Signature Page

The mechanisms underlying nuptial colouration in the white Threespine Stickleback (*Gasterosteus aculeatus*)

By
Morgan Lindsay MacDonald

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

April 2020, Halifax, Nova Scotia

Approved: _____
Dr. Anne Dalziel
Supervisor

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Dr. Laura Weir
Reader

Date: May 1st, 2020

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Abstract

Often the most striking displays of colour in animals arise from interactions during mating. Nuptial colouration is exclusively associated with mating and typically functions in either female mate choice by signalling male quality, or male competition by asserting dominance and possession of a territory. Animal colouration is the result of various chromatophores found within the integument. Changes in the number and distribution of chromatophores and pigments over time causes seasonal colour change associated with nuptial colouration. The Threespine Stickleback (*Gasterosteus aculeatus*) is a teleost fish found throughout the northern hemisphere that displays large variation in nuptial colouration among populations. The white Threespine Stickleback, endemic to Nova Scotia, displays a pearlescent white colour during breeding season in contrast to the common Threespine Stickleback which is a dark green/brown colour. Although Threespine Sticklebacks are a model system for many evolutionary studies, the mechanisms involved in the evolution of the contrasting nuptial colouration of the white and common males are not well understood. The purpose of this study was to determine the cellular mechanisms underlying the differences in nuptial colouration of these ecotypes. In particular, I looked at both melanophore and iridophore coverage as well as melanophore number and percent of isolated iridophores in the two dermal chromatophore layers of stickleback. My results indicate that common males have a higher melanophore coverage and fewer isolated iridophores in the deep dermal layer than the white males. However, the number of melanophores and the iridophore coverage in each layer, as well as the surface melanophore coverage, did not significantly differ between the two groups. These finding indicate that the integumental chromatophores in these males are quite morphologically similar in number and distribution. Future studies should investigate the thickness of chromatophores and the orientation and spacing of platelet crystals within iridophores among white and common stickleback.

April 2020

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Acknowledgments

I would like to thank my supervisor Dr. Anne Dalziel for all her support and guidance throughout the past year, as well as my reader Dr. Laura Weir for her helpful feedback. Thank you to the Dalziel/Weir Fish lab and Caila Lebans for always being available and eager to assist me, whether in writing or fish care. I would also like to thank Dr. Tamara Franz-Oodendal and Dr. Shirine Jeradi from Mount Saint Vincent University for welcoming me into their lab and allowing me to use their cryostat, without which this project would not be possible. Thank you to Terrell Roulston for helping me learn how to code, and Alexa Tymkiw for motivating and supporting me throughout this process. Finally, funding was provided by the Natural Sciences and Engineering Research Council of Canada and is gratefully acknowledged.

Introduction

1.1 Speciation and Divergent Sexual Selection

One of the goals of evolutionary biology is to understand the processes that lead to the formation of new species (Butlin et al., 2012; Lowry, 2012; Ravinet et al., 2017; Schluter, 2001). According to Ernst Mayr's biological species concept, species can be defined as "groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 1969). Therefore, to understand speciation in sexually reproducing organisms it is imperative to study the causes of reproductive isolation (Schluter, 2001). Reproductive isolation can occur at different stages during the mating process. For sympatric populations, mate choice is often an important reproductive barrier between species (Butlin et al., 2012; Kozak, Rudolph, Colon, & Fuller, 2012). If individuals within a population possess a preference for a certain phenotype, then sexual selection may occur (Servedio & Boughman, 2017).

Sexual selection is an evolutionary process that arises from differences in individual mating success due to a particular trait, leading to a change in that trait's frequency (Servedio & Boughman, 2017). Divergence in sexual selection between populations can lead to speciation (Butlin et al., 2012; Schluter, 2009; Servedio & Boughman, 2017) by causing the formation of different groups within a population, which then become reproductively isolated, further reducing gene flow between the two groups (Servedio & Boughman, 2017). Therefore, the evolution of divergence in mate choice can be closely related to speciation, and understanding the mechanisms underlying differences in sexually selected traits may help to better understand speciation (Boughman, Rundle, & Schluter,

2005; Marques et al., 2017). Male nuptial colouration is a sexually selected trait that can be an important factor influencing mate choice and eventually lead to speciation in many species, such as in cichlid fishes (Boughman et al., 2005; Marques et al., 2017).

1.2 Nuptial Colouration

Some of the most incredible colouration in animals arises from the interaction between males and females in the pursuit of mates (Kronforst et al., 2012). Nuptial colouration is colouring that is exclusively associated with mating, and typically evolves in one of two ways: from female mate choice (intersexual selection) or by male/male competition (intrasexual selection) (Hunt, Breuker, Sadowski, & Moore, 2009). However, in some circumstances the males are choosey and the females compete for access to males (Amundsen & Forsgren, 2001). Nuptial colouration is often important in mate choice and therefore, may commonly evolve during speciation by sexual selection (Dijkstra, Seehausen, Pierotti, & Groothuis, 2007; Kodric-Brown, 1998; Price, Weadick, Shim, & Rodd, 2008; Seehausen & Schluter, 2004). An example of speciation driven by sexual selection on nuptial colouration occurs in cichlid fishes (Allender, Seehausen, Knight, Turner, & Maclean, 2003). Cichlids have diverged into over 1000 different species, many of which are sympatric and differ in nuptial colouration (Allender et al., 2003). For these fish, nuptial colouration is extremely important in mate choice and may contribute more strongly to reproductive isolation than other ecological factors, and therefore is an important mechanism in their rapid speciation (Allender et al., 2003).

Nuptial colouration can sometimes be indicative of an individual's ability to ward off predators (Hill, 1991), as bright colouration can indicate a healthier, stronger mate. Because of this, females often initiate and drive the evolution of varying colour displays in

males (Kronforst et al., 2012). House finches are an excellent example of an animal in which male nuptial colouration is an honest signal to prospective mates. The red and orange pigments throughout the bird's plumage are carotenoid based, and they must acquire this pigment from food (Hill, 1991; Hill & Farmer, 2005; Lendvai, Giraudeau, Németh, Bakó, & McGraw, 2013). A deeper red colour indicates the male has a better food source, is more resistant to certain parasites, and is more attentive to the female during breeding, all of which indicate a better mate (Hill, 1991; Hill & Farmer, 2005). Alternatively, female colour preference can also be the result of sensory drive, whereby environmental cues promote selection of features within the sensory system important in a particular habitat, which may lead to runaway selection or indirect benefits (Cummings & Endler, 2018; Maan, Hofker, Van Alphen, & Seehausen, 2006). Females may gain indirect benefits by detecting colour if the selected colours indicate male genetic quality (Montoya & Torres, 2014).

1.3 Mechanisms Underlying Nuptial Colouration in Fishes

In fishes, amphibians, reptiles, crustaceans and cephalopods most external colouration is the result of pigment cells known as chromatophores (Cal, Suarez-Bregua, Cerdá-Reverter, Braasch, & Rotllant, 2017; Fujii, 2000). Changes in chromatophore number, size, distribution, and pigment content result in the varying nuptial colourations among fish (Sköld, Aspengren, Cheney, & Wallin, 2016). There are several types of chromatophores, each varying in the type of pigment they contain (light reflecting or absorbing), the colour of pigment they contain, their shape, arrangement, and response to hormones (Schartl et al., 2016; Sköld et al., 2016). In particular, fish have a number of different chromatophore types, including melanophores, iridophores, leucophores, xanthophores, and erythrophores (Sköld et al., 2016). Melanophores are light absorbing

dendritic cells containing brown or black eumelanin pigments (Schartl et al., 2016; Sköld et al., 2016). Iridophores are light reflecting cells that contain thin, flat, reflecting platelets containing purines, particularly guanine platelet crystals (Oliphant & Hudon, 1993; Schartl et al., 2016). Iridophores appear iridescent when the platelets are highly organized in stacked rows oriented in the same direction and more white when platelets are less organized and randomly scattered at different angles (Schartl et al., 2016). Additionally, the structure of iridophores can influence colour by altering the way light is reflected, producing colours ranging from blue to red, such as in the paradise whiptail (Price et al., 2008). Leucophores are light scattering dendritic cells containing mainly uric acids that also appear white in colour (Schartl et al., 2016; Sköld et al., 2016). Xanthophores and erythrophores are light absorbing dendritic cells with pteridine and carotenoid pigments that range in colour from yellow to red (Schartl et al., 2016; Sköld et al., 2016). Varying densities and distributions of the different types of chromatophores, as well as the pigments within, produce different skin colour patterns (Sköld et al., 2016).

In fish, chromatophores are most often found in the dermis but can also be found in the epidermis, scales, and other tissues (Fujii, 2000; Sköld et al., 2016). Teleost fish can possess a variety of colours and patterns, but share a common structural organization of their chromatophores (Leclercq, Taylor, & Migaud, 2010). Chromatophores in teleosts are typically arranged in layers of cell types (Price et al., 2008; Sköld et al., 2016). However teleosts with scales have a more complex distribution of chromatophores as these cells are not restricted to layers (Leclercq et al., 2010). In salmonids, iridophores and melanophores are located in the dermis just beneath the epidermis as well as below the *stratum compactum* on the lower boundary of the dermis (Djurdjevič, Kreft, & Sušnik Bajec, 2015;

Leclercq et al., 2010). Melanophores are frequently found in association with iridophores forming a melano-iridophore complex, usually below the iridophores but with their dendritic processes extending around them (Leclercq et al., 2010).

