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Environmental and Physiological Regulation of Yellow Stingray Color Change

Theresa Rose Gunn

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ENVIRONMENTAL AND PHYSIOLOGICAL REGULATION OF YELLOW STINGRAY COLOR CHANGE

by

THERESA ROSE GUNN

(Under the Direction of Christine Bedore)

ABSTRACT

Many reef fishes exhibit dynamic coloration and body patterns that can change under nervous or hormonal control. Several species of benthic sharks and rays likely alter melanin in the skin to provide background matching for camouflage. The yellow stingray (*Urobatis jamaicensis*) is a benthically-oriented elasmobranch with elaborate spot patterns that provide effective camouflage within its habitats. This patterning, when coupled with the ability to alter melanin in response to background color, could increase background matching effectiveness in these species. The yellow stingray has been anecdotally noted to lighten or darken skin color. However, it is unclear whether this type of change is controlled by Morphological or Physiological change. Manipulation melanin content to produce a color change is termed Morphological color change. The movement of pigment granules alone to produce a color change is termed Physiological color change. Despite the wide array of studies conducted on color change for enhanced background-matching capabilities in bony fish, this ability and its mechanism remains understudied among elasmobranchs. To investigate this, we housed rays in either black or white tanks for one week and photographed the rays daily. On the last day, blood and skin samples were taken to quantify melanin concentrations, and cell morphology. Stingrays in black tanks significantly darkened skin color over the seven-day period whereas rays in white tanks significantly lightened their skin color during the same period. However, skin melanin concentrations did not differ between rays maintained in black or white tanks after seven days. Furthermore, stingrays had the same cell density after seven days but the melanophore index values (Hogben and Slome, 1931) were different between tank treatments. These results demonstrate that yellow stingrays do regulate their body color in response to background color. Furthermore, the results suggest that stingrays undergo physiological (not morphological) color change in order to regulate body color. This is the first study to both quantify the background matching response and evaluate the physiological regulation of that response in a stingray species.

INDEX WORDS: Stingray, Elasmobranch, Color change, Physiological color change, Melanin, Melanophore, Regulation, Background color, Background matching.

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B.S., Florida Atlantic University, 2013

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MASTER OF SCIENCE

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INTRODUCTION

Camouflage is the ability for an organism to conceal itself in its environment and render itself undetectable and is widespread throughout the animal kingdom (Stevens and Merilaita, 2011). This ability for animals to avoid detection greatly reduces the probability of predation and increases success of prey capture (Stevens, 2007). Camouflage is especially important for animals that are easily preyed upon and that are limited in their ability to defend themselves. For example, cuttlefishes are soft bodied animals without overt mechanical defenses, but are equipped with multiple dynamic modes of camouflage which aid in predator avoidance against a wide range of predator foraging strategies (Bedore et al. 2015; Hanlon and Messenger, 1988). Therefore, the ability to avoid detection by predators, rather than attempt escape after detection, is likely highly favored in this group of animals (Stuart-Fox and Moussalli, 2009).

Color change in fishes

Many fishes, including several species of flounder, also exhibit camouflage and rely heavily on avoiding detection by employing a technique called background matching (Burton and Vokey, 2000). Background matching is a form of camouflage in which an organism's body color, texture, or shape, mimics the environment in which it lives (Stevens and Merilaita, 2011). Background matching is especially useful when an animal can manipulate some of these features, such as body color, to match a wider variety of environments seamlessly.

Background matching via color change in animals is achieved through manipulation of pigment containing cells called chromatophores. Melanophores are a specific type of chromatophore which contain the brown/black pigment melanin. These chromatophores are associated with lightening or darkening the overall body color to appear more similar to the

brightness of the environment or background (Skold et al., 2013). Manipulation of melanophores for color change occurs through either morphological or physiological color change. Morphological color change occurs when pigment granules or the pigment cells themselves are being synthesized or degraded in order to modulate an animals color (Sugimoto, 2002). For example, red seabream (*Pagrus major*) exhibit morphological color change when held in shallow (20 meter) outdoor net cages (Adachi et al. 2005). These ‘sun tanned’ bony fish increase melanin concentrations, darkening their skin color in some areas to half the lightness of wild type fish, likely as a protective mechanism when exposed to the high levels of ultraviolet (UV) radiation which can permeate their shallow water cages. This type of color change is commonly a response to stimuli that require long-term responses (days to months), such as protection from extended exposure to harmful UV radiation.

Alternatively, physiological color change occurs when the pigment granules themselves are moved throughout the chromatophore to produce a color change through aggregation or dispersion. Physiological color change is common in many bony fishes (teleosts) (Skold et al., 2013). For example, zebrafish (*Danio rerio*) lighten or darken their body color in response to changes in background color by aggregating or dispersing melanin granules within melanophores (Logan et al. 2006). This type of color change is used on a short term scale (seconds to hours), where an animal may need to manipulate color in response to changing environments or to mediate predator-prey interactions (Stuart-Fox and Moussalli, 2009).

Prior studies on fish have evaluated these melanophore changes by examining melanophores in skin samples under a microscope and assigning the skin a value based upon the state of dispersion or aggregation of the cells (Figure 1). The disadvantage of this method is that there are often intermediate states in which the reviewer decides the MI value using personal

judgement which is subjective by nature. One way to minimize subjectivity when evaluating color change in an organism is to extract melanin pigment from the cells and quantify the concentration of melanin in the skin. Additionally, as Hogben and Slome's method only describes the appearance of melanophores it cannot indicate whether more pigment is made or degraded in order to generate a color change. Using these techniques together can help to elucidate the type of color change that has occurred through evaluating both the appearance of the melanophore as well as the amount of melanin present.

Color change in elasmobranchs

Despite a lack of many published studies, some elasmobranchs (sharks, skates, and rays) have been reported to change their skin color in a captive setting. Specifically, small-spotted catsharks and three species of skates demonstrate a darkening or lightening of skin color when placed in either black or all white tanks, effectively background-matching to their environments (Hogben and Slome, 1931, Waring, 1938; Chevins & Dodd, 1970). Color change for background matching in marine elasmobranchs is considered to be mediated primarily through melanophores (Visconti et al. 1999). The mechanism is hypothesized to occur by expansion/contraction of melanophores (physiological color change) similar to some teleosts, reptiles, and amphibians through manipulation of hormone concentrations (Visconti et al. 1999).

