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Original Article

The meaning of melanin, carotenoid, and pterin pigments in the bluefin killifish, *Lucania goodei*

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Male bluefin killifish (*Lucania goodei*) exhibit extensive color variation in their fins, but the utility of this variation has not yet been determined. We collected males from multiple populations and spectrophotometrically determined the pigment types responsible for fin coloration. We determined that the orange coloration in the caudal fin is caused by carotenoid pigmentation. In contrast, color in the anal fin is either pterin based (yellow and red) or structural (blue) with a melanic fin border. As these colors have different developmental origins, the potential for complex signaling is high. Therefore, we sought to determine whether behavior, reproductive success, or health correlated with pigmentation. Males with more melanin on the anal fin were more dominant and had higher spawning success. Male–male aggression was greater between males with similar-sized melanic borders, indicating that melanic markings function as badges of status between males. Caudal carotenoid pigmentation did not correlate with dominance, but this highly labile ornament was correlated with body condition, parasite infection, and spawning success, suggesting a role in intersexual selection by signaling health to potential mates. Similar results were found for caudal fin coloration using digital photography. Pterin pigmentation in the anal fin was not related to dominance but was related to overall spawning levels and parasite infection, suggesting that pterin pigmentation may also signal immune status. Thus, the coloration of male bluefin killifish provides multiple messages to multiple receivers through these 3 pigments (melanin, pterin, and carotenoid) that have distinct developmental origins.

Key words: **badge of status, carotenoid, digital photography, dominance, melanin, parasite, pigment extraction, pterin.**

INTRODUCTION

Often times, animals differ in multiple aspects of coloration, and in these cases, the obvious question is why multiple ornaments have evolved where perhaps one would do. Moller and Pomiankowski (1993) examined why birds have multiple ornaments and put forth 3 hypothesis: 1) the multiple message hypothesis, where each ornament signals a different quality of the animal, 2) the redundant signal hypothesis, where the ornaments combine to give a more obvious signal, and finally 3) the unreliable signal hypothesis, where signals are unreliable and exist in multiples because they are cheap to produce and may have a small benefit (see also Johnstone 1996; Hebets and Papaj 2005 for a review). Møller and Pomiankowski focused on intersexual signals when they developed their hypotheses, but Andersson et al. (2002) pointed out that intrasexual selection may also produce signals. They proposed the multiple receiver hypothesis, in which multiple ornaments exist as signals to different classes of receivers. The developmental origins of each ornament may point toward which hypothesis explains the origin of the multiple ornaments/signals. If all ornaments are produced the same way, then they may combine into one overall "redundant signal." However, if each signal has a different developmental pathway, then each may be responsive to different aspects of the animal's status and in turn send "multiple messages" to "multiple receivers."

There is an extensive literature on the informational content of signals composed of carotenoids and melanins that is predicated in part upon the physiology behind their development (McGraw 2005; Searcy and Nowicki 2005; Price et al. 2008). For example, carotenoid-derived ornaments are frequently assumed to be honest signals because animals cannot synthesize carotenoids de novo and must obtain them via diet (Olson and Owens 1998). At their simplest carotenoid signals may convey information to potential mates about foraging ability or condition, especially in carotenoid-limited environments (Brush and Power 1976; Kodric-Brown 1989; Hill 1992; Hill and Montgomerie 1994; Grether 2000). Carotenoids can also be antioxidants (McGraw 2005) or linked to pathways related to immune function (Hill and Johnson 2012), and their role

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in immune function suggests that carotenoid-based signals may indicate current health or parasite infection status (Hamilton and Zuk 1982) or may predict the ability to fight off future infection (Lozano 1994; Hill and Farmer 2005). In fact, an extensive literature exists that links carotenoid levels to immunocompetence in birds and fish (Blount et al. 2003; Faivre et al. 2003; Grether et al. 2004). Multiple studies have suggested carotenoid-based ornaments do influence female mate choice (Houde 1987; Hill 1991; Milinski and Bakker 1990), and a recent study links carotenoid-based ornaments to male competitive ability in a lizard (Hamilton et al. 2013).

The proposed signaling functions of diet-derived carotenoid ornaments stand in contrast to another well-studied pigment, melanin. Melanin-based color patterns are synthesized de novo by the organism and are thought to be cheap signals to manufacture (Gonzalez et al. 1999; McGraw et al. 2002). Thus, even low-condition individuals should be able to manufacture them. However, these supposedly inexpensive and extraneous signals are often used in socially critical dominance interactions (Jarvi et al. 1987; Senar et al. 1993; Horth 2003). As cheaply produced, long lasting signals of dominance (or more aptly fighting ability), they have been labeled "badges of status." It has been hypothesized that the honesty of these signals is maintained because of the high cost of lying in intrasexual interactions where "cheaters" are subject to increased aggression (Tibbetts and Dale 2004; Searcy and Nowicki 2005).

The relationship between pigment origin and function is not always clear, however. In addition to dominance interactions, melanin has also been associated with immune function and oxidative stress (Fitze and Richner 2002; McGraw 2005; Galván and Alonso-Alvarez 2009), and carotenoid signals sometimes function as badges of status (Pryke et al. 2002; Pryke and Andersson 2003). Further, a meta-analysis also revealed that the relationship between male condition and pigmentation did not vary depending on whether the pigment was carotenoid or melanin based (Griffith et al. 2006). In addition, the qualities of lesser known pigments, such as pterins, are even more unclear. Pterins, which like melanins can be synthesized de novo, have also been identified as antioxidants (McGraw 2005). However, only a very limited number of studies in penguins and lizards have thus-far linked pterins with condition (McGraw et al. 2009; Weiss et al. 2012) or immunocompetence (Nolan et al. 2006).