The distribution and combination of these cell layers produces different colours in fish skin, but there are multiple ways to achieve the same colour (Sköld et al., 2016). For example, the blue colour in the blue damselfish is created by a single layer of iridophores overlaying melanophores whereas blue colour in the common surgeonfish has two layers of iridophores (Sköld et al., 2016). White colouration in fish skin can be caused by the presence of leucophores, such as in the Japanese medaka (Sköld et al., 2016), or alternatively, white colour may be caused by the presence of iridophores, such as in the domino damselfish (Goda & Fujii, 2001). Additionally, the combination of iridophores overlaying melanophores or xanthophores is known to produce the black and yellow stripes respectively, in zebrafish, displaying the importance of the relationship between chromatophores in skin (Sköld et al., 2016).

Colour change associated with nuptial colouration in fish can be categorized in one of three ways: permanent, long term, or rapid colour change (Kodric-Brown, 1998; Price et al., 2008). Tropical fish such as cichlids and guppies maintain breeding grounds year-round and thus display permanent nuptial colouration (Galis & Metz, 1998; Kottler, Fadeev, Weige, & Dreyer, 2013; Price et al., 2008). In contrast, fishes with restricted breeding seasons, such as mummichogs and Threespine Stickleback, display long-term seasonal nuptial colouration (Marques et al., 2017; McKinnon, 1995; McKinnon & Rundle, 2002; Reimchen, 1989). Changes in the number of chromatophores, chromatophore morphology, and/or deposition of pigments occurring over longer time periods (weeks to

months) is what causes seasonal colour change (Bagnara & Matsumoto, 2007; Price et al., 2008; Sköld et al., 2016). Darkening the skin related to seasonal colour change is often achieved when light absorbing chromatophores (such as melanophores) disperse their pigment granules or when light reflecting chromatophores (such as iridophores) display a an aggregation response (Sugimoto, Yuki, Miyakoshi, & Maruko, 2005). The opposite occurs when lightening the skin. Additionally, melanophores can develop or degenerate over the long-term to more effectively change colour (Sugimoto et al., 2005).

An excellent model for studying the evolution of nuptial colouration is the Threespine Stickleback because there is considerable variation in male nuptial colouration among populations within this species (reviewed by Bell & Foster, 1995). Because the Threespine Stickleback has a restricted breeding season, the males of this species are ideal for studying the mechanisms of long term colour change in relation to sexual selection (reviewed by Haley, 2018).

1.4 Threespine Stickleback as a Model System to Study the Evolution of Nuptial Coloration

The Threespine Stickleback (*Gasterosteus aculeatus*) is a teleost fish that is used as a model organism in many evolutionary studies (Wootton, 2009). Studies involving the Threespine Stickleback commonly focus on animal behaviour and in particular male breeding behaviours, as well as animal physiology and morphology (Wootton, 2009). Populations of Threespine Stickleback vary in many traits including body size, breeding colouration, number of bony plates, and spine length, which has made it the subject of many studies involving adaptation and speciation (Bell & Foster, 1995). Studying the

Threespine Stickleback has led to significant progress in understanding how speciation occurs in nature (McKinnon & Rundle, 2002). Speciation in the Threespine Stickleback is driven by natural selection in a number of populations (Barrett, Rogers, & Schluter, 2008; Conte & Schluter, 2013; Nagel & Schluter, 1998); however, differential sexual selection for traits such as breeding colouration, mating strategies, and parental care also contribute to assortative mating and may maintain or promote diversification (Marques et al., 2017; Ólafsdóttir, Ritchie, & Snorrason, 2006). Male nuptial colouration can result in sexual isolation when females have a strongly divergent colour preference (Boughman et al., 2005). In the Threespine Stickleback, ecotypes have evolved to recognize mates based on body size and nuptial colouration (Boughman et al., 2005). Sexual isolation based on colour arises from females of each ecotype choosing to spawn with bright homospecific males (Boughman et al., 2005). Sexual selection on nuptial colouration can be divergent between some populations of Threespine Stickleback (Marques et al., 2017).

The white Threespine Stickleback is an ecotype in which males have an atypical white nuptial colouration and are thought to have recently diverged from the typical marine form (McKinnon & Rundle, 2002). The white and common Threespine Sticklebacks occur sympatrically, but differ in traits such as breeding behaviour, size, and nuptial colouration (Blouw & Hagen, 1990; Haley, Dalziel, & Weir, 2019; McKinnon & Rundle, 2002). White males are more active than common males in regard to breeding behaviour, particularly in courtship behaviour (Blouw & Hagen, 1990; Blouw, 1996; Haley et al., 2019). Additionally, white males do not invest in parental care as the common males do (Blouw & Hagen, 1990; Blouw, 1996; Haley et al., 2019). Common males are generally larger than white males and because of their contrasting nuptial colours they can be distinguished by

eye (Haley et al., 2019). During the breeding season, both the common and white males develop a red throat and blue iridescent eyes (Blouw & Hagen, 1990; Haley et al., 2019). However, the white males additionally develop a very conspicuous shimmering white dorsum, in contrast to the common males which develop a colour ranging from a dark blue-green to brown shade on their dorsum (Figure 1; Blouw & Hagen, 1990; Haley et al., 2019). Outside of the breeding season, both white and common Threespine Stickleback exhibit olive green-brown and silver colouring that resembles the females and can be difficult to distinguish (Blouw & Hagen, 1990). The development of these bright colours during the breeding season in males implies that this trait is important during mating (Gumm, Feller, & Mendelson, 2011). Both laboratory tests and field observations indicate that the white Threespine Stickleback and the common Threespine Stickleback are reproductively isolated from each other, and that the differences in breeding colouration and behaviour of the two ecotypes has a genetic basis (Blouw & Hagen, 1990; Blouw, 1996; Samuk, 2016; Samuk, Iritani, & Schluter, 2014; Samuk et al., 2017). Although sticklebacks have long been a model system, the mechanisms involved in the evolution of the contrasting nuptial colouration of the white and common Threespine Stickleback are not fully understood (but see Haley et al. 2019).

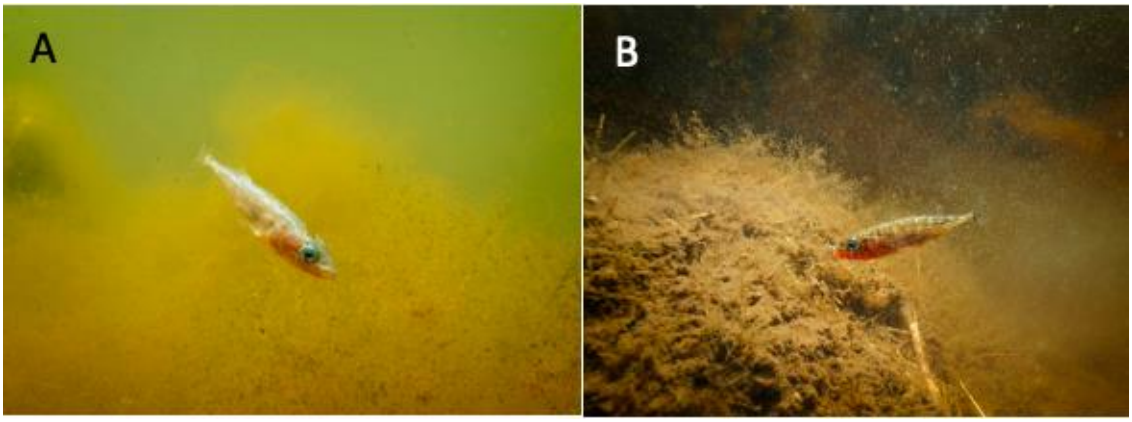

Figure 1. Breeding male Threespine Stickleback from the Atlantic coast of Nova Scotia. White ecotype from Rainbow Haven Beach (A) and common ecotype from Canal Lake (B). Photographs by Dr. Paul Bentzen, reproduced from Haley et al. (2019).

The contrasting nuptial colouration of the white and common Threespine Stickleback is likely due to differences in the chromatophore content and distribution in their skin, particularly differences in melanophores and iridophores. Although leucophores are also known to produce white coloured skin, they have only been found in a few groups of fishes, such as medaka (Kimura et al., 2014), and have not been found in stickleback (Haley et al., 2019). Iridophores however are present, and several studies provide examples of how iridophores produce white colouration (e.g. Frohnhöfer, Krauss, Maischein, & Nüsslein-Volhard, 2013; Goda & Fujii, 2001; Salis et al., 2019; Sugimoto et al., 2005). As predicted, breeding white males have lower overall melanophore density and coverage compared to breeding common males (Haley et al., 2019). However, stickleback skin has two layers of dermal melanophores (surface and deep; Burton, 1975, 1978) and the distinction between each melanophore layer has not been investigated. Since

chromatophores deep within the dermis of teleost fish produce the overall pigment pattern such as stripes or spots, whereas surface chromatophores provide an overall tint to the body (Sugimoto et al., 2005), these two layers may have differing influences on dorsal 'whiteness'.

