2 B). The model also retained a location effect indicating a slight increase in zig-zag frequency in Baddeck populations. The model that best explained material retrieval demonstrated considerable uncertainty; all models were within 2 AICc values of one another, but the effects of ecotype and location held the most model weight with white males showing a higher frequency of material retrieval than common males (Table 3.1.2, Fig. 3.1.2 C). In terms of nest-tending, only the intercept was retained although there were weak effects of ecotype and location (Table 3.1.2, Fig. 3.1.2 D).

Table 3.1.1 Generalized linear mixed effects models with Poisson distribution indicating the effect of ecotype, location, and their interaction on frequency of behaviours (courtship, nestbuilding, aggression). Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), the difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Location can be either Mainland or Baddeck, and ecotype is either common or white. Models with the lowest AICc scores by 2 or more are shown in bold.

Behaviour	Models	*df*	AICc	Δ AICc	Weight
Courtship	**Ecotype + Location + Ecotype × Location**	5	943.5	0.00	0.521
	Ecotype	3	944.7	1.17	0.291
	Ecotype + Location	4	945.6	2.04	0.188
	Intercept	2	962.2	18.63	0.000
	Location	3	962.5	18.96	0.000
Nest-building	**Intercept**	2	1222.9	0.00	0.374
	Ecotype	3	1223.3	0.30	0.321
	Location	3	1225.0	2.01	0.137
	Ecotype + Location	4	1225.2	2.23	0.123
	Ecotype + Location + Ecotype × Location	5	1227.2	4.22	0.045
Aggression	**Intercept**	2	604.4	0.00	0.417
	Location	3	605.3	0.97	0.256
	Ecotype	3	606.1	1.78	0.171
	Ecotype + Location	4	606.9	2.57	0.115
	Ecotype + Location + Ecotype × Location	5	609.0	4.67	0.040

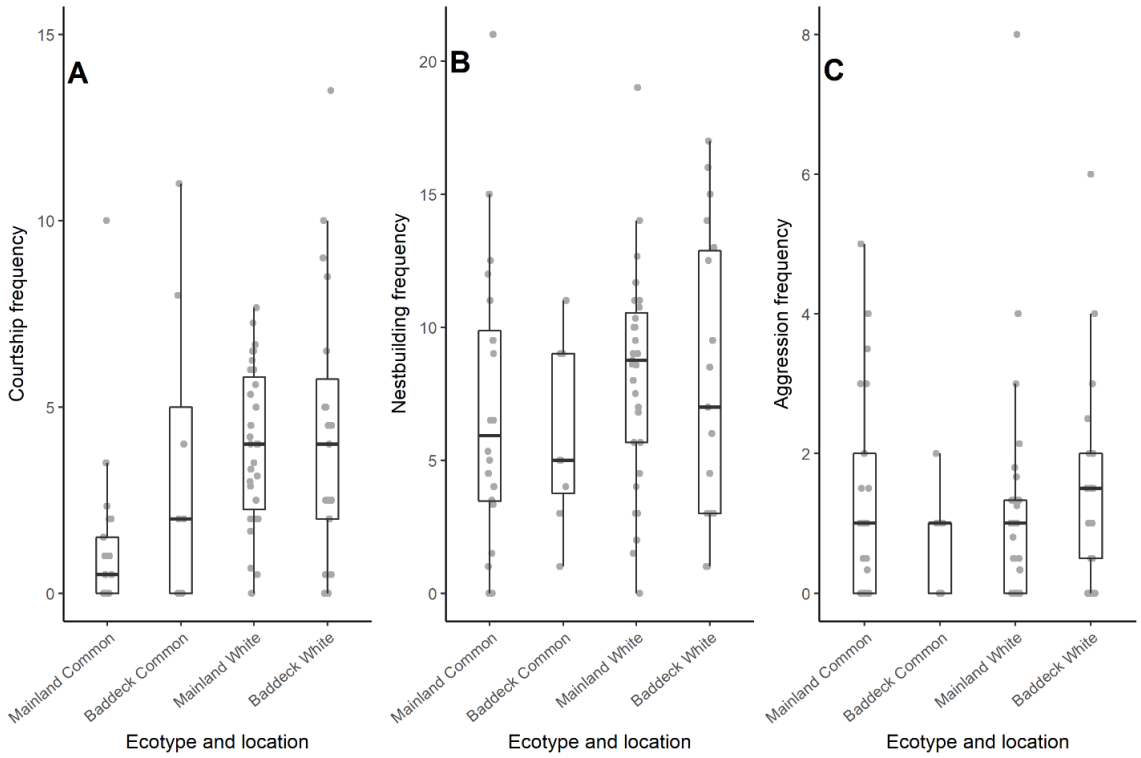

Figure 3.1.1 Frequency of (A) courtship, (B) nestbuilding, and (C) aggressive behaviours over 5-minute observation periods in both white (n = 31) and common ecotypes (n = 21) from mainland (Canal Lake and Rainbow Haven), and white (n = 19) and common (n = 19) and common (n = 8) from the Bras d'Or population (Baddeck). Black bars represent the median and grey dots represent means for all observations periods for an individual fish. Mean individual recordings and the effect of individual is accounted for in the statistical analysis as a random factor.

Table 3.1.2 Generalized linear mixed effects models with Poisson distribution indicating the effect of ecotype, location, and their interaction on frequency of behaviours (leads to the nest, zig-zags, material retrieval, and nest-tending). Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), the difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Location can be either Mainland or Baddeck, and ecotype is either common or white. Models with the lowest AICc scores by 2 or more are shown in bold.

Focal Behaviour	Models	*df*	AICc	Δ AICc	Weight
Leads to the nest	**Ecotype**	3	183.4	0.00	0.494
	Ecotype + Location + Ecotype × Location	5	184.5	1.06	0.290
	Ecotype + Location	4	185.4	1.94	0.187
	Intercept	2	189.8	6.42	0.020
	Location	3	191.6	8.22	0.008
Zig-Zags	**Ecotype**	3	857.5	0.00	0.624
	Ecotype + Location	4	859.3	1.75	0.260
	Ecotype + Location + Ecotype × Location	5	860.9	3.35	0.117
	Intercept	2	880.8	23.33	0.000
	Location	3	883.0	24.46	0.000
Material Retrieval	**Ecotype + Location**	4	885.3	0.00	0.351
	Location	3	886.2	0.94	0.219
	Ecotype	3	886.9	1.67	0.153
	Intercept	2	887.1	1.82	0.141
	Ecotype + Location + Ecotype × Location	5	887.2	1.90	0.136

Table 3.1.2 (continued) Generalized linear mixed effects models with Poisson distribution indicating the effect of ecotype, location, and their interaction on frequency of behaviours (leads to the nest, zig-zags, material retrieval, and nest-tending). Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), the difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Location can be either Mainland or Baddeck, and ecotype is either common or white. Models with the lowest AICc scores by 2 or more are shown in bold.

Focal Behaviour	Models	*df*	AICc	Δ AICc	Weight
Nest-tending	**Intercept**		1030.4	0.00	0.427
	Ecotype		1031.5	1.15	0.241
	Location		103.20	1.58	0.194
	Ecotype + Location		1033.3	2.89	0.101
	Ecotype + Location + Ecotype × Location		1035.3	4.87	0.038

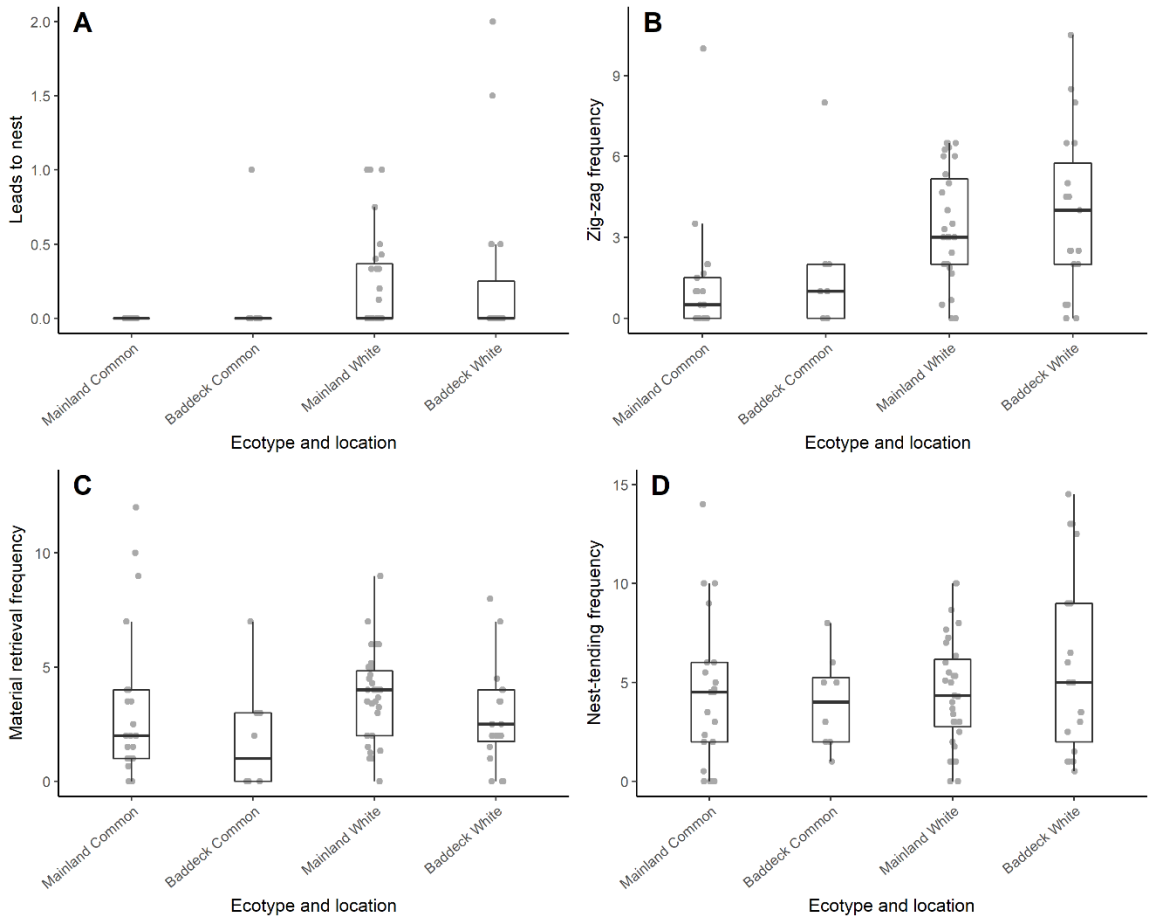

Figure 3.1.2 Frequency of (A) leads to nest, (B) Zig-zag frequency, (C) material retrieval, and (D) nest-tending behaviours over 5-minute observation periods in both white (n = 31) and common ecotypes (n = 21) from mainland (Canal Lake and Rainbow Haven), and white (n = 19) and common (n = 8) from the Bras d'Or population (Baddeck). Black bars represent the median and grey dots represent means for all observations periods for an individual fish. Mean individual recordings and the effect of individual is accounted for in the statistical analysis as a random factor.