Here, we use the bluefin killifish (*Lucania goodei*) to examine whether multiple ornaments in this species convey redundant messages, multiple messages, or no messages. The bluefin killifish presents a tremendous opportunity to study the nature of multiple ornaments due to the unique nature of fin coloration in this species. While females are largely colorless, male fins exhibit extensive variation in coloration across discrete locations (Figure 1). First, males maintain a polymorphism within and across populations in anal fin color (red, yellow, or blue) and saturation of that color (Fuller 2002). Second, the anal fin also varies in the extent of black outlining the distal end of the fin. Third, males have an orange caudal fin that can vary widely in amount of orange present. The caudal fin is orange at the base and is yellow on the distal portions. In addition to having multiple discrete ornaments, this small freshwater fish found in the southeast United States has an easily quantifiable social system. Males are territorial. Dominance hierarchies are largely stable, and aggressiveness toward other males is moderately heritable ($h^2 = 0.17$) (McGhee and Travis 2010, 2012). Females visit multiple males, are courted, and allocate their eggs across several males throughout the breeding season (Breder and Rosen 1966; Fuller 2001).

The multiple types of fin ornamentation make the killifish an ideal system to study multiple signals. The informational content of the orange coloration on the caudal fin and the black coloration on the anal fin has never before been examined, and the function of the multiple anal fin color morphs has remained elusive in the studies that have tried to address it. The anal fin polymorphism is not linked to any obvious behavioral types (McGhee et al. 2007; McGhee and Travis 2010), and while a slight female preference for males with red over yellow anal fins has been detected in some studies (Fuller and Johnson 2009; Fuller and Noa 2010), others have failed to show this pattern (McGhee et al. 2007; McGhee and Travis 2010). While little is known about the fitness correlates of these colors, the genetic/environmental control of the anal fin color morphs has been examined in some detail. The red/yellow anal fin polymorphism is largely genetically determined with a single locus of large effect in which yellow is dominant to red; blue is orthogonal to the red/yellow polymorphism, and its prevalence is affected by both genetics and lighting environment (Fuller and Travis 2004).

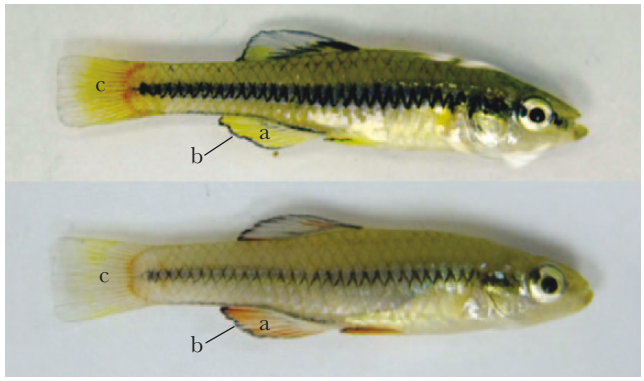

Figure 1
Variation in anal fin pterins (a), anal fin melanin (b), and caudal fin carotenoids (c) in male *Lucania goodei*.

The goals of this study were to 1) determine which pigment types are used in *L. goodei* anal and caudal fins and to 2) determine whether these pigments predict male dominance, male spawning success, or male health, which would indicate signal function for the ornamentation. In our first study, we determined the pigment classes responsible for the red, yellow, blue, and orange fin coloration from individuals across multiple populations across Florida. In our second study, we performed behavioral observations where we allowed 2 males to repeatedly compete for a female over 4 days and measured male dominance and courting behaviors. By monitoring male–male–female interactions in behavioral trials and quantifying coloration and pigment levels in the anal and caudal fins, we were able to determine the informational content of these fin ornaments. Following the behavioral trials, we determined male body condition and macroparasite loads. Hence, this study allowed us to elucidate the relationships between pigmentation, male behaviors, and male health.

METHODS

Pigment class identification

The adult male fish used to identify pigment class were collected with dipnets and seines from 5 populations in Florida: Upper Bridge and St. Marks Refuge in the Wakulla drainage ($N = 11$ and 8, respectively); Delks Bluff in the Oklawaha drainage ($N = 11$); Wacissa Springs in the Aucilla drainage ($N = 8$); and 26-Mile Bend in the Everglades ($N = 8$). Fish were held in water collected from

their site of origin without food and were euthanized within 5 days of collection. Fins were removed and frozen until pigments could be analyzed. To identify pigment class, individual anal and caudal fins were thoroughly ground with a mortar and pestle in 1 mL 1% NH_4OH. One milliliter of a 1:1 hexane:tert-butyl methyl ether solvent was added to elute carotenoids. The absorption spectra of both solvent layers were examined to determine pigment class. While eumelanic and structural coloration did not go into solution, pigments identified as belonging to the pterin class of pigments were identified by a strong UV absorption in the NH_4OH layer (McGraw 2006), and carotenoids were identified by a characteristic pattern of absorbance in the hexane:tert-butyl methyl ether solvent (Zang et al. 1997; McGraw et al. 2005). To further confirm the presence of pterins, we used chromatographic methods as described in Narayanan and Weir (1964). Pigment was extracted from anal fins in 1% NH_4OH for 2-dimensional TLC. The Rf values and fluorescence of the fins were compared to pigment from eyes from *Drosophila melanogaster* and a xanthopterin standard (Schircks Laboratories).

Behavioral trials

We employed observations of 2 males in competition over a female in order to discern any behavioral correlates with male ornamentation. The fish utilized in these behavioral trials were collected with seines and dip nets from the Upper Bridge of the Wakulla River, Wakulla County, Florida population near Tallahassee, Florida. Fish were housed in a communal stock tank (~300 L) located in a climate-controlled greenhouse at the University of Illinois at Urbana-Champaign with supplemental light from Xenon lamps (which supplement the ultraviolet portion of the spectrum) providing a 14 h light, 10 h dark schedule. Fish were fed frozen adult *Artemia* and flake food. Fish also had access to naturally occurring invertebrates and algae growing in the tank.

Fifty behavioral trials were conducted over January, February, July, and August of 2010. Because blue morphs are very rare in the focal population, only red and yellow morph males were utilized in the behavioral trials. For each set of trials, adult male fish were selected at random and isolated visually from each other in individual 19 L tanks. After 3 days of isolation, the males were randomly paired and anesthetized with a 0.025% MS-222 solution in the late afternoon/evening. Each pair was moved to a petri dish filled with a small amount of water, placed against a white background with a color standard, and had a picture taken of their left sides. Each pair was then placed in a 114 L tank with a female from the stock tank and 5 yarn mops that the fish use as shelter and as spawning substrate. During each of the following 4 mornings, behavioral observations were taken for 2 non-consecutive 20-min bouts between 0700 and 1100.

