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A comparison of nuptial coloration and breeding behaviour in white and common marine threespine stickleback (*Gasterosteus aculeatus*) ecotypes

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ABSTRACT

Background: In Nova Scotia, Canada, an endemic 'white' ecotype has evolved in sympatry with 'common' marine stickleback. Common males develop blue-green or brown dorsal coloration during mating, whereas white males become bright pearlescent white. White males also differ behaviourally from common males; they court females at a higher intensity and do not provide parental care. Both ecotypes occur along the oceanic coast of Nova Scotia and in the Bras d'Or Lake, an inland body of water on Cape Breton Island. Common marine stickleback from these two locations are genetically and morphologically distinct, but the phylogenetic relationship between white populations is not known. Furthermore, direct comparisons of mating behaviour and nuptial coloration between oceanic and Bras d'Or Lake stickleback have not been conducted for either ecotype.

Questions: Do Bras d'Or and Atlantic oceanic populations of white and common stickleback ecotypes display similar patterns of divergence in nuptial coloration and mating behaviour?

Methods: We measured melanophore density and coverage and quantified male breeding behaviour (courtship, nest-building, and intra-sexual aggression) in wild white and common males from Atlantic oceanic sites and the Bras d'Or Lake.

Results: Breeding white males from both geographic locations had a lower density of melanophores and reduced melanophore coverage compared with sympatric common males. White males from the two regions also behaved in a similar manner and had higher rates of courtship than common males but did not vary in other breeding behaviours. For both melanophore and behavioural data, there were some weak differences among white populations from the two locations but breeding white males in both areas use very similar strategies to attract females.

Keywords: Atlantic Canada, convergent evolution, melanophores, parental care, sexual selection, speciation.

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INTRODUCTION

When selective pressures differ among populations with limited gene flow, they can diverge over evolutionary time to such an extent that they become reproductively isolated and form different species (reviewed by Nosil *et al.*, 2003; Kitano *et al.*, 2007; Ritchie, 2007). In the case of sympatric populations, strong disruptive selection is required to cause divergence, and assortative mating can maintain reproductive isolation by preventing gene flow (reviewed by Bolnick and Fitzpatrick, 2007). Disruptive selective pressures may result from either natural or sexual selection, and recent work has highlighted how interactions between these two types of selection may promote speciation, particularly when sexual selection is involved in the initial stages of divergence (reviewed by Maan and Seehausen, 2011; Servedio and Boughman, 2017).

The mechanisms by which sexual selection can act during speciation vary, and more than one of these mechanisms can be implicated in the initiation or maintenance of reproductive isolation (Maan and Seehausen, 2011). For example, both female choice (e.g. Dieckmann and Doebeli, 1999; van Doorn *et al.*, 2004; Verzijden *et al.*, 2005; Hohenlohe and Arnold, 2010) and male–male competition have been considered as potential drivers of speciation (Seehausen and Schluter, 2004; Dijkstra *et al.*, 2007, 2017; Keagy *et al.*, 2016; Tinghitella *et al.*, 2018). Indeed, differences in sexual selection pressures associated with female choice (Galis and Metz, 1998; Wilson *et al.*, 2000; Allender *et al.*, 2003) and negative-frequency dependent selection during male–male competition (Seehausen and Schluter, 2004; Dijkstra *et al.*, 2007) are jointly implicated in the speciation process in the classic sympatric radiation of African crater lakes cichlids. In particular, these cichlids display a remarkable diversity of male nuptial coloration across species, and the maintenance of this variation is attributed to both inter- and intra-sexual selective pressures and assortative mating (Maan and Sefc, 2013).

Nuptial coloration, the colour patterning associated only with mating, can be an important trait that evolves during speciation caused by sexual selection (Kodric-Brown, 1998; Seehausen and Schluter, 2004; Dijkstra *et al.*, 2007; Price *et al.*, 2008). In the case of assortative mating and disruptive sexual selection, divergent patterns of coloration within populations may be indicative of incipient speciation (Kodric-Brown, 1998; Seehausen and Van Alphen, 1998; Price *et al.*, 2008). While colour polymorphism can be linked to sexual selection and ultimately speciation in several cases (reviewed by Gray and McKinnon, 2007), colour divergence may be caused and maintained by a combination of sexually selected morphological and behavioural traits through linkage or pleiotropy (e.g. Keagy *et al.*, 2016), which often act in concert with other ecologically important characteristics (Galis and Metz, 1998; Boughman *et al.*, 2005).

To better understand the role of sexual selection in speciation, model systems comprised of populations at different stages in the speciation continuum or with independent, repeated evolution of reproductive isolation are required (Servedio and Boughman, 2017). One potential empirical model system occurs in the Atlantic Canadian province of Nova Scotia: the sympatric marine 'white' and 'common' threespine stickleback (*Gasterosteus aculeatus*) ecotypes (Blouw and Hagen, 1990). Breeding white and common males display marked differences in body size, nuptial coloration, nest-site preference, mating behaviour, and parental care. In particular, breeding white males develop a conspicuous bright white dorsal and lateral coloration, a pale red throat, and a white-blue iris (Blouw and Hagen, 1990) (Fig. 1A). This differs from typical common marine males that acquire a bright red throat, blue eyes, and a blue-green or brown dorsal colour during the breeding season (e.g. van Iersel, 1953) (Fig. 1B). Both ecotypes aggressively defend a territory in which they build a nest; however, white males build their nests in patches of filamentous algae, while common males typically use muddy or sandy substrate (Blouw and Hagen, 1990). Differences in courtship behaviour also occur

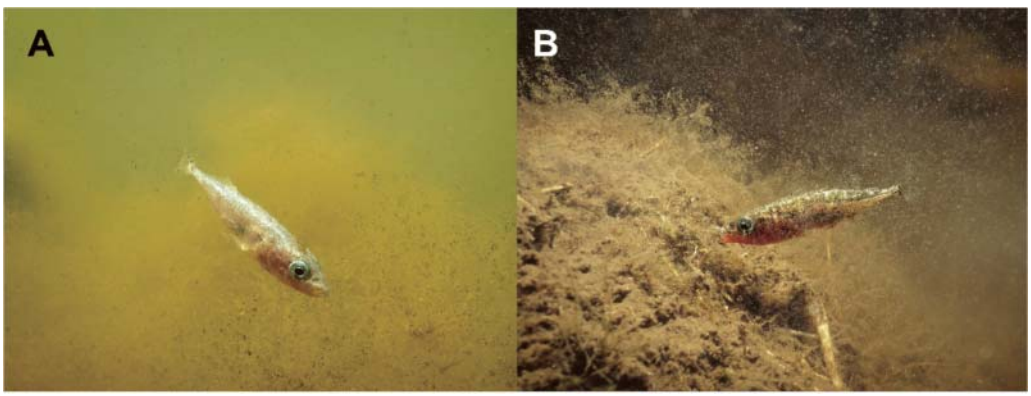

Fig. 1. A breeding white male from Rainbow Haven Beach (A) and breeding common male from Canal Lake (B). Both Canal Lake and Rainbow Haven Beach are along the Atlantic coast of Nova Scotia (see Fig. 2). Photographs by Dr. Paul Bentzen.

between ecotypes; common threespine stickleback males perform zig-zag, dorsal pricking and circling rituals (Wootton, 1976), whereas white males perform only the zig-zag dance (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 males remove embryos from their nests after breeding to scatter them in filamentous algae and return to their nest to breed, at which time courting and spawning is resumed (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a; Blouw, 1996). Thus, no parental care is provided by white stickleback males. By contrast, after a bout of successful spawnings, common males cease courtship and remain at the nest to fan and guard the eggs. Males do not resume courtship for a number of weeks until offspring have vacated the nest (van Iersel, 1953; Wootton, 1976; Jamieson *et al.*, 1992b).