3.2 Chromatophore counts and densities

In the lateral region, two-way interactive effects that included breeding condition, ecotype, and location were retained in the best model (Table 3.2.1, Fig. 3.2.1, A, B). The model that best explained melanophore density in the ventral region included the interactive effect of breeding condition, ecotype, and location (Table 3.2.1, Fig. 3.2.1 C, D). The model that best fit dorsal melanophore coverage included the two-way interactive effects including breeding condition, ecotype, and location (Table 3.2.2, Fig. 3.2.2 A, B). In the lateral region, the model that best fit the data retained the interaction of breeding condition and location only (Table 3.2.2, Fig. 3.2.1 C, D). Ventral melanophore coverage was best explained by the three-way interaction effect of breeding condition, ecotype, and location (Table 3.2.2, Fig. 3.2.1 E, F).

Both breeding and non-breeding Bras d'Or white males had significantly fewer melanophores than common males in lateral and ventral regions (Fig. 3.2.1). In Atlantic samples, breeding white males had significantly fewer melanophores than non-breeding white males in the ventral region only, while breeding white males had significantly fewer melanophores than all common males in the lateral region. Breeding white males from both the Atlantic populations and Bras d'Or Lake had significantly less dorsal melanophore coverage than non-breeding white males, as well as their common counterparts in both breeding conditions, from both locations (Fig. 3.2.1 A, B). Interestingly, breeding common males from Bras d'Or had more melanophore coverage than non-breeding conspecifics in both dorsal and lateral regions (Fig. 3.2.2 A, C), while Atlantic breeding commons had more melanophore coverage than non-breeding commons in the lateral region (Fig. 3.2.2 D).

Table 3.2.1. Generalized linear models with Poisson distribution indicating the effect of colour, location, and ecotype on the number of melanophores in lateral and ventral regions. Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Colour is either breeding or non-breeding condition, location is either Atlantic or Bras d'Or, and ecotype is white or common. Models with lowest AICc scores are shown in bold.

Melanophore region	Models	*df*	AICc	Δ AICc	Weight
Lateral count	**Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype**	7	1599.7	0.00	0.509
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype + Location × Ecotype × Colour	8	1599.7	0.07	0.491
	Colour + Location + Ecotype + Colour × Ecotype + Location × Ecotype	6	1620.9	21.19	0.000
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype	6	1625.3	25.59	0.000
	Colour + Location + Ecotype + Colour × Location + Location × Ecotype	6	1630.3	30.66	0.000
Ventral count	**Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype + Location × Ecotype × Colour**	8	712.1	0.000	0.525
	Colour + Location + Ecotype + Colour x Location + Colour ×Ecotype + Location × Ecotype	7	712.3	0.200	0.475
	Colour + Location + Ecotype + Colour × Ecotype + Location × Ecotype	6	730.3	18.180	0.000
	Colour + Location + Ecotype + Colour × Location + Location × Ecotype	6	751.4	39.280	0.000
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype	6	804.3	92.200	0.000

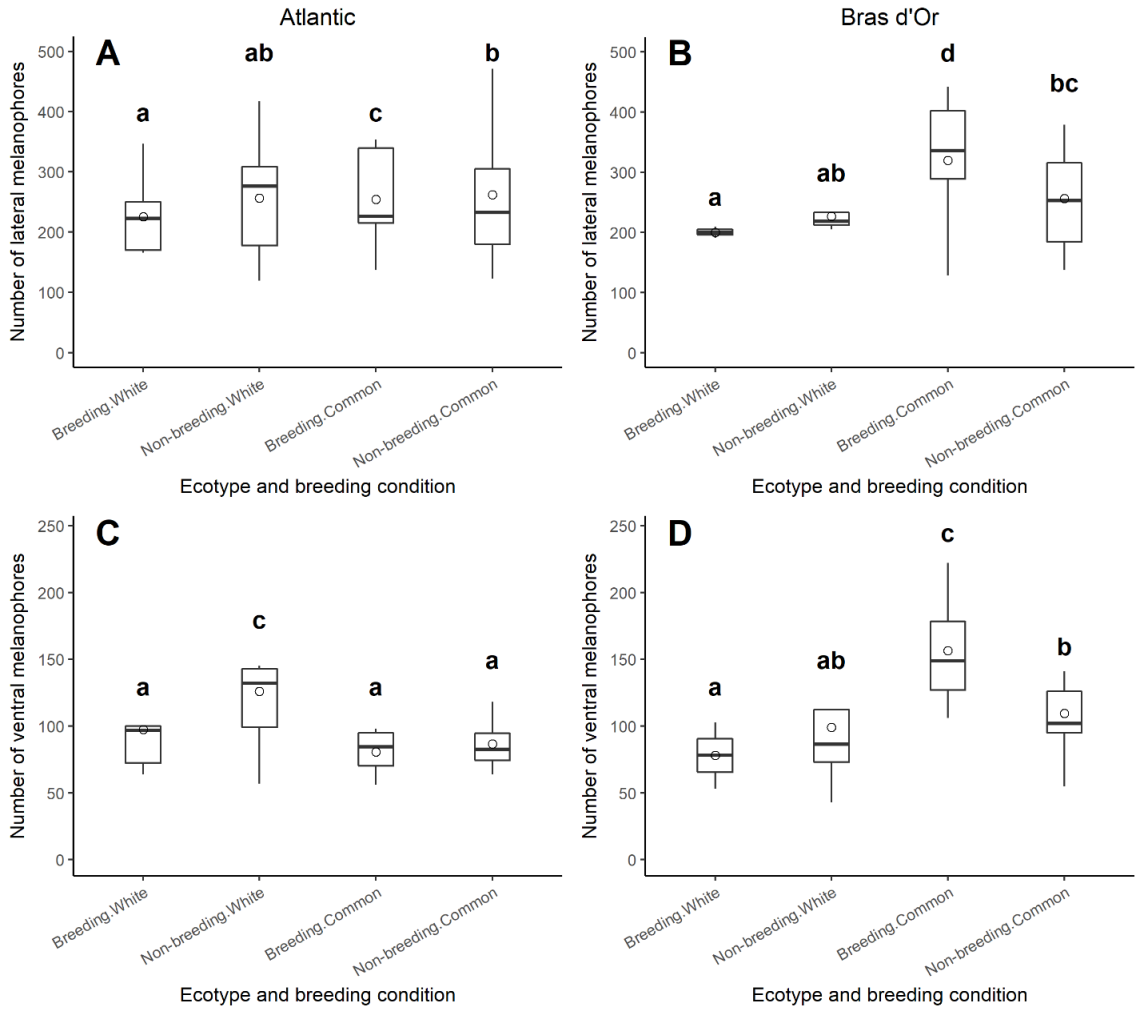

Figure 3.2.1 Ecotype and breeding condition effects on number of lateral (A, B) and ventral (C, D) melanophores per area in (A, C) Atlantic white (n = 15) and common (n = 9) males and (B, D) Bras d'Or white (n = 5) and common (n = 11) males. Bars represent the median and circles represent the mean. Box edges represent the 25th and 75th percentile, and lines represent outliers. Significant differences between groups are indicated by different letters ($p < 0.05$).

Table 3.2.2 Generalized linear models with Binomial distribution indicating the effect of colour, location, and ecotype on melanophore coverage in dorsal, lateral, and ventral regions. Model degrees of freedom (df), Akaike Information Criterion corrected sample size (AICc), difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Colour is either breeding or non-breeding condition, location is either Atlantic or Bras d'Or, and ecotype is white or common. Models with lowest AICc scores are shown in bold.

Melanophore region	Models	*df*	AICc	Δ AICc	Weight
Dorsal coverage	**Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype**	7	652.5	0.00	0.674
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype + Colour × Ecotype × Location	8	654.0	1.48	0.321
	Colour + Location + Ecotype + Colour × Ecotype + Location × Ecotype	6	662.3	9.82	0.005
	Colour + Ecotype + Location + Colour × Location + Location × Ecotype	6	670.0	17.53	0.000
	Colour + Ecotype + Location + Location × Ecotype	5	671.9	19.43	0.000
Lateral coverage	**Colour + Ecotype + Colour × Ecotype**	4	373.6	0.00	0.503
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype	6	375.8	2.22	0.166
	Colour + Location + Ecotype + Colour × Ecotype	5	375.8	2.23	0.165
	Colour + Location + Ecotype + Colour × Ecotype + Location × Ecotype	6	377.5	3.89	0.072
	Colour + Location + Ecotype + Colour × Ecotype + Location × Ecotype + Colour × Location	7	377.7	4.08	0.066

Table 3.2.2 (continued) Generalized linear models with Binomial distribution indicating the effect of colour, location, and ecotype on melanophore coverage in dorsal, lateral, and ventral regions. Model degrees of freedom (df), Akaike Information Criterion corrected sample size (AICc), difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Colour is either breeding or non-breeding condition, location is either Atlantic or Bras d'Or, and ecotype is white or common. Models with lowest AICc scores are shown in bold.