Individual males were identified by size, shape, and coloration during the observations. Counts of male–male aggressive behaviors were recorded for each individual. These behaviors included fin flares, chases, sigmoids (a pre-attack stance), and attacks resulting in physical contact. We also noted each male's interactions with the female by recording fin flares, physical attacks, chases, and courting bouts (head flicks directed at the female). A male was recorded as having successfully spawned with a female when he was observed in contact with a female while in the yarn mops and when eggs were subsequently found in the tank. Circle fights (both males attack each other repeatedly while circling each other) and chases involving all 3 fish were also recorded for the observation, although these behaviors were not assigned to a particular male. At the end of each morning's observations, we searched the mops for eggs and discarded any found.

Two separate observations over each of the 4 days of the trial yielded a total of 8 observations for each pair. Each observation was evaluated separately, and the dominant individual was determined for the observation. An individual was scored as dominant for an observation if he exhibited more aggressive actions than his partner. For some observational bouts (19 in 400), neither male performed any behaviors, and in these cases, neither male was recorded as dominant for that observation. Thus, each male could be scored as dominant up to 8 times, and this provided his overall dominance score. The male with the higher dominance score was labeled as the overall dominant male of the pair. There were no ties in dominance score observed across our 50 pairs, and switching dominance between the first and fourth days was very rare.

Pigmentation and color

Following the 4 days' observations, the male pairs were euthanized with an overdose of MS-222. The standard length and wet weight of each male were recorded, and the males were again photographed in their pairs. The anal and caudal fins were removed from the fish and stored at −80 °C. The fish were placed in ethanol and also stored at −80 °C. Males were later examined under a dissecting scope for acanthocephalan macro-parasites in their body cavity.

To measure carotenoid pigment content in the caudal fin, fins were ground in 1 mL 1% NH_4OH using a mortar and pestle. The pigment was then transferred to 1 mL of a 1:1 hexane:tert-butyl methyl ether solvent via vortexing and measured on a spectrophotometer. Absorption at peak wavelength (445 nm) was recorded to determine the amount of carotenoid. To determine relative pterin pigment content in the anal fin, the fin was ground using a mortar and pestle in 400 µL of 1% NH_4OH, and absorption was measured at 398 nm (yellow) and 498 nm (red) in a spectrophotometer as these were the pigment peaks. To account for the fact that our putative red pigment, drosopterin, also has some absorbance at the yellow peak in 1% NH_4OH (Figure 3), the amount of yellow pigment in red individuals was adjusted by subtracting 20% of the absorption measured at the red peak.

In addition to measuring carotenoid coloration via pigment extraction, we also used digital photography to assess male coloration. The advantage of photography is that coloration can be assessed both before and after the behavioral trials. Photographs of the fish from before and after the behavioral trials were used to quantify change in caudal fin coloration over the course of the trial. Picture light and color levels were standardized using the in Camera PicoColor 4.5 Photoshop plug-in, which used the color standard in the image for calibration in order to create a new, color-corrected digital profile of the image. This allowed us to standardize each picture for deviations attributable to alterations in the lighting conditions or camera set up. Subsequently, in ImageJ (U.S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/), the caudal fin was outlined using the freehand selection tool. All colors in the caudal fin besides orange were removed using the "Threshold_Color" plug-in, and the number of pixels was counted to measure amount of orange. The number of pixels was adjusted by using a size standard to account for minor differences in magnification between pictures. The difference in pixel number between pictures taken before and after the trials indicated the change in amount of orange over the course of the trial.

We also used digital photographs to assess black coloration (i.e., melanin) on the male anal fins before and after the behavioral trials.

Using ImageJ, the distal ends of the anal fins, which contain a black band, were isolated using the freehand selection tool. The image was converted to black and white by using the adjust threshold function and selecting black and white threshold color. The histogram tool was used to count the number of black pixels on each anal fin.

Using these methods, we obtained several measures of pigmentation: black anal coloration (pictures both before and after the trial); orange on the caudal fin (levels of carotenoid pigmentation assessed via absorbance at 445 nm and orange coloration assessed via digital photography both before and after the trials); and anal red/yellow pigmentation (yellow pterin measured as corrected absorbance at 398 nm, red pterin measured as absorbance at 498 nm, total pterin levels as those absorption levels summed, and color morphs as assigned by AMJ). Table 1 lists and defines the color variables measured for each fish. We used SAS v 9.3 (SAS Institute, Cary, NC) to analyze these variables. We used Pearsons correlations to determine if the pigments were correlated with each other. When necessary, we also obtained residual pigment values by regressing overall pigment levels on standard length (hereafter length). This helped to account for size discrepancies between fish. We could not measure fin mass directly to quantify pigment concentration because wet-weight was too variable and drying the fins destroyed the pigment. To determine if the anal fin morphs differed in their amount of each pigment, we used general linear models (proc glm) to compare them.

Our first goal was to determine which elements were correlated with male dominance and spawning success. Because we were able to assign male dominance for every pair, we used simple paired *t*-tests to ask which elements varied between dominant and subdominant pair members. We also looked at which pigments affected overall dominance score. In this case, we used generalized linear models (proc genmod) where the distribution of the data was modeled as binomial with a logit link function. Because the observations were not independent (i.e., the behavior/score of 1 male depended on the behavior of another), we included male pair as a repeated factor in the analysis. We performed a similar analysis on spawning where the model considered the total number of times a male spawned given the summed number of spawns observed for both males as a function of male coloration, where male pair was treated as a repeated factor. When analyzing overall counts of spawning, we also used generalized linear models with a negative binomial distribution due to the high number of zeros and male pair as a repeated factor. When considering other behaviors, we used the same process with a normal distribution.

Our second goal was to determine the relationship between male health and pigmentation. We measured body condition as the residuals of the regression of log10 of weight on log10 of length (Bolger and Connoly 1989) and used Pearsons correlations to determine if body condition was related to pigmentation. As another measure of health, we used infection with acanthocephalan parasites. We modeled infection as either binary (parasitized or not), in which case we used logistic regression (proc logistic), or we modeled the number of parasites each individual was infected with (parasite load) as a generalized linear model (proc genmod) using a negative binomial distribution to account for the high number of zeros.