Laboratory breeding studies have found that differences in breeding coloration and behaviour between white and common ecotypes have a genetic basis (Jamieson *et al.*, 1992a; Blouw, 1996), and field and laboratory studies suggest that mating is completely assortative (Blouw and Hagen, 1990; Blouw, 1996). These findings are supported by population genomic studies indicating that white threespine stickleback found along the coast of mainland Nova Scotia can be genetically distinguished from co-occurring common threespine stickleback populations (Samuk *et al.*, 2014, 2017; Samuk, 2016). However, the overall level of genetic differentiation between the two ecotypes is relatively low (Haglund *et al.*, 1990; Samuk *et al.*, 2014, 2017; 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 low genetic differentiation suggests ongoing gene flow and/or recent divergence (Samuk *et al.*, 2014; Samuk, 2016).

The Atlantic Canadian white and common ecotypes represent an opportunity to investigate the role of sexual selection at a relatively early stage of the speciation process. The utility of this system for such an investigation would be augmented if the white ecotype had evolved repeatedly, as this would allow tests for parallelism, which is suggestive of speciation by selection (Servedio and Boughman, 2017). Recent genomic studies have found that populations of common stickleback residing along the oceanic coast of Nova Scotia are more closely related to the white ecotype from the same geographic range than they are to common stickleback in the Bras d'Or Lake, a large inland brackish water lake located on

Cape Breton Island (Samuk, 2016). These data suggest that the common marine Bras d'Or fish are a genetically distinct group and raise the possibility that the white ecotype present in the Bras d'Or Lake (Jamieson *et al.*, 1992a; Blouw, 1996; Samuk, 2016) may have evolved independently from coastal Nova Scotian white stickleback. However, genetic data for Bras d'Or white stickleback are currently lacking. Furthermore, differences in breeding behaviour and nuptial coloration, two potentially sexually selected traits, have not been directly compared between white and common threespine stickleback populations from mainland Nova Scotia and in the Bras d'Or Lake.

The overall goal of this study was to directly compare divergence in male nuptial coloration and mating behaviour among populations of white and common threespine stickleback from the Bras d'Or Lake and coastal oceanic Atlantic Canada. We compared nuptial coloration by assessing the density and overall skin coverage of one type of chromatophore (pigment-containing cell) that might contribute to the white phenotype: melanophores. These dendritic, eumelanin-containing cells are found in the fish's dermis or epidermis (reviewed by Fujii, 1993). We predicted that the dark black melanophores would be reduced in density, size, or pigment content in white stickleback males compared with common stickleback males. We only quantified melanophores in this study, but it is likely that chromatophores that produce white coloration due to reflectance, such as iridophores and leucophores (reviewed by Sköld *et al.*, 2016), also contribute to the iridescent nuptial coloration in breeding white males. We examined behavioural differences between breeding white and common males in the Bras d'Or Lake and coastal oceanic sites by quantifying breeding behaviour in the field. Because white males should experience an increased intensity of sexual selection due to the potential for a higher mating rate in the absence of time-consuming parental care, we predicted that breeding white threespine stickleback males would show an increased intensity of breeding behaviour (courtship, nest-building, and intra-sexual aggression). In particular, we predicted that white males would initiate courtship and competition more often than common males, and display higher rates of mating behaviour than the common ecotype, as noted by Blouw and Hagen (1990) and Jamieson *et al.* (1992a). Our direct comparison of the Bras d'Or Lake white and common ecotypes with those residing along the oceanic coast of Atlantic Canada also allowed us to determine if white males in these two locations use similar mechanisms to produce their nuptial coloration and conduct similar behavioural displays during mating.

MATERIALS AND METHODS

Breeding habitat

We collected and observed breeding male white and common threespine sticklebacks from a variety of locations throughout Nova Scotia and one site in Newfoundland (commons only; Table 1, Fig. 2). We measured the standard length of all fish that were caught or collected to compare body sizes among populations. As indicated in previous studies (Blouw and Hagen, 1990; Samuk, 2016), white stickleback males are smaller than common males; this has been shown to be the case for both the Atlantic and Bras d'Or populations (evolutionary-ecology.com/data/3172Appendix.pdf: Table A1, Fig. A1). Nova Scotian Atlantic males of both ecotypes were smaller than males from the same ecotype in Bras d'Or Lake. However, Newfoundland common males were larger than Nova Scotian Atlantic common males and were similar in size to Bras d'Or common males (Table A1, Fig. A1). We included

Table 1. Description of sites in Nova Scotia and Newfoundland where we observed breeding male behaviour (B) or collected breeding males to assess melanophore density and coverage (M) of both white (W) and common (C) ecotypes

Site	Study	GPS coordinates	Ecotype(s) present	Water temperature (°C)	Salinity at depth (ppt)	Water clarity	Substrate
Canal Lake, Nova Scotia	B, M	44.50°N, 63.90°W	W, C	13.0–28.0	10.3–22.6	Tea-coloured	Mud, gravel, filamentous algae
Lawrencetown, Nova Scotia	M	44.65°N, 63.32°W	W	NA	NA	Tea-coloured	Mud, filamentous algae
Rainbow Haven, Nova Scotia	B, M	44.65°N, 63.42°W	W, C	NA	NA	Tea-coloured	Mud, rocks, filamentous algae
Blues Cove, Nova Scotia	M	45.90°N, 61.09°W	C	22.6–24.8	15.9–16.6	Clear	Mud, filamentous algae
Middle River, Nova Scotia	M	45.99°N, 60.98°W	C	NA	5.0	Clear	Mud, gravel
Baddeck, Nova Scotia	B, M	46.10°N, 60.74°W	W, C	21.4–24.1	17.8–18.4	Tea-coloured	Mud, filamentous algae
Corner Brook, Newfoundland	M	48.95°N 57.95°W	C	13	16.4	Clear	Mud, gravel
Maitland, Nova Scotia	M	45.25°N 63.46°W	C	NA	NA	Clear	Mud

Note: We record ranges in water temperatures and salinities at depth during observations and detail the type of substrate and water turbidity present at each site. Note that the Canal Lake site was previously studied by Samuk (2016).

