Nuptial coloration is unaffected by the immune response fibrosis in threespine stickleback.

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Abstract

To understand why immune traits affect the fitness of its host is an important part of understanding why and how immune responses evolve. In threespine stickleback (*Gasterosteus aculeatus*), fibrosis is an immune response evolved to combat infection of the tapeworm parasite *Schistocephalus solidus.* Already known is that fibrosis leads to a reduced mating success for males. Here I investigated whether the immune response, fibrosis, reduces the nuptial coloration of male threespine stickleback (*Gasterosteus aculeatus*), and whether this is the reason behind the reduction in mating success for fish expressing fibrosis. The software ImageJ was used to analyse the difference in coloration between males with and without a *S. solidus* infection and with and without fibrosis. Although strong sex and lake differences in coloration were recovered, no evidence that fibrosis causes a reduction in nuptial coloration was found.

Introduction

To mitigate the fitness costs that a parasite infection has on its host, the immune system has evolved. But the immune system also has costs of its own. Energy that could have been allocated to e.g. growth or nuptial coloration, is instead allocated to the immune response (Verhulst & Sheldon, 1996). Another cost of the immune system is that it can attack the host's own tissue (Billi et al., 2019).

The cestode parasite *Schistocephalus solidus* has the fish species threespine stickleback (*Gasterosteus aculeatus*) as an obligate intermediate host (Nosil & Reimchen, 2001). This means that the tapeworm has to inhabit a threespine stickleback to fully develop. *S. solidus* is a trophically transmitted tapeworm parasite, distributed world wide. Their eggs hatch in the water and then infect cyclopoid copepods, which then are consumed by the threespine sticklebacks. The tapeworm penetrates the intestinal wall of the fish and enters its body cavity. There it grows to its final size. In the case of a bird consuming the stickleback the tapeworm then sexually matures in the intestine of the bird. (Orr & Hopkins, 1969) To increase the risk of a stickleback being predated on by a bird, *S. solidus* is able to alter the behaviour, buoyancy and coloration of the fish (Heins et al., 2010).

In order to mitigate the fitness cost that *S. solidus* has on its host, resistance has evolved in certain stickleback populations. Threespine stickleback inhabits both salt, fresh and brackish waters. The species is circumpolar, which means that it can be found in northern Europe, Asia and in North America. For predatory fish such as salmon and char the threespine stickleback is an important prey (Ljunghager, 2014). Some populations of the threespine stickleback develop peritoneal fibrosis as an immune response to being infected with the cestode. This response reduces the growth of the cestode and can in some cases even trap and kill the parasite (Weber et al., 2017). The fibrotic response in stickleback is similar to the one in humans during repair of tissue (Mutsaers et al., 1997). This immune response is costly. It both reduces female fecundity and the mating success of males (De Lisle & Bolnick, 2020).

During breeding season male threespine stickleback display a conspicuous red coloration, which is carotenoid-based. This is called nuptial coloration and is a social signaling. During courtship and in the competition for breeding territories this display of coloration is important (Baube, 1997). Female sticklebacks have been found to prefer to mate with males who have a more intense coloration (Barber & Braithwaite, 2000). Thus, males that can produce brighter and more intense coloration will have a higher mating success. Previous studies by Bakker and Milinski (1991) have shown that the intensity of the nuptial coloration decreases following the experimentally induced infection of *Ichthyophthirius multifiliis*, a protozoan parasite. The brighter males also showed a greater resistance to the infection than males who were dull. A parasitic infection does not by definition mean that the male in question will be duller. However, Folstad *et al.* (1994) have found that parasites transmitted through copepods are associated with differences in coloration of the stickleback.

Mating success among male sticklebacks has been shown to correlate with the level of fibrosis. Males who were caught defending active nests had a lower level of fibrosis than males who were sampled randomly (De Lisle & Bolnick, 2020). So, is it possible that fibrosis decreases or in some way affects the nuptial coloration?

Method

Adult threespine sticklebacks were captured between June 2 and 8 in 2019. They were captured in the two lakes, Boot Lake and Roselle Lake, on Vancouver Island, British Columbia, Canada. A total of 387 sticklebacks were analysed here, of which 289 were males. The lakes were chosen because the populations of sticklebacks there had been observed to be infected with *Schistocephalus solidus* and respond with fibrosis. To be able to compare reproductive success between males with and without fibrosis, both nesting and random males were caught. Nesting males were identified by snorkeling to observe nesting behaviour and egg fanning. All fish were frozen and brought back to the lab, where they were photographed under standardized lighting conditions, examined for parasites and their fibrosis was scored on a scale from 0-3, where 0 corresponds to no apparent fibrosis and 3 corresponds to excessive fibrosis (Bolnick & De Lisle, 2020). The fibrosis-scoring method has been shown to be highly repeatable between independent observers (Goldzmid & Trinchieri, 2012).). Photographs were taken using a Canon Rebel XTi with external flash at the same manual exposure settings, in raw file format. Raw files were batch-converted to jpeg using Adobe Camera Raw for later analysis.