The raw data for this study can be found at Dryad (doi:10.5061/dryad.85k8m).

Ethical note

These experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois (protocol numbers # 11143 and #08183).

RESULTS

Pigment identification

Though roughly similar in coloration, the caudal and anal fin pigmentation in *L. goodei* results from different classes of pigments. The orange pigment extracted from the caudal fins in all populations was isolated in the hexane:tert-butyl methyl ether solvent and had an absorption spectrum characteristic of a carotenoid (Figure 2). No pigment was observed in the hypophase. In contrast, the yellow and red pigments extracted from the anal fins were isolated in the NH_4OH layer and had absorption spectra characteristic of pterins (McGraw 2006). Yellow morph males had a single pterin absorption peak of ~398 nm, indicating the presence of a yellow pterin while red morph males had a red peak at ~498 nm in addition to the yellow peak at 398 nm, indicating that red-morph males produce 2 separate pterin pigments (Figure 3). No pigment was observed in the epiphase of the anal fins. The results from TLC on the fins also supported the identification of the yellow and pigments as pterins, with the Rf values and fluorescence (yellow and

Table 1

Variables related to coloration, their definitions, and major results

Variable	Definition	Results
$Black_{pre\text{-}trial}$	Number of black pixels in digital image of anal fin; pictures taken prior to behavioral trials	Positively correlated with dominance and dominance score
$Black_{post\text{-}trial}$	Number of black pixels in digital image of anal fin; pictures taken after behavioral trials	Positively correlated with dominance and dominance score. Predicts spawning success
Similarity in anal fin melanin	$Black_{post\text{-}trial}$ of less melanic fish divided by $black_{post\text{-}trial}$ of more melanic trial partner	Positively correlated with total male–male aggression
Caudal carotenoid pigment	Absorbance of the caudal fin at 445 nm	Predicts spawning success. Correlated with body condition. Predicts parasite infection status and parasite load
Size-corrected carotenoid pigment	Residuals from the regression of caudal carotenoid pigment on standard length	Positively correlated with body condition
$Orange_{pre\text{-}trial}$	Number of orange pixels in a digital image of caudal fin; pictures taken prior to behavioral trials	Predicts parasite infection status and parasite load
$Orange_{post\text{-}trial}$	Number of orange pixels in a digital image of caudal fin; pictures taken after behavioral trials	Positively correlated with condition. Predicts parasite infection status and parasite load
Total pterin pigment	Summed absorbance of anal fin at 398 and 498 nm, representing sum of yellow and red pterin pigments	Predicts spawning events. Predicts infection status and parasite load.

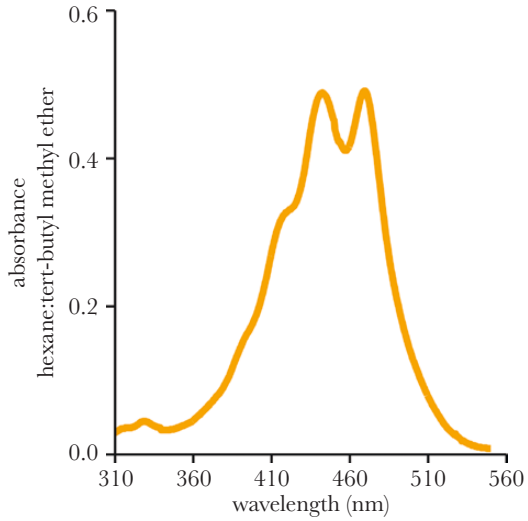

Figure 2
A representative caudal fin absorption spectra indicating carotenoid pigmentation.

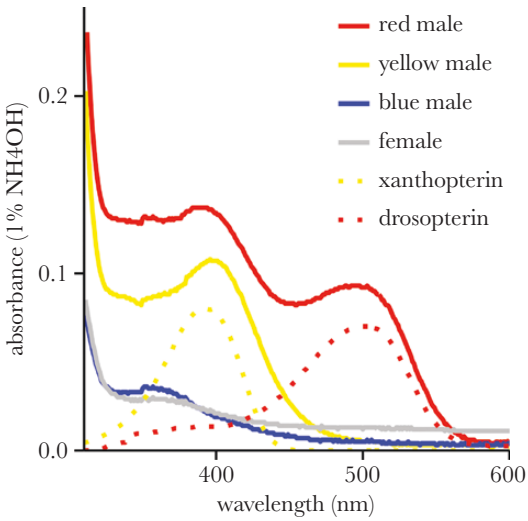

Figure 3
Representative absorption spectra of *Lucania goodei* anal fins indicate pterin pigment content in red and yellow morphs. Yellow pigment absorption peaks at 398 nm while red pigment peaks at 498 nm. The anal fins of blue males and females lack discernible pigments. The pterins xanthopterin (Schirck Laboratories) and drosopterin (isolated by TLC from *Drosophila melanogaster*) are shown for comparison.

orange, respectively) matching those we ran of known standards of xanthopterin and drosopterin. No pigment was detected in males with blue anal fins, where the absorption spectra matched colorless females (Figure 3), suggesting that blue coloration was due to structural reflectance properties rather than pigmentation. Black coloration on the anal fin was not soluble in the solvents used here, indicating that it was melanin.