Previous studies did not investigate the environmental variables associated with the purported color change in combination with the physiological regulatory mechanisms in the same species. Many of the studies completed on background matching abilities in elasmobranchs have been focused on small shark species and a comprehensive study on a single stingray species has yet to be completed (Waring, 1938; Chevins & Dodd, 1970; Hogben and Slome, 1931). This is

problematic as there are several differences between the two groups, for example sharks are covered with placoid scales (dermal denticles) while they are sparsely distributed in most batoids. This difference in skin morphology could affect the location or action of dermal melanophores between the two species. Furthermore, sharks and rays have various morphological (i.e. body shape, locomotion) and life history differences (i.e. trophic level) which could affect which environmental cues initiate color change. Considering the various differences in shark and rays, it is difficult to draw conclusions about physiological mechanisms or functions of color change in elasmobranchs with current knowledge.

Study species

Many stingrays are benthically oriented, are commonly prey species of large sharks, and are patterned, suggesting that they rely heavily on camouflage. Considering these life history traits, stingray species have an ecological need to maintain camouflage as it could aid certain stingray species which already rely heavily on camouflage to increase success of prey capture and for predator avoidance. The yellow stingray (*Urobatis jamaicensis*) is a small stingray species (less than 25 cm disc width (Fahy, 2004)) that is cryptically patterned, and as such, likely relies upon their coloration for camouflage on shallow patch reefs. Yellow stingrays also been anecdotally noted to lighten or darken skin color. Prior studies have documented the spectral reflectance of overall body color of yellow stingrays (showing they have peaks in the yellow and brown region of the visible light spectrum) but did not evaluate lightening or darkening of body color (Bedore et al, 2013). To address the mechanistic and functional significance of color change across cellular and organismal levels, I investigated yellow stingray responses to colored tank environments through maintenance of stingrays in black and white tanks and evaluation of dermal melanophore responses.

Hypotheses and predictions

H₁: Yellow stingrays will change the color of their skin (through lightening or darkening) to match different tank color environments (black or white).

Prediction₁: Yellow stingrays will be significantly different from initial in brightness after seven days in each tank environment (black or white).

Prediction₂: Yellow stingrays maintained in white tanks will be significantly lighter than yellow stingrays in black tanks on day seven.

H₂: Stingrays will undergo physiological color change to regulate melanin granules and thus color change.

Prediction₁: Stingrays maintained in black tanks will have the same concentration of melanin as stingrays in white tanks after seven days.

Prediction₂: At the end of experiments (seven days) stingrays maintained in black tanks will have more dispersed melanophores (MI 4-5) than stingrays in white tanks (1-2).

Prediction₃: Stingrays will have the same number of melanophores regardless of tank environment (black or white).

METHODS

Experimental animals and housing

Individual yellow stingrays were captured by an aquarium distributor from waters of Palm Beach and Broward County, Florida. Stingrays were transported in black plastic bags filled with buffered and oxygenated sea water to the aquarium facilities at Georgia Southern University within three days of capture. Transport was completed via an air conditioned vehicle. Upon arrival to the lab, stingrays were acclimated to lab conditions in a 3-gallon black tank for one to three hours.

Rays were housed individually in 40-gallon black or white tanks which were identical except for tank color, each with a 20-gallon sump, supplemental aeration, and a multistage canister filter (Marineland C-530). Each tank was illuminated by broadband LED reef light fixtures (Marineland reef capable LED aquarium strip light) and maintained on a 12hr:12hr light:dark cycle. Each tank was painted either white or black using aquarium safe black or white paint. Apart from color, black or white tanks were identical, and contained sand that matched the paint color (black or white Carib Sea sand). Up to four individuals were housed simultaneously, each in their own tank.

Photographs and sample collection

After acclimation, but before the stingrays were introduced to their experimental tanks, each ray was photographed in a white photograph tank to quantify initial coloration (described below). After photographs were taken, stingrays were randomly assigned to a black or white tank treatment, where the ray remained for the duration of the experiment (black tank n=8; white tank n=9). Rays were photographed daily in the laboratory for seven days and photos were taken at

the same time each day (± 3 hours) to control for the potential influence of circadian rhythm on coloration. Following photographs on the seventh day, all rays were sacrificed by tricane overdose ($>0.5\text{mg/L}$) to obtain samples for physiological analyses (Table 1). Control animals ($n=4$) were acquired to evaluate the color and melanophore state of yellow stingrays before being exposed to tank treatments. These rays were acquired and acclimated identically to treatment rays, but were not introduced to treatment tanks and were instead sacrificed and sampled following photographs.

In order to evaluate plasticity of the color change response to varying tank color environments on individual stingrays, two stingrays were acquired and maintained as described above (one ray in each tank color), however, on day seven, rays were moved to the opposite tank color, where photographs were taken daily for an additional seven days. All experiments were conducted in accordance with IACUC standards and protocols (Protocol #: I16004).

Photographic analysis

Photographs were taken in a 30-gallon tank, which was painted white, using the same white aquarium safe paint as the holding tanks. Two white LED lights, positioned 45cm above the rim on both sides of the tank, illuminated the tank evenly. Photographs were taken with a Cannon Powershot G12 camera suspended approximately 61 cm above the rim of the photo tank. Inside the photo tank, grey color standards (Color aid gray set 9x12) were placed in four corners of the tank. The gray standards are matte sheets that contain a set of 10 shades ranging from black to white and each color has a known reflectance value. RGB values (intensities) are obtained by sampling each of the grey standards using the “Eyedropper tool” in Photoshop. This tool measures the RGB (Red, Green, and Blue) values for any particular point on an image. The

RGB values were plotted against the known reflectances for each of the 10 shades creating a standard curve following methods of Tedore and Johnsen (2012).