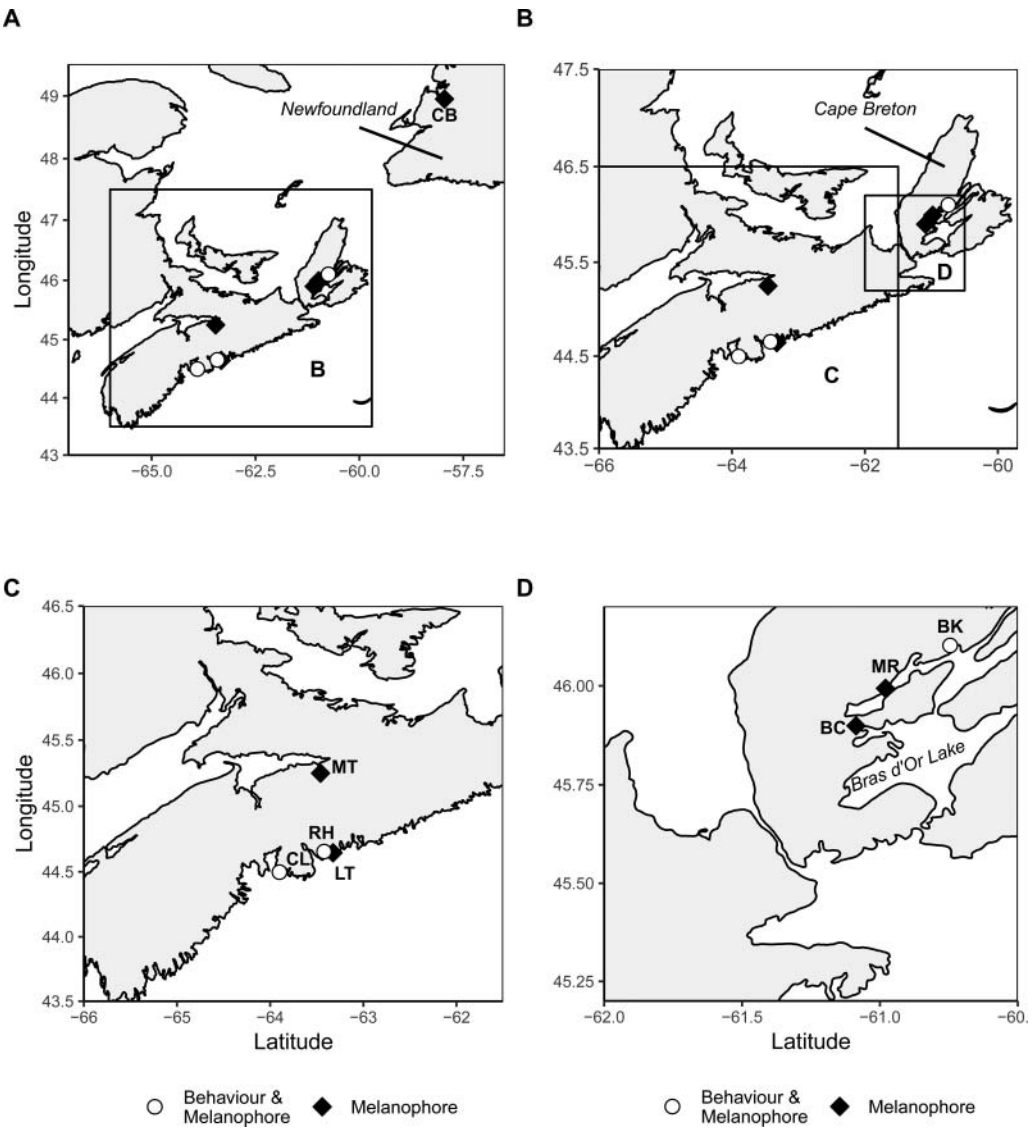

Fig. 2. Map outlining all sites where stickleback were collected for melanophore quantification (◆) and the sites where fish were collected for melanophore quantification and observed for male breeding behaviour (○). (A) All sites in this study; (B) Nova Scotia sites only; (C) Nova Scotia mainland sites that include Canal Lake (CL), Rainbow Haven (RH), Lawrencetown (LT), and Maitland (MT); (D) Bras d'Or Lake sites that include Blues Cove (BC), Middle River (MR), and Baddeck (BK). The single Newfoundland site, Corner Brook (CB), is outlined in panel (A).

Newfoundland common males because we had difficulty finding Nova Scotian common breeding males, and migratory common marine stickleback from Corner Brook, Newfoundland are genetically similar to mainland Nova Scotian coastal marine populations (Antoine Paccard, McGill University, personal communication). Therefore, we combined chromatophore

data from breeding males from mainland and Newfoundland locations and hereafter refer to this group as 'Atlantic' fish.

To locate white and common breeding males, we visited sites where previous behavioural (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a) and genetic (Samuk *et al.*, 2014; Samuk, 2016) studies occurred. We did not find breeding males from both ecotypes at most of these locations, but only visited the majority of sites once during the summer, so may have missed the stickleback breeding season. The breeding cycles of white and common threespine stickleback overlap (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a), and it is not known if there is any temporal isolation between ecotypes. We also discovered new white stickleback populations (Table 1) by targeting brackish tidal basins predicted to have slow water flow, filamentous algae, and muddy substrates (as described by Blouw and Hagen, 1990; Jamieson *et al.*, 1992a, 1992b; MacDonald *et al.*, 1995). We observed filamentous algae, such as *Cladophora* sp., at all sites where we found white stickleback (Blouw and Hagen, 1990) (Table 1). Temperature and salinity fluctuated throughout the day at all of our observation and collection sites.

Melanophore quantification

Fish collection, husbandry, and sampling

We collected breeding male threespine stickleback from May to July of 2017 and 2018 following methods approved by the Saint Mary's University Animal Care Committee (Protocol 17-18), with Department of Fisheries and Oceans Scientific Collection Permits #343930 (Maritime Region) and NL-4111-17 (Newfoundland). Mature stickleback were caught via dip netting after careful observation of male breeding colour and behaviour to classify fish as common or white stickleback.

We housed 31 wild fish in the aquatic facilities at Saint Mary's University prior to preserving fish for melanophore measurements. Fish were held at 10 ppt and 19–21°C with a photoperiod of 10 hours dark and 14 hours light to mimic the natural environment during the mating season. We fed the stickleback a combination of brine shrimp nauplii, frozen bloodworms, and *Mysis* shrimp twice a day. Nitrogenous waste concentrations were maintained within recommended levels and we conducted water changes at least once per week. We selected males to study breeding coloration if they had a red throat and blue eyes, traits found in both white and common males. We selected white males that also had a bright white dorsal body colour, while common males were blue-green or brown dorsally (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a).

We euthanized breeding male fish with a lethal concentration of buffered tricaine methane sulfonate (MS-222) and preserved them in a 50-mL falcon tube filled with formalin (37% formaldehyde) where they incubated for 2 weeks to dissolve iridophores and facilitate melanophore measurements (Greenwood *et al.*, 2011, 2012).

Melanophore density and coverage

We quantified melanophore density and coverage in the dorsal, lateral, and ventral regions of the fish by analysing photographs of the left lateral flank (Fig. 3A). Prior to taking photographs, we bisected the fish longitudinally and removed the internal organs to prevent shading that might influence our measures of melanophore coverage. We placed the left flank on the stage of an Olympus SZ61 dissecting microscope with a ruler and used trans-illumination to photograph the area adjacent to the second dorsal spine at

1.5× magnification. We used ImageJ 1.51s to conduct all analyses and converted images to 8-bit greyscale. To increase the contrast of dark pigment cells against the light skin, we used the automatic contrast function and set the scale using the 'Set Scale' function.