Melanophore region	Models	*df*	AICc	Δ AICc	Weight
Ventral coverage	**Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype + Colour × Ecotype × Location**	8	308.1	0.00	0.723
	Colour + Location + Ecotype + Colour × Ecotype + Location × Ecotype	6	312.2	4.09	0.093
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype + Location × Ecotype	7	312.3	4.18	0.089
	Colour + Location + Ecotype + Colour × Location + Colour × Ecotype	6	315.1	7.00	0.022
	Colour + Location + Ecotype + Colour × Ecotype	5	315.3	7.20	0.020

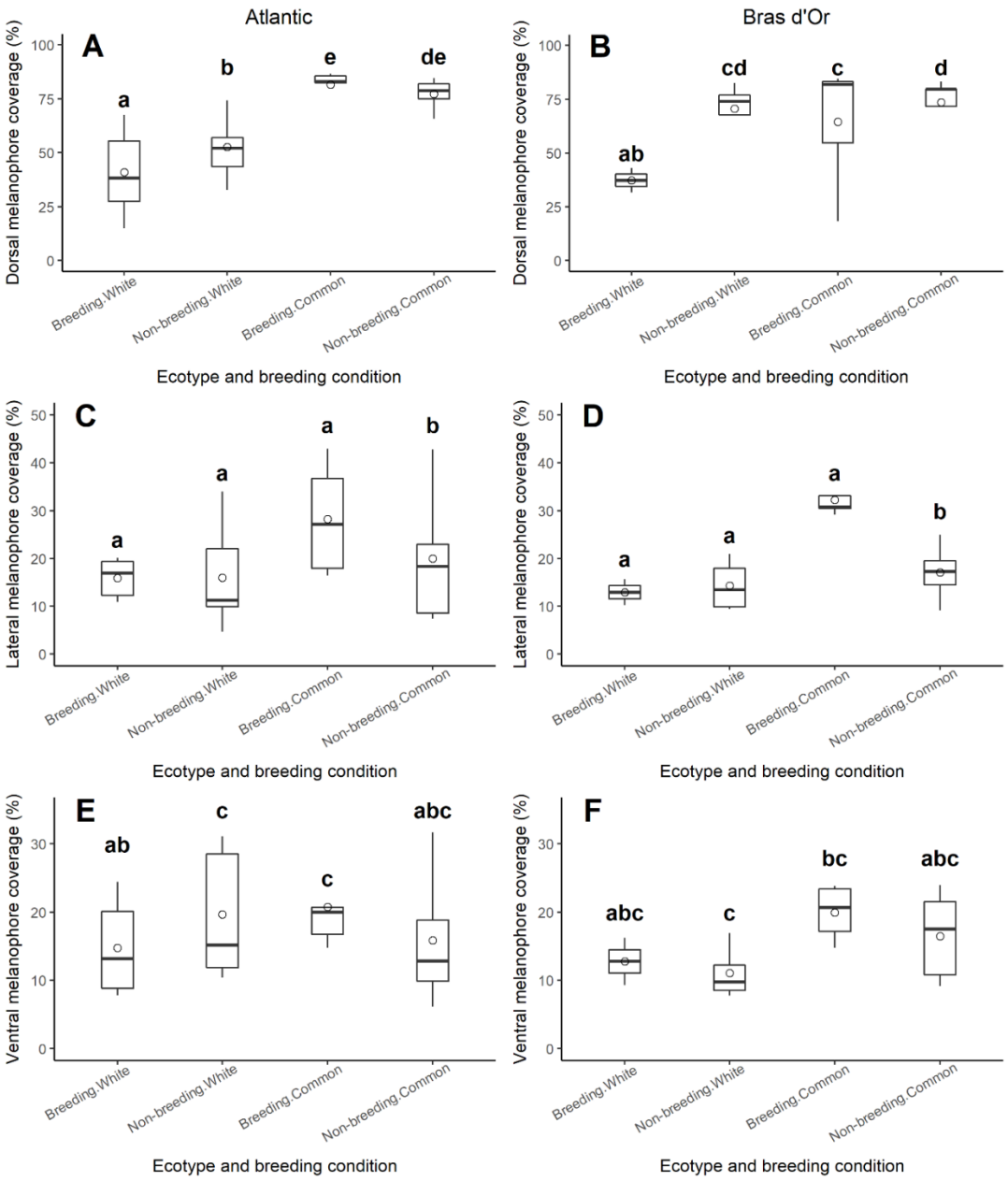

Figure 3.2.2 Ecotype and breeding condition effects on dorsal (A, B), lateral (C, D) and ventral (E, F) melanophore coverage in (A, C, E) Atlantic white (n = 15) and common (n = 9) males and (B, D, F) Bras d'Or white (n = 5) and common (n = 11) males. Bars represent the median and circles represent the mean. Box edges represent the 25th and 75th percentile, and lines represent outliers. Significant differences between groups are indicated by different letters ($p < 0.05$).

3.3 Correlation of colour and behaviour

The correlation between the intensity of white colouration and different breeding behaviour were based on white males only, and the means of behavioural frequencies, visual colour scores, and contrast value per fish calculated from field photographs over the five-minute observation period described in section 2.2.1. The model that best explained the frequency of courtship behaviour included only the effect of score for the intensity of brightness (Table 3.3.1, Fig. 3.3.1 B, C). The model that employed field photographic contrast values included the effects of brightness, location and their interaction (Table 3.3.1, Fig. 3.3.1. E, F). Both scores and values indicated that courtship frequencies increased with the intensity of brightness, although this relationship differed between the mainland and Cape Breton populations for the model using field photographic contrast values.

Similarly, when score was used to denote intensity of brightness in the nestbuilding models, score was retained as the main effect (Table 3.3.2, Fig. 3.3.2 A, B). However, when brightness was evaluated from the field photographs, the model with location as the main effect best explained the data (Table 3.3.2, Fig. 3.3.2 C, D). This discrepancy is best explained by differences between fish given a brightness score of '2'. When these animals were removed from the data set, the best model to explain the data retained only the intercept.

When visual score was used to determine intensity of brightness in the aggression models, only the intercept was retained but there is considerable uncertainty identified in this analysis (Table 3.3.3, Fig. 3.3.3 A, B). Similarly, when aggression was correlated

with the field photograph values, contrast value and location did not have a strong effect on the model (Table 3.3.3, Fig. 3.3.3 C, D).

Table 3.3.1 Generalized linear mixed effects models with Poisson distribution indicating the effect of location, score, and contrast value on the frequency of courtship behaviours. Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), difference between models with lowest AICc values and all other models (ΔAICc), and model weight are shown here. Location is either Canal Lake or Baddeck, score is the intensity of brightness as denoted in the field, and contrast value is the intensity of brightness synthesized from photographs. Models with the lowest AICc scores are shown in bold.

Colour variable	Models	*df*	AICc	Δ AICc	Weight
Score	**Score**	3	687.7	0.00	0.658
	Location + Score	4	689.6	1.89	0.255
	Location + Score + Location × Score	5	691.7	4.05	0.087
	Intercept	2	754.0	66.34	0.000
	Location	3	756.1	68.43	0.000
Contrast	**Location + Contrast + Location × Contrast**	5	642.3	0.00	0.496
	Contrast	3	643.7	1.38	0.249
	Location + Contrast	4	645.2	2.94	0.114
	Intercept	2	645.5	3.18	0.101
	Location	3	647.3	5.02	0.040

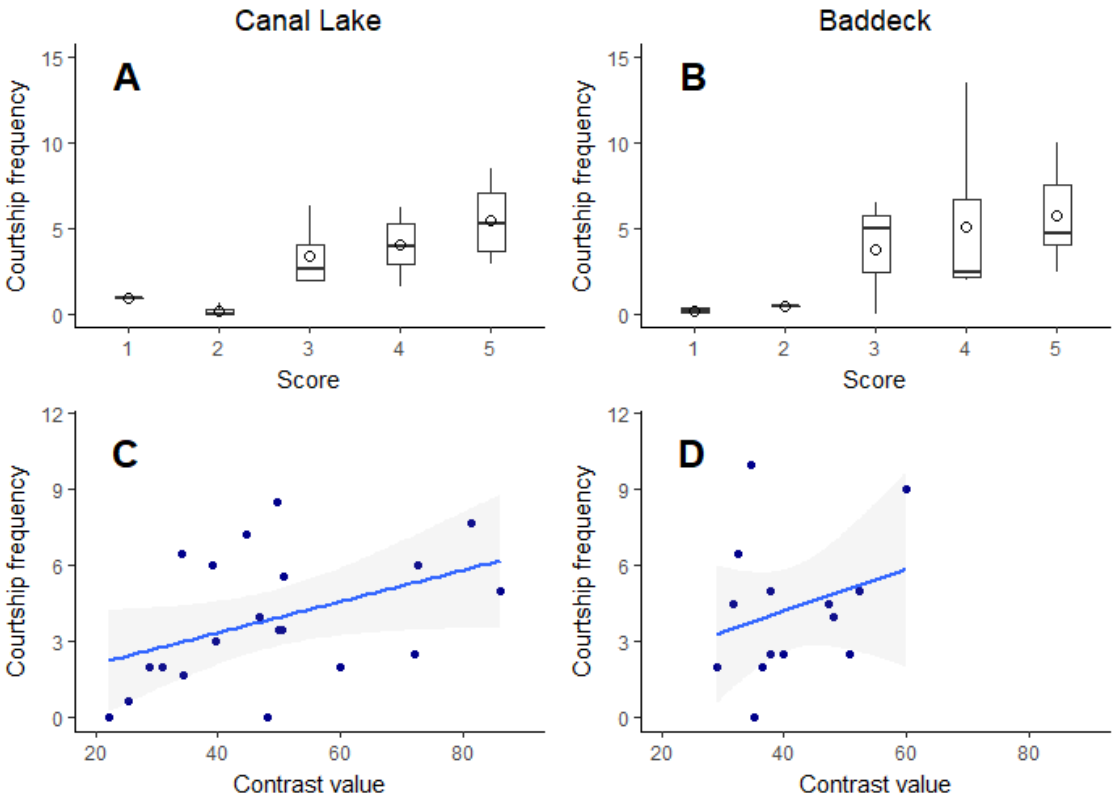

Figure 3.3.1 Intensity of brightness evaluated visually (A, B: scores where 5 is the brightest white.) or from field photographs (C, D: contrast values) correlated with the frequency of courtship behaviour. Courtship behaviour is depicted in Canal Lake Lake (Figs. A, C) scores: 1 (n = 1), 2 (n = 3), 3 (n = 4), 4 (n = 13), 5 (n = 10), and Baddeck in the Bras d'Or Lake (Figs. B, D) scores: 1 (n = 2), 2 (n = 1), 3 (n = 3), 4 (n = 7), 5 (n = 7). Bars represent the median and circles represent the mean. Shaded areas represent the 95% confidence interval of the line of best fit.

Table 3.3.2 Generalized linear mixed effects models with Poisson distribution indicating the effect of location, score, and contrast value on the frequency of nestbuilding behaviours. Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), difference between models with lowest AICc values and all other models (ΔAICc), and model weight are shown here. Location is either Canal Lake or Baddeck, score is the intensity of brightness as denoted in the field, and contrast value is the intensity of brightness synthesized from photographs. Models with the lowest AICc scores are shown in bold.