In order to evaluate the background matching response, we calculated the brightness of each individual for each day of the experiment. The brightness value acts as an indicator of how light (white = 255) or dark (black = 0) a particular object is. Photos of stingrays were analyzed in Photoshop by averaging a 100x150 inch square in 11 predetermined areas along the dorsum of the stingray and then obtaining RGB values (intensities). The RGB values of the averaged areas were measured using the “Eyedropper tool” in Photoshop and a brightness value for each of the 11 areas was calculated by comparing these RGB intensities to the standard curve. After the brightness values for each of the 11 spots had been determined they were averaged together in order to assign an overall brightness value to the stingray.

General least squares regression (GLS) was used to assess if final brightness on day seven differed between rays in the two tank color environments, using the *gls* function in the *nlme* package in R v.3.3.2 (Pinheiro and Bates 2000, R Core Team, 2016). This modeling framework was used as the assumption of equal variance for the same model using simple linear regression was violated (i.e. variance increased with increasing fitted values), and GLS allows for the inclusion of a variance structure to fix patterned residuals (Zuur et al., 2009). Model selection was conducted using Akaike’s information criterion corrected for small sample sizes (AICc) to compare a simple linear model to three GLS models, each with a different variance structure included (Burnham and Anderson, 2010). A GLS model with the power of the covariate variance structure was the most supported model (AICc = 118.59) compared to a simple linear model (AICc 134.19), and GLS models with a fixed variance structure (AICc = 127.28),

exponential variance structure (AICc = 122.28), and a variance structure for each tank color environment (AIC = 124.29), respectively.

Melanin quantification

To quantify differences in melanin concentration between rays held in black tanks, rays held in white tanks, and control rays, melanin was extracted from skin samples by serially washing following Pavlidis et al. (2006) and Wilson and Dodd (1973). Skin samples were thawed and soaked in 95% EtOH overnight, after which, they were samples boiled in 1% HCL for 1 hour. The HCL was then removed from vials by washing with distilled water. Samples were then boiled in 1.5mL of a NaOH- H₂O₂ (100ml of 0.2% NaOH with 1.5 ml of 3% H₂O₂) for 1 hour before centrifugation at 13,000 rpm for 15 minutes. The resulting supernatant was removed and melanin was resuspended in 1.5 mL of the NaOH- H₂O₂ solution by boiling until dissolved (~30 minutes). The absorbance of each sample was read on a spectrophotometer at 360nm (Spectronic spec200 ThermoFisher Scientific). Concentration for each sample was calculated using a standard curve of serial dilutions of synthetic melanin pigment (Sigma Aldrich). Melanin concentration was compared across tank treatments using a one-way anova with $\alpha=0.05$.

Melanophore Index and melanophore cell counts

In order to evaluate differences in dispersal state of melanophores from rays held in black tanks compared to those in white tanks, skin samples stored in 4% paraformaldehyde were analyzed microscopically. Post-fixation, skins were sliced from underlying muscle tissue with

care taken to not damage the epidermal layer. The resulting skin sample was then placed on a slide with a photoetched gridded coverslip with 0.25mm^2 square grids and examined under 40x magnification using a light microscope for cell counts and assigning dispersion state. Five randomly selected square cells (0.25mm^2 each) within the gridded coverslip were selected for cell density and MI analysis.

To quantify cell density, the number of melanophores for each skin sample was recorded from each grid in the coverslip in which the skin was fully enclosed by a square. Then the total number of cells counted per individual was divided by the total area of the skin evaluated (1.25mm^2) resulting in the cell density (cell/mm^2). The melanophore cell density values were compared using a student's t-test in order to test for significant differences in the density of cells between black and white tank treatments (JMP).

To compare dispersion state of melanophores from rays in black tanks and rays in white tanks, a melanophore index (MI; Hogben and Slome 1931) was used to assign a number from 1-5, according to the state of contraction/expansion of the melanophore (Figure 1). Any cells with intermediate states were rounded up to the higher MI value. This MI value was assigned to every cell within the five randomized grids chosen. The median MI value per individual was calculated and tank treatments were compared using a Mann-Whitney-U test (white tanks $n=6$; black tanks $n=7$; controls $n=4$). A non-parametric analysis was chosen due to a violation of the assumption of normality (JMP).

Also, 25 randomly selected cells per individual were used in to evaluate frequency of each MI state and a chi square contingency analysis on the proportions of MI per tank treatment was completed (JMP).

RESULTS

Photographic analysis

Our best model found that initial brightness was not different between the two tank color environments indicating that all rays were the same brightness before being exposed to their tank treatments (GLS, $F_{3,12} = 6.22$, $p > 0.05$). Rays were significantly different with respect to whole-body brightness on day seven compared to day zero for both tank treatments. Specifically, rays in black tanks were significantly different in final brightness (mean = 27.303 ± 2.284) than initial brightness (mean = 34.056 ± 2.284) (paired t-test, $t(8) = -2.956$, $p = 0.018$) and rays in white tanks were significantly different in final brightness (mean = 68.703 ± 5.587) than initial brightness (mean = 32.340 ± 5.587) (paired t-test, $t(7) = 6.507$, $p < 0.001$) (Figure 3). This indicates that the rays did change their brightness in response to tank color. When evaluating final brightness alone, rays in white tank and black tanks were found to be significantly different from each other on day seven with final brightness found to be 40.54 units higher in rays in white tanks compared to rays in black tanks (GLS, $F_{3,12} = 6.22$, $p < 0.001$). This shows that rays in white tanks have a higher brightness indicating a lighter skin color than rays in black tanks which statistically confirms the phenotypes which were visually observed (Figure 3). Furthermore, our model indicated that stingrays in white tanks exhibited a greater change in overall brightness compared to stingrays in black tanks over the seven-day period (Figure 4). Rays in white tanks significantly changed their brightness faster than those in black tanks, where rays in white tanks were significantly different after only 1 day while rays in black tanks were not (paired t-test, $t(6) = 5.43$, $p = 0.0016$). Rays in black tanks were not significantly different in final brightness from initial brightness until >3 days. Although not analyzed statistically, rays ($n=2$) that were placed in one tank color for one week and

then in the opposite color for an additional did change their brightness according to tank treatment each time they were moved (Figure 5).