Using the shape tool, we added two 3 × 1 mm rectangles to the lateral and ventral body flank regions to count melanophore density and added these to the region of interest (ROI) manager prior to placement in the appropriate locations. We were not able to accurately count melanophore density in the dorsal flank of the fish because melanophores were at such a high density in all common and many white males that individual melanophores could not be distinguished. We measured nine common Atlantic and 11 common Bras d'Or males and 15 white Atlantic and five white Bras d'Or males. The bottom left-hand corner of the 'ventral' rectangle was placed on the insertion point of the pelvic spine and the 'lateral' rectangle was placed directly above the ventral box and just below the midline of the fish (Fig. 3). We then used the 'find maxima' tool in ImageJ, which highlights the darkest points in a region of interest, to highlight and count the pigment clusters. We also visually inspected each box after highlighting maxima to check for overlapping melanophores and, if needed, added in individual cells with the 'cell counter plugin' in ImageJ (Roberts *et al.*, 2017).

To measure the percentage of skin covered by dark pigment along the fish's dorsal, lateral, and ventral flank, we followed the methods of Rodgers *et al.* (2010) and Kelley *et al.* (2016) with some modifications. This measure of the percentage of the fish flank covered by melanophores does not distinguish the role of variation in chromatophore number, pigment dispersion, or the amount of pigment deposited in the cells, on melanophore coverage. We created 1.5 mm tall and 3.5 mm wide rectangles, which were slightly larger than the rectangles used to measure melanophore density, as we hoped to incorporate a larger skin surface area to measure percent cover. For the dorsal ROI, we used the insertion of the second dorsal spine as a landmark by positioning the top left corner of the rectangle at the insertion point of the dorsal spine and the lateral and ventral boxes were placed using the same landmarks as for melanophore density.

To avoid user bias, we chose the Bernsen automatic local thresholding method as the most appropriate for this study after many trials with all local thresholding methods available in ImageJ. Not only did this method best reflect what we saw, the Bernsen method exploits algorithms that set the threshold at a mid-range value calculated from the mean of the minimum and maximum grey values in the local area (Sezgin and Sankur, 2004). This ensures that each pixel depicting the melanophore patterns on the small sample regions we chose are considered, accurately reflecting the melanophore pattern. Following conversion to its binarized form, we measured the percentage of dark pixels in the selected ROI using the 'threshold analysis' tool in ImageJ.

Behavioural patterns

Field observations

Behavioural observations of both ecotypes were conducted in mainland Nova Scotia at Canal Lake and Rainbow Haven and in Bras d'Or Lake we observed fish at Baddeck. Prior to observations in Canal Lake, we captured and tagged male white and common threespine stickleback using subcutaneous visual implant elastomer tags (Northwest Marine Technologies). We recorded standard length using Vernier callipers, photographed the fish next to a colour standard, and took anal fin clips that were stored in ethanol for later genetic

work. Following tagging, we returned individuals to their territories and did not resume behavioural observations until the following day to give the male time to return and re-acclimate to his nest site. We did not tag or fin clip fish from Rainbow Haven (mainland) or Baddeck (Bras d'Or Lake), and began observations on territorial males once we arrived on site. The tagging and field observation procedures were approved under the SMU Animal Care Committee Protocol 16-16.

We quantified eight focal behaviours (Table 2) related to courtship, nest-building, and aggression that have been the focus of previous work on stickleback (van Iersel, 1953; Blouw and Hagen, 1990; Jamieson *et al.*, 1992a; Wootton and Fletcher, 2009). Prior to the start of a behavioural observation, two observers agreed upon the ecotype of the male, based upon nuptial colour and nest-site location. Therefore, the observers were aware of the male ecotype during observations. We then conducted 5-minute visual observations of male mating behaviour adapted from the procedure outlined by MacDonald *et al.* (1995). During the observation period, the observer tallied the frequency of each behaviour in a waterproof field book; each behavioural category was outlined prior to observation. Because males are very active, we could observe all breeding behaviours within a 5-minute period. The same observer performed all behavioural tallies.

The total sample size for mainland Nova Scotia sites (Canal Lake and Rainbow Haven) was $n = 50$, $N = 96$ for white males (where n represents the number of unique nest-sites and N represents the total number of observations, including multiple observations of males at the same nest) and $n = 21$, $N = 34$ for common males. For Bras d'Or Lake fish from Baddeck, total sample size was $n = 19$, $N = 36$ for white males and $n = 8$, $N = 8$ for common males.

Focal observations of individual male fish behaviour occurred at least once a day and, if visibility and nest attendance allowed, we observed an individual a second time during a

Table 2. Focal behaviours measured in the field to assess male courtship, nest-building, and aggressive behaviour based on previous work (van Iersel, 1953; Blouw and Hagen, 1990; Jamieson *et al.*, 1992a; Wootton and Fletcher, 2009)

Behavioural category	Focal behaviours	Description of male behaviour
Courtship	Zig-zag dance	Swims rapidly back and forth in a 'Z-shaped' pattern
	Dorsal prick	Pricks female in the abdomen with dorsal spines
	Circle	Swims around female in a circular pattern
	Leads to nest	Turns with flank facing the surface of the water and swims in the direction of his nest as the female follows
Nest-building	Material retrieval	Picks up nest-building material (e.g. algae) with mouth and returns to nest
	Nest tend	Uses spiggin (glue produced by kidneys) to maintain nest integrity. Male swims through nest to maintain tunnel shape
Aggression	Chase conspecifics (white and common stickleback)	Swims rapidly towards intruder to defend nest and territory as intruder flees
	Bite	Charges then bites intruder. Observed when chasing is unsuccessful (intruder does not flee)

different tidal cycle to account for potential behavioural variability resulting from tidal influence. We began our observations at the beginning of the breeding season, when males appeared to have established a territory with a nest. We observed the males on nests within a site on a given day in random order. In general, white males were more active than common males and displayed all types of mating behaviours in the 5-minute observation period, and tended to court gravid females of any stickleback species, a behaviour also noted by Jamieson *et al.* (1992a). This difference in activity levels has been observed previously (Jamieson *et al.*, 1992a), and is likely an inherent difference between the two ecotypes. Female stickleback of both ecotypes were present in the nesting areas, and both common and white males were in the proximity of gravid females when they were on their nests. Furthermore, both ecotypes of females will visit white and common males (Blouw, 1996), and males court both types of females. We recorded each instance of behaviour 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, we recorded it as two separate instances. However, when males engaged in zig-zag courtship behaviour for the entire observation period without pause, we recorded each zig-zag to a female as a separate occurrence. Within the aggression category, we recorded two instances of chasing if a male returned to its territory after a chase and chose to leave its territory again to charge at the same intruder.

Because white males do not conduct parental care or fan embryos, parental fanning behaviour was not included in our analyses. However, fanning behaviour related to 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 throughout the 5-minute observation period, we recorded the behavioural frequency as zero. We recorded the behaviour of all individuals possessing territories at the site in a randomized order each day.