Prior research examining the mechanisms underlying skin coloration in fish have found that altering either melanophore and/or iridophore properties can cause lightening or darkening of skin. For example, Sugitmoto et. al., (2005) found that changes in dorsal darkness in zebrafish are the result of alterations in the density of surface melanophores, such that an increase in the density causes a dark black tint. The distinction of each layer is important because the melanophores in each layer are regulated by different pathways within the neuroendocrine system (Burton, 1975, 1978). Understanding the differences between these two layers reveal which physiological control mechanisms and biochemical pathways lead to white colouration to better understand how the white ecotype evolved. In some species it has been found that the melanophores are the variable factor determining the lightness of a fish with iridophores remaining the same. The number of iridophores (which are aligned in a single layer) in the gray/brown turbot is the same as in the dark turbot, however the dark turbot has more melanophores (Faílde, Bermúdez, Vigliano, Coscelli, & Quiroga, 2014). Alternatively, in other fish, it is the alteration of iridophores responsible for lighter colouration. In clownfish, a higher density of iridophores are present in the matte white stripes in comparison to the orange skin (Salis et al., 2019). In another study, it was found that an increase in the thickness of the dermal iridophore layer is responsible for the bright white spot on the domino damselfish (Goda & Fujii, 2001). However, location of the iridophores is also an important factor. In a study with zebrafish,

it was found that iridophores located underneath the melanophores are not reflective in the overall tint of the skin because they are not able to reflect light through the melanophores, whereas iridophores found above the melanophores contribute to bright shiny skin by reflecting light (Frohnhöfer et al., 2013; Sugimoto et al., 2005). Therefore, changes in iridophore properties such as the location in the skin could be another factor leading to the difference in nuptial colouration of the white and common Threespine Stickleback.

1.5 Thesis Goals

The goal of this thesis is to determine the cellular mechanisms underlying differences in nuptial coloration between white and common male Threespine Stickleback. In particular, I will test whether or not variation in the size and distribution of surface and deep dermal melanophores and distribution and position of iridophores exists between these two ecotypes of the Threespine Stickleback. I predict that the combination of a decrease in overall melanophore size and coverage, fewer melanophores in the surface layer, and an increase in iridophore distribution contribute to the bright white colouration of the male white Threespine Stickleback (Haley et al., 2019). Determining the difference in skin chromatophore morphology of these two ecotypes will contribute to understanding how the white sticklebacks evolved. I will accomplish this goal by comparing cross sections of frozen skin samples of both white and common Threespine Stickleback in breeding colour and measuring the distribution and coverage of iridophores and melanophores in the surface and deep dermal layers.

Materials and Methods

2.1 Sampling Sites and Collection of Threespine Stickleback

Fish were collected between May-August 2019, from two locations on the Nova Scotia mainland. A permit from the Department of Fisheries and Oceans was obtained (Fishing Licence #343920) for fish collection. Male white Threespine Stickleback were collected from Rainbow Haven Beach (44.654799°N, 63.421140°W) and common Threespine Stickleback were collected from Antigonish Landing (45.63243°N, 61.9603°W). Rainbow Haven was chosen as a collection site because both white and common Threespine Stickleback have been observed in this location (Haley, 2018), and the goal was to collect sympatric populations of the ecotypes. However, in the summer of 2019 no common Threespine stickleback were found at Rainbow Haven. Another site on the Atlantic coast, Canal Lake (44.497627° N, 63.900449° W), has also been used as a site to collect sympatric populations of common and white Threespine Stickleback in previous studies (Haley, 2018; Samuk, 2016). However, like Rainbow Haven, in the summer of 2019 no common Threespine Stickleback were found. Therefore, Antigonish Landing was chosen as a collection location for common Threespine Stickleback because it has previously been studied and was found to only contain common Threespine Stickleback (Samuk, 2016).

Prior to collecting males, I observed them visiting their nest and courting females, and qualitatively assessed their breeding colours to ensure I was sampling the desired ecotype. Adult males were caught using dip nets in accordance to SMU Animal Care protocol 17-18A “Collection of sticklebacks and killifish to study the evolution of fish

physiology" to confirm we caught the fish we observed and classified as white or common. Males were also caught with minnow traps at Antigonish Landing, due to the presence of only the common ecotype at this location (Samuk 2016). In total, 11 white males and 12 common males were transported back to the aquarium facilities at Saint Mary's University.

Figure 2. Map of Rainbow Haven (blue marker) and Antigonish Landing (red marker), Nova Scotia, Canada (A); Satellite view of Antigonish Landing sampling site denoted by a red marker (B); Satellite view of Rainbow Haven sampling site denoted by a blue marker (C).

2.2 Husbandry and Laboratory Housing

Fish were housed in 15 gallon tanks equipped with a waterfall filter at a temperature between 20-22°C and salinity of 10ppt ± 1 ppt in the Saint Mary's University Aquarium

facilities. These conditions reflect the approximate temperature and salinity of the locations the fish were collected from during breeding season. The light conditions were also similar to the natural environment during breeding season, with the photoperiod set to 14 hours light and 10 hours dark (Haley et al., 2019). The white and common ecotypes were housed in separate tanks and allowed at least two weeks to acclimate to the lab prior to sampling. The acclimation period is important in chromatophore studies because wild caught fish typically lose some of their colouration in the lab from stress and handling, so this buffer period allows all fish to equally habituate to lab environment (Corney, 2019; Haley et al., 2019). Tanks were supplemented with aquarium gravel, plastic plants, and clay pots for environmental enhancement. Fish were given a diet of frozen bloodworms, brine shrimp nauplii, and mysis shrimp twice daily. Hagen water quality test kits were used to monitor levels of ammonia, pH, nitrate and nitrite and 20% water changes were performed weekly or whenever nitrogenous waste measured was above healthy levels.

2.3 Sampling Procedures

Fish were sampled to obtain histological skin samples to quantify chromatophore content in white and common males following the SMU Animal Care protocol 19-08 “Evolution of skin coloration in stickleback fishes”. Sampling took place in the laboratory from July-September 2019 after the fish had acclimated in the aquaria, but before the males lost their breeding colouration. Prior to sampling, fish were given a qualitative score of 1-5 reflecting their breeding condition (Haley, 2018). The score indicates the intensity of breeding colouration, with 5 being the brightest coloration, near to what would be seen in the field during breeding season, and 1 being non-breeding colour as previously described (section 1.4). After scoring, the fish were removed from their tank using a dip net and

euthanized with a lethal concentration of buffered tricaine methanesulfonate (MS-222). Each fish was removed from the MS-222 and rinsed with water before being photographed next to a colour checker with an Olympus Tough TG-4 camera. Files of photographs were saved in JPG format.

After taking photographs, 3-4 transverse sections of ~3mm thick were cut using a razor blade behind the second spine of the fish. The head and tail were discarded. The first section cut behind the spine was put into a tissue mould and filled with Tissue-Tek® Optimal Cutting Temperature (O.C.T.) compound before being submerged into a beaker containing cold isopentane to flash freeze it. The isopentane was chilled to about -150°C, indicated by a small rim of frozen isopentane present, by submerging the beaker in liquid nitrogen. The mould was held in the isopentane for about 10 seconds, until the O.C.T was completely frozen, appearing white in colour. These samples were stored in a -80°C freezer until ready for sectioning. The second and third sections cut behind the spine were placed into separate microcentrifuge tubes containing potassium rich saline solution for thirty minutes to contract the melanophores. The fourth section was placed into a microcentrifuge tube containing physiological saline solution for thirty minutes to expand the melanophores. After the thirty minutes, the second section was frozen with O.C.T in the same procedure as the first and stored in a -80°C freezer until ready for sectioning. The third and fourth sections were placed into microcentrifuge tubes containing 10% neutral buffered formalin and were stored at room temperature before being decalcified, dehydrated, and embedded in paraffin as back-up samples. Only the frozen samples that had been soaked in potassium rich saline were used for sectioning, due to time constraints.

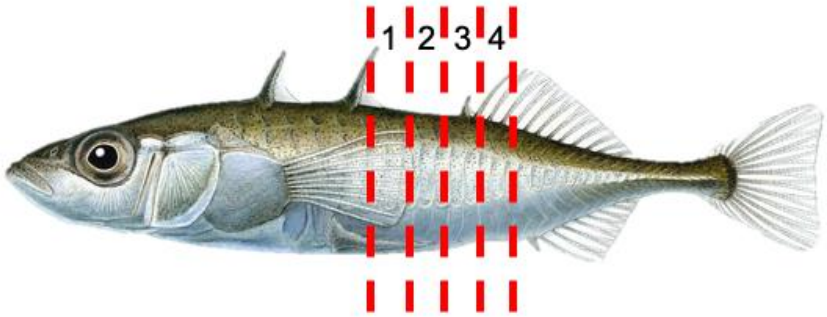

Figure 3. A diagram of how the four transverse sections were cut behind the second spine of the fish. Sections were prepared and fixed in the following ways: 1) untreated, flash frozen; 2) contracted melanophores, flash frozen; 3) contracted melanophores, paraffin embedded; 4) expanded melanophores, paraffin embedded. Only the second sections (contracted melanophores, flash frozen) were used for further analysis. Stickleback drawing by Emily S. Damstra.