To analyse the color differences between fish with and without fibrosis the color histogram in ImageJ was used. ImageJ is a scientific imaging software and can be used in a wide variety of ways (Eliceiri et al., 2012). To determine the brightness level the RGB color histogram function in ImageJ was used (Andrew et al. 2000). The selected area was underneath the eye to the ventral end of the fish. It stretched from the beginning of the eye to the end of the operculum of the gills (Fig. 1A). This is the area where nuptial coloration on stickleback occurs. A control area where no nuptial coloration occurs was also chosen. This area strecthed from the end of the pectoral fin to the beginning of the first anal fin (Fig. 1B). The color histogram provides data from each selected pixel, at 256 (0-255) different brightness levels for the colors red, green and blue separately (Frischknecht, 1993). It is a binary code in 8 bits (8^2 = 256). Since the binary code provides an integer score, the means of each color are unitless. High values indicate light hues, while low values indicate dark hues. A mean value for each of the colors (reg, green and blue) was obtained that provides an overall estimate of average coloration in the selected area.

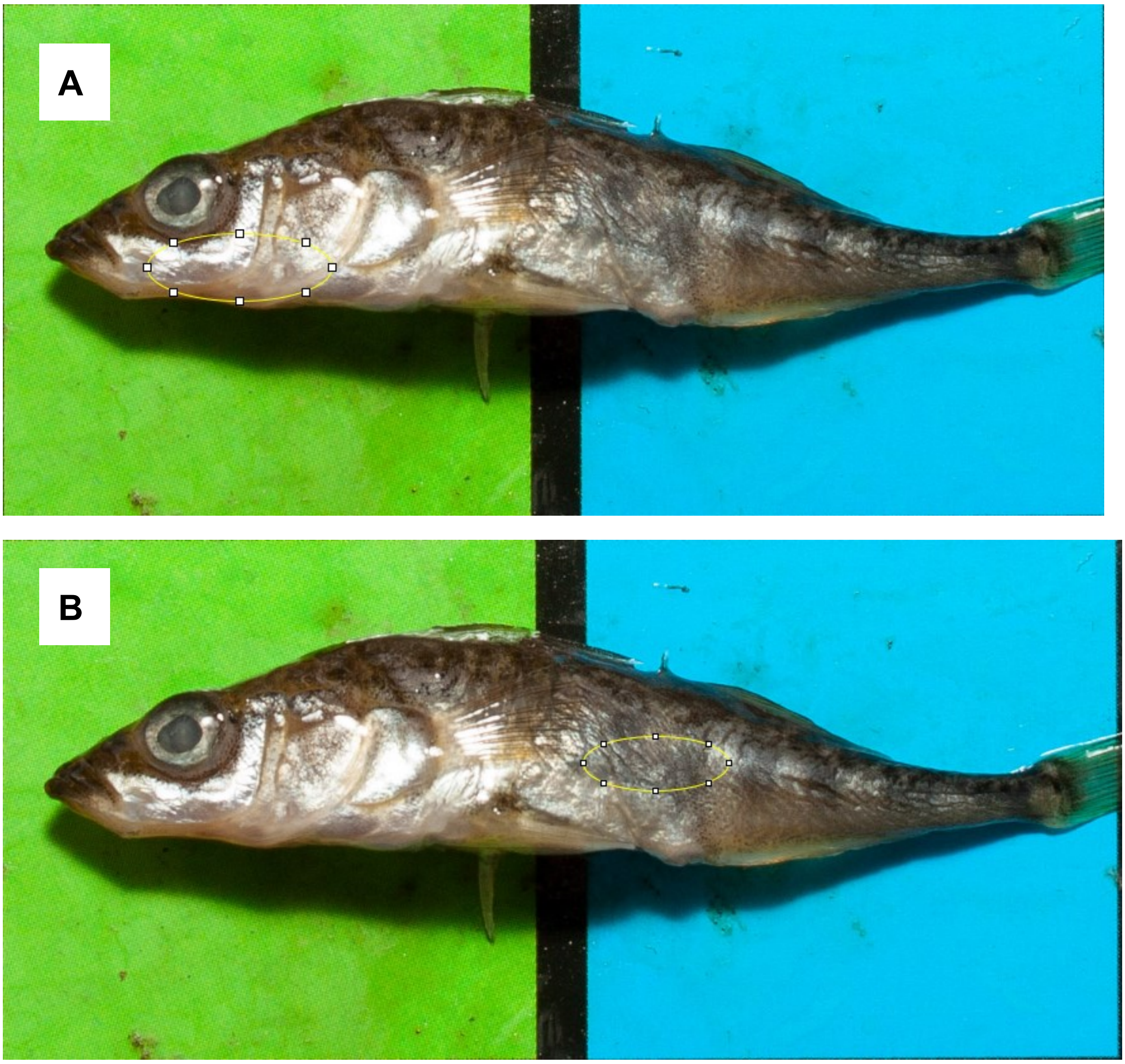

Figure 1. *Example of where on the threespine stickleback coloration was measured. The first picture (A) shows the area of possible nuptial coloration while the second (B) shows where the control was measured.*

Statistical analysis

The following series of two-way ANOVA models were fitted to assess the color variation across lakes, sexes and level of fibrosis. From past work and casual observation, it is known that there is a difference in color between sexes and in coloration between the two lakes. To confirm this a two-way ANOVA with the fixed factors sex and