Statistical analyses

Analyses were carried out in R v.3.4.3 (R Core Team, 2017). For all model comparisons, we used Akaike Information Criterion values corrected for small sample sizes (AICc) and compared all possible models. To determine that a single model best fit the data, we chose the model with the lowest AICc, provided that the AICc difference between the best model and other models was greater than 2.

Melanophore data were analysed using generalized linear models (GLM). To assess the potential effects of ecotype (white, common) and location (Bras d'Or or Atlantic) on the number of melanophores present in ventral and lateral areas on the left side of the fish, we competed models with Poisson error distributions appropriate for count data. We constructed similar models for the percent coverage of melanophores in the dorsal, lateral, and ventral regions, but used GLMs with binomial error distributions as the data were proportions.

We used the lme4 package (Bates *et al.*, 2015) to construct generalized linear mixed models (GLMM) with Poisson error distributions to examine the effects of ecotype (white, common) and location (Bras d'Or or Atlantic) on the frequencies of courtship, nest-building, and aggressive behaviours. We entered each nest site as a random factor to account for unknown differences among individuals and the repeated observations that were made in the experiment. Using this approach, we analysed the data in two ways: first, we treated the dependent variable as the sum of all focal behaviour that occurred in one of the three main categories outlined in Table 2 (i.e. courtship, nest-building, and aggression); second, we

examined each of the individual behaviours within each category in isolation (results in Tables A2 and A3).

RESULTS

Melanophore quantification

White males from both coastal oceanic and the Bras d'Or Lake populations had lower melanophore densities and percent coverage than sympatric common males in all areas for which measurements were made (Table 3; Fig. 3B–F; melanophore density was too high in the dorsal region for an accurate assessment of density). In addition, there was an interactive effect of ecotype and location on some melanophore measures. Atlantic white males

Table 3. Generalized linear model comparison of the effect of geographic location (Bras d'Or, Atlantic) and ecotype (common, white) on the density and percent coverage of melanophores in different body regions

Body region	Model	*df*	AICc	ΔAICc	ω_i
Density					
Lateral	**Ecotype + Location**	**3**	**1296.7**	**0.0**	**0.64**
	Ecotype + Location + Ecotype × Location	**4**	**1298.0**	**1.3**	**0.33**
	Ecotype	2	1302.9	6.2	0.03
	Location	2	1661.1	364.4	0.00
Ventral	**Ecotype + Location + Ecotype × Location**	**4**	**725.5**	**0.0**	**1.00**
	Ecotype	2	747.6	22.1	0.00
	Ecotype + Location	3	749.9	24.4	0.00
	Location	2	816.1	90.6	0.00
	Intercept	1	816.9	91.4	0.00
Percent coverage					
Dorsal	**Ecotype + Location + Ecotype × Location**	**4**	**587.6**	**0.0**	**1.00**
	Ecotype	2	618.9	31.3	0.00
	Ecotype + Location	3	620.7	33.0	0.00
	Location	2	889.9	302.2	0.00
	Intercept	1	898.1	310.5	0.00
Lateral	**Ecotype + Location + Ecotype × Location**	**4**	**415.6**	**0.0**	**0.83**
	Ecotype + Location	3	418.7	3.1	0.17
	Ecotype	2	437.9	22.3	0.00
	Location	2	483.7	68.1	0.00
	Intercept	1	488.2	72.6	0.00
Ventral	**Ecotype + Location**	**3**	**316.9**	**0.0**	**0.62**
	Ecotype + Location + Ecotype × Location	4	319.0	2.1	0.22
	Ecotype	2	319.5	2.6	0.00
	Intercept	1	345.9	29.0	0.00
	Location	2	346.1	29.2	0.00

Note: df = model degrees of freedom; AICc = Akaike Information Criterion corrected for sample size; ΔAICc = difference between models with lowest AICc values and all other models; and ω_i = model weight. Models with the lowest AICc scores by a value of 2 or more are shown in **bold**.

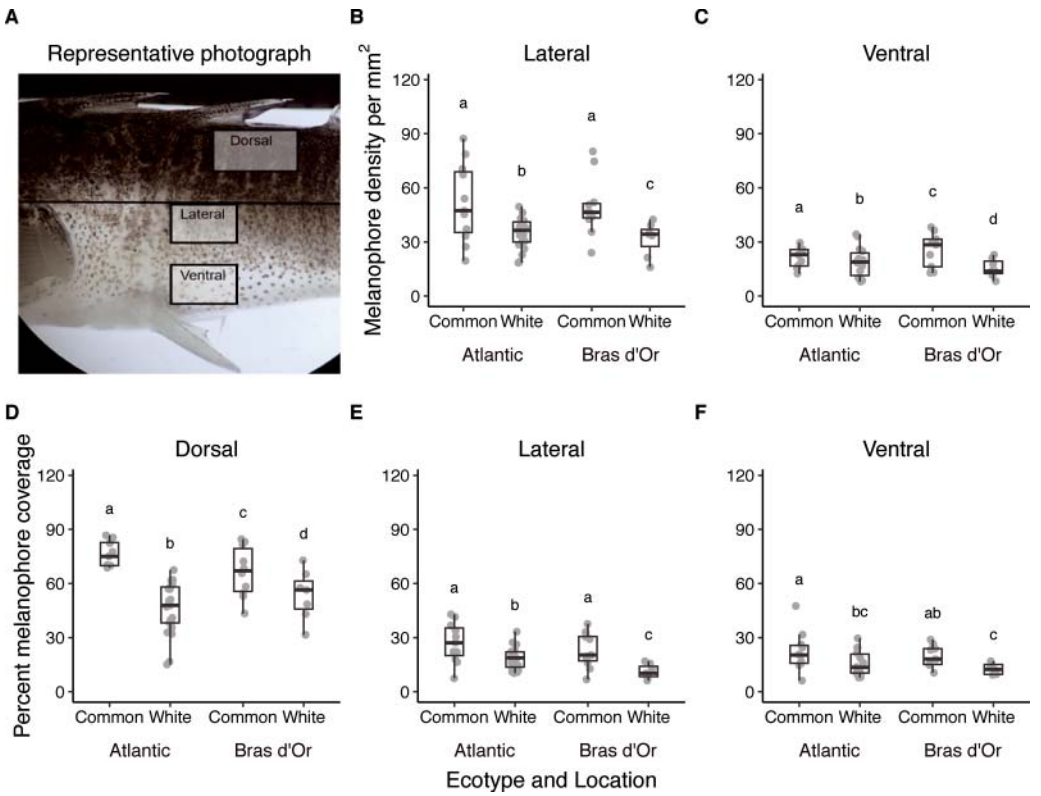

Fig. 3. Representative photograph of the preserved flank of a white stickleback from the Bras d'Or Lake (A), lateral melanophore density (B), ventral melanophore density (C), and dorsal (D), lateral (E), and ventral (F) melanophore coverage of Atlantic white ($n = 15$) and common ($n = 9$) males and Bras d'Or white ($n = 5$) and common ($n = 11$) males. Bars represent the median, box edges represent the 25th and 75th percentiles, and grey dots represent individual samples. Differences between groups determined by Tukey *post-hoc* tests are indicated by different letters.

had a higher density and coverage of lateral melanophores and higher ventral melanophore density than Bras d'Or white males (Table 3; Fig. 3B, C, E). However, Bras d'Or white males had a higher dorsal melanophore coverage than Atlantic white males (Table 3; Fig. 3D). Bras d'Or common males had a higher ventral melanophore density, but a lower dorsal melanophore coverage, than coastal Atlantic common males (Table 3; Fig. 3C, D). In all cases, male stickleback had the highest average melanophore coverage dorsally and the lowest density ventrally (Fig. 3D, F).