2.4 Slide Preparation

Frozen samples were transported to Mount Saint Vincent University on dry ice. A LEICA CM1850 cryostat set to -20°C +/- 1°C was used to cut 10 µm thick sections of each sample. Fisherbrand® Superfrost™ Plus slides were used to ensure samples adhered to slides. Two to three slides containing five to ten sections on each slide were cut from every sample (each fish). Slides were transported back to Saint Mary's University on dry ice and stored in a -80°C freezer.

The slides were then haematoxylin and eosin (H&E) stained, using the following staining and mounting protocol: slides were allowed two minutes to briefly thaw at room temperature before being placed in -20°C VWR® acetone for eight minutes to fix the tissue. They were then washed with phosphate-buffered saline (PBS) for ten minutes and stained with VWR® Harris's haematoxylin for one minute. Next, the slides were washed in distilled water for five minutes and stained with VWR® eosin Y 1% for 30 seconds. Finally, the slides followed a dehydration series of 70% ethanol, 80% ethanol, 90% ethanol, and 100% ethanol for five minutes each before being cleared twice with Fisherbrand® SafeClear™ xylene substitute (2-methylnonane) for five minutes each. Slides were cover-slipped with Fisher Chemical™ Permount® and stored at room temperature.

2.5 Data Collection and Analysis

The slides were visualized using bright field microscopy enhanced with condenser differential interference microscopy (DIC) prisms with integrated polarizers. A ZEISS Axioplan 2 imaging microscope with an Axiocam camera (ZEISS) mounted to the top, and the program Axiovision (ZEISS) was used to take photographs of the samples and set the scale. Two to three photographs were taken of the dorso-lateral portion of transverse sections of each fish for analysis. In total, 67 photographs of cross sections and 21 photographs of the full fish were analysed. All photographs were given a randomized number as file names so that the analysis could be completed "blind".

The full body photos of the fish were analysed using ImageJ version 1.52. First, the photos were converted to 8-bit grayscale. The photos were then standardized by calibrating to the mean gray value of the white square in the colour checker. In 8-bit gray scale the lowest value, 0, indicates the colour black and the highest value, 255, indicates the colour

white. The typical gray value of the white square was between 150-200, so the value of 185 was chosen as the gray value to calibrate the white squares to. White squares were not calibrated to the true white value of 255 in order to avoid over exposure of the photos. The calibration was done by measuring the mean gray value of the white square in each photo using the "Measure" function, dividing that number into 185, and using the product in the "Multiply" function to adjust the gray value of each photo. After being standardized, the average dorsal brightness of each fish was measured. This was done by first drawing straight line from the top of the fish's eye through the caudal peduncle. Then, a small rectangle was drawn on the dorso-lateral region between the second and third spine down to the horizontal line. This area was chosen because it is the same region where the frozen cross-section samples taken, and is often the brightest region of the fish (Corney, 2019). The rectangle was then measured for average gray value to represent the brightness of the fish.

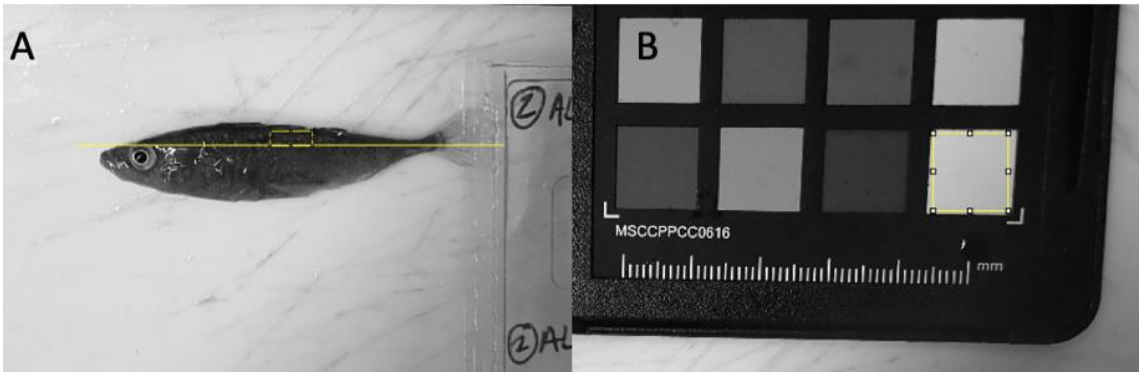

Figure 4. An example of how the section of the dorsal skin (A) and colour checker (B) were selected in each photo to be analyzed.

The photos of hematoxylin and eosin stained skin cross-sections were also analysed using ImageJ version 1.52. First, a global scale was set with the "Set Scale" function in ImageJ. The number of melanophores present in the surface and deep layers were counted by eye and recorded. Next, several measurements were taken using the "Segmented Line" tool. The length of a transect line along the surface and deep layers of dermis where the chromatophores are found (Burton, 1978) was measured. Then, the area of each transect line covered by melanophores and iridophores was measured. This was completed by individually measuring the length of each melanophore or iridophore cluster present on the transect line and adding them up. Additionally, the area of each transect line covered by iridophores in isolation of melanophores was measured. Percent cover of chromatophores integrates the chromatophore number, the amount of pigment deposited in the cell as well as the dispersion of the cell itself. Notes were taken about each photo of general qualitative observations regarding the presence or absence of melanophores and iridophores in each layer, the degree of melanophore contraction, and the location of iridophores in relation to the melanophore

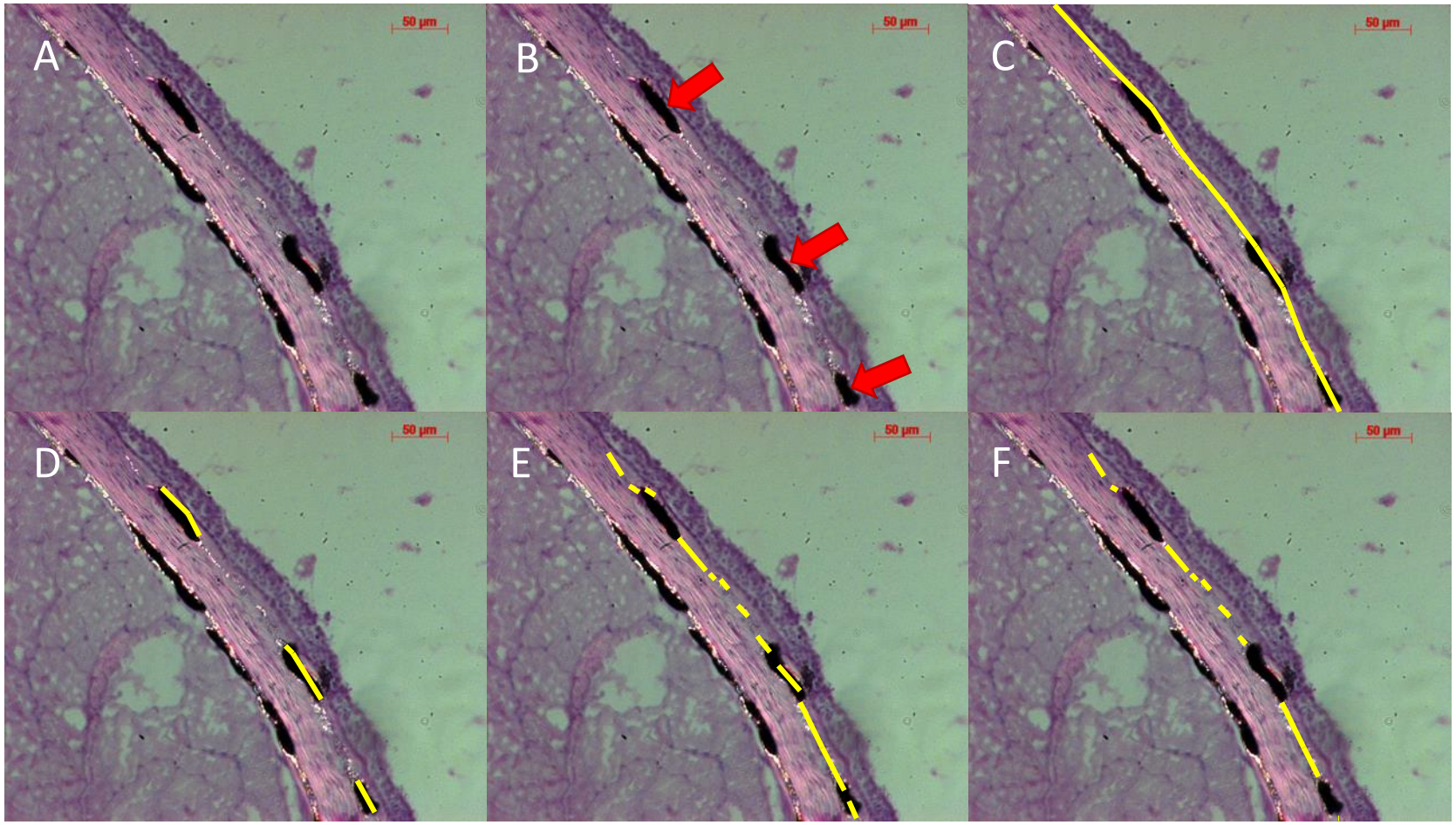

Figure 5. Representative photographs of transverse histological sections through male stickleback integument (A). An example of how the number of surface melanophores (B), surface transect line (C), surface melanophores (D), surface iridophores (E), and surface "free"

iridophores (F) were quantified. Transect lines are represented by yellow lines; note that in some cases these yellow lines hide iridophores.