lake, and mean red as the dependent variable was performed. The total number of fish, female and male, was 387. The rest of the tests were also two-way ANOVAs, but only the data for males was used (289 males). Past work also gives the knowledge that sexual selection in part is based on the brightness of the nuptial coloration, and that nuptial colors and female preference for them differ across lakes (De Lisle & Bolnick, 2020). In order to confirm this, each of the three colors (red, green and blue) was tested with the fixed factors mating status (caught in trap/ caught protecting active nest) and lake. Thus, these first two models allow confirmation of effects we fully expect to see and know are consistently present, allowing us to ensure that our image analyses were indeed capturing biological reality. Finally, our main interest lies in understanding if fibrosis affects the nuptial coloration of a male. To determine this, separate two-way ANOVAs were conducted for each of the three colors as dependent variables, with fibrosis and lake as fixed factors. Fibrosis was set to two levels. No apparent fibrosis was named 0 and presence of fibrosis named 1. The effects of *S. solidus* infection on nuptial coloration was tested in the same way as the effect of fibrosis. Infected males were given a 1 and uninfected were given a 0. A two-way ANOVA was also conducted for the mass of *S. solidus* as a fixed factor.

Results

Effect of sex and lake on coloration

A significant difference in coloration was observed both between sexes ($F_{1, 382} = 64.381$, $p < 0.001$) and between the two lakes Boot Lake and Roselle Lake ($F_{1, 382}= 45.8$, $p < 0.001$) (Fig. 2). Individuals from Boot Lake were significantly lighter than those from Roselle Lake, which are darker red. The females are overall lighter in coloration than the males (Fig. 2).