Behavioural patterns

Eight focal behaviours were compiled to compare courtship (points and leads to nest, zig-zags, and dorsal pricking and circling), nest-building (material retrieval and nest maintenance), and aggression (chasing and biting conspecifics), in white and common males from both coastal Atlantic and Bras d'Or sites (Tables 2 and 4; Fig. 4).

Both ecotype and location were important predictors of total courtship frequency (sum of zig-zags, leads and points, dorsal pricking and circling in a 5-minute observation period; Table 4; Fig. 4A). The effect of location on the frequency of total courtship varied based upon ecotype, with similar courtship frequencies by white males from the Bras d'Or Lake and coastal Atlantic sites, and slightly higher courtship rates for common Bras d'Or males than common Atlantic males (Fig 4A). In both locations, white males had higher total courtship rates than sympatric common males (Table 4; Fig. 4A) because of a higher rate of zig-zags (Fig. 4A; Table A2) and more leads and points to the nests (Fig. 4A; Table A2). As previously noted by Blouw and Hagen (1990), white males did not exhibit dorsal pricking behaviour (Fig. 4A).

Two models best fit the data for total nest-building frequency (sum of material retrieval and nest-tending); one that included no fixed effects, and an almost equivalent model that included ecotype. White males tended to perform nest-building behaviours at a slightly higher frequency than common males (Table 4; Fig. 4B; Table A2). White and common males did not show large differences in nest-tending frequency, but white males from Bras d'Or had slightly higher nest-tending frequencies than all other males (weak effects of ecotype and location retained in the model; Fig. 4B; Table A2).

Aggression frequency was similar in both ecotypes and locations; however, males from Bras d'Or were slightly more aggressive than other males, as indicated by the weak effect of location in the second-best model (Table 4; Fig. 4C).

Table 4. Generalized linear mixed effects models with Poisson distribution indicating the effect of ecotype (common, white), geographic location (Mainland, Bras d'Or), and their interaction on frequency of mating behaviours

Behaviour	Models	*df*	AICc	ΔAICc	ω_i
Courtship	**Ecotype + Location + Ecotype × Location**	**5**	**943.5**	**0.0**	**0.52**
	Ecotype	**3**	**944.7**	**1.2**	**0.29**
	Ecotype + Location	4	945.6	2.1	0.19
	Intercept	2	962.2	18.7	0.00
	Location	3	962.5	19.0	0.00
Nest-building	**Intercept**	**2**	**1222.9**	**0.0**	**0.37**
	Ecotype	**3**	**1223.3**	**0.4**	**0.32**
	Location	3	1225.0	2.1	0.14
	Ecotype + Location	4	1225.2	2.3	0.12
	Ecotype + Location + Ecotype × Location	5	1227.2	4.3	0.04
Aggression	**Intercept**	**2**	**604.4**	**0.0**	**0.42**
	Location	**3**	**605.3**	**0.9**	**0.26**
	Ecotype	**3**	**606.1**	**1.7**	**0.17**
	Ecotype + Location	4	606.9	2.5	0.115
	Ecotype + Location + Ecotype × Location	5	609.0	4.6	0.04

Note: Courtship and nest-building are sums of the different behaviours in these categories. *df* = model degrees of freedom; AICc = Akaike Information Criterion corrected for sample size; ΔAICc = difference between models with lowest AICc values and all other models; and ω_i = model weight. Models with the lowest AICc scores by a value of 2 or more are shown in **bold**.