2.6 Statistical Analysis

Data was analysed using R v.3.5.2 with ggplot2 package. Several welch two sample t-tests with a Bonferroni correction were conducted to examine differences between the common and white ecotypes in each of the chromatophore measures described. Bonferroni correction was used because multiple comparisons were completed for each sample. The adjusted critical p-value for these measures was 0.005. To test for an effect of melanophores on dorsal brightness, linear regressions were conducted between dorsal gray value and melanophore number and percent coverage. Due to the small sample size, the data for the white and common male melanophore measures was pooled. The assumption was made that the ecotypes would have the same slope and regression, however variation among ecotypes should be tested for using an ANCOVA in the future using a larger sample size.

Results

The common and white Threespine Stickleback are ecotypes that differ in the nuptial colouration of males. To determine the morphological basis of this colouration, fish were collected and sampled from two locations in Nova Scotia: the white males from Rainbow Haven Beach and the common males from Antigonish Landing. Histological skin samples were soaked in K+ rich saline to contract melanophores before being flash frozen and H&E stained. Skin samples were prepared on slides and then visualized using light microscopy. Figure 6 depicts representative images of the skin samples used for analyses.

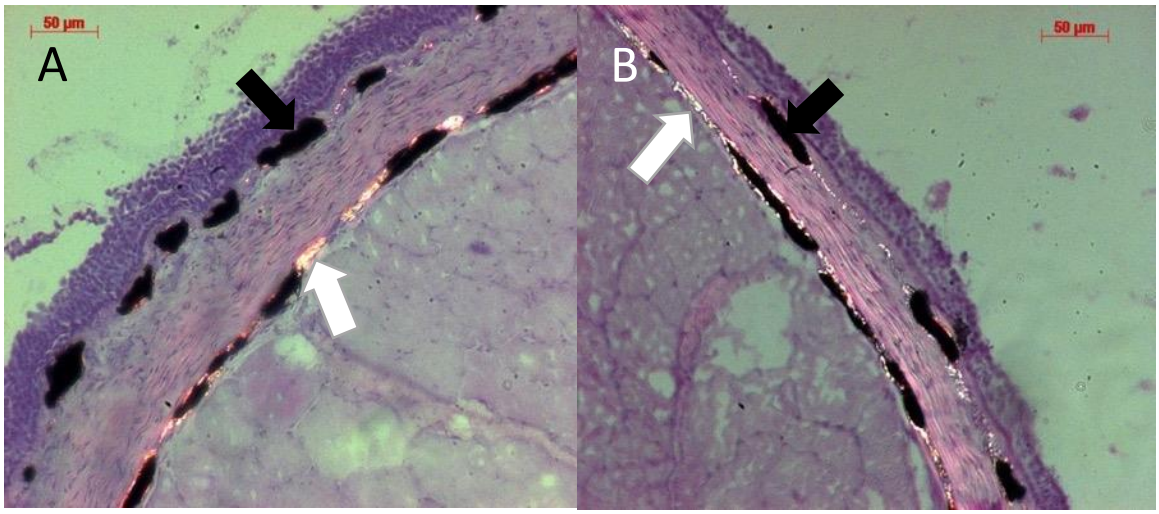

Figure 6. Microscope images of transverse cut histological sections through the integument on the dorso-lateral portion of common (A) and white (B) Threespine Stickleback. Black arrows indicate melanophores and white arrows indicate iridophores.

3.1 Skin Brightness (Dorsal Gray Value) in Threespine Stickleback

All data were found to be normally distributed using a Shapiro-Wilk test for normality. The common Threespine Stickleback had a significantly lower dorsal gray value than white males, indicating a darker colour (Figure 7). I use dorsal gray value as a measure of skin brightness, so this indicates that the white males typically have brighter skin than the common males. Prior to sampling fish were given a qualitative score from one to five reflecting their breeding condition (data not shown) (Haley, 2018). Eight out of the ten white males were scored a 3.5 or above, with the remaining two scored at 2.5, meaning most of the white males were in breeding colouration. In contrast, one common male was scored 4.5 and the remaining 11 scored 1.5 and below, meaning most of the common males were not in breeding colouration. Threespine Stickleback are rarely as brightly coloured in the laboratory facilities as they are in the field and so do not reflect the level of contrast between these breeding males in the wild (Blouw & Hagen, 1990).

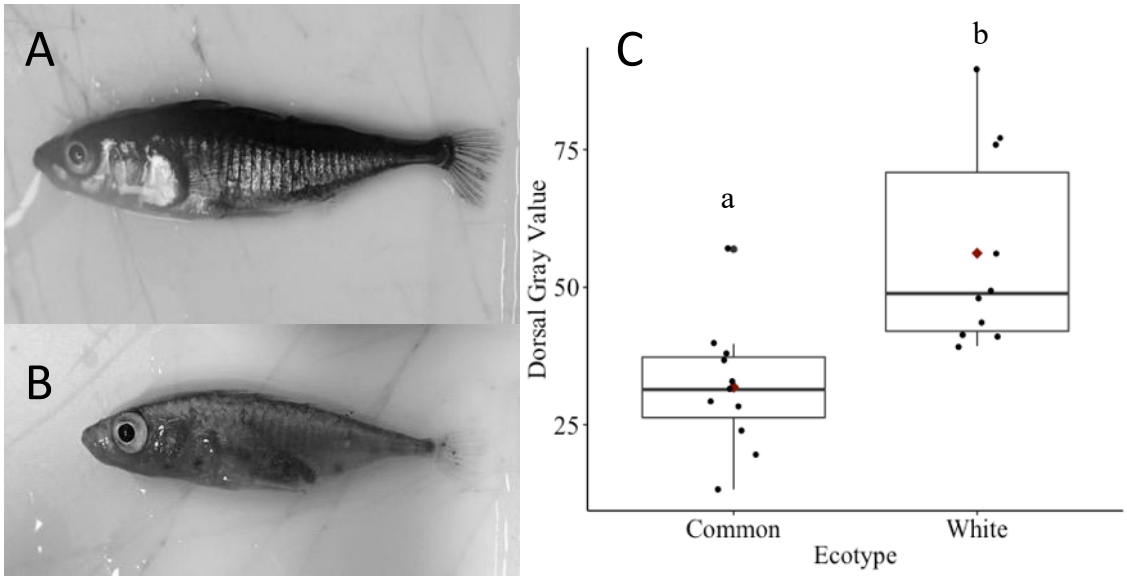

Figure 7. Dorsal gray values of common and white male Threespine Stickleback. Example fish of a common male with a dorsal gray value of 13.215 (A) and a white male with a dorsal gray value 77.157 (B). Note that 0 is black and 255 is white. Red diamonds represent the mean, black bars represent the median, and black dots represent the average melanophore coverage for each individual fish. Boxes with different letters are significantly different from each other (Welch's two sample t-test with Bonferroni correction, $n_{white} = 10$, $n_{common} = 11$, $t = -3.6337$, $df = 15$, $p = 0.0024$) (C).

3.2 Histological Analysis of Chromatophore Morphology in Threespine Stickleback

Examination of cross-sections of the skin under the light microscope revealed the two predicted dermal melanophore layers (Burton, 1978), in addition to two dermal iridophore layers, previously undocumented in stickleback (Figure 5). Each chromatophore measure was taken from two to three serial sections with two to three photos of each fish

and then averaged for analysis (Figure 5). Some sections showed stretching or tearing present within the dermis and muscle tissue; if this was predicted to affect my measures, these photos were not used, resulting in only two photos for some individuals. One fish had no chromatophores in the surface layer in all three replicate photos (Figure 8A).

The common males had a slightly higher average melanophore number in both the surface and deep dermal layers than the white males, but this was not significantly different between groups (Figure 8).