Relationships among pigments

Pigmentation was examined in greater depth using the fish in our behavioral study. As we had many measures of pigmentation, we have defined these measurements and summarized our results in Table 1 for clarity. In addition, Table 2 defines the variables for

Table 2
Definitions of variables related to behaviors and condition

Dominance score	Number of times (out of 8 observations) the male exhibited more aggressive behaviors than his tank mate
Dominant male	Male of the tank pair with higher dominance score
Total male–male aggression	Male–male fin flares, sigmoids, chases, and attacks summed across both males
Total male–female aggression	Male–female chases and attacks, summed across both males
Spawning events	Number of spawning events observed
Spawning success	Number of spawning events male obtained/ total number of spawning events in male's tank
Body condition	Residuals from the regression of $\log_{10}$ (mass) on $\log_{10}$(standard length)
Infection status	Yes/no infection with one or more acanthocephalans
Parasite load	Total number of acanthocephalans

which we looked for associations with pigmentation. Different measures of the same color element were correlated with one another. The photographic pre-trial and post-trial measures were correlated for both caudal orange ($r = 0.77$, $P < 0.0001$) and anal black ($r = 0.56$, $P < 0.0001$). The photographic measures of orange were also correlated with carotenoid pigment measures ($\text{orange}_{\text{pre-trial}}$ and carotenoid pigment: $r = 0.53$, $P < 0.0001$; $\text{orange}_{\text{post-trial}}$ and carotenoid pigment: $r = 0.58$, $P < 0.0001$), indicating that the photographs captured the degree of orange pigmentation well. As expected, anal fin morphs visually categorized "red" by AMJ had significantly more red pterin pigment ($F_{1,98} = 34.23$, $P < 0.0001$) than yellow morphs, while there was no difference between the morphs in amount of yellow pterin ($F_{1,98} = 0.47$, $P = 0.49$).

In these fish, the regressions of carotenoid and total pterin levels (red and yellow absorption summed) on standard length of the fish were highly significant (carotenoid: $T_1 = 10.59$, $P < 0.0001$; total pterin: $T_1 = 5.61$, $P < 0.0001$), with larger fish having higher values, though this was not the case for melanin ($P = 0.0947$). We sought to determine if each fish's pigment levels were independent of each other after correcting for size of the fish by using fish length as a partial correlate. The amount of black on the anal fin and total pterin on the anal fin were correlated ($r = 0.20$, $P = 0.043$), probably as a result of differential fin sizes relative to length. However, caudal carotenoid levels were not correlated with either of the other classes of pigments in the anal fin (melanin: $r = 0.11$, $P = 0.29$; total pterin: $r = 0.09$, $P = 0.37$). Thus, caudal carotenoid pigmentation was independent of anal pterin and anal melanin pigmentation in these fish. We also checked if the red and yellow morphs had different amounts of carotenoid and melanin. We found that there was no difference in the amount of melanin ($\text{black}_{\text{post-trial}}$ $F_{1,98} = 0.27$, $P = 0.6$; $\text{black}_{\text{pre-trial}}$ $F_{1,98} = 1.38$, $P = 0.24$) or carotenoid pigment ($F_{1,98} = 2.72$, $P = 0.1$).

Pigments as predictors of dominance

The numbers of aggressive and courting behaviors strongly differed between males identified as dominant and subdominant (Table 3), yielding a clear differentiation between dominant and subdominant individual dominance scores (Figure 4). Not only was the behavior of dominants and subdominants different, but dominant males spawned more than subdomiant males (57 total spawning events observed, $X_1^2 = 9.31$, $P = 0.0023$), indicating that assigned dominance directly affected fitness. Dominant males differed only

Table 3

Average measurements and behavioral counts (standard deviations) for dominant and subdominant fish in the 50 trials conducted

	Dominant male	Subdominant male
Length (mm)	34.2 (4.1)	33.3 (4.7)
Mass (g)	0.604 (0.23)	0.555 (0.23)
Male–male flare	63.3 (72.7)	20.7 (20.7)
Male–male chase	32.6 (31.1)	8.6 (17.2)
Male–male attack	24.9 (22.4)	7.6 (15.5)
Male–male sigmoid	2.6 (3.4)	0.7 (1.5)
Circle fight	0.8 (2.1)	
Male–female flare	24.2 (21.4)	8.8 (19.5)
Male–female chase	11.6 (14.7)	3.3 (7.8)
Male–female attack	8.8 (10.1)	3.6 (7.2)
Courting bout	38.1 (32.8)	14.5 (31.2)
Spawn	0.9 (1.2)	0.24 (2.1)
Male–male–female chase	0.92 (2.3)	

Behaviors are summed totals from the 8 observations in a trial.

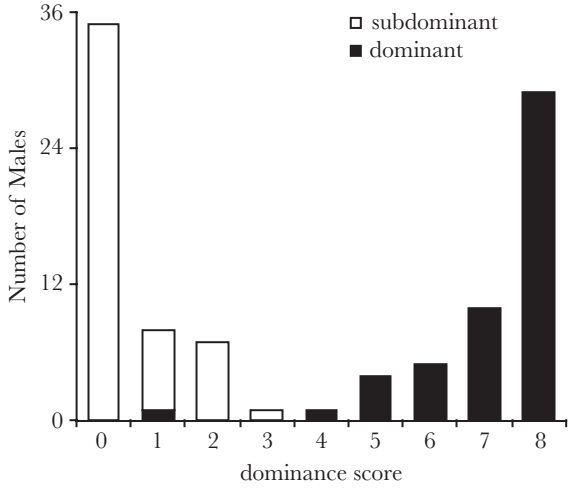

Figure 4

Number of times in 8 observations that a male was scored as the dominant member of the male–male pair. When no activity from either male was recorded, neither male was scored as dominant. The male with the higher dominance score of the pair was identified as the dominant male.

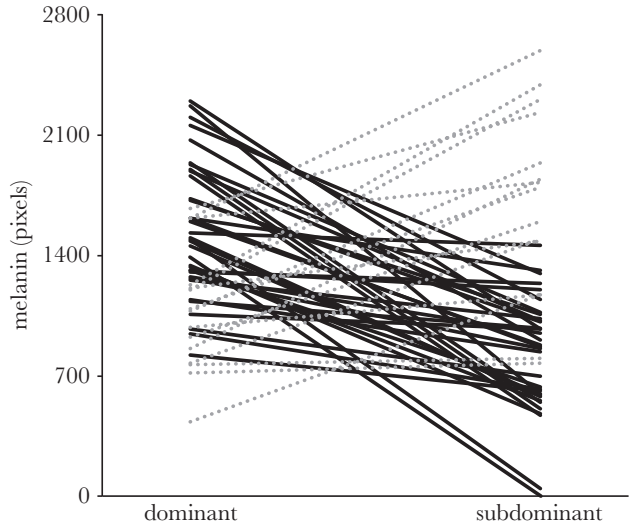

Figure 5

Dominant males have more melanin than their subdominant partners (connected by lines drawn) on the distal portion of their anal fins in photographs taken after behavioral observations. Dotted lines show trials that deviate from this pattern.