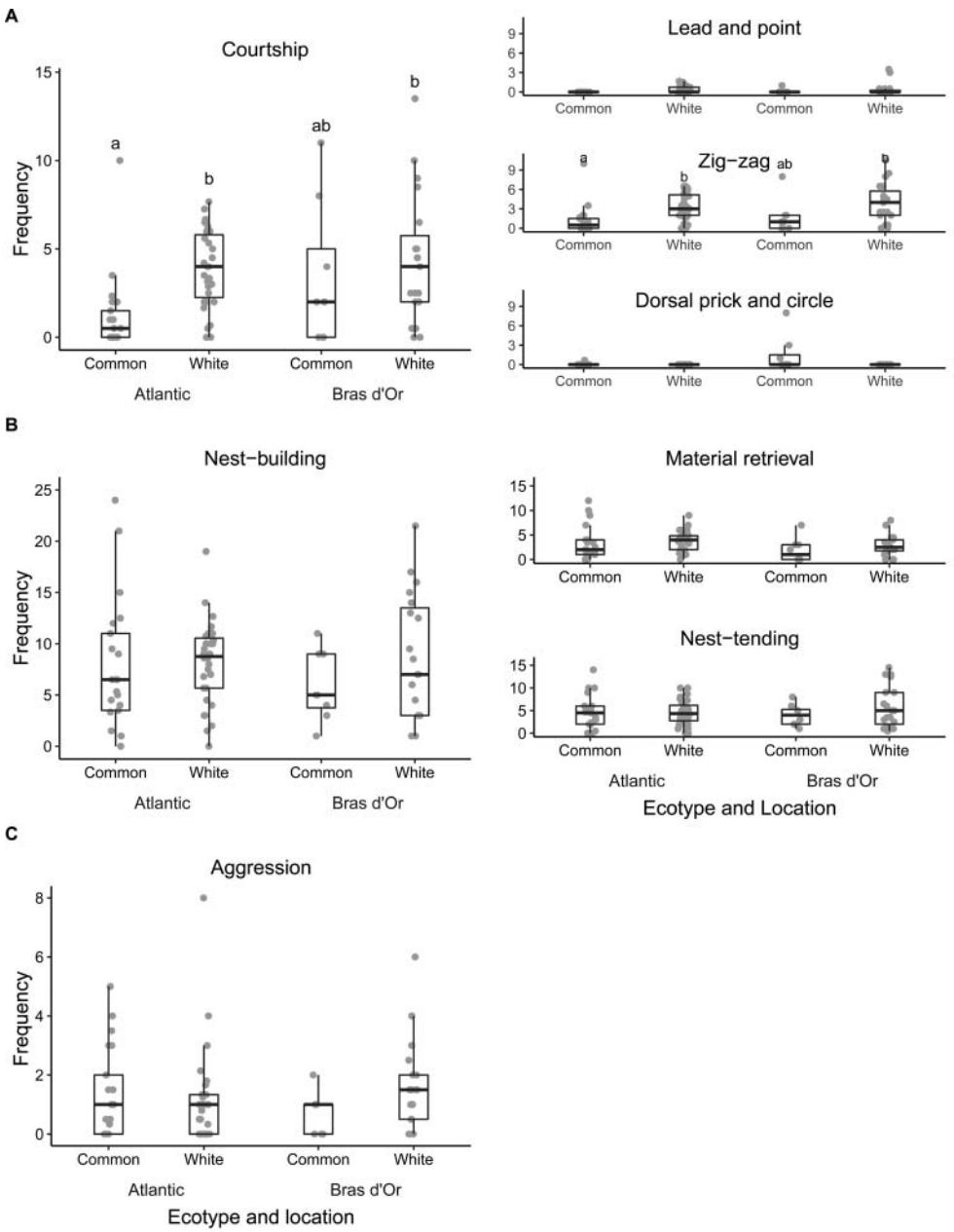

Fig. 4. Frequency of mating behaviour over 5-minute observation periods in male threespine stickleback for white ($n = 50$, $N = 96$) and common ecotypes ($n = 21$, $N = 34$) from the mainland (Canal Lake and Rainbow Haven), and for white ($n = 19$, $N = 36$) and common ecotypes ($n = 8$, $N = 8$) from Bras d'Or Lake. (A) Total courtship behaviours (zig-zag, lead and point to nest, dorsal pricking, circling); (B) total nest-building behaviour (material retrieval, nest-tending); and (C) aggression. Black bars represent the median and grey dots represent means for all observation periods for an individual fish. Mean individual recordings and the effect of individual are accounted for in the statistical analysis as a random factor.

DISCUSSION

In this study, we compared male nuptial coloration and breeding behaviour in white and common threespine stickleback ecotypes from two potentially distinct population sets (Atlantic oceanic vs. Bras d'Or Lake) in Atlantic Canada. To better understand the cellular basis for white nuptial coloration, we quantified the density and coverage of melanophores (chromatophores containing black and brown pigments) in white and common males. We found that breeding white males in both geographic locations have less skin covered by melanophores, and a lower melanophore density, than sympatrically breeding common males. We also studied multiple aspects of breeding behaviour in the field (courtship, nest-building, and intra-sexual aggression) and found that white males from both populations courted females more frequently than sympatric common males. However, the frequency of nest-building and aggressive behaviour did not vary markedly between ecotypes or locations. Overall, our data suggest that a decrease in melanophore coverage contributes to white nuptial coloration in both Bras d'Or Lake and coastal oceanic white males, and that white males from both populations use similar behavioural strategies (increased courtship) to attract females.

Divergence in nuptial coloration between white and common breeding males

The pearlescent white body colour displayed by breeding white stickleback males could result from a variety of cellular mechanisms associated with one or more of the different fish chromatophores. For example, the lightening of skin colour in white males compared with common males might be due to a reduction in melanophore coverage (reviewed by Sköld *et al.*, 2016). In this study, we found that breeding white males from both the Atlantic coast and the Bras d'Or Lake have a lower percentage of dorsal, lateral, and ventral skin covered by melanophores than sympatric common males. Breeding white males also have a lower density of lateral and ventral melanophores. (Note that dorsal melanophore density could not be measured, as cells were too closely packed together to distinguish individual cells.) Differences in overall coverage could be due to decreases in melanophore number or size, a reduction in the amount of eumelanin deposited per melanophore, or increased pigment aggregation; our data suggest that reduced melanophore coverage in white compared with common males is at least partially explained by a reduction in cell number. In sticklebacks, melanophores are restricted to the dermis and are found in two layers – superficial and deep (Burton, 1978, 1979). We did not distinguish between these two melanophore types, so future work will aim to quantify superficial and deep melanophore densities and assess melanophore size, eumelanin content, and pigment dispersion to hone in on the specific mechanisms underlying the reduction in melanophore coverage in white compared with common stickleback males.

While a reduction in melanophore density can lighten a fish's skin, this alone cannot produce the bright coloration seen in breeding white males. Thus, changes in melanophore characteristics must act in concert with changes in white-producing chromatophores in the white stickleback. Both leucophores and iridophores can produce a pearlescent whiteness (reviewed Sköld *et al.*, 2016). Leucophores are a type of dendritic cell containing light-reflecting pigments made of uric acid or other purines, and iridophores are normally non-dendritic cells containing reflecting platelets made of purines or pteridine crystals that can lead to the production of a variety of iridescent colours, including white, if the multi-layered thin-film

interference is ideal (reviewed by Fujii, 1993; Bagnara and Matsumoto, 2007). Leucophores are phylogenetically restricted to only a few groups of fishes [e.g. medaka (Kimura *et al.*, 2014)], and we did not observe them in our stickleback males. Therefore, we hypothesize that increases in iridophore density, reflecting platelet density, or iridophore size act in combination with reductions in melanophore coverage to produce the bright white dorsal coloration of breeding white stickleback males. Indeed, Goda and Fujii (2001) found that an increase in the thickness of the dermal iridophore layer is associated with the bright white spots of the domino damselfish.

While this research focused on differences in breeding coloration among white and common ecotypes, we also observed a great deal of colour plasticity. We found that bright white males become drably coloured, and more cryptic, within seconds of a disturbance (Blouw and Hagen, 1990; Haley, 2018). Alternatively, common males blanch rapidly when disturbed, as occurs in many fishes when stressed (reviewed by Sköld *et al.*, 2013), making the two ecotypes difficult to distinguish when caught. The rapid blanching of common stickleback is the result of deep melanophore pigment aggregation (Burton, 1978), and it is possible melanophore pigment dispersal leads to the development of cryptic coloration in whites. This rapid colour change might allow the white stickleback, and other species with bright white coloration (e.g. Galván-Villa and Hastings, 2018), to balance nuptial signalling with potential predation risks. It is not yet clear if the white breeding coloration is a signal involved in male–male competition or female choice. However, there is evidence that the degree of iridescent white coloration is correlated with higher courtship frequency, and not aggression, in breeding white males, suggesting that it is associated with mate choice in the white ecotype (Haley, 2018).

Breeding ecology of white and common males

Our results indicate that both populations of white males are more active in breeding than common males, particularly with respect to courtship behaviour, as was found in previous studies (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a; MacDonald *et al.*, 1995). Thus, these behavioural differences among ecotypes have remained stable for over 25 years (Blouw and Hagen, 1990). The increased energetic investment in breeding behaviour in white males may be possible because of corresponding physiological and behavioural adaptations (Blouw and Hagen, 1990). For example, white males do not invest in parental care (Jamieson *et al.*, 1992b; Blouw, 1996), and can theoretically expend more energy on pre-mating courtship behaviour to maximize their number of mates and offspring, if all other energetic costs are equal. This relationship between parental care and courtship has been observed in marine common males; males who spent less time fanning their nests were more likely to court more females (Gross and Sargent, 1985). Similarly, von Hippel (2000) found that increased rates of courtship were correlated with reduced fanning and decreased egg survival in an estuarine stickleback population. This lack of parental care is not seen in any other ecotype of threespine stickleback, with the possible exception of lighter coloured, 'hard-bottom' males in the Baltic that breed on hard substrate or algae and show a reduction in fanning behaviour (Borg, 1985).