Variable	Models	*df*	AICc	Δ AICc	Weight
Score	**Score**	3	906.2	0.00	0.564
	Location + Score	4	908.3	2.13	0.194
	Location + Score + Location × Score	5	908.5	2.36	0.173
	Intercept	2	911.0	4.84	0.050
	Location	3	913.1	6.90	0.018
Contrast	**Location**	3	626.0	0.00	0.631
	Location + Contrast	4	628.1	2.07	0.224
	Location + Contrast + Location × Contrast	5	629.0	3.03	0.139
	Intercept	2	636.0	10.02	0.004
	Contrast	3	638.1	12.12	0.001

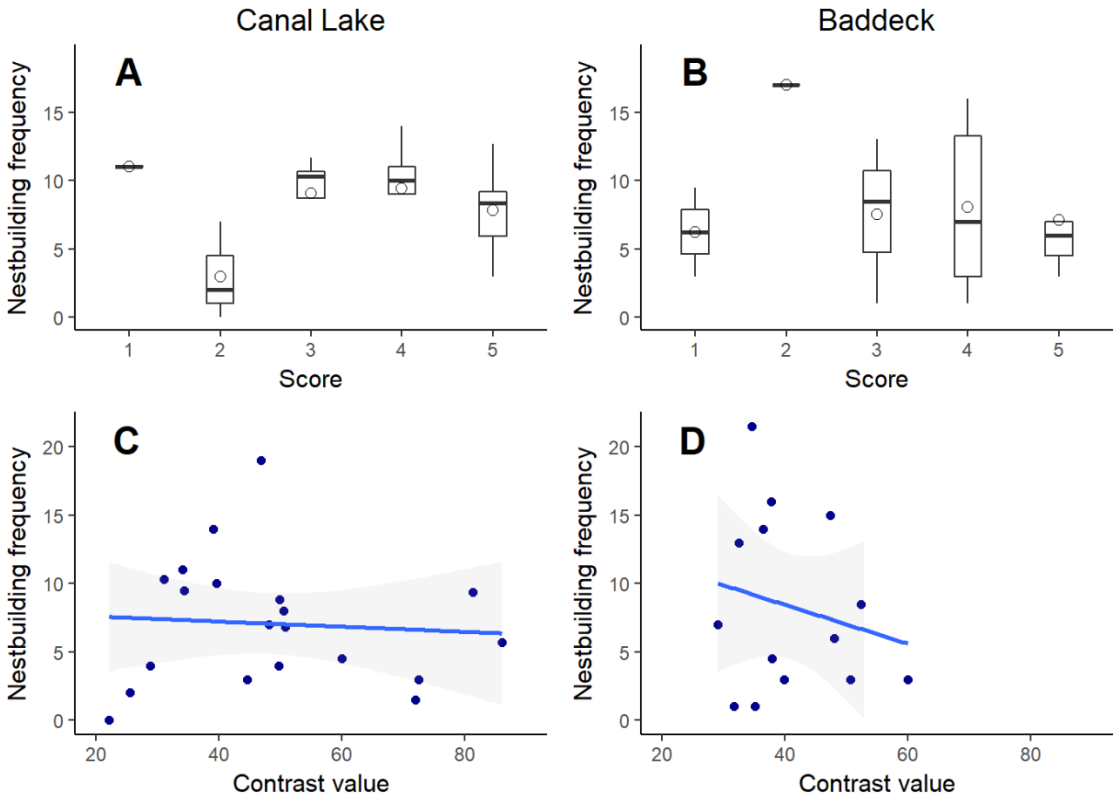

Figure 3.3.2 Intensity of brightness evaluated visually (A, B: scores, where 5 is the brightest white) or from field photographs (C, D: contrast values) correlated with the frequency of nestbuilding behaviour. Nestbuilding behaviour is depicted in Canal Lake (Figs. A, C) scores: 1 (n = 1), 2 (n = 3), 3 (n = 4), 4 (n = 13), 5 (n = 10), and Baddeck in the Bras d'Or Lake (Figs. B, D) scores: 1 (n = 2), 2 (n = 1), 3 (n = 3), 4 (n = 7), 5 (n = 7). Bars represent the median and circles represent the mean. Shaded areas represent the 95% confidence interval of the line of best fit.

Table 3.3.3 Generalized linear mixed effects models with Poisson distribution indicating the effect of location, score, and contrast value on the frequency of aggression. Model degrees of freedom (df), Akaike Information Criterion corrected for sample size (AICc), difference between models with lowest AICc values and all other models (ΔAICc), and model weight are shown here. Location is either Canal Lake or Baddeck, score is the intensity of brightness as denoted in the field, and contrast value is the intensity of brightness synthesized from photographs. Models with the lowest AICc scores are shown in bold.

Variable	Models	*df*	AICc	Δ AICc	Weight
Score	**Intercept**	2	421.2	0.00	0.414
	Location	3	422.2	0.75	0.284
	Score	3	423.2	1.93	0.157
	Location + Score	4	423.9	2.69	0.108
	Location + Score + Location ×	5	426.1	4.86	0.036
Contrast	**Contrast**	3	379.0	0.00	0.320
	Location + Contrast	4	379.4	0.42	0.259
	Location	3	380.3	1.28	0.169
	Intercept	2	380.4	1.36	0.162
	Location + Contrast + Location × Contrast	5	381.6	2.57	0.089

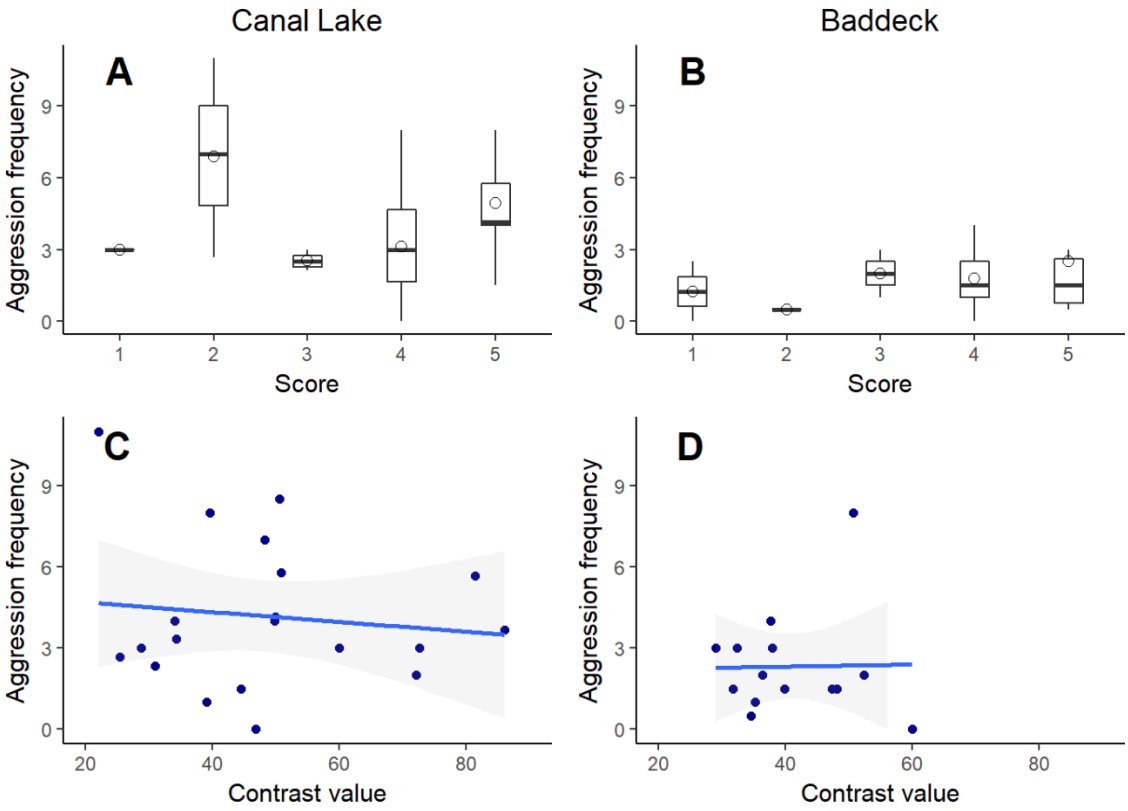

Figure 3.3.3 Intensity of brightness evaluated visually (A, B: scores, where 5 is the brightest white) or from field photographs (C, D: contrast values) correlated with the frequency of aggression. Aggression is depicted in Canal Lake (Figs. A, C) scores: 1 (n = 1), 2 (n = 3), 3 (n = 4), 4 (n = 13), 5 (n = 10), and Baddeck in the Bras d'Or Lake (Figs. B, D) scores: 1 (n = 2), 2 (n = 1), 3 (n = 3), 4 (n = 7), 5 (n = 7). Bars represent the median and circles represent the mean. Shaded areas represent the 95% confidence interval of the line of best fit.

4. DISCUSSION

White and common ecotypes of the Threespine Stickleback represent a comparative model for breeding ecology as well as behavioural and physiological factors that influence male nuptial colouration. In the province of Nova Scotia, populations of white and common ecotypes inhabit both the mainland and Bras d'Or Lake, but gene flow between these two areas is limited (Samuk 2016). In this study, I compared behavioural differences between ecotypes, quantified melanophore number and density to understand the cellular basis for seasonal white nuptial colouration, and correlated breeding behaviour with nuptial colouration. My results indicate that: 1) breeding activity in white males is higher than in common males; 2) breeding white males have fewer melanophores and less melanophore coverage than breeding common males; and 3) courtship frequency is positively correlated with increased brightness in white nuptial colouration.

4.1 Behavioural differences between ecotypes

My results indicate that white males are more active in breeding than common males as was found in previous studies (Blouw and Hagen 1990; Jamieson et al. 1992a; MacDonald et al. 1995). I found that white males are also more successful in leading females to the nest, which matches the data from the Jamieson et al. (1992a) field study. I also measured aggression frequency towards conspecifics, an important aspect of intra-sexual selection that has not been previously quantified.

The increased energetic investment in breeding behaviour in white males may be associated with naturally and/or sexually selected physiological and behavioural adaptations (Blouw and Hagen 1990). As white males do not invest in parental care (Jamieson et al. 1992b; Blouw 1996), they can theoretically expend more energy on pre-

mating courtship behaviour to maximize the number of mates and offspring, if all other energetic costs are equal. This lack of parental care is not seen in any other ecotype of the Threespine Stickleback complex with the exception of the lighter coloured males of the Swedish population in the Baltic that breed on hard substrate or algae and show a reduction in fanning behaviour (Borg 1985). It is possible that the algae upon which some males of this Baltic population breed may provide an adequate substitute for parental care allowing for decreased parental care, which is also observed in the Nova Scotia white ecotype. Another possibility may be that the atypical nesting sites of the Baltic and Nova Scotia white male are more desirable to females than the open substrate nesting sites typically used by common males. The algae with which white males and 'hard-bottom' Swedish males build nests provides cover during the breeding season (Blouw and Hagen 1990). Both 'hard-bottom' Baltic and Nova Scotia white males may have an advantage if the increased potential for offspring protection, provided by the algae, entices white females to the nest more often than common females to common male nests, thereby increasing the frequency of courtship behaviour observed in white males. Furthermore, white males may also court more energetically and display brighter nuptial colouration in a breeding ground that provides protection (Jamieson et al. 1992a), as opposed to common males (breeding in open areas) that show decreased courtship and nuptial colouration in areas of increased predation (FitzGerald, Gerard and Dutil 1981; Pressley 1981).