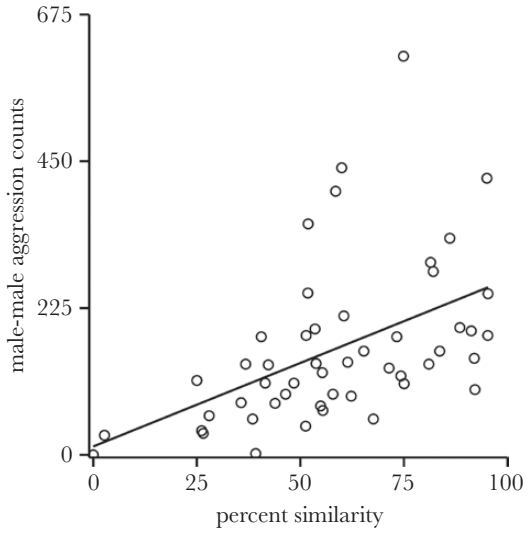

Figure 6

Percent similarity in anal fin melanin in photographs is correlated with the total number of aggressive interactions (male–male fin flares, sigmoids, chases, and attacks summed for both males).

slightly in length and weight from subdominant males, and these differences were not statistically significant (paired *t*-test, length: $T_{49} = 1.82$, $P = 0.076$; weight: $T_{49} = 1.75$, $P = 0.086$) (Table 3).

We looked for associations between each pigment class and dominance. Melanin was strongly correlated with dominance. Dominant individuals had significantly more melanin on the distal portion of their anal fin in photographs taken from both before the trial began (paired *t*-test, $T_{49} = 2.64$, $P = 0.01$) and at the conclusion of the observations (paired *t*-test, $T_{49} = 2.89$, $P = 0.0057$) (Figure 5). The amount of black was also significantly indicative of overall dominance score in the 8 observations ($\text{black}_{\text{pre-trial}}$: $X_1^2 = 4.41$, $P = 0.036$; $\text{black}_{\text{post-trial}}$: $X_1^2 = 4.93$, $P = 0.024$).

Overall aggression between a pair of males was highest when the 2 males had similar melanin levels. There was a significant positive correlation between percent similarity in anal fin melanin (calculated as male with lower melanin/male with higher melanin) and total male–male aggression (Spearman correlation, $r = 0.59$, $P < 0.0001$) (Figure 6). There was no correlation between percent similarity in anal fin melanin and total male–female aggression (spearman correlation, $r = 0.01$, $P = 0.9$).

Anal fin color morph was not related to dominance. Of the 50 trials recorded, 25 were yellow–yellow, 5 were red–red, and 20 were red–yellow male pairs. Among the 20 red-yellow pairs, yellow was dominant over red 13 times, but this was not significantly different than what was expected by chance (binomial test, 2-tailed, $P = 0.2632$). Total pterin did not predict dominance either in raw amount (paired *t*-test $T_{49} = 1.67$, $P = 0.10$) or after using residuals corrected for length ($T_{49} = 1.06$, $P = 0.29$). Yellow males did tend to exhibit more aggressive behavior (total fin flares, chases, attacks, and sigmoids) toward their tankmate than red males ($X_1^2 = 3.81$, $P = 0.051$), and this tendency was especially pronounced after

including similarity in anal fin melanin in the model (morph: $X_1^2 = 5.33$, $P = 0.021$; anal fin melanin similarity: $X_1^2 = 11.56$, $P = 0.0007$).

Caudal fin carotenoid pigmentation was also not related to male dominance. There was no difference between dominant and subdominant fish in carotenoid pigment (paired *t*-test, $T_{49} = 1.67$, $P = 0.10$) or size-corrected carotenoid pigment (paired *t*-test, $T_{49} = 0.77$, $P = 0.44$). Interestingly, the visual appearance of carotenoid pigment in the caudal fin was conspicuously labile. Males placed in isolation lost orange pigmentation, and pictures taken before the behavioral trials reflected this loss. However, during the ~15 h between being placed in the observation tank and the initiation of behavioral observations the following morning, the appearance of orange rapidly increased (Johnson AM, personal observation). This plasticity was reflected in a significant increase in caudal fin pigmentation captured in photographs from before and after the trials (paired *t*-test, $T_{99} = 6.64$, $P < 0.0001$). Dominant males did not have a larger percent increase in the amount of caudal orange than subdominant males either as a group (*t*-test, $T_{98} = 0.08$, $P = 0.9$) or correcting for trial partner (paired *t*-test, $T_{49} = 0.09$, $P = 0.93$).

Pigments as predictors of spawning success

In addition to being correlated with dominance, the amount of melanin also correlated with spawning success ($\text{black}_{\text{post-trial}}$ $X_1^2 = 6.7$, $P = 0.0096$). Males with higher levels of carotenoid also had higher spawning success ($X_1^2 = 4.24$, $P = 0.0395$), although this relationship did not remain significant when using size-corrected carotenoid ($P = 0.0904$). There was no difference between anal fin morphs in spawning success ($X_1^2 = 1.03$, $P = 0.3$). Total pterin was not related to proportion of successful spawns either before ($X_1^2 = 3.15$, $P = 0.076$) or after correcting for length ($X_1^2 = 1.80$, $P = 0.18$).

As another measure of spawning, we also considered whether the number of spawning events observed overall (rather than proportion within each male pair) was related to pigmentation. In this case, more melanic males did not have more spawning events attributed to them ($\text{black}_{\text{post-trial}}$ $X_1^2 = 3.33$, $P = 0.068$), nor did males with more carotenoid have a higher number (carotenoid $X_1^2 = 0.76$, $P = 0.38$; size-corrected carotenoid $X_1^2 = 0.05$, $P = 0.82$). However, males with higher total pterin did have a higher number of spawning events (total pterin $X_1^2 = 5.79$, $P = 0.016$; size-corrected total pterin $X_1^2 = 6.00$, $P = 0.014$).