Melanin quantification

Melanin concentration (mg/mm² of skin) did not differ significantly across tank treatments (including procedural control) (ANOVA, $F_{2,17}=1.510$, $p=0.252$) (Figure 6). Black tank rays had a mean concentration (mean \pm SE) of 0.0015 ± 0.0001 mg/mm² of skin, while white tanks had a mean of 0.0010 ± 0.0001 mg/mm² of skin, and controls had a mean of 0.0012 ± 0.0002 mg/mm² of skin. There was no relationship between brightness and melanin concentration (Linear Regression, $F_{1,17}=4.11$, $p=0.059$) (Figure 6).

Melanophore Index and melanophore cell counts

Melanophores of black tank rays were significantly more dispersed than those of white tank rays, as indicated by their median melanophore index (mean \pm SE), (MI black tanks mean = 4.285 ± 0.285 ; MI white tanks mean = 2 ± 0.447) (Mann-Whitney U, $\chi^2(1, N=7) = 7.6402$, $p=0.005$) (Figure 7). Melanophores of control rays were intermediate (3.250 ± 0.629) (contingency analysis, $\chi^2(8, N=17) = 82.273$, $p < 0.001$) (Figure 7). Although melanophores were more dispersed in black tank rays, there was no difference in cell density among tank treatments (ANOVA, $F_{2,16} = 2.8329$, $p=0.09$; (mean \pm SE) white tanks 164.00 ± 14.89 cells/mm², black tanks 145.48 ± 13.79 cells/mm², control tanks 108.15 ± 18.24 cells/mm² (Figure 8)).

DISCUSSION

We have shown in this study that yellow stingrays are able to change their overall body color with respect to their environment (tank color). After placing stingrays in all black or all white tanks, rays reliably lightened or darkened their body color correspondingly. The lack of difference in melanin concentration and cell density across tank colors indicates that stingrays undergo physiological color change when regulating body color in response to tank color. Therefore, the stingrays were not making or breaking down melanin or melanophores in order to create differences in overall body color, but were instead moving pigment around within the cell to create the change. This conclusion is further supported by the difference in dispersion state of the melanophores, in which rays from black tanks had significantly more dispersed melanophores than white tank rays.

Background matching

Rays housed in white tanks were 149.8% brighter on day seven than on day zero, whereas stingrays housed in black tanks were -20.4% darker. One explanation for the difference in percent change in brightness is that without a uniform background stimulus (black or white) stingrays maintain a high contrast coloration. This high contrast (areas of dark adjacent to areas of white/yellow) could be the most advantageous coloration maintained to avoid detection in their naturally colorful and variable rock reef or brown/green sea grass bed environments (Ward-Paige et al, 2010). In this state, stingrays may maintain melanophores at a mostly dispersed state (~4 on MI scale) in which they are closer to their darkest possible physiology. Meaning that when the stingrays are placed in the white tanks their melanophores have to undergo a much larger change to background match (from MI ~4 to MI~1) to their new tank environments. While stingrays in that are placed in black tanks have a far smaller change to facilitate background

matching (from MI~4 to MI~5). This disparity in distance between initial MI values and their maximums could explain why rays in white tanks became so much lighter compared to their initial values when compared to stingrays in black tanks. Alternatively, preliminary data suggested that stingrays changed their color within approximately one day placed into white tanks while rays in black tanks took greater than three days to be significantly different in color. This could indicate that it is more energetically costly to expand melanophores from the intermediate state (3 on MI scale) than to aggregate them.

If the rays were not creating or breaking down melanophores, as our data suggest, there is a limited amount of melanin to be moved around which would directly limit the ability for the ray to be lighter or darker. Therefore, there may be a physiological limit to how much the stingray can lighten or darken over seven days. There is likely a large metabolic cost to changing skin color which could outweigh the benefit of lightening or darkening skin color past the maximums observed in this study. This could be another reason for why the rays did not become as proportionally darker as the rays that became lighter. Interestingly, stingrays also became lighter faster than they became darker which could indicate that it is metabolically less expensive for the melanophores to contract than it is for them to disperse. However, although the cells are believed to move melanin granules around using microtubules, the mechanism of moving melanin granules within a melanophore has yet to be fully elucidated. Therefore, it is difficult to determine what the energetic costs and benefits may be. There could have been an effect of transport (from capture to the facility) on body color which led the rays to maintain the high contrast coloration as a response. However, all stingrays the same brightness before entering into the treatment tanks, so initial coloration did not have an effect on the final results (Figure 4).

Regulation of body color in response to background color has been categorized directly in a few small shark species and a skate species (Waring, 1936; Chevins and Dodd, 1970; Wilson and Dodd, 1973). However, no study to our knowledge has documented the background matching response in a stingray. Visconti and Castrucci (1993) investigated key hormones which were determined to regulate color change using the skins of the freshwater stingray, *Potamotrygon reticulatus*. However, despite determining the key hormones implicated in whole body color change in the freshwater stingray, the isolated cellular responses were not evaluated with the consideration of background color. Future studies should evaluate the role these hormones play in whole body color change of stingrays that are adapted to colored environments.

Physiological color change

Data from this study suggest that yellow stingrays underwent physiological color change to regulate body color in response to changes in background color. This was concluded as there were no significant differences in final brightness and MI values between tank color but no differences in cell density or melanin content between tank color (Figure 4, Figure 6, Figure 7, Figure 8). However, as background matching experiments have only lasted from four to seven days, it is possible that the rays could undergo morphological color change given more time (on the scale of weeks-months) (Waring, 1963). Despite no significant differences in melanin concentration between treatments, there was less melanin in skins of rays in the white treatment (mean= 0.0010 mg/mm² of skin \pm 0.0001) than in rays in black tanks or control rays (Figure 4). Furthermore, rays in black tanks had more melanin in their skins (mean= 0.0015 mg/mm² of skin \pm 0.0001) than rays in white tanks or control rays (Figure 4). These differences were not found to be significant statistically but the trend could imply that the rays have begun to undergo morphological color

change but have not changed enough to be detected with statistics. It is also worth noting that our sample sizes may have been insufficient considering the amount of individual variation to detect the trend. However, although it is possible that the rays could undergo morphological color change it may not be ecologically advantageous nor necessary to lighten or darken beyond the extremes which the rays are able to achieve using physiological color change. Nevertheless, future studies should consider this possibility and factor it into future evaluations of stingray color change.