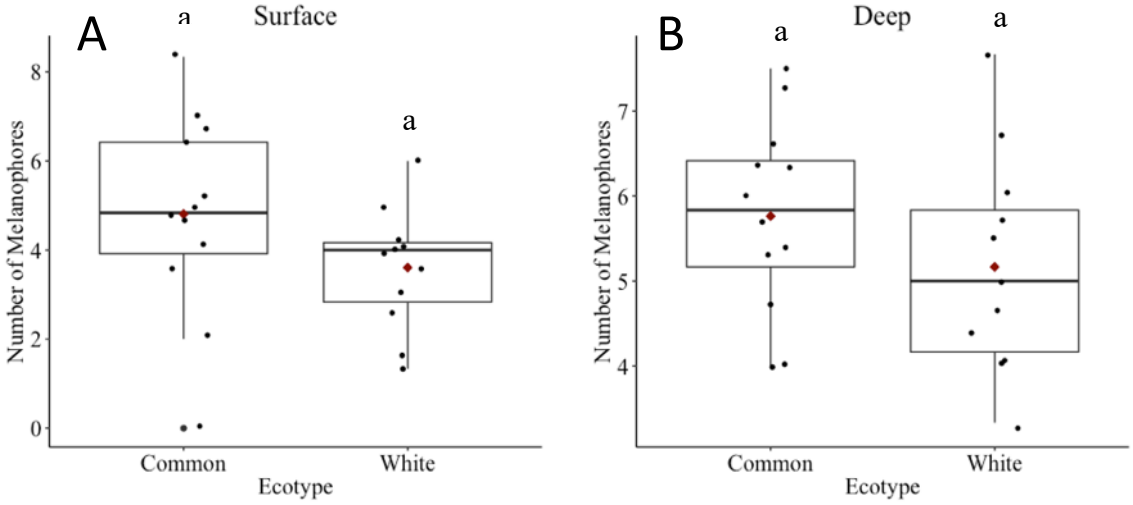

Figure 8. Melanophore number in the surface (A) and deep (B) dermal layers of white and common male Threespine Sticklebacks. Red diamonds represent the mean, black bars represent the median, and black dots represent the average melanophore coverage for each individual fish. Boxes with shared letters are not statistically significant from each other (Welch's two sample t-test with Bonferonni correction, $n_{white} = 11$, $n_{common} = 12$, $t = 1.5511$, $df = 18.353$, $p = 0.138$ (A); $t = 1.1642$, $df = 20.227$, $p = 0.2579$ (B)).

Several measurements of melanophore and iridophore coverage were taken. The common male Threespine Sticklebacks were found to have a higher average surface melanophore coverage than the white males, but this was not significantly different between groups (Figure 9A). Common Threespine Sticklebacks were also found to have a significantly higher average melanophore coverage in the deep layer compared to the whites (Figure 9B).

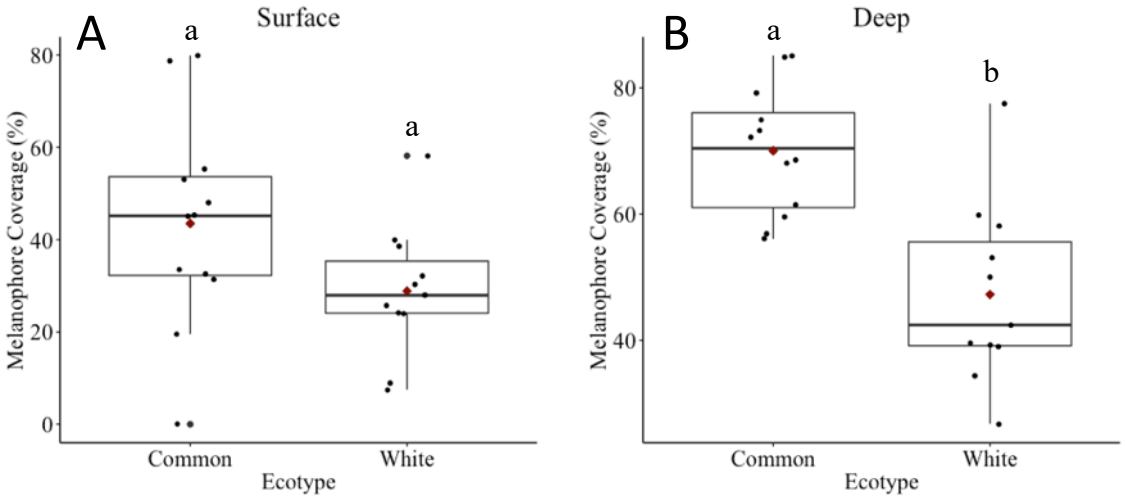

Figure 9. Melanophore coverage in the surface (A) and deep (B) dermal layers of white and common male Threespine Stickleback. Red diamonds represent the mean, black bars represent the median, and black dots represent the average melanophore coverage for each individual fish. Boxes with shared letters are not statistically different from each other (Welch's two sample t-test with Bonferroni correction, $n_{white} = 11$, $n_{common} = 12$, $t = 1.8764$, $df = 18$, $p = 0.07635$ (A); $t = 4.3805$, $df = 17.897$, $p = 0.0003651$ (B)).

The common Threespine Stickleback males were found to have a slightly lower average iridophore coverage in both the surface and deep layers than the white males, but neither of these results were significantly different among groups (Figure 10).

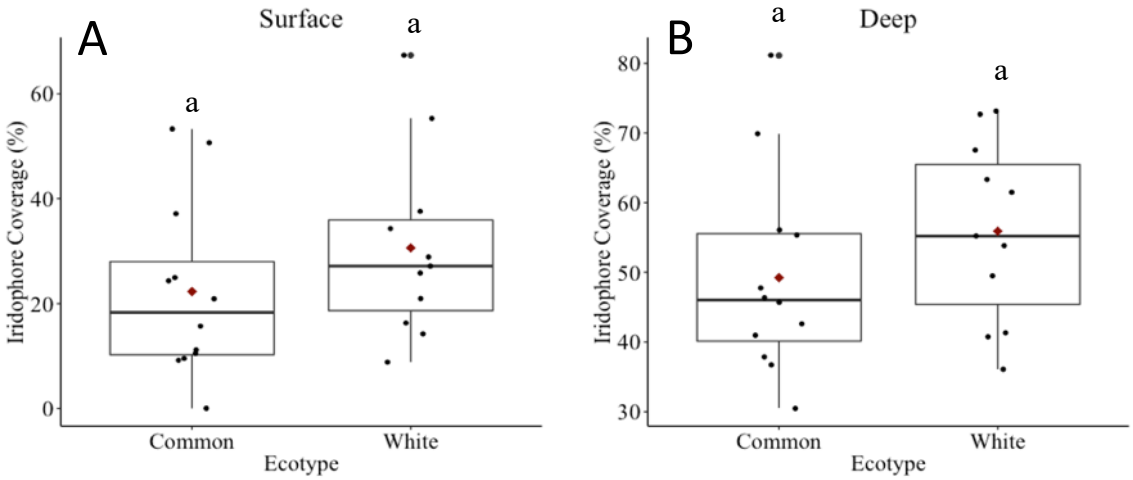

Figure 10. Iridophore coverage in the surface (A) and deep (B) dermal layers of white and common male Threespine Stickleback. Red diamonds represent the mean, black bars represent the median, and black dots represent the average melanophore coverage for each individual fish. Boxes with shared letters are not statistically different from each other (Welch's two sample t-test with Bonferroni correction, $n_{white} = 11$, $n_{common} = 12$, $t = -1.1553$, $df = 20.634$, $p = 0.2612$ (A); $t = 1.1644$, $df = 20.994$, $p = 0.2565$ (B)).

The percent coverage of 'free' iridophores that were not associated with melanophores was also measured. It was found that the common Threespine Stickleback had fewer 'free' iridophores than the white Threespine Stickleback in the surface dermal layer, but this was not significant (Figure 11A). It was also found that the common

Threespine stickleback had significantly less ‘free’ iridophores in the deep dermal layer (Figure 11B).

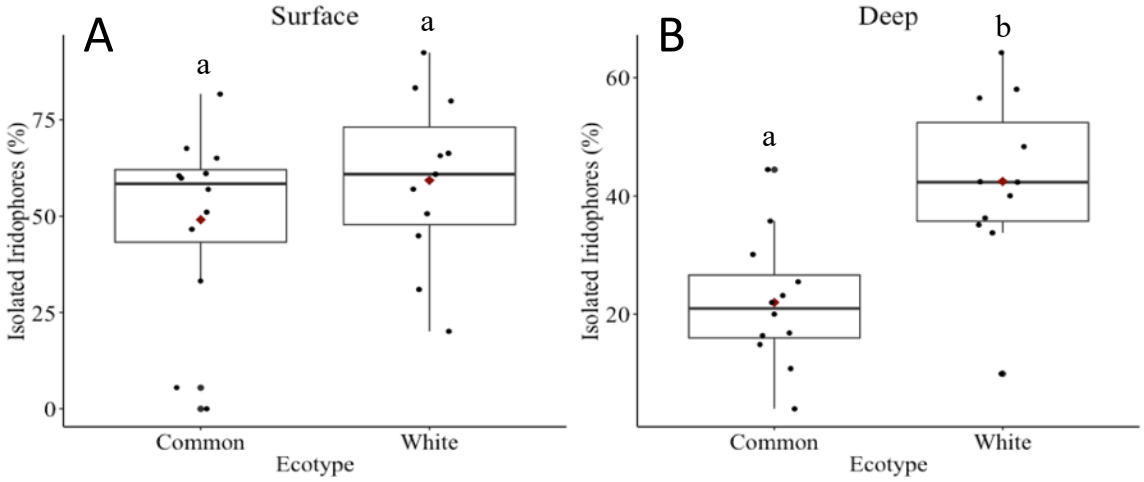

Figure 11. Iridophore coverage in isolation of melanophores in the surface (A) and deep (B) dermal layers of white and common male Threespine Stickleback. Red diamonds represent the mean, black bars represent the median, and black dots represent the average melanophore coverage for each individual fish. Boxes with shared letters are not statistically different from each other (Welch’s two sample t-test with Bonferroni correction, $n_{white} = 11$, $n_{common} = 12$, $t = -1.0505$, $df = 20.986$, $p = 0.3055$ (A); $t = -3.7413$, $df = 18.44$, $p = 0.001443$ (B)).