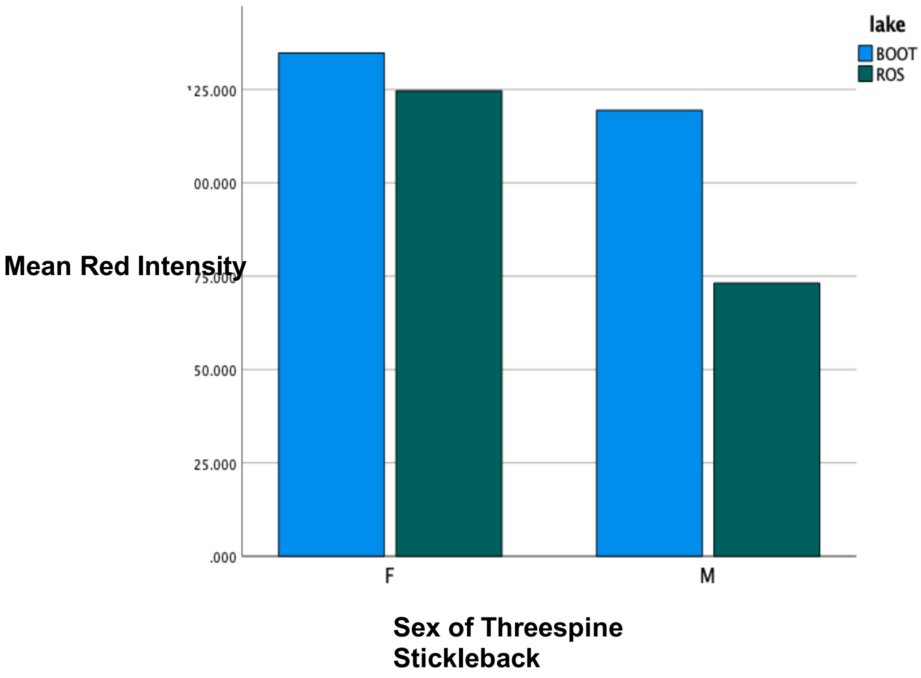

Figure 2. *The mean red (of the bit measure on the RGB color channel) of females (F) and males (M) ($F_{1,\,382}$ = 64.381, p < 0.001) in the two lakes Boot Lake (BOOT) and Roselle Lake (ROS) (F1, 382= 45.8, p < 0.001).*

Difference in coloration depending on mating status

It can be observed that a significant difference in coloration exists between males who were caught protecting an active nest and males who were caught in traps (Fig. 3). Of the 289 males in this study, 98 were caught protecting active nests. The significant difference is observed in all three colors, red ($F_{1,\,281}$ = 35.833, p < 0.001), green ($F_{1,\,281}$ = 34.759 , p < 0.001) and blue ($F_{1,\,281}$ = 54.625 , p < 0.001).

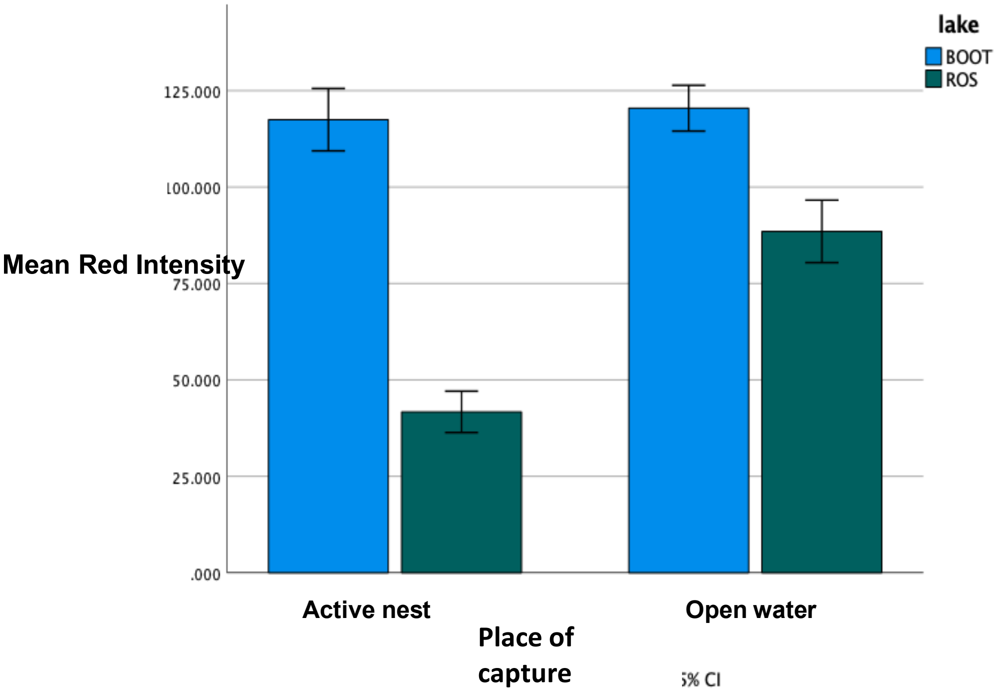

Figure 3. *Mean red (of the bit measure on the RGB color channel) for males trapped in each lake, Boot Lake (BOOT) and Roselle Lake (ROS) ($F_{1,281}$ = 176.839, $p < 0.001$) depending on their mating status ($F_{1,\,281}$ = 35.833 $p < 0.001$).*

The effects of fibrosis on coloration

For the mean of red ($F_{1,\,281} = 0.485$, $p = 0.487$) no significant effect of fibrosis is observed (Fig. 4). Neither is a significant observed effect for the colors green ($F_{1,\,281}$= 0.470, $p = 0.493$) (Fig. 5) or blue ($F_{1,\,281} = 0.015$, $p = 0.904$) (Fig. 6).