Ecological and evolutionary considerations

Prior studies have indicated that some bony fish employ physiological color change over short term, and then over long term may switch to morphological color change (Sugimoto, 2002). Morphological color change has been documented in a shark species (young of year scalloped hammerheads) that was exposed to high levels of UV-radiation via outdoor cages over a 215-day period (Lowe and Goodman-Lowe, 1996). This study did not test a background matching response but did demonstrate the ability for elasmobranchs increase melanin concentration in response to an environmental variable. The findings of the current study showed that stingrays modulated their color in response to background color. These rays also inhabit high UV environments so it would be interesting to see which environmental cue would prove to be the larger cue regulating color change. Understanding how these environmental cues influence color change could help us better understand the challenges this species faces in its natural environment.

As the background matching response has been documented in a benthic shark, a skate, and now a stingray species, more questions regarding this conserved response among

elasmobranchs arise. We have observed another stingray species, the smooth butterfly ray (*Gymnura micrura*) exhibiting a similar background matching response (Unpublished data). Further evaluation of this response and its mechanism are currently being investigated. This response is of particular interest because yellow stingrays and smooth butterfly rays inhabit different spectral environments. Yellow stingrays inhabit clear marine environments with generally high visibility where it may greatly benefit the stingrays to be cryptically colored and modulate their background color to conceal themselves visually. The smooth butterfly rays frequent turbid estuarine environments where it is often difficult to see more than a couple meters in any direction. This would suggest that the stingrays are likely not targeted visually and making their background matching ability even more interesting. Considering that multiple stingray species from different visual environments seem to show a similar background matching response leads to some interesting questions. Primarily, it is unclear how widespread the background matching response is among elasmobranchs. It has been documented in small benthic shark species, noted in a skate species, and this study shows a stingray species. However, the small scalloped hammerhead sharks darkened even over sandy bottoms in their enclosures. It may be possible that some species of elasmobranchs do not and would not need to undergo physiological color change to background match. From an ecological perspective, pelagic species such as the scalloped hammerhead may not benefit greatly from the ability to regulate its color and may instead use morphological color change to combat a larger problem faced by the species, excess levels of UV radiation. UV radiation has been found to damage cells leading to some forms of cancer which is problematic for long lived species who spend the entire lives at sea far from any form of shelter. Despite this, a species that may not need to background match in its environment (the smooth butterfly ray) seems to have conserved this background matching

response. Future studies should evaluate the background matching response in species that are not strictly benthically oriented prey species in order to determine if this background matching response is a conserved ability across all elasmobranchs or if it adapted to certain groups with specific life histories.

Conclusions

The ability for stingrays to regulate body color in response to background color presumably aids them in predator evasion and prey capture. As yellow stingrays likely already use their cryptically patterned bodies for camouflage, it is reasonable that stingrays would benefit from a phenotypically plastic response. This study has demonstrated that background color is a strong driver of body color in yellow stingrays. Yellow stingrays use physiological color change to reorganize melanin already present in melanophores instead of creating or breaking down melanophores, in response to background color. It is possible that if rays are maintained for a period of many months that they could begin using morphological color change. However, morphological color change may be more energetically costly than physiological color change and may not serve an ecological purpose. Regardless of the mechanism, the background matching response seems to be spread throughout elasmobranchs. This study documents the first stingray species regulating color in response to background. Furthermore, this is the first study which has confirmed an environmental driver of color regulation in a stingray species while also evaluating the resulting cellular response.

Table 1: Samples collected for analysis. All biopsy punches were taken from the perimeter of the stingrays pectoral fins (Figure 2).

Sample type	Number of samples	Use	Stored
4mm Biopsy punch	4 sets of 8 punches	Melanin quantification	-80 °C
4mm Biopsy punch	1 set of 4 punches	Cell density, cell morphology	4% paraformaldehyde

FIGURES

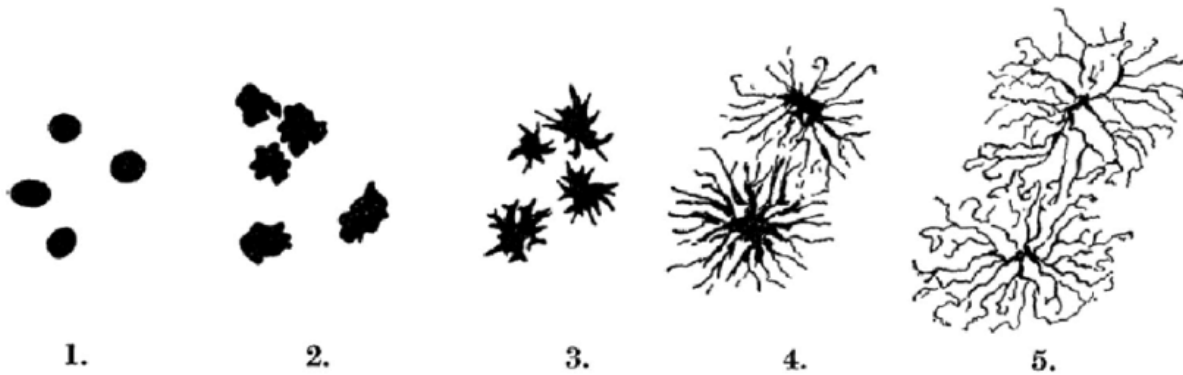


Figure 1: Hogben and Slome's Melanophore Index scale. Melanophore Index created by Hogben and Slome in 1931. This study created these categories for the purposes of evaluating the state of dispersion of melanophores in the African clawed frog (*Xenopus laevis*). Elasmobranch melanophores were later evaluated using the same scale as their melanophores are functionally and morphologically similar (Waring, 1936). Stingray melanophores were similar in shape and showed the same states of dispersion as those detailed above and therefore, the same index was used for analyses in this study. A fully dispersed melanophore is stellate in shape with melanin pigment granules moved away from the center of the cell and is indicated by a MI value of 5 (Hogben and Slome, 1931). A fully contracted melanophore is much smaller and circular, with all of the melanin pigment retained in the center of the cell and is indicated by a MI value of 1.