Marine threespine stickleback males show decreased courtship and nuptial coloration in areas with increased predation (FitzGerald and Dutil, 1981; Pressley, 1981). Therefore, white males may be able to court more energetically and display brighter nuptial coloration because their breeding ground is densely vegetated and provides additional protection from predators, compared with common males breeding in open areas (Jamieson *et al.*, 1992a). Both 'hard-bottom' Baltic and Nova Scotia white males may have an advantage over males breeding in

open sites if the increased potential for offspring protection provided by the algae entices white females to the nest, thereby increasing the frequency of courtship behaviour observed in white males.

In addition to differences between white and common male courtship frequency, we observed differences in the type of courtship behaviour exhibited by the two ecotypes. White males primarily use zig-zag behaviour to attract females, and rarely performed dorsal pricking; this result supports previous findings on white stickleback male courtship behaviour (Jamieson *et al.*, 1992a). Dorsal pricking may serve as a less conspicuous form of courtship in areas of high cannibalism, aid the male in displaying dorsal spines, or allow the male to assess a potential female and induce spawning (Bell and Foster, 1994; Kitano *et al.*, 2009).

We observed a difference in courtship behaviour between white males from the Atlantic and Bras d'Or populations, whereby the Bras d'Or fish courted less frequently than the Atlantic fish. While this may be an indication of population differences, we cannot rule out other correlated ecological factors that may influence courtship rate. For example, we did not measure female encounter rates, and our observations in the Bras d'Or population occurred over a short time towards the later end of the breeding season. Thus, we interpret this result with caution, especially in light of the relatively small sample size in the Bras d'Or population compared with the Atlantic population, and the fact that we only observed one population in each region.

We did not find that the frequencies of intra-sexual aggression and nest-building showed consistent differences between white and common males. Both common and white males establish and defend a territory prior to the arrival of females; females tend to enter and leave the spawning areas in groups, possibly associated with tidal changes. Within common males, nest-building tends to peak before males have eggs in their nests, although occasional nest maintenance is still performed while tending fertilized eggs to ensure proper nest ventilation and the removal of mouldy eggs (van Iersel, 1953; reviewed by Wootton, 1976). Similarly, common males remain at their nest during the parental phase to defend it from predators (van Iersel, 1953; reviewed by Wootton, 1976). By contrast, white males do not defend their nests, but rather return to nest-building after scattering eggs in the algal substrate after spawning (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a; Blouw, 1996). Both types of males are highly territorial, and similar levels of aggression are not unexpected. Territory establishment is the first phase of the mating cycle, and the quality of the territory may influence mating success; however, territorial defence is necessary for both white and common males, and in this study the two ecotypes had similar frequencies of aggressive behaviour. These similarities in aggressive behaviour, in conjunction with the differences in courtship between the two ecotypes, suggest that white males may increase their mating success mainly through inter-sexual selection, rather than male–male competition.

Evolution of the white ecotype

A major goal of this study was to directly compare white and common ecotypes from the Bras d'Or Lake and the rest of Atlantic Canada to determine if white males from these two areas use similar breeding strategies. This interest stemmed from Samuk's (2016) finding that common and white stickleback from the Atlantic form a separate clade from Bras d'Or commons. At present, the phylogenetic relationships of the white sticklebacks from these two locations are not known, so we hypothesized that the white stickleback in the Bras d'Or might be phenotypically and genetically divergent from coastal Atlantic whites. These geo-

graphical differences are reflected in larger body size in Bras d'Or common and white males compared with their Nova Scotian Atlantic counterparts (Samuk, 2016; this study). However, our melanophore and behavioural data largely suggest this is not the case; we observed some differences in melanophore characteristics and breeding behaviour between white males from the mainland and Bras d'Or Lake, but these traits were generally similar within ecotypes in both locations. These findings are consistent with several different hypotheses about the origin of the white stickleback. For example, our finding of similar behavioural and melanophore phenotypes in Bras d'Or and coastal white males might occur because the white ecotype has a single, common origin or because the white form evolved independently in the two locations and the breeding phenotypes we studied have converged as a result of new mutations with similar phenotypic effects selected due to comparable ecological pressures or gene flow. Further population genetic studies are required to tease apart the evolutionary history of the Bras d'Or and coastal Atlantic white and common threespine stickleback populations.

Another goal of this study was to further characterize the 'white' phenotype to gain insight into the potential physiological mechanisms underlying the correlated divergence in nuptial coloration and behaviour. The evolution of colour divergence among populations and colour polymorphisms within a species are often associated with changes in behaviour or physiology that might occur because of genetic linkage or pleiotropy (reviewed by McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). The ability of hormonal signalling to exert control on multiple systems throughout an organism's body means that evolutionary variation in the neuroendocrine system often has pleiotropic effects (reviewed by Zera *et al.*, 2007). Such pleiotropic hormonal effects are predicted to underlie many associations among coloration and other traits (McKinnon and Pierotti, 2010). For example, changes in both behaviour and body coloration stem from variation in the melanocortin system in colour polymorphic cichlids (Dijkstra *et al.*, 2017). We focused on melanophores because increased melanin-based body coloration is often correlated with variation in a variety of other behavioural and physiological traits (reviewed by Ducrest *et al.*, 2008; San-Jose and Roulin, 2018). Indeed, darker coloration resulting from higher overall melanocortin and receptor levels is generally associated with increased sexual activity, fecundity, stress resistance, aggressiveness, and body size in fishes (reviewed by San-Jose and Roulin, 2018). White males are smaller, as predicted if decreased melanocortin system activity leads to white skin colour, but have similar aggressiveness and higher sexual activity than commons (Blouw and Hagen, 1990; Jamieson *et al.*, 1992a; Blouw, 1996; Samuk *et al.*, 2014), opposite to what occurs in other fishes displaying melanocortin system variation (San-Jose and Roulin, 2018). If melanocortin system evolution is the mechanism underlying the white phenotype, these deviations from the trends summarized by San-Jose and Roulin (2018) could occur for a variety of reasons; for example, if only a sub-set of melanocortin receptors have evolved, or if evolutionary variation in melanocortin antagonist expression is tissue-specific. In addition, because the white males do not provide parental care, it is possible that these pleiotropic effects normally associated with melanocortin system variation may be uncoupled because of strong female choice for increased rates of courtship as a signal of reproductive vigour to ensure mating success.

The potential shared regulatory control of skin coloration and the behaviours (increased courtship, loss of parental care) and morphological traits (smaller body size) associated with the evolution of the white phenotype suggests that teasing out the selective pressures responsible for the initial evolution of white coloration will require a comprehensive approach. Future research examining the mechanisms leading to white breeding coloration

should test the hypothesis that hormonal pleiotropy leads to a suite of characteristics that have evolved in this ecotype. Interestingly, McLennan and McPhail (1989) found that anadromous male threespine stickleback turn snowy white just after creeping through the nest or gluing, and then actively zig-zag. The authors suggest that this 'white flush' can predict spawning readiness. If this is the case, and if females are more likely to spawn with 'flushed white' males, white nuptial coloration may be a direct target of selection. As such, the evolution of white dorsal breeding coloration might have occurred by genetic assimilation of this plastic colour change. Indeed, the intensity of white coloration is correlated with increased courtship rates in the wild (Haley, 2018). Thus, the degree to which white coloration and mating behaviour are preferred by females, and are co-regulated by hormonal changes, warrants further study.

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