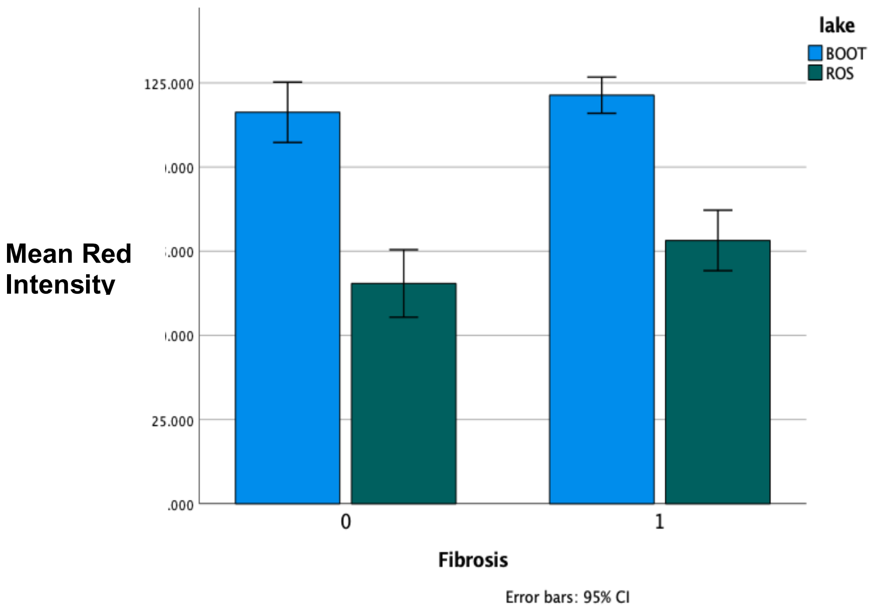

Figure 4. *The effect of fibrosis in male threespine stickleback on the mean of red (of the bit measure on the RGB color channel) ($F_{1,\,281}$ = 0.485, $p = 0.487$). A comparison between males with fibrosis (1) and males without fibrosis (0) for the two lakes Boot Lake (BOOT) and Roselle Lake (ROS).*

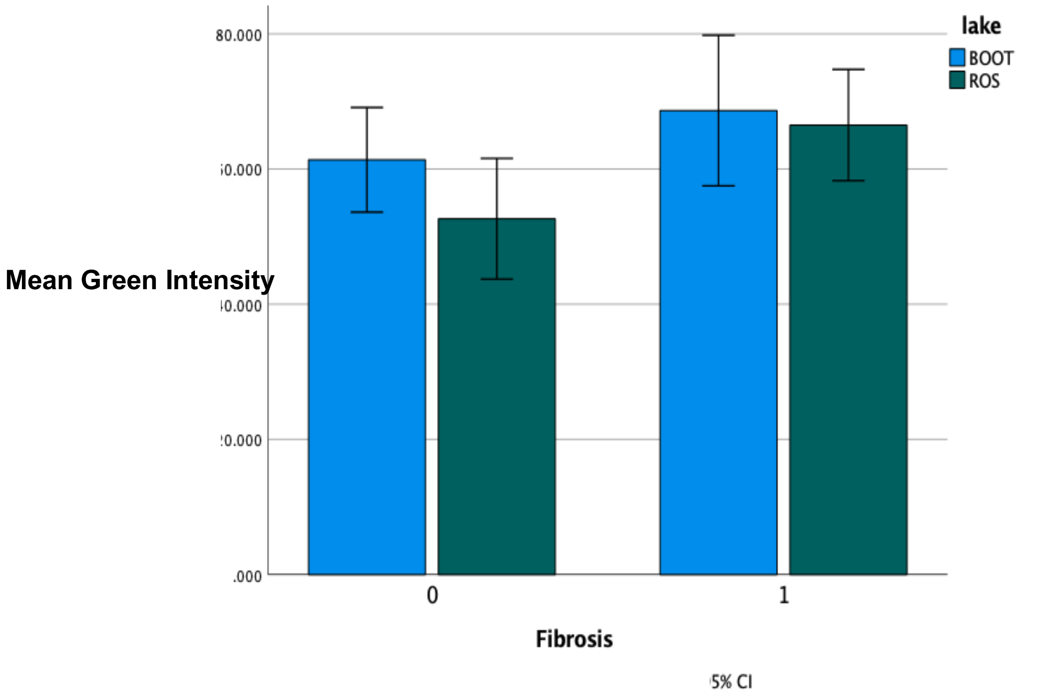

Figure 5. *The effect of fibrosis in male threespine stickleback on the mean of green (of the bit measure on the RGB color channel) (F1, 281= 0.470, p = 0.493) . A comparison between males with fibrosis (1) and males without fibrosis (0) for the two lakes Boot Lake (BOOT) and Roselle Lake (ROS).*