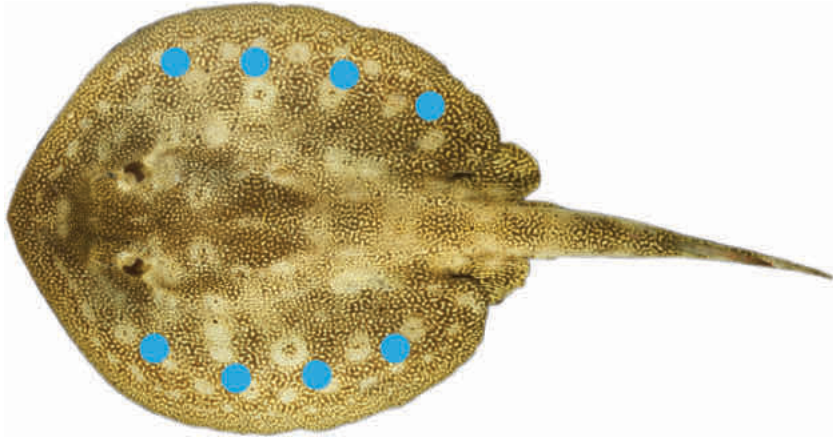


Figure 2: Stingray Biopsy Punch Locations. Samples were collected along the pectoral fin margin for melanin quantification, MI analyses, and cell density analyses.



Figure 3: Yellow stingray background matching. Photographs show stingray color change after being maintained for one week in treatment tanks, a) left panel shows initial stingray coloration after transport and acclimation and right panel shows stingray after seven days in an all-white tank. b) left panel shows initial stingray coloration after transport and acclimation and right panel shows stingray after seven days in an all-black tank.

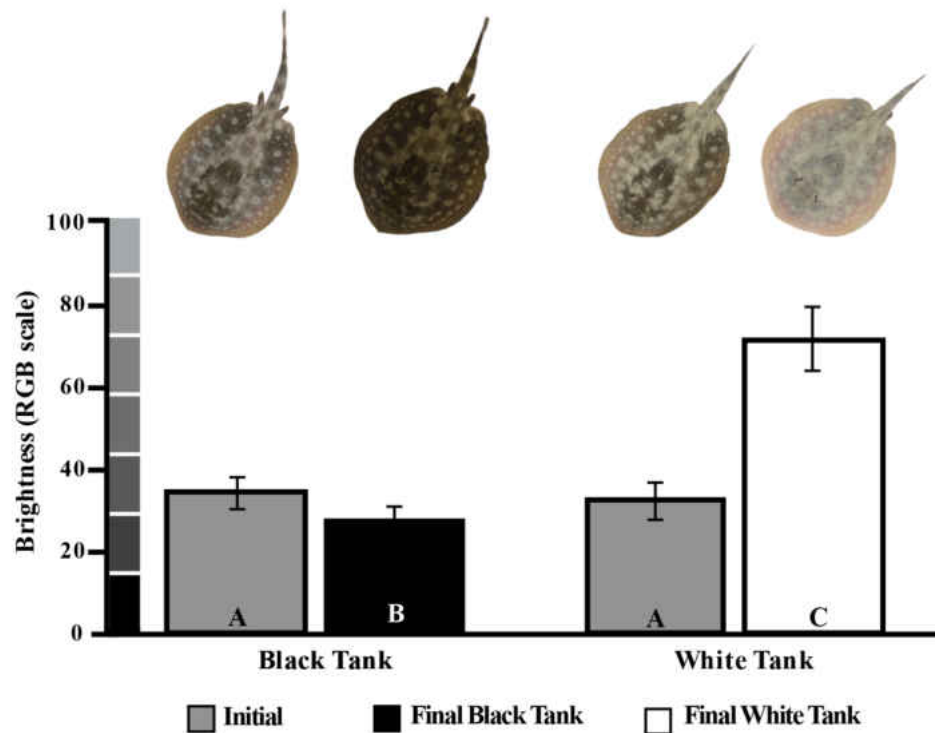


Figure 4: Yellow stingray brightness in black and white tanks. Overall brightness in white (n=8) and black (n=9) tank adapted rays. Day zero values indicate initial brightness before being placed in a treatment tank. Day seven values show brightness on the last day of exposure to either black or white tank conditions. White and black tank adapted rays were significantly different in brightness on day seven than on day zero (paired t-test $* < 0.05$). Rays in white tanks became lighter and rays in black tanks became darker. Brightness values are represented on an RGB scale where 0=black and 255=white. Bars that share the same letter were not significantly different from each other.