3.3 Correlation of Melanophore Measures to Skin Brightness (Dorsal Gray Value) in Threespine Sticklebacks

To test if my histological measures of melanophore number and coverage correlated with overall brightness, I conducted several linear regressions. I found that melanophore

number in the surface dermal layer could not predict dorsal brightness (Figure 12A). Melanophore number in the deep dermal layer also could not predict dorsal brightness (Figure 12B). The combination of the number of melanophores in the surface and deep layer also could not predict dorsal brightness (Figure 12C). I found that the percent coverage of melanophores in the surface layer can predict dorsal brightness, but only weakly (Figure 12D). Percent coverage of melanophores in the deep layer can predict dorsal brightness, and this result was slightly stronger (Figure 12E). The combination of melanophore coverage in the surface and deep layers also predicts dorsal darkness but this was not any stronger than the melanophores in the deep layer alone (Figure 12F).

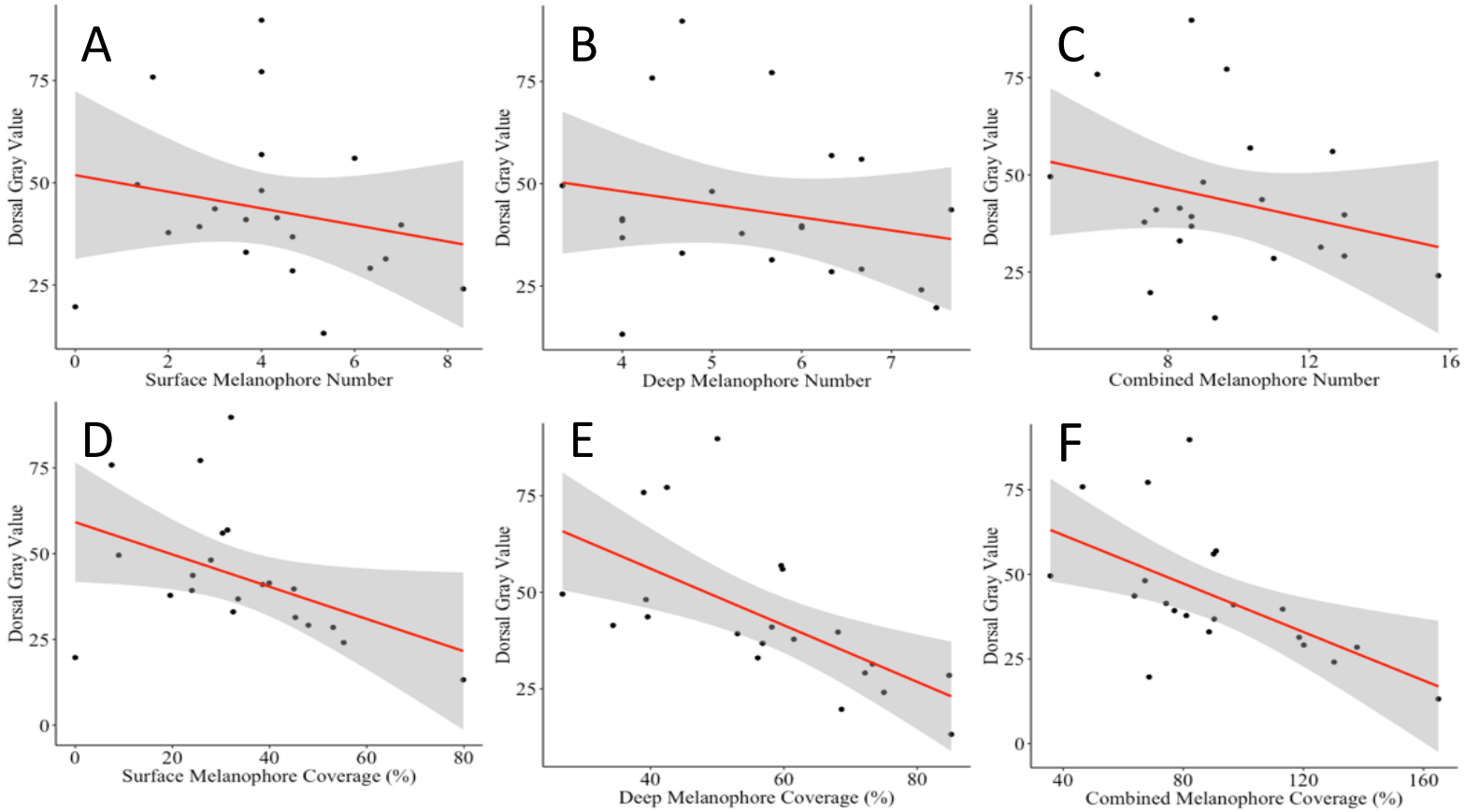

Figure 12. Relationship between surface melanophore number and dorsal gray value in Threespine Stickleback males (A) (linear regression, n = 21, t = -0.956, df = 19, F = 0.9149, p = 0.3508, adjusted R_2 = -0.0043); relationship between deep melanophore number

and dorsal gray value in Threespine Stickleback males (B) (linear regression, n = 21, t = -0.957, df = 19, F = 0.9154, p = 0.3509, adjusted R_2 = -0.0042); relationship between the combination of melanophores in the surface and deep dermal layers and dorsal gray value of Threespine Stickleback males (C) (linear regression, n = 21, df = 18, F = 0.742, p = 0.4902, adjusted R_2 = -0.0265); relationship between percent melanophore coverage in the surface dermal layer and dorsal gray value of Threespine Stickleback males (D) (linear regression, n = 21, t = -2.131, df = 19, F = 4.541 p = 0.0464, adjusted R_2 = 0.1504); relationship between percent melanophore coverage in the deep dermal layer and dorsal gray value of Threespine Stickleback males (E) (linear regression, n = 21, t = -3.465, df = 19, F = 12, p = 0.0026, adjusted R_2 = 0.3549); relationship between the combination of percent coverage in the surface and percent coverage in the deep layers and dorsal gray value (F) (linear regression, n = 21, $t_{surface}$ = -0.247, t_{deep} = -2.405, df = 18, F = 5.735, p = 0.0118, adjusted R_2 = 0.3214).

Discussion

White and common ecotypes of Threespine Sticklebacks represent a useful model species to study how male nuptial colouration might evolve (Haley, 2018). In this study, I quantified the number and distribution of melanophores, as well as the distribution of iridophores to better understand the cellular mechanisms underlying white nuptial colouration. My results indicate that: 1) as predicted, stickleback possess two chromatophore layers; 2) the number of melanophores in both layers are not significantly different between white and common ecotypes; 3) common males have a significantly higher melanophore coverage in the deep dermal layer, but not the surface; 4) the coverage of iridophores in each layer in the common and white males is not significantly different but white males had more "free" iridophores in the deep layer; 5) the percent coverage of melanophores in the deep dermal layer was the best predictor of dorsal brightness in surface skin photographs.

4.1 Skin Brightness in White and Common Threespine Stickleback

My results indicated that the common males had significantly darker dorsum than the white males (Figure 7). This result was expected, knowing that the white males become white and the common males become dark blue-green, so dorsal brightness was measured as a "control" to confirm what is known from previous studies (Corney, 2019; Haley et al., 2019; Jamieson, Blouw, & Colgan, 1992). However, it is important to note the qualitative breeding colour scores given to the fish prior to sampling. Despite finding a large difference in the dorsal brightness, many of these fish were not in full nuptial colouration. As mentioned previously, the nuptial colouration of Threespine Stickleback housed in the

laboratory is rarely as bright as it is in the field (Blouw & Hagen, 1990), so these observations do not reflect the level of contrast between breeding males in nature and are thus a conservative estimate of differences in dorsal brightness among ecotypes. The fish were brought back to the lab to acclimate to the same conditions and surroundings, however the time in the lab for some of the fish (mostly the common males) was long enough that they had lost most of their breeding colouration. Additionally, the common males were collected later in the breeding season than the white males, due to the difficulty in finding sites with commons. Therefore, it is more accurate to state that this study compared the chromatophores of lab-acclimated breeding white males to lab-acclimated common males that were no longer displaying nuptial colouration.