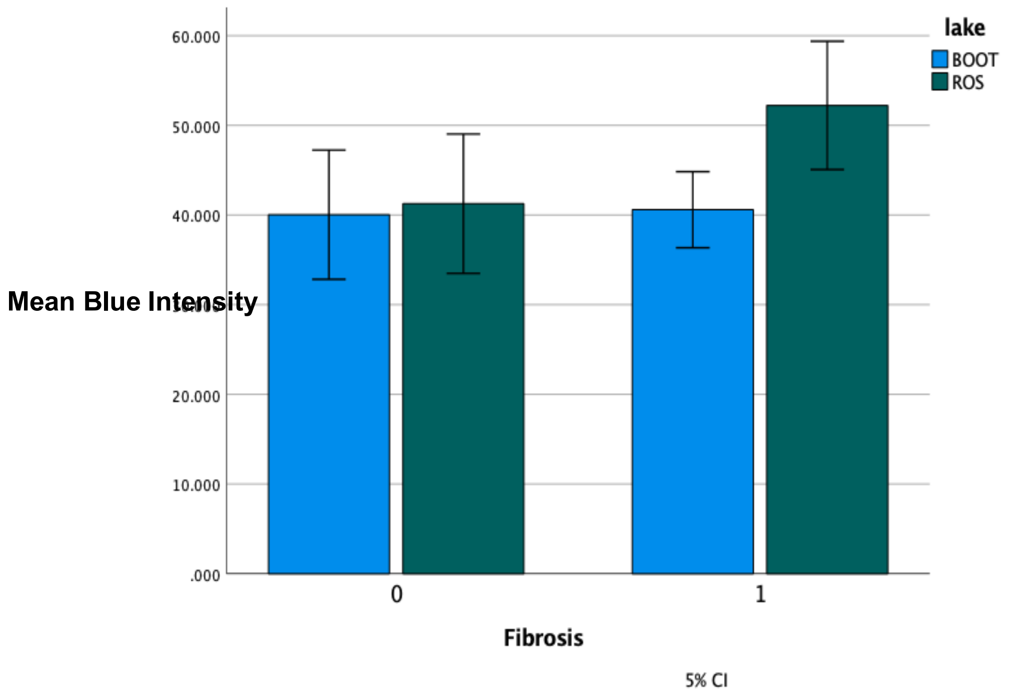

Figure 6. *The effect of fibrosis in male threespine stickleback on the mean of blue (of the bit measure on the RGB color channel) (F1, 281 = 0.015, p = 0.904). A comparison between males with fibrosis (1) and males without fibrosis (0) for the two lakes Boot Lake (BOOT) and Roselle Lake (ROS).*

Schistocephalus solidus effect on coloration

The male threespine stickleback were tested for an effect on nuptial coloration depending on whether they were infected with the tapeworm *S. solidus* or not. Neither of the colors red ($F_{1, 281} = 1.113$, $p = 0.292$) (Fig. 7) nor green ($F_{1, 218} = 3.093$, $p = 0.080$) (Fig. 8) is shown to be significantly affected by the tapeworm infection. A small statistically significant effect is observed for the color blue ($F_{1, 281} = 4.265$, $p = 0.040$) where the infected males have a slightly brighter color (Fig. 9).