My results indicated a weak effect of ecotype on nestbuilding frequency, in which white males tended to their nests at a slightly higher frequency than common males. The slight difference observed between ecotypes may be related to the length of the sexual

phase in which males attempt to court females and spawn successfully (Jamieson et al. 1992b). Common males cease courtship behaviour after successful spawning events to engage in parental care, while white males continue to breed after dispersing embryos in algae (Jamieson et al. 1992a). Although white males may need to construct and maintain nest integrity more often than common males if they are successful in spawning more often, common males continue to tend to the nest even when the eggs are present and will return to breeding once offspring have vacated the nest (Blouw and Hagen 1990; Jamieson et al. 1992a). Therefore, this slight increase in nest-building frequency is likely related to the material retrieval aspect of nest-building and not the length of the common and white male's sexual phase. When nest-building behaviour was further broken down into material retrieval and nest-tending, the data indicated that common and white males tended to their nests at the same frequency, but white males retrieved nesting materials at a higher frequency. This increase in material retrieval may be related to the differences in the nests that the common and white males construct and occupy. I also noted that white males in Canal Lake (which were tagged and thus identified) switched nest sites during the breeding season and constructed new nests; this may also be a contributing factor to the slight difference in nest-building frequency observed between ecotypes.

During my observations, I found that white males more vigorously defended their territory from neighbouring white males than common males did. This finding may indicate that the intensity of intra-sexual selection is higher for white males than for common males, as competition for mates is extremely intense over the course of the breeding season (Jamieson et al. 1992a). White males do not guard their eggs in the nest after fertilization, which is the main driver of stickleback aggression for males that

provide parental care (Wootton et al. 1995). Increased aggression in white males may occur because white males appeared to breed at higher densities (A. Haley, personal observation), resulting in more male-male competition (Blouw and Hagen 1990). White and common males rarely used biting in their aggressive bouts relying almost solely on chases. Furthermore, egg-eating predators such as killifish (*Fundulus* sp.) were present in both my sites. In Baddeck, I observed a killifish destroy a white stickleback nest where a female had recently laid eggs. Thus, although males do not shelter eggs for long periods of time after successful spawning, eggs may still be at risk in the nest during the short time -usually within 120 seconds (observed in the laboratory; Blouw 1996) - that they remain there. The relatively high density of killifish in Baddeck may also explain the higher aggression frequency I observed in comparison with Canal Lake white males. Once males have collected and dispersed embryos in the surrounding algae, predation risks may decrease.

A second possibility for the observed increase in breeding behaviour demonstrated by the white males is that the energetic costs of parental care may be too great for common males to act as intensely as the white males, or the benefits of increased mating outweigh the potential cost to offspring from the loss of parental care (Jamieson et al. 1992a,b). This trade-off was observed with respect to the common ecotype; Sargent (1985) concluded that competitive Threespine Stickleback males that spent less time fanning were more likely to court more females than less competitive males. von Hippel (2000) also concluded increased courtship and intense nuptial colouration depleted energy reserves, leading to reduced fanning and decreased egg survival. A lacustrine Threespine Stickleback population of Wapato Lake may also support the idea that males lacking

energy reserves may show a trade-off with parental care (Kynard 1978). The Wapato population exhibits little parental care during the first two days after mating, when males conceal their eggs and remain mostly absent from their nests. Concealment is likely a response to raider packs of fish and neighbouring males that steal eggs from nests. Males leave their eggs concealed and unattended in the nest, perhaps to reduce conspicuousness, avoid conflict with predators, or to obtain food (Kynard 1978). Jamieson et al. (1992a) observed that white males also leave their territories. It would be interesting to investigate where these males go, for how long, the extent of their territories, and the purpose behind vacating their nests.

Further studies of the reproductive success of the white ecotype, their physiological responses to the environment, and the use of filamentous algae as parental care replacement are needed to determine whether energetic trade-offs may be related to the increased frequency of breeding behavior (Jamieson et al. 1992a; Head et al. 2016). In addition, sample sizes of common males were small in this study, and further investigation observed in differing locations would be beneficial in understanding the factors leading to reproductive isolation between these ecotypes.

4.2 Chromatophores, long-term colour change, and rapid colour change

My results indicated that breeding white males have fewer lateral melanophores and less dorsal melanophore coverage than breeding common males. These data support the prediction that white nuptial colouration is partly owing to a reduction in dark pigmentation. In non-breeding males, melanophore number and coverage between ecotypes from both locations was similar in all regions of the body, except for ecotypic differences observed in dorsal melanophore coverage. These findings support the observation that males are morphologically similar outside of the breeding season (Blouw and Hagen 1990) and evidence of long-term nuptial colour change observed in Threespine Stickleback ecotypes (Price et al. 2008). An interesting finding was that common males increase melanophore density during the breeding season. This may occur to accentuate the iridophores necessary for the dark blue dorsal colouration observed in common males (A. Haley, personal observation). Blue colouration in fishes can occur through changes in iridophore structure (Bagnara and Matsumoto 2007), and in some cases, iridophores are neuronally regulated and intertwined with melanophores (Sköld et al. 2016). However, non-breeding sample sizes were small in this study, and future work should address the comparison of breeding condition (breeding and non-breeding) in more detail. This study provides the first investigation of melanophore number and cover between ecotypes displaying differing nuptial colouration and provides insight into the long-term colour change that occurs in both white and common ecotypes during the breeding season.

Differences in darkness can occur from changes in the number of melanophores, the amount of pigment deposited in each cell, and/or the aggregation and dispersion of

pigment-containing dendritic cells (reviewed by Sköld et al. 2016). I counted melanophores to quantify the number of pigment cells in each region and calculated the percent cover of melanophores. Melanophore coverage is an interesting measure because it incorporates chromatophore density, cell dispersion and darkness. Below, I discuss my results in the context of long-term colour change and hypothesize what the cellular basis for rapid colour change during the breeding season might be.

Long-term colour change

Melanophore pigments are endogenously synthesized and increase in number through differentiation from precursor cells (Sugimoto 2002). Additionally, melanic colouration is regulated through hormonal and neuronal processes that may draw on an individual's energy reserves (Price et al. 2008). As such, white nuptial colouration may be less expensive than typical colouration owing to a reduction in the synthesis of melanin. Another possibility may be that the chromatophores themselves provide useful functions (Djurdjevič et al. 2015); the pigments leading to white colouration (likely the platelets observed in iridophores) may function in thermoregulation through light reflectance, allowing white males to breed more often in warmer temperatures (Djurdjevič et al. 2015). In addition to melanophores and light-reflecting chromatophores, it would be interesting to investigate the presence of other pigment-based chromatophores that are acquired from the diet. For example, white males display red throats during the breeding season, but these are much lighter than what is typically seen in a common breeding male (Blouw and Hagen 1990). Carotenoids for example, are responsible for the red throat colour that is typically an intra-sexual signal indicating aggression and male health, but also an inter-sexual signal to attract females (Kodric-brown 1998). Because carotenoids

are energetically expensive to obtain and may compromise the immune system (Kodric-brown 1998), a decrease in the expression of carotenoid pigments may allow for the energetic courtship behaviour and white nuptial colouration observed in the white ecotype.

In addition to reducing the number and coverage of melanophores in the integument, structural colour change affected by the light-reflecting platelets of iridophores may be responsible for the bright iridescent colour characteristic of the white ecotype. Leucophores, which are another type of chromatophore responsible for white colouration, were not observed in the skin of white stickleback males during my preliminary observations, and are not known to be present in this clade of fishes (Appendix F; Kimura et al. 2014).

Rapid colour change

Rapid changes between melanic and blanched colouration has previously been observed in many vertebrates (Price et al. 2008). Short-term colour change can occur within seconds through the dispersion of pigment granules in the cell or changes in the orientation of reflecting platelets in iridophores and is physiologically regulated by a melanocyte-stimulating hormone and a number of other hormonal and neural signals (Nery and de Lauro Castrucci 1997; Price et al. 2008). Furthermore, melanin-based colour change is often pleiotropically linked to physiological and behavioural traits, such as colouration, courtship and aggression (Ducrest et al. 2008). For example, in the cichlid fish *Astatotilapia burtoni* blue and yellow morphs (the yellow morph being more aggressive than the blue morph) exist in this species, and the melanocortin system simultaneously regulates colour and behaviour (Dijkstra et al. 2017). The addition of the

melanocortin system peptide α-melanocyte stimulating hormone (*a*-MSH), which stimulates melanin and carotenoid pigment dispersal in the skin, increases aggressive behaviour and colouration in a morph-specific manner (Dijkstra et al. 2017). *a*-MSH increases yellow colouration in both morphs through the dispersion of xanthophores (chromatophores related to yellow colour) and increases aggressive behaviour in blue morphs.

Changes in the reflectance and structure of iridophores may also be the catalyst for rapid colour change observed during the breeding season (Schartl et al. 2016). It would be interesting to determine if there are differences in the number and layer of iridophores between ecotypes (Teyssier et al. 2015), and if the physiological pathways that regulate iridophore density and orientation are similar. Further comparative studies should be done to investigate physiological pathways leading to rapid colour change in white males and determine if differing ecotypes exhibit similar pathways.

Melanophores, in combination with iridophores, are likely responsible for both long-term white colouration and rapid changes in the intensity of brightness during the breeding season. In this study, the sample sizes of white males from Bras d'Or were low, and it would be useful to supplement this study with further investigation into the chromatophore differences between ecotypes and populations to better understand the cellular basis for nuptial colouration and gain insight into the mechanisms underlying the evolution of the white ecotype.

4.3 Selective pressures on breeding behaviour and nuptial colouration

I found that behavioural correlates indicate courtship is positively related to the intensity of brightness displayed by breeding white males. This is consistent with the

hypothesis that white nuptial colouration is under female-choice driven selective pressure and might be the mechanism by which assortative mating occurs. This hypothesis is matched by previous studies that indicated positive assortative mating in the field and laboratory, and genetically-based white nuptial colouration and energetic courtship behaviour exhibited by breeding males (Blouw and Hagen 1990; Jamieson et al. 1992a,b).

Rapid colour changes are typically observed in monochromatic or dichromatic male fishes to signal courtship and/or aggression, and may aid in female mate recognition (Kodric-brown 1998). Furthermore, energetic courtship and aggression behaviour normally enhances rapid colour change in other fishes (Kodric-brown 1998), which is supported by my data that indicate a positive relationship between courtship and the intensity of brightness. Thus, white iridescent colour observed in both mainland and Bras d'Or populations, coupled with enthusiastic courtship behaviour on the part of the white ecotype, likely provides a prominent signal to gravid females (Jamieson et al. 1992a).