This may have affected the results because perhaps these males were in the lab long enough to implement some of the long-term mechanisms of colour change, such as altering the number or dispersal of melanophores. Common Threespine Stickleback males increase melanophore density during the breeding season (Haley, 2018), so the fact that the common males were not in breeding colouration when they were sampled could mean that the density of melanophores was lower than what would have been found in breeding males, therefore reducing the difference in this trait between the common and white ecotypes. Although I did find a trend of higher melanophore number in common males in both the surface and deep layers (Figure 8), neither of these results were significantly different. Therefore, the fact that the common males had lost their breeding colour could have significantly impacted the results of this study. Despite this, the high points in the melanophore coverage data (Figure 9) were not the fish with the highest qualitative breeding colour score. Future work should complete the skin sampling procedures on site

in the field to avoid any long-term chromatophore changes related to lab acclimation that could affect the study. This should focus on sampling males at their maximal nuptial colouration, as well as males outside of the breeding season to investigate how the chromatophores change in the field over time.

4.2 Chromatophore Morphology in Threespine Stickleback

My results indicate that common males have a higher melanophore coverage and fewer isolated iridophores in the deep dermal layer than the white males. However, most of my results were not significantly different between the groups. The number of melanophores in each layer and the surface melanophore coverage was not significantly different between the common and white males. Additionally, the iridophore coverage in each layer and the percent of isolated iridophores in the surface layer did not differ between the two groups. These finding indicate that the skin of these males is notably more morphologically similar than predicted. This result is similar to what is seen in turbot; the number of iridophores in the brown/gray turbot is the same as in the dark turbot but the dark turbot has a greater number of melanophores (Faílde et al., 2014).

My results showed the predicted two dermal melanophore layers (Burton, 1978), in addition to two dermal iridophore layers, previously undocumented in stickleback (Figure 5). The iridophores and melanophores were also very closely associated with each other in a melano-iridophore complex. Iridophores were found both above and below melanophores in each layer as if the melanophores were embedded within the iridophores. This melano-iridophore complex arranged in two dermal layers is seen in many other teleost fish including salmonids (Djurdjevič et al., 2015; Leclercq et al., 2010).

Another observation was that the melanophores present in the deep dermal layer of chromatophores appeared larger in diameter than in the surface layer in both ecotypes, but the difference in the layers appeared greater in the commons. Previously, the deep layer of melanophores has been described as continuous (Burton, 1978), but my results show that the deep layer still consists of visible discrete melanophores. Perhaps the morphology of the deep chromatophores are simply wider than those of the surface layer, but it is also possible that the K+ rich saline solution was unable to penetrate through the dermis into the deep layer as effectively. Another study that also aimed to completely aggregate pigment granules was performed with zebrafish, a teleost fish with scales (Sugimoto et al., 2005). In this study, the scales were removed before being submerged in norepinephrine to contract the melanophores, therefore complete submersion would allow complete aggregation (Sugimoto et al., 2005). In future work, thinner skin samples should be taken in order to allow solutions to penetrate throughout the entire sample. Additionally, alternative chemicals, such as norepinephrine, should be explored to find the most effective solution for contracting chromatophores.

Other morphological differences in the chromatophores of the common and white Threespine Stickleback that were not measured in this study, may reveal differences between the ecotypes. One example is the thickness of the iridophores. In many of the photos, the dermis of the fish appeared to be stretched so we could not measure the thickness of the chromatophores or the dermis layer. In another fish, the domino damselfish, an increase in the thickness of the iridophore layer has been found to be responsible for the bright white spot on the fishes' forehead (Goda & Fujii, 2001). Although

this did not appear to be the case from qualitative observations, it would be worth-while to further look into the thickness of the iridophore layer.

4.3 Future Directions

In this study I have found that common males have a higher deep melanophore coverage and fewer isolated iridophores in the deep dermal layer than the white males. However, the number of melanophores in each layer, the surface melanophore coverage, the iridophore coverage in each layer, and the percent of isolated iridophores in the surface layer did not differ between the two groups. These findings indicate that the morphology of the chromatophores in the white and common Threespine Stickleback are more similar than predicted, but more studies should be done to ensure all fish are in maximal nuptial colouration in addition to incorporating other measures, such as the thickness of the skin and chromatophore layers. Furthermore, the orientation of and spacing between the platelet crystals within iridophore cells are an important factor influencing skin colour (Goda, 2017; Mäthger, Land, Siebeck, & Marshall, 2003; Szydłowski, Madej, & Mazurkiewicz-Kania, 2017; Teyssier, Saenko, Van Der Marel, & Milinkovitch, 2015). Transmission Electron Microscopy (TEM) has been used in several other studies to reveal the orientation and spacing of iridophore platelet crystals and their influence on animal skin colour (Goda, 2017; Mäthger et al., 2003; Szydłowski et al., 2017). Iridophores have also been found to be the chromatophore responsible for colour change in chameleons (Teyssier et al., 2015), so it would be fitting to further investigate the morphology of iridophore platelets within sticklebacks.

Future studies should also aim to measure the hormones that regulate skin pigment content in fish, to determine which pathways are most likely responsible for the nuptial colouration of Threespine Sticklebacks. It is also important to note that the common and white Threespine Stickleback males possess a great deal of plasticity when it comes to their colouration (Haley et al., 2019). With disturbance, bright white males become darker and more cryptically coloured whereas common males rapidly blanche, making them difficult to distinguish between after being caught (Blouw & Hagen, 1990; Haley, 2018). Therefore, determining which regulators are involved in this colour change will allow us to pre-treat samples to look at white males in their maximal and minimal brightness. Many chromatophores in teleost fish are known to disperse when regulated by increases in the levels of the neuropeptide *a*-melanocyte stimulating hormone (*a*-MSH), and in some fish, such as the cichlid, it also causes more aggressive behaviour (Djurdjevič et al., 2015). Increased overall melanocortin and receptor levels associated with darker colouration are generally correlated with higher sexual activity and aggressiveness, however, several studies have found that white Threespine Stickleback males have higher sexual activity and similar aggressiveness (Blouw & Hagen, 1990; Blouw, 1996; Haley, 2018; Samuk et al., 2014). Determining which pathways are responsible for colouration can lead to increased understanding of the mechanistic links between colouration and animal behaviour.

Furthermore, this Threespine Stickleback species complex is a good model system for determining the genetic basis of colouration evolution. There are several candidate genes involved in the evolution of colour patterns identified from previous research (Irion & Nüsslein-Volhard, 2019). For example, the countershading colour present in many fish is dependent on Agouti signalling, encoded by the ASIP gene (Cal et al., 2019). A low level

of expression of *asip1* dorsally leads to a dark dorsum in the Zebrafish (Cal et al., 2019). In the Japanese flounder, the expression of the three melanocortin receptors *mc1r*, *mc5r*, and *melanin-concentrating hormone receptor 2*, are increased in darker, more pigmented areas (Matsuda et al., 2018). The gene responsible for darker skin in a marine population of sticklebacks compared to a freshwater population was found to be the *Kit ligand* (Miller et al., 2007), however this has not been investigated in the common and white marine Threespine Stickleback. Future research should investigate whether these same genes have repeatedly evolved in stickleback, or if different populations use different mechanisms to vary skin colouration.

Furthermore, candidate genes for iridophore content in stickleback should be investigated. In zebrafish, endothelin signalling is required for iridophore development and proliferation (Frohnhöfer et al., 2013). Mutations in the *shady* gene (encoding leukocyte tyrosine kinase) as well as the *rose* gene (encoding endothelin receptor b1a) are known to inhibit iridophore development in zebrafish (Frohnhöfer et al., 2013), and zebrafish with mutations in *fhl2a*/*fhl2b*, gpnmb, and *saiyan* display significantly fewer iridophores (Salis et al., 2019). Additionally, the overexpression of *edn3b* has been shown to increase iridophore coverage in zebrafish (Spiewak et al., 2018). A number of other genes (*pnp4a*, *prtfdc1*, *tfec*, etc.) are also known to be important in the function and development of iridophores in teleost fish (Salis et al., 2019), and these genes should be investigated in the white and common Threespine Stickleback in order to gain a more cohesive understanding on the evolution of white nuptial colouration.

4.4 Conclusion

The white and common Threespine Stickleback are a species complex that possess contrasting nuptial colouration making them a good model species for studying how male nuptial colouration might evolve (Haley, 2018). In this study, I quantified several characteristics of melanophores and iridophores to better understand the cellular mechanisms underlying white nuptial colouration. My results indicate that the integumental chromatophores in both white and common males are more morphologically similar in number and distribution than predicted. Future studies investigating further chromatophore measures such as thickness of chromatophores and skin, and the orientation of the platelets in iridophores is warranted. Additionally, investigating the genes and hormones responsible for white colouration would be beneficial to gain a complete understanding of the evolution of white nuptial colouration.

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