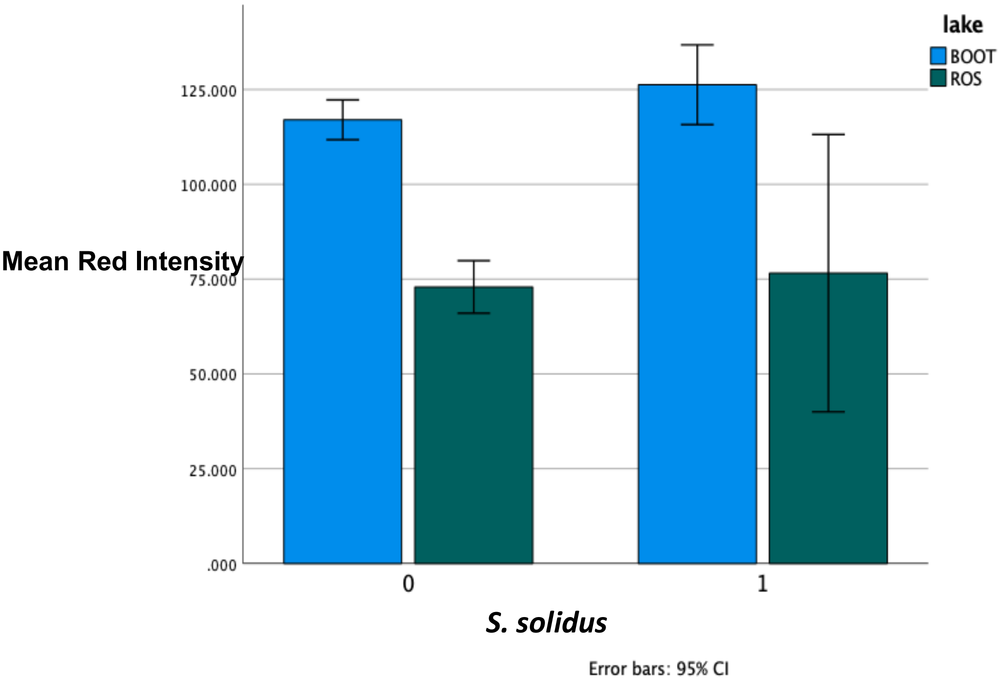

***Figure** 7. Mean red (of the bit measure on the RGB color channel) for males with (1) and without (0) S. solidus ($F_{1, 281}$= 1.113, p = 0.292) in the two lakes Boot Lake (BOOT) and Roselle Lake (ROS).*

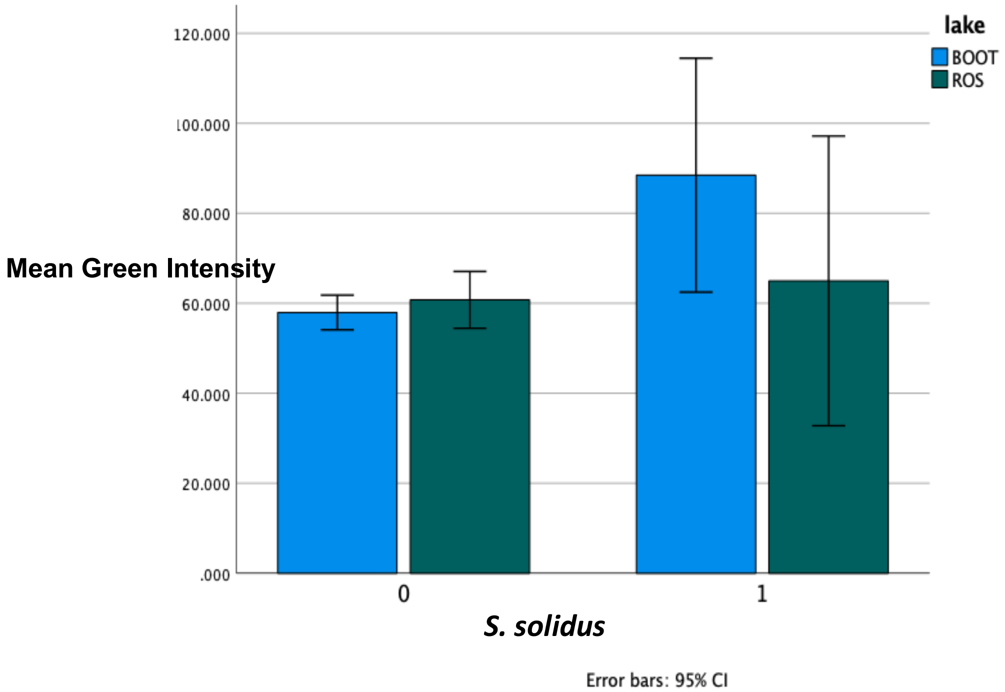

Figure 8. *Mean green (of the bit measure on the RGB color channel) for males with (1) and without (0) S. solidus ($F_{1, 218} = 3.093$, $p = 0.080$) in the two lakes Boot Lake (BOOT) and Roselle Lake (ROS).*