Although my results suggest that white nuptial colouration is associated with female mate choice, other processes, such as the loss of parental care and increased aggression, may have played a role in the divergence between the white and common ecotypes before the selective pressure of female choice, based upon white nuptial colouration, had an effect. Moreover, colour patterns are often found to covary with other traits, such as courtship and aggression, owing to pleiotropic effects regulated by neuronal processes (McKinnon and Pierotti 2010). As such, both naturally and sexually selected forces may have led to the evolution of white nuptial colouration in males and disentangling these effects should be taken into consideration in future studies.

For example, the unique iridescent nuptial colouration of the white ecotype contrasts with the green or brown colour of the algae upon which they build nests (Blouw and Hagen 1990) and makes the male white Threespine Sticklebacks highly visible, even from long distances (Blouw and Hagen 1990). Thus, breeding colour of the white ecotype has the potential to attract predators as well as potential mates. Conspicuous colouration and behaviour is predicted to lead to differential predation on common Threespine Stickleback in a region of the St. Lawrence Estuary, where four species of Stickleback co-occur; Threespine Stickleback, Blackspotted Stickleback (*G. wheatlandi),* Fourspine Stickleback *(A. quadracus)* and the Ninespine Stickleback (*P. pungitius*: FitzGerald, Gerard and Dutil 1981). Of these four species, only *G. aculeatus* is preyed upon by the black-crowned night heron (*N. nycticorax*), a phenomenon thought to be associated with its larger size and more energetic breeding behaviour (Fitzgerald 1983). In particular, Threespine stickleback are larger, nest in open areas (as opposed to in algae), and males display brighter nuptial colouration than the other species (Fitzgerald 1983), making them more susceptible to predation. The white ecotype is even more conspicuous than the common Threespine Stickleback, but it is smaller and uses the algae present in the breeding grounds as cover. Indeed, white males blend in with clumps of bladder wrack that have similar colouration and are not as conspicuous on the breeding grounds as originally believed (A. Haley, personal observation). Additionally, white males can quickly (within seconds) dull their colouration and become better camouflaged, which is useful in the presence of predators. Piscivorous fishes such as eels, trout, and sculpin, also pose predation risks to Threespine Stickleback (Pressley 1981; Blouw and Hagen 1984, 1990). Interestingly, Blouw and Hagen (1984) observed that the white ecotype is present in sites with fewer piscivorous fish, indicating that the white Threespine Stickleback does

not exist in areas of high predation pressure, allowing for the observed increased energetic behaviour and conspicuous colouration. Further investigation of differential predation on common and white Threespine Stickleback would provide useful information about whether white colouration increases susceptibility to predators.

Much like the white ecotype of Nova Scotia, males of the Swedish population that nest in hard-bottom substrate or algae lack common red nuptial colouration, show reduced fanning behaviour, and display a greenish-blue tint (Borg 1985). Conspicuous white colouring may increase the possibility of mating (Endler 1983; Sköld et al. 2016), and much like other conspicuous traits, may be an honest indicator of fitness. This theory, in which potential mates advertise quality through costly sexual signals (Zahavi 1975), is observed in many other fishes such as guppies, cichlids, salmonids, and *Betta splendens* that display bright colours and complex ornaments (Endler 1983; Allender et al. 2003; Price et al. 2008). The unique colour of the Nova Scotia white Threespine Stickleback attracts females of both ecotypes, although 'common' females do not mate with 'white' males in the wild (Jamieson et al. 1992a).

In addition to trade-offs associated with predation, it would be interesting to test the hypothesis that white nuptial colouration is a naturally and sexually selected trait associated with the effects of parasitism. Sticklebacks are adversely affected by parasites, which causes weight loss and delays sexual maturity in males and females (reviewed by Wootton 1976). Furthermore, the effect of parasitism on male nuptial colouration influences female mate choice, as females select males that have not been exposed to parasites and are capable of signalling health with bright nuptial colouration (Milinski and Bakker 1990). Interestingly, Poulin and FitzGerald (1987) observed parasitism effects on

three host populations, which included *G. aculeatus*, and found that parasites have the potential to re-structure stickleback communities through preferential host selection, much like the effects of predation. Female Threespine Sticklebacks also choose males with a high diversity of major histocompatibility proteins (Kurtz et al. 2004), thereby selecting males that are not infected by tapeworms and microsporidians which cause white tumours on the skin and gills of sticklebacks. These microsporidians are prevalent and noticeable in Nova Scotia Threespine Stickleback (A. Haley, personal observation). The conspicuousness of white colouration might not lead to adverse predation effects in communities where other individuals are infected by white spores that are just as visible. In this case, white nuptial colouration, and the white colour of the tumours seen on individuals infected by microsporidia, may signal infection and unprofitable prey to predators. Perhaps these parasites play a role in the structure of the white and common ecotype as they breed in sympatry and provide an example of how ecological processes in combination with female mate choice drives pre-mating isolation.

While predation and parasitism may both contribute selective pressures on white and common Threespine Stickleback populations, the habitat in which white males breed may also shed some light on the evolution of this unique breeding behaviour and colour. Interestingly, in Bras d'Or Lake which is largely geographically isolated from the ocean, white males were found to be breeding during the months characterized by the least amount of freshwater influx (Petrie and Bugden 2002), when salinity and temperature variation mirrored that of the mainland sites. Because sites on the mainland and in Bras d'Or are ecologically similar (Blouw and Hagen 1990; A. Haley, personal observation), this may facilitate the radiation of the white ecotype in both locations. Additionally, water

turbidity in some locations may affect female perception of nuptial colouration, leading to the selection of conspicuous colouration and breeding behaviour. For example, in the Haida Gwaii archipelago, stickleback inhabit either clear or black freshwater lakes (Reimchen 1989; Peichel and Marques 2017). In black freshwater lakes, black nuptial colouration enhances blue eye colouration during the breeding season, a trait driven by female mate choice (Reimchen 1989). By contrast, typical male nuptial colouration is observed in clear freshwater lakes of the same region. The evolution of a red-shifted opsin, a light-sensitive protein, in the blackwater populations may be under natural and sexual selection as colour vision adapts to the blackwater light spectrum allowing for better visibility of prey and potential mates (Marques et al. 2017).

Further studies are needed to determine if female mate choice may have led to the evolution of white nuptial colouration and energetic courtship, and if male-male competition is related to the increased frequency of aggression observed in breeding white stickleback males. Increased aggression may afford males an advantage during the breeding season (e.g., better nest sites). Additionally, although contrast values are not as reliable as *in situ* 'brightness' scores, values obtained from photographs during timed behavioural observations are important evidence of rapid colour change and provide useful information on the behaviour and nuptial colouration of individual fish. Further work should develop a method that better correlates brightness with behaviour from photographic values to reduce the variation observed from the calculated contrast values. Although I standardized for brightness and cloud cover, environmental changes likely contributed to the variability observed in this study.

5. CONCLUSION

In this study, I found that white males are more active in courtship, nest-building, and aggression than common males in the field, in agreement with the qualitative observations of Blouw and Hagen (1990) and Jamieson et al. (1992a). I found that courtship was particularly divergent among white and common ecotypes, with white males courting at a significantly higher rate. Investigation into the cellular basis of nuptial colouration suggests that white colouration is related to a reduction of the number of melanophores and overall melanophore coverage. Furthermore, my finding that the intensity of brightness and courtship rate are positively correlated is consistent with the idea that brightness is a sexually selected trait driven by female mate choice. Recent population genetic analyses did not include the Bras d'Or white form (Samuk 2016). Therefore, the goal of this study was to determine breeding behaviour differences and skin colouration divergence among mainland and Bras d'Or populations of both ecotypes.

Although there are some observed differences between the breeding behaviour and nuptial colouration of white males from the mainland and Bras d'Or Lake, preliminary evidence suggests that these traits are generally similar in both locations. This indicates that the Bras d'Or white Threespine ecotype may have evolved from the Bras d'Or common Threespine ecotype independently from mainland white populations, or, the white colouration in Bras d'Or and mainland Nova Scotia stickleback populations may have a shared genetic basis if there is gene flow between mainland Nova Scotia and the Bras d'Or Lake populations. Further population genetic studies would be useful to tease apart how the Bras d'Or white Threespine Stickleback population fits in with other Threespine Stickleback populations across the province.

6. LITERATURE CITED

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APPENDIX A

Examples of local adaptive thresholding methods for melanophore percent cover calculations

Prior to melanophore coverage analysis, I conducted preliminary trials to determine the best threshold method suitable for my samples. I chose to proceed with an automatic local adaptive thresholding method to reduce user bias and to account for the high degree of detail needed to distinguish dark cells (melanophores) from the light background (skin without melanophores), especially in the dorsal region of common breeding males (Fig. A1). I first looked at all possible methods available in ImageJ on the dorsal region, where melanophore density is highest, of both common (Fig. A1) and white (Fig. A2) representative samples. I then narrowed the possibilities down to three methods that best fit my samples (Fig. A3). The Global method lacked definition around the edges of each sample, but the Bernsen and MidGrey methods were nearly identical (Fig. A3), with only a ~2% observed difference after percent cover was calculated. Both the Bernsen and MidGrey methods were conservative measures because they did not overestimate dark areas, yet were useful because they retained definition around the edges of the sample. Of these two methods, the Bernsen method was chosen for this study.