Pigments as predictors of health

We used 2 measures of male health. We measured body condition as the residuals of log10 of mass on log10 of length. None of the measures of melanin correlated with condition and neither did total pterin. However, all measures of caudal carotenoid positively correlated with body condition except those from the pre-trial photographs, which were taken when fish were least orange (carotenoid: $r = 0.21$, $P = 0.040$; size-corrected carotenoid: $r = 0.29$, $P = 0.0036$; $\text{orange}_{\text{post-trial}}$: $r = 0.24$, $P = 0.016$; size-corrected $\text{orange}_{\text{post-trial}}$: $r = 0.29$, $P = 0.0037$) (Figure 7).

As another measure of male health, we looked at infection with acanthocephalan parasites. There was no relationship between any measure of melanin and infection, which was reflected in the fact that dominant males were no more likely to be infected than subdominant males ($P = 0.92$). However, pterin pigment levels had an interaction with length that correlated with acanthocephalan

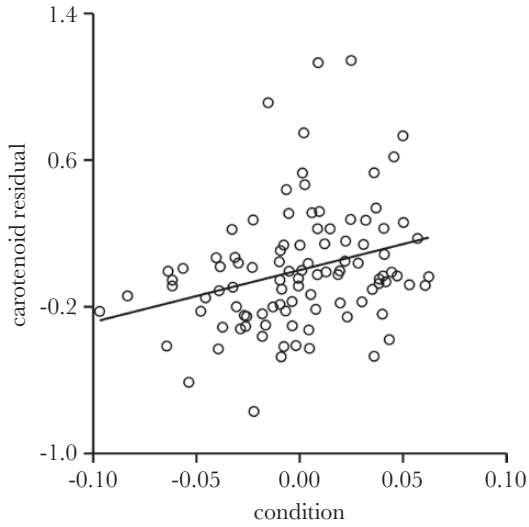

Figure 7
Male body condition is correlated with size-corrected caudal carotenoid pigment.

infection (logistic regression, pterin: $X_1^2 = 7.61$, $P = 0.006$, length: $X_1^2 = 9.71$, $P = 0.002$, total pterin × length: $X_1^2 = 7.69$, $P = 0.006$) (Figure 8a). Caudal pigmentation also correlated with infection (logistic regression, carotenoid: $X_1^2 = 3.24$, $P = 0.072$, length: $X_1^2 = 8.83$, $P = 0.003$, carotenoid × length: $X_1^2 = 3.9$, $P = 0.048$; $\text{orange}_{\text{post-trial}}$: $X_1^2 = 4.30$, $P = 0.038$, length: $X_1^2 = 13.1$, $P = 0.0003$, $\text{orange}_{\text{post-trial}}$ × length: $X_1^2 = 5.56$, $P = 0.018$, $\text{orange}_{\text{pre-trial}}$: $X_1^2 = 4.28$, $P = 0.039$, length: $X_1^2 = 7.56$, $P = 0.006$) (Figure 8b). In both of these cases, larger males that were infected had less pigment than expected. Results were the same when using parasite load (total pterin: $X_1^2 = 5.21$, $P = 0.02$, length: $X_1^2 = 7.53$, $P = 0.006$, total pterin × length: $X_1^2 = 5.07$, $P = 0.024$; carotenoid: $X_1^2 = 4.88$, $P = 0.027$, length: $X_1^2 = 9.23$, $P = 0.002$, carotenoid × length: $X_1^2 = 5.27$, $P = 0.021$; $\text{orange}_{\text{post-trial}}$: $X_1^2 = 4.16$, $P = 0.04$, length: $X_1^2 = 13.71$, $P = 0.0002$, $\text{orange}_{\text{post-trial}}$ × length: $X_1^2 = 5.58$, $P = 0.018$, $\text{orange}_{\text{pre-trial}}$: $X_1^2 = 6.25$, $P = 0.012$, length: $X_1^2 = 9.15$, $P = 0.0025$).

DISCUSSION

Our results show that coloration in the bluefin killifish (*L. goodei*) originates from multiple classes of pigments. Coloration in the anal fin involves melanin, pterin, and structural elements: blue morphs utilize structural coloration, yellow morphs utilize a yellow pterin pigment, and red morphs utilize both a red and yellow pterin pigment. In addition, the anal fins are accented by a melanic border. In contrast to the anal fin, orange color variation in the caudal fin is caused by varying amounts of carotenoid(s). This pattern of pigment utilization is similar in all the populations we have examined thus far.

This is one of the rare cases where similar colors (to our eyes) in the same organism are produced by completely different pigments in different body parts. It stands in contrast to the guppy (*Poecilia reticulata*), which uses both pterins and carotenoids within a single orange spot to maintain a desired hue (Grether et al. 2001, 2005). While the evolution of pterin and carotenoid pigmentation in guppies may potentially be constrained by each other, *L. goodei* pigment evolution is unique in that it appears to be uncoupled and allows us

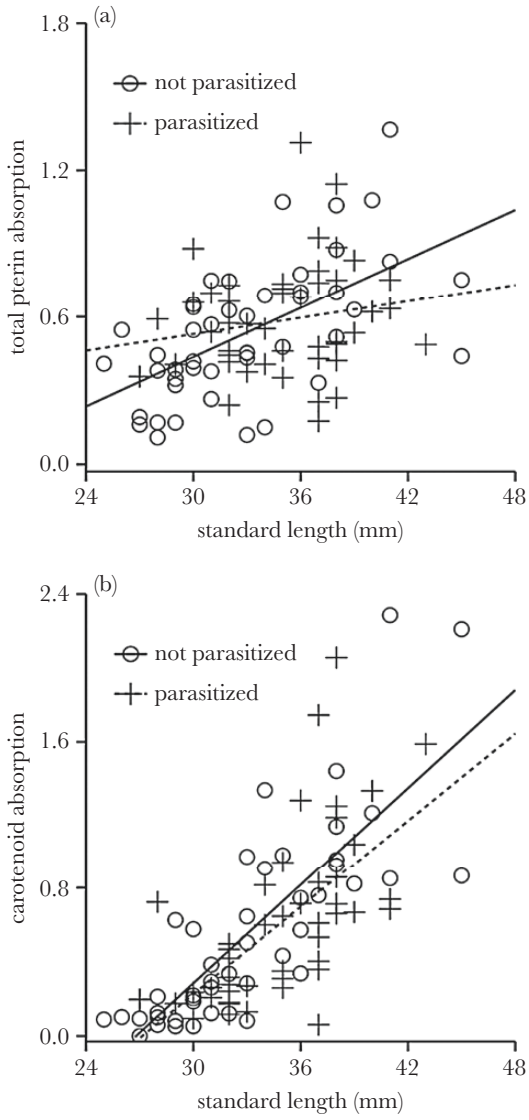

Figure 8
Interaction between total pterin (a) and carotenoid pigmentation (b) and length and their effects on acanthocephalan infection. The solid line is for uninfected individuals and the dotted line is for infected individuals.

to examine the potentially disparate functions for which carotenoid, pterin and melanin-derived ornaments might have evolved.