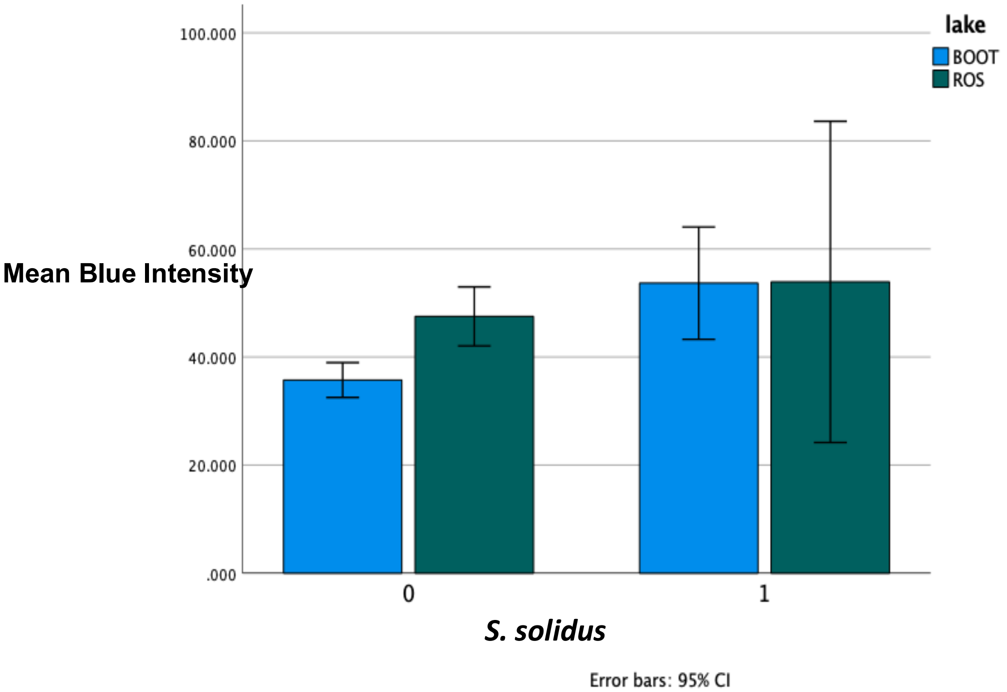

Figure 9. *Mean blue (of the bit measure on the RGB color channel) for males with (1) and without (0) S. solidus ($F_{1, 281}= 4.265$, $p = 0{,}040$) in the two lakes Boot Lake (BOOT) and Roselle Lake (ROS).*

If the coloration of the males depended on the total mass of *S. solidus* was also investigated. The brightness of the mean red ($F_{1, 242} = 1.164$, $p = 0.238$) was not significantly affected. Both the green ($F_{1, 242} = 8.027$, $p < 0.001$) and the blue ($F_{1, 242} = 2.137$, $p < 0.001$) means were significantly affected by the mass of the tapeworm.

Discussion

In this study I analyzed color variation in wild caught fish that exhibit a natural and measurable immune response to a common parasite. Past work from De Lisle & Bolnick (2020) has shown that this fibrosis immune response carries costs for host male nesting success, and my hypothesis was that this nesting success cost may be the result of reduced nuptial coloration in fish that exhibited this immune response. I found out that this was not the case. No relationship between fibrosis and nuptial coloration was found.

What could be the reason behind that there is no significant effect of fibrosis on the nuptial coloration of male threespine stickleback? Could these results be the product of not enough data? That is unlikely. Already known patterns, like the difference in coloration between males and females, difference in coloration between the lakes and the color difference between nesting males and males caught at random, could be observed with this set of data and the color analysis techniques I employed. This indicates that the results where fibrosis plays no role in the nuptial coloration is well supported.

Why is it then that the immune response fibrosis causes a lower fitness? This could be due to any number of reasons. It could be a result of reduced swimming performance, leading to a poor ability to defend a nest. Nest defense requires a lot of energy and swimming performance of the fish. The male is constantly on the lookout for other males that want to fight and even swarms of females that would happily eat all the eggs in the nest. So any slight physiological/physical swimming performance disadvantage could be very costly for nesting success. It could also be that fibrosis affects other traits that are important for mate choice, but not color. For example, perhaps it affects chemical cues, but not the visual coloration.

These are of course speculations. However, my findings are also consistent with recent unpublished work (by S. Tsuruo) in the D. Bolnick group at the University of Connecticut, which found no evidence of reduced sex hormone expression in male fish that had artificially-induced fibrosis immune response in the lab. Both this work and my work indicate that fibrosis has little to no effect on the development of secondary sex traits in stickleback. To fully understand the reasons and causes of immune response and their fitness effects, more research is needed.

Understanding why immune traits affect fitness is key for understanding if and how they evolve. In this study, I show that there is little evidence that fibrosis affects male nuptial coloration. This indicates that more work, particularly experiments may be important for understanding why fibrosis leads to a lower mating success in affected males.

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