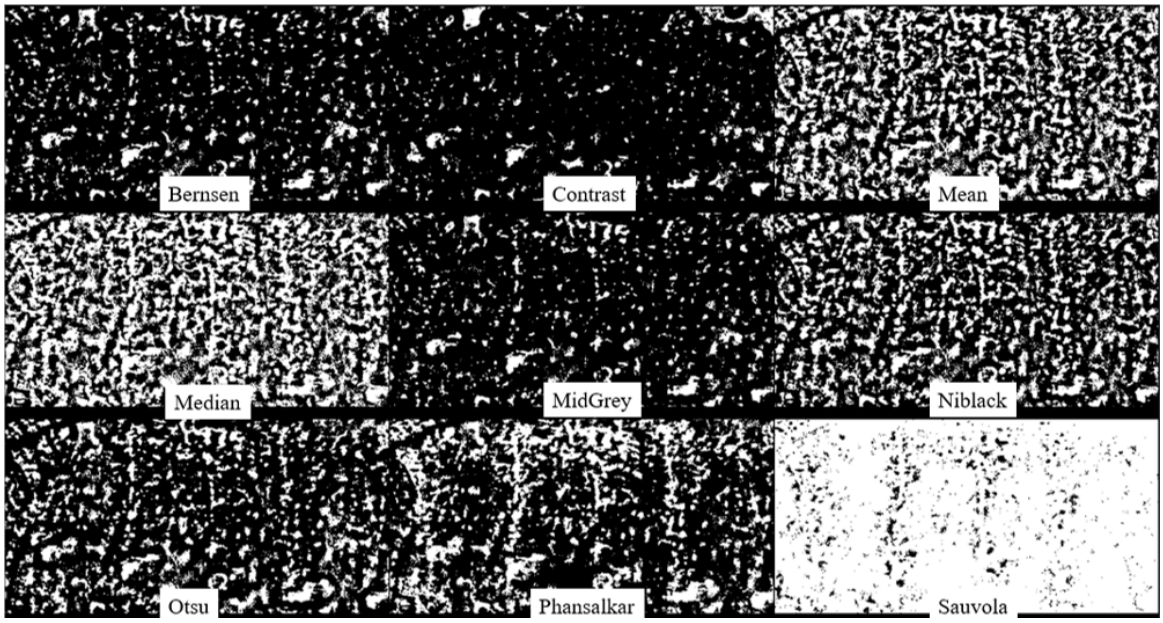

Figure A1. All possible local adaptive threshold methods available in ImageJ performed on the dorsal region of a common breeding male.

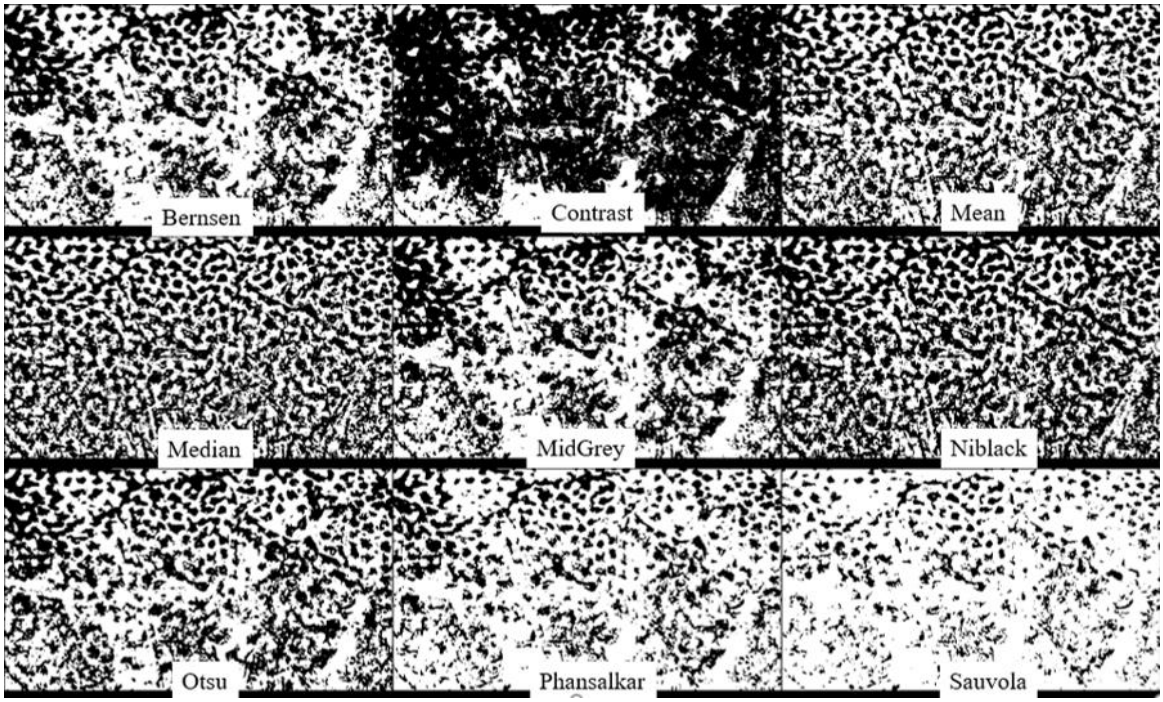

Figure A2. All possible local adaptive threshold methods available in ImageJ performed on the dorsal region of a white breeding male.

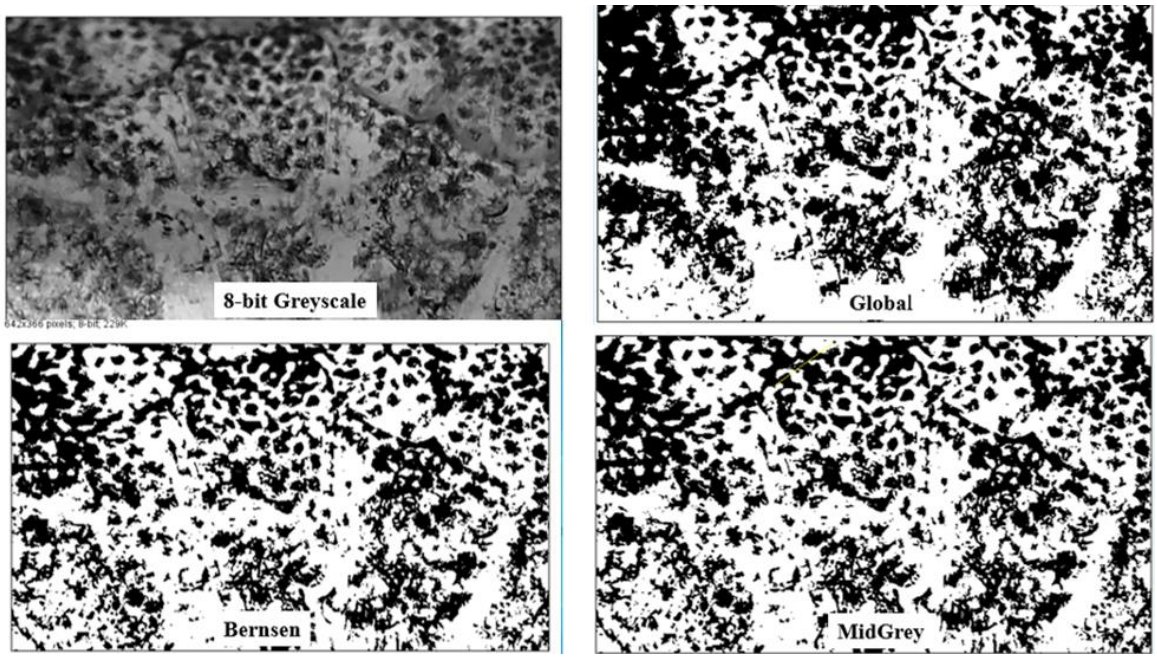

Figure A3. Examples of the three local automatic thresholding methods that best represented the samples.

APPENDIX B

Photographic contrast value correlates with visual scores from the field as measures to determine the intensity of brightness in male white Stickleback

I correlated visual scores of brightness and contrast values calculated from photographs to determine if the intensity of brightness was found to be similar with both methods (Fig. B1). Although the photographic contrast values are variable, likely owing to environmental variation in the field, there is a positive correlation between both mean score and mean contrast value and I used both measurements in this study.

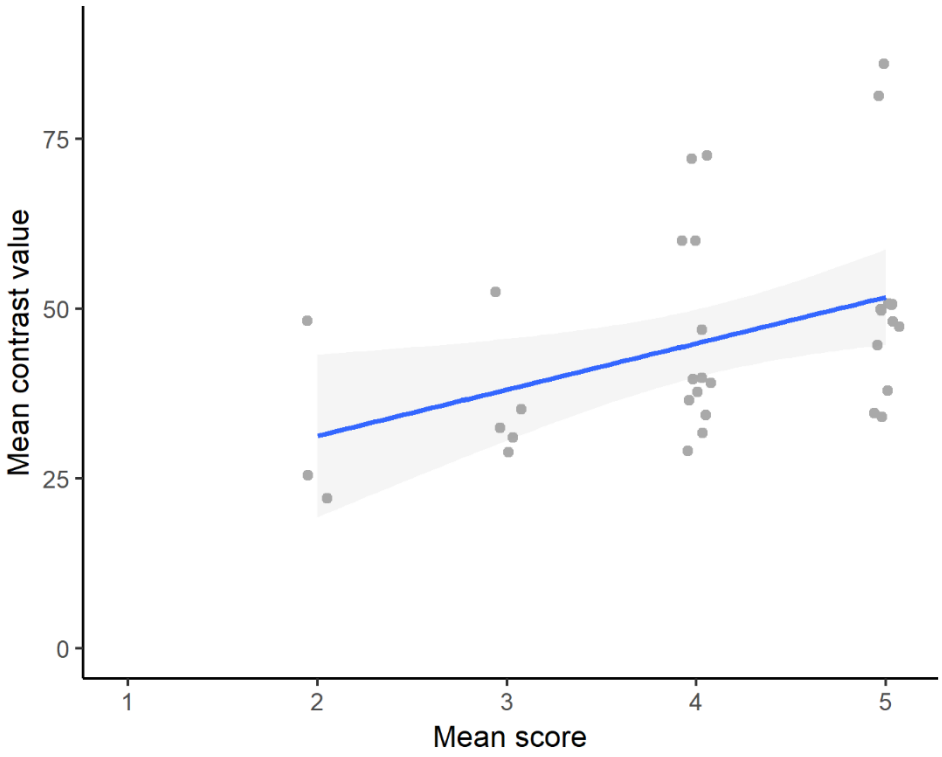

Figure B1. Intensity of brightness evaluated visually in the field (mean score from one to five, with five indicating the brightest white) correlated with the intensity of brightness calculated from photographs (mean contrast value, higher values are brighter white), LM: $p = 0.0003$, $R^2 = 0.12$. Note that contrast values of individuals with a score of 1 (dull grey colouration) could not be calculated from photographs because individuals were not bright enough to be identified.

APPENDIX C

Proportion data for correlates of behaviour and intensity of brightness

I plotted the relationship between colour and courtship, nestbuilding and aggression behaviour as a proportion of total behaviour to account for the effect of brightness on behaviour in both active and non-active fish (Table C1, Fig. C1). The observed patterns mirrored those for the behavioural correlates of mean frequency and mean score of individuals, which is what used in this study.

Table C1. Generalized linear mixed effects models with Binomial distribution indicating the effect of location and score of courtship, nestbuilding, and aggression as a proportion of total behaviour. Location is either Canal Lake or Baddeck, and score is the intensity of brightness as denoted in the field. Model degrees of freedom (*df*), Akaike Information Criterion corrected for sample size (AICc), difference between models with lowest AICc values and all other models (ΔAICc), and model weight are shown here. Models with lowest AICc scores are shown in bold.