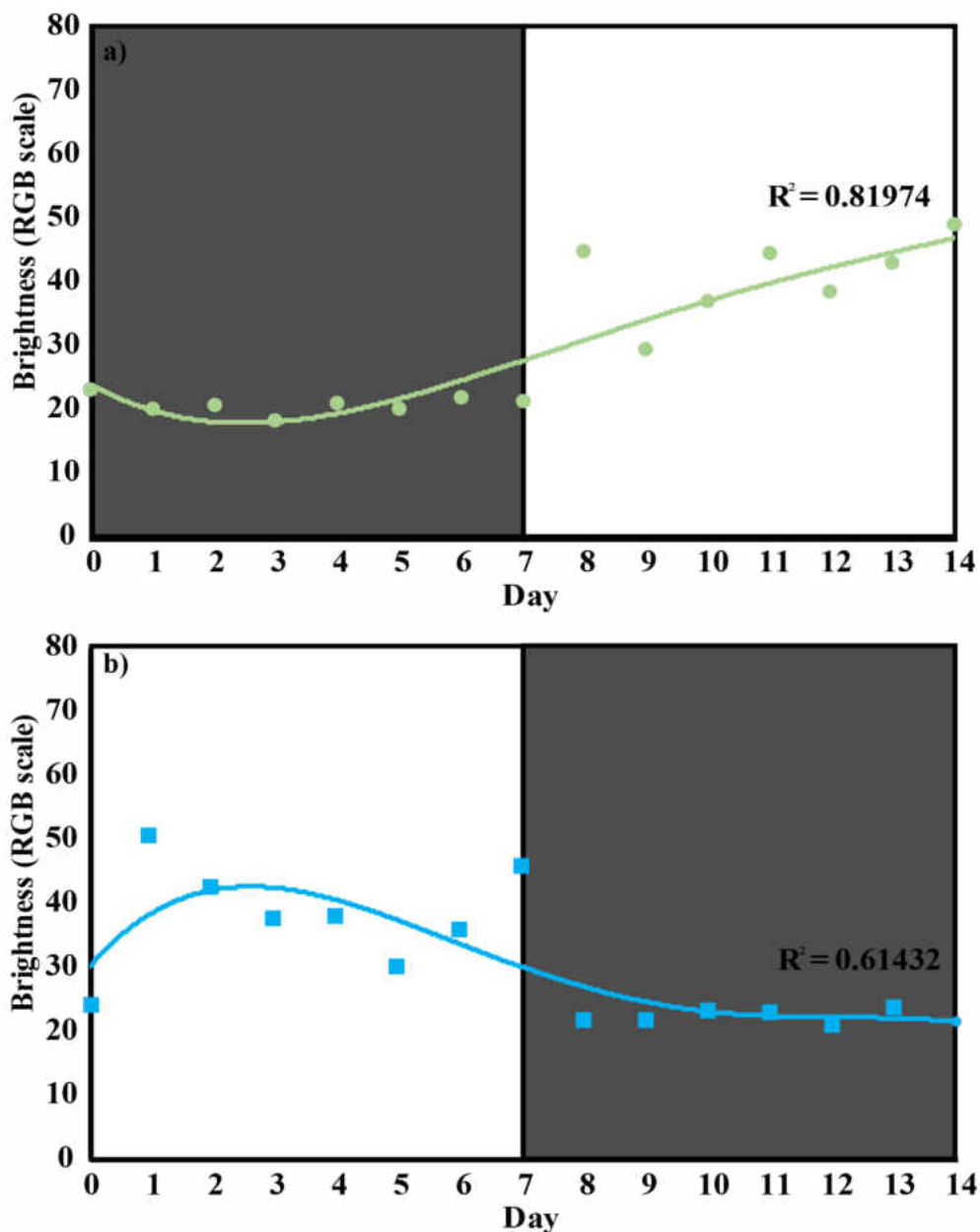


Figure 5: Individual yellow stingray adaptability to multiple tank environments. a)

Representative yellow stingray brightness values shown over a 14-day period where the ray was switched from a black tank to a white tank on day seven. Stingray brightness was not significantly different after 24 hours in a black tank. Stingray brightness was different after 24 hours when put into a white tank. b) Representative yellow stingray (n=1) brightness values shown over a 14-day period where the ray was switched from a white tank to a black tank on day seven.

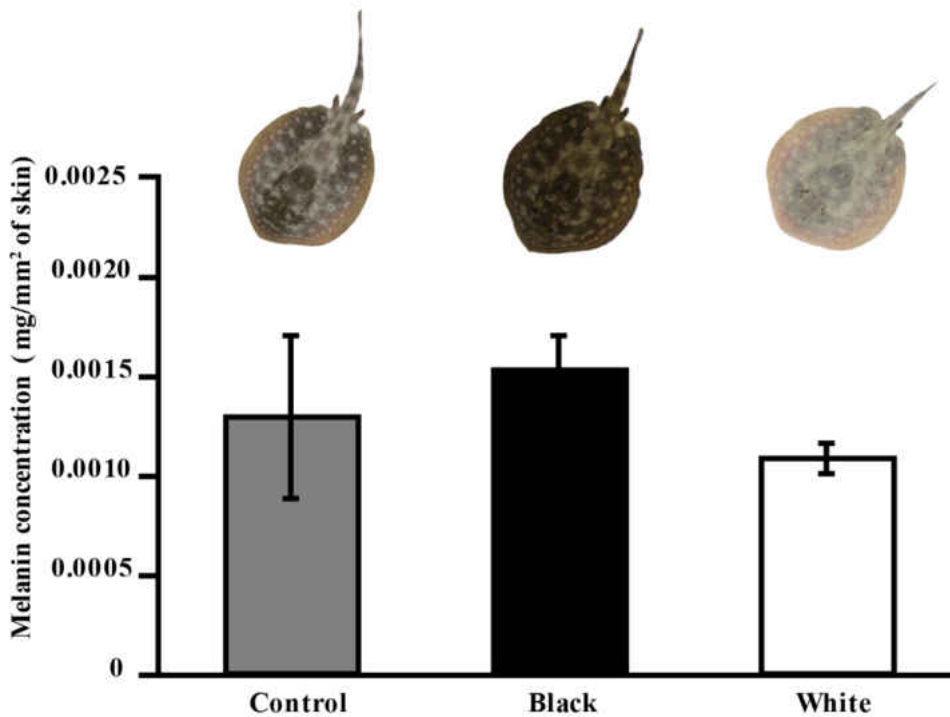


Figure 6: Yellow stingray skin melanin concentrations. Skin melanin concentrations (mg/mm^2 of skin) of control rays ($n=4$), white tank rays ($n=7$) and black tank rays ($n=7$) for seven days were not significantly different from each other (t -test, $p>0.5$). Black tank rays had a mean concentration (mean \pm SE) of $0.0015 \pm 0.0001 \text{ mg}/\text{mm}^2$ of skin, while white tanks had a mean of $0.0010 \pm 0.0001 \text{ mg}/\text{mm}^2$ of skin, and controls had a mean of $0.0012 \pm 0.0002 \text{ mg}/\text{mm}^2$ of skin. This indicates that rays changed color using physiological color change and therefore by moving melanin pigment instead of production or degradation of pigment.