Melanin

We show that melanin is implicated in dominance interactions in the bluefin killifish; the more melanic male in each behavioral trial was more likely to be dominant (Figure 5) and obtained a higher proportion of the spawns with the female. In a natural setting, males must first compete with each other to establish territories before courting females. Thus, establishing dominance quickly and efficiently is likely highly important in these fish, and the melanic border may serve as a signal to facilitate these male–male interactions.

It is possible that the melanic fin border, while correlated with fighting ability, has no actual effect on receiver behavior and is not a signal to other males. Rather, a link between melanism and aggression could be induced by pleiotropic effects of melanocortins. For example, melanocortins bind not just to the melanocortin 1 receptor in the skin, but also have weak affinity for the 4 other melanocortin receptors in other organs throughout the body. It has been suggested that pleiotropic binding effects could substantially alter behavior (Ducrest et al. 2008). However, we show that aggressive interactions between male pairs are increased when the ornaments are of similar size, while having no effect on aggressive actions towards females. This targeted escalation suggests that the ornament is functioning as a "badge of status" (Hurd 1997).

If, as our results indicate, it is highly advantageous to be a dominant male, what keeps males from evolving dishonest badges to improve their dominance status? Melanin is easily synthesized (Gonzalez et al. 1999; McGraw et al. 2002), and as a result, the honesty of this signal is most likely not derived from the cost of manufacturing it. However, honesty in badges may be maintained by other costs. For example, testosterone has been proposed to increase badge size in birds (Evans et al. 2000; Buchanan et al. 2001) while lowering immune function (Folstad and Karter 1992). This would make the badge an immunocompetence handicap that only high quality males could afford. However, our experiments found no link between badge size and condition or immune function in killifish, suggesting that this is not the case. Rather, it seems more likely that honesty in the killifish badge is maintained by "social control" via the consequences of deception (Rohwer 1977; Tibbetts and Izzo 2010). Interestingly, it has been suggested that in systems like this one, where aggression is increased among individuals with similar sized badges, social control is an especially effective method of maintaining honesty (Hurd 1997) because males who forge a larger badge suffer more from conflicts with truly high status males.

Carotenoid

The caudal carotenoid-based ornament appears to signal different information than the anal melanic stripe. Though the ornament was not associated with dominance, males with higher levels of carotenoids did obtain a higher proportion of the spawnings in their tank. Carotenoid levels were positively correlated with body condition (Figure 7), a relationship one might expect given the pigment's association with diet. In addition, parasitized males had lower levels of carotenoid pigmentation (Figure 8b).

This pattern might be explained by female choice. In a natural setting, a female evaluates multiple males and selectively spreads her eggs across them. The signals males use to advertise themselves to females might be distinct from those used in dominance interactions with other males. We found that carotenoid levels are associated with body condition. Females might therefore use caudal carotenoid levels to facilitate evaluation of male condition. As carotenoid levels in males are presumably honest, mate choice decisions by females may strongly factor in the evolution of this signal. The fact that carotenoid coloration is reduced in isolation but increased in the presence of other fish (including females) suggests that carotenoids function in signaling–perhaps to females.

Pterins

No relationships between dominance and pterin morph or total pterin levels were found. However, pterin pigmentation was related to parasite levels (Figure 8a). Larger males that were infected with acanthocephalan parasites had less anal fin pigmentation than males that were uninfected. Pterin pigmentation is just beginning to be studied. While pterins, like melanins, are presumed to be easy pigments to manufacture, studies are beginning to link pterins to immune function due to their potential antioxidant properties (McGraw 2005).

Males with higher levels of pterins did spawn at higher levels overall. These results suggest that females may use pterin pigmentation in addition to carotenoid pigmentation to evaluate the immunocompetence of potential mates. This research is an important step in beginning to understand the functions of these pigments.

CONCLUSIONS

Our results clearly show that the melanic anal fin border is related to male–male dominance in *L. goodei*. As our study was primarily designed to assess dominance, our results in regard to female choice are less robust. However, it appears that females prefer males with high carotenoid and pterin levels, perhaps because they serve as signals of condition and immunocompetence. Our results in this regard are conservative because the focal animals had been living in captivity under an abundant diet and without exposure to parasites for some time (i.e., acanthocephalan parasites were obtained in the field). This suggests that carotenoid and pterin signals may be more important for intersexual interactions than our study could reveal. The relationship between dominance and spawning success might be inflated in the lab because a single male can monopolize an entire tank (McGhee et al. 2007; McGhee and Travis 2010). However, in nature females can freely travel between territories, and in this case, females may use caudal carotenoid and pterin content to assess the condition or immunocompetence of potential mates as they lay their eggs across multiple males. To truly interpret the meanings behind these various pigments would require manipulation of the ornaments (Sheldon and Verhulst 1996). Therefore, cautious interpretation of these results is required.

The natural world is complex. Animals like the bluefin killifish balance at a precarious point between attracting mates, battling rivals, and avoiding predation. Our work, the first to simultaneously examine the potential informational content in melanin, carotenoid, and pterin-based ornaments, suggests that killifish males compartmentalize these competing tasks. The multiple receivers hypothesis best explains our results. Melanin appears to be a badge of status that signals dominance to other males during territorial conflicts. Caudal carotenoid levels signal condition to females and perhaps imunocompetence. Anal pterin levels may also signal imunocompetance to females. Each of these ornaments has a different developmental origin and independent evolutionary path that shapes its signal function.

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