Behaviour	Models	*df*	AICc	Δ AICc	Weight
Courtship	**Score**	3	631.9	0.00	0.675
	Location + Score	4	634.0	2.13	0.232
	Location + Score + Location × Score	5	635.9	3.97	0.093
	Intercept	2	672.9	41.04	0.000
	Location	3	675.0	43.07	0.000
Nestbuilding	**Score**	3	737.3	0.00	0.623
	Location + Score	4	738.8	1.59	0.281
	Location + Score + Location × Score	5	741.0	3.75	0.096
	Intercept	2	754.3	17.00	0.000
	Location	3	756.1	18.80	0.000
Aggression	**Score**	3	456.9	0.00	0.413
	Location + Score	4	458.3	1.37	0.208
	Intercept	3	458.5	1.59	0.186
	Location	3	459.8	2.89	0.097
	Location + Score + Location × Score	5	459.9	2.92	0.096

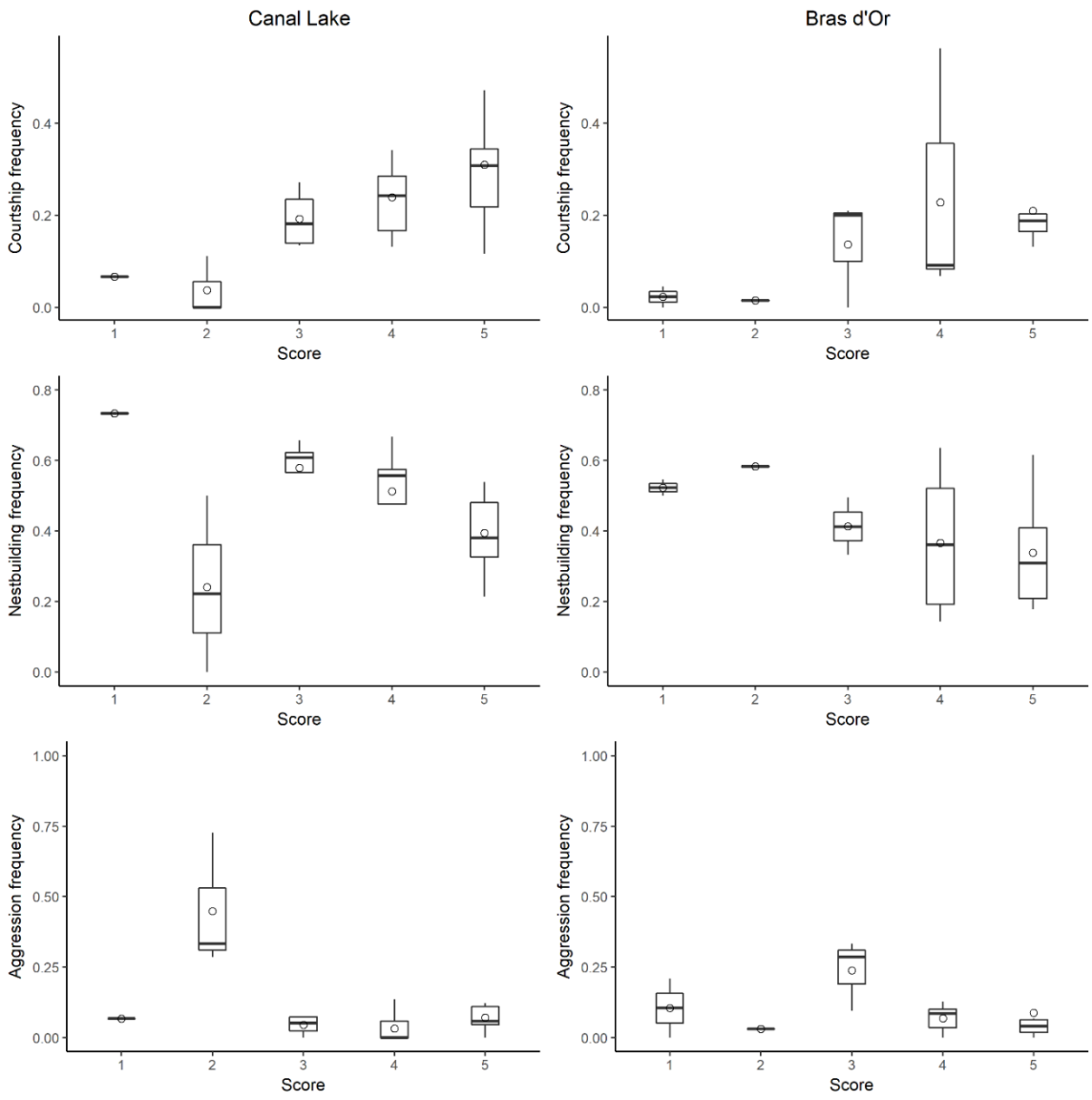

Figure C1. Intensity of brightness (evaluated visually) correlated with the frequency of courtship, aggression, and nestbuilding, modelled as proportions of total behaviour. Bars represent the median and circles represent the mean.

APPENDIX D

Effect of *Fundulus* sp. on white Threespine Stickleback male aggression frequency

To investigate the aggression frequency differences of mainland and Baddeck males (Table D1, Fig. D1), I added the number of potential egg-eating predators such as *Fundulus* sp., Blackspotted Stickleback and Fourspine Stickleback chases from the aggression category. I was interested in the possibility that the observed increased aggression frequency by white males from Baddeck was related to a higher density of *Fundulus* sp. at the site. The model that best explained the data included the effects of ecotype and location, with white males from Baddeck performing more aggressive bouts than common males and white males from Canal Lake. This indicates that the increased frequency of aggression observed in Baddeck white males is likely associated with a higher density of *Fundulus* sp. in that site (Table D1, Fig. D1).

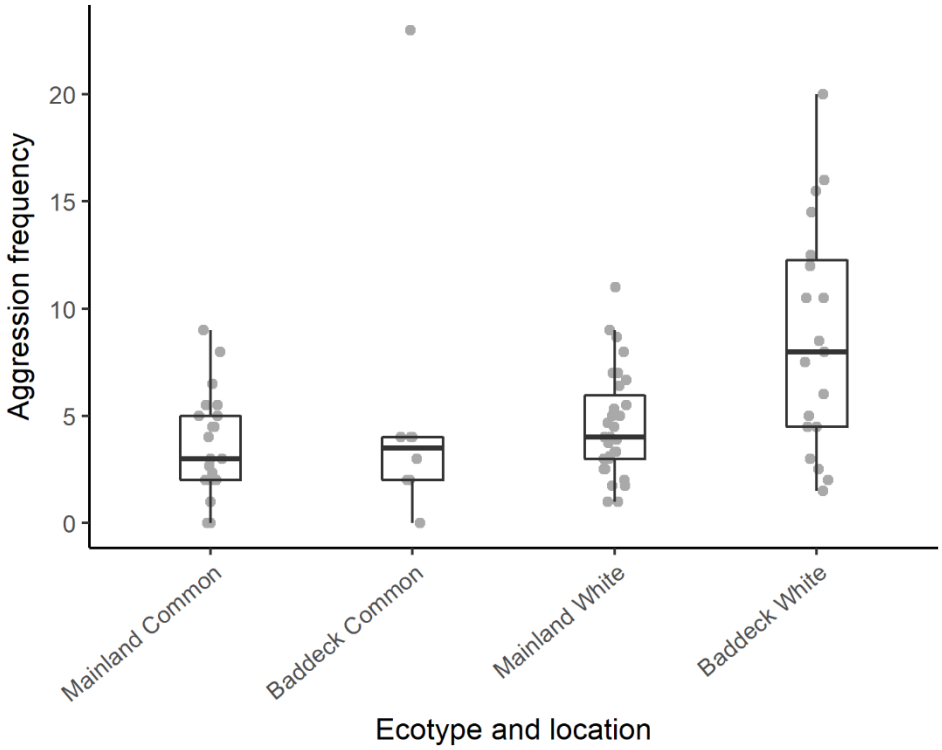

Figure D. Frequency of aggressive behaviour of white Threespine Stickleback males from mainland (Canal Lake and Rainbow Haven) and Bras d'Or Lake (Baddeck) with the effect of potential egg-eating predators (Killifish, Blackspotted Stickleback, Fourspine Stickleback) included. Bars represent the median and grey dots represent mean individual behavioural frequencies from all observations which were accounted for in the analysis as a random factor.

Table D. Generalized linear mixed effects models with Poisson distribution indicating the effect of ecotype, location, and their interaction on the frequency of aggression. Model degrees of freedom (*df*), Akaike Information Criterion corrected for sample size (AICc), the difference between models with lowest AICc values and all other models (Δ AICc), and model weight are shown here. Location can be either Canal Lake or Baddeck.

Behaviour	Models	*df*	AICc	Δ AICc	Weight
Aggression	Location + Ecotype	4	1001.5	0.00	0.446
	Location + Ecotype + Location × Ecotype	5	1001.8	0.30	0.384
	Location	3	1003.6	2.09	0.157
	Score	3	1008.8	7.29	0.012
	Intercept	2	1012.3	10.76	0.002

APPENDIX E

Experiments to maximize the dispersal and aggregation of chromatophores and identification of leucophores

Prior to chromatophore sampling, I carried out preliminary experiments to maximize visibility of melanophores, leucophores and iridophores. This work consisted of putting fish skin and/or scales in different solutions, counting the number of visible chromatophores, and determining if melanophores had aggregated, leucophores had dispersed and iridophores platelets became more reflective (Menter et al. 1979; Mathger 2003). I tested for melanophore aggregation and iridophore changes using 10µm epinephrine solution and K+ physiological saline and melanophore dispersion with physiological saline (Oshima et al. 2001; Mathger 2003). During this preliminary sampling, I also isolated and incubated scales of Japanese medaka (*Oryzis Latipes*) and Mummichog (*Fundulus heteroclitus*), animals that have leucophores, in K^+ rich physiological saline solution to properly identify these cells prior to identification in stickleback (Menter et al. 1979; Fujii 1993; Fujita and Fujii 1997). Leucophores have not been previously identified in common Threespine Stickleback (Kimura et al. 2014) and thus preliminary studies were necessary to determine if leucophores, in combination with iridophores, contribute to the nuptial colouration of the white ecotype. I did not succeed in identifying leucophores in either Japanese medaka or in the Mummichog, as I only identified one possible leucophore. This difficulty in identification may have resulted from environmental conditions under which the fish were held, as acclimation (or lack therof) to a white background influences leucophore production and dispersion (Menter et

al. 1979; Oshima et al. 2001). Therefore, I cannot equivocally determine if Threespine Stickleback have leucophores, although it is unlikely, as this chromatophore type has only been identified in the Ovalentaria to date (Kimura et al. 2014).