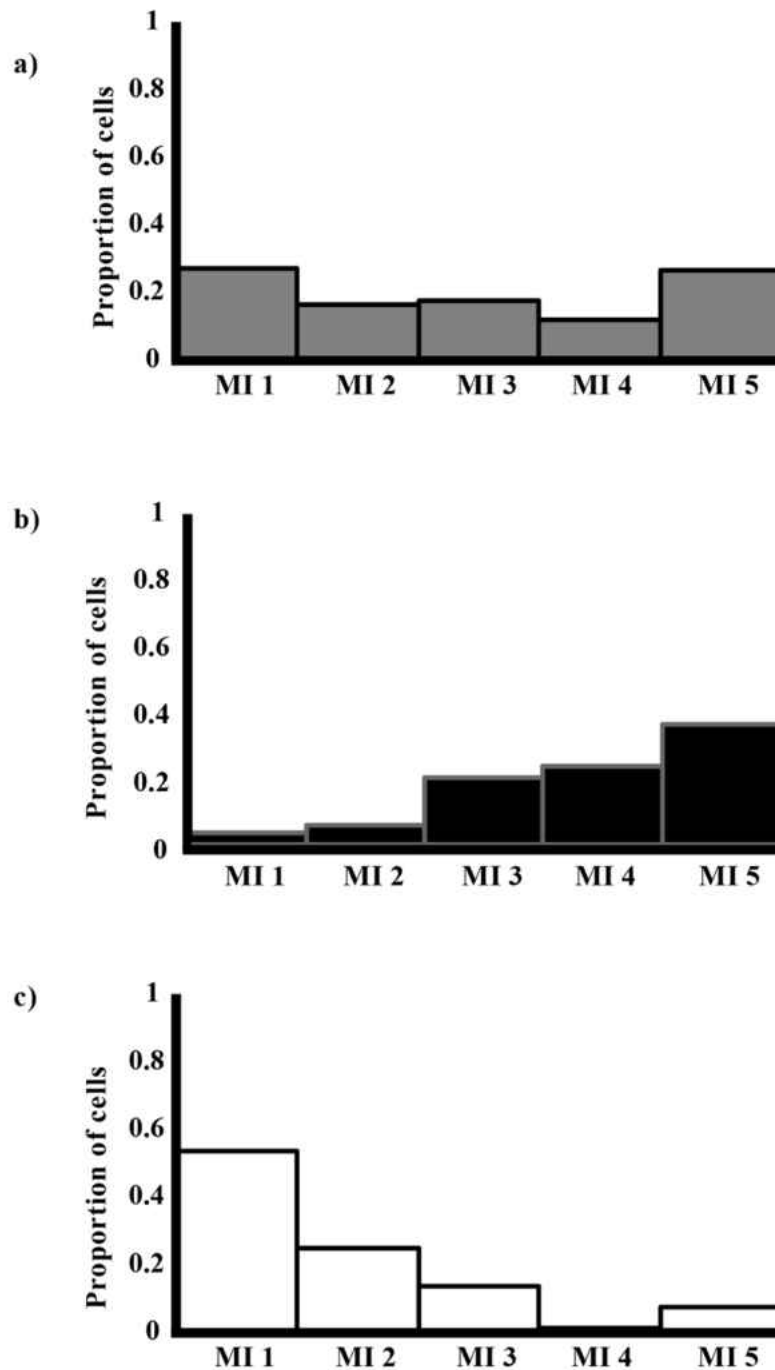


Figure 7: Proportion of cells in varying states of dispersion (MI values). Melanophore index values shown for a) control rays, b) rays in black tanks, and c) rays in white tanks. There was a higher proportion of MI values of 4 or more (dispersed) in rays in black tanks while rays in white tanks had a much larger proportion of cells with MI values of 2 or less (aggregated). Tank treatments were significantly different (chisquare contingency analysis, $p < 0.001$).

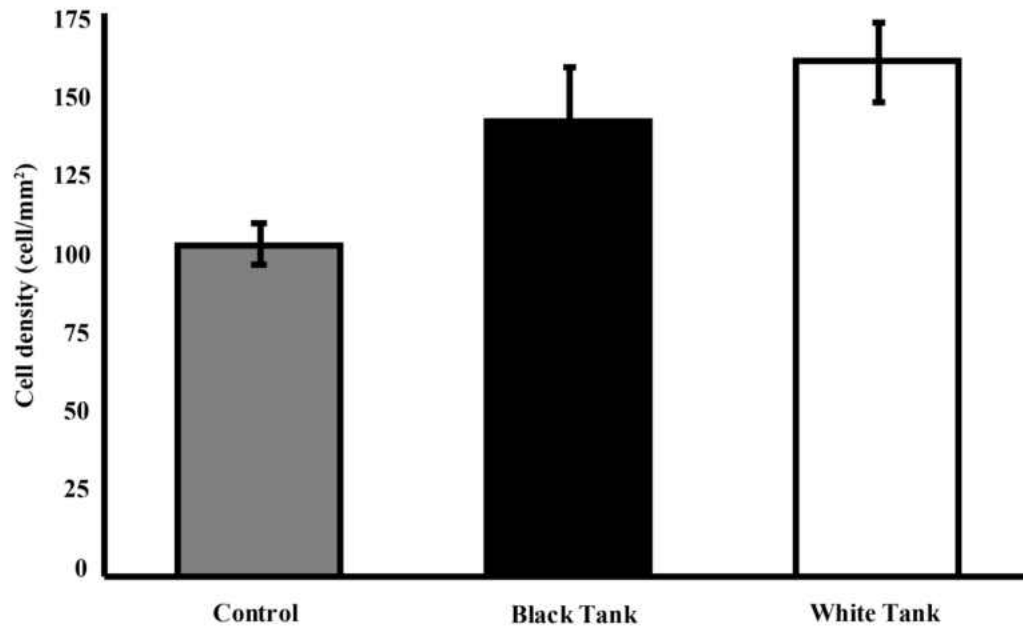


Figure 8: Melanophore cell density by tank treatment. Mean cell density (cell/mm²) for rays in white and black tanks. Rays did not differ in the amount of cells per treatment indicating physiological color change as no cells are being created or destroyed (ANOVA, $F_{2,16} = 2.8329$, $p = 0.09$). This suggests that stingrays used physiological color change to regulate color as each treatment maintained the same cell density.

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