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Developmental order of a secondary sexual trait reflects gonadal development in male sheepshead minnows (*Cyprinodon variegatus*)

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ABSTRACT

Question: How can one demonstrate that coloration is an honest signal of sexual development?

Hypothesis: Coloration in a cyprinid fish is positively related to the development of testes.

Species and location: Sheepshead minnows (*Cyprinodon variegatus*) from South Carolina and Connecticut moved to Santa Cruz, California.

Methods: Common garden experiment involving individually grown fish, colour pattern assessment, and the development of male gonads.

Results: Males developed bright iridescent blue coloration on their upper parts in front of the dorsal fin through four stages that were positively related to the growth of testes. Such parallel development between sexual coloration and gonad mass was consistent between populations.

Conclusions: Coloration in male sheepshead minnows provides an accurate signal of the maturity status of males, which can thus inform both mate choice and intrasexual competition.

Keywords: development, gonads, secondary sexual trait, sexual colour.

INTRODUCTION

Fishes are known for their display of colours (e.g. Kodric-Brown, 1998; Love *et al.*, 2002; Quinn, 2005), which has prompted many studies of both the mechanism and function of coloration. A long-standing theory posits that, in general, sexual hormones that respond to environmental resources control the development of secondary sexual traits, so that secondary sexual development is correlated with body condition (Bakker *et al.*, 1999). Studies in several taxa have revealed that there is a direct relationship between the development of these traits and other factors (Andersson *et al.*, 2007), and have sought to understand the effect of environment on developmental mechanisms (Kotiaho, 2001). Understanding the developmental order of traits

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and relative gonadal growth producing sexual hormones (Kokko *et al.*, 2006) means interpreting external signal traits in terms of internal gonadal development.

In sheepshead minnows (*Cyprinodon variegatus*), we tested how closely secondary colour development is associated with gonad development and thus represents an honest signal of male reproductive status. In this species, mature fish are sexually dimorphic and males develop iridescent blue spots on their dorsal surface as a form of secondary sexual coloration (Page and Burr, 1991). Sheepshead minnows are small fish common to nearshore marine and estuarine waters along the US east coast and the Caribbean, and are an integral part of estuarine food webs (Raimondo *et al.*, 2009). They become mature within approximately 2–3 months and can live up to 3 years. Using populations from South Carolina and Connecticut in a common garden experiment, we focused on the development of secondary coloration and gonadal maturation. Doing so allowed us to test how external sexual signal traits are associated with internal sexual development prior to the breeding season.

MATERIALS AND METHODS

We collected wild juveniles from estuarine waters in South Carolina (SC: 32°45′2″N, 79°53′50″W) and Connecticut (CT: 41°20′9.7″N, 72°2′3.14″W) in August and November 2014 respectively, and transferred them to the Fisheries Ecology Division, NOAA Fisheries Science Center, Santa Cruz, California. We used these two geographically distinct populations to infer the generality of our findings.

We followed the protocol of Raimando *et al.* (2009). We grew the fish in a common environment, in which temperature and salinity were maintained at 24°C and 20 ppt respectively, with a photoperiod of 14 hours light/10 hours dark. We fed adult fish TetraMin® flakes (Tetra Holding, Blacksburg, VA, USA) *ad libitum*. Beginning 20 May 2015, we randomly sorted F1 fish into breeding groups of six adult females (SC: 3.52 ± 0.14 cm; CT: 3.55 ± 0.16 cm) and four adult males (SC: 3.60 ± 0.13 cm; CT: 3.56 ± 0.15 cm). We maintained three breeding groups per population in a 120-litre sea table (241.3 × 290.2 × 63.5 cm) with aeration and a filter. We placed breeding groups on net-breeders (45 × 50 × 25 cm) with egg-collecting mats and vertical dividers to separate males and females.

After 2 weeks of acclimation, during which egg-collecting mats were covered with a fine screen to prevent collection, we reduced the salinity to 10 ppt for 2 days to induce spawning. We removed the vertical divider 30 minutes before turning on the lights and collected eggs after 2 hours of spawning. We collected approximately 100 eggs from each breeding group and transferred them to fine-mesh net-breeders (13.4 × 17.2 × 13.4 cm) for hatching. Two weeks after hatching (at roughly 7 mm on average), we randomly selected 150 larvae from each population (50 larvae from each net-breeder). In order to aid identification and to prevent food and social stresses, we reared fish individually in a cylindrical growth chamber (diameter 8.5 cm, height 20 cm) with mesh walls and a solid bottom. We measured standard length (± 0.1 mm) weekly until 12 weeks post-hatching using a Canon 40D digital camera (Canon, Japan) with Image J software.

We checked weekly for the onset of sexual coloration and recorded the numbers of males in each of five colour stages ($n = 103$ and 94 for the SC and CT population respectively; see Results for detailed description of the stages). As each stage became most prevalent, we randomly collected 12 males in that stage and sacrificed them by tricaine methanesulfonate (MS-222) immersion. We measured standard length (±0.01 mm) and wet mass (±0.001 g),

and then removed and weighed the testes. We calculated the gonadosomatic index [GSI = (gonad mass/total mass) × 100] for 60 individuals of the SC and CT population, respectively. In order to compare GSI in Stage 4 to fully mature individuals, we sacrificed eight additional males from each breeding population.

To assess the relationship between the developmental order of secondary sexual traits and the GSI, we used Polyserial correlation, which is the inferred latent correlation between an ordered categorical variable (developmental order) and a continuous variable (GSI). To determine whether this relationship was different between populations, we used the Fisher *R*-to-*Z* transformation to compare the correlation coefficient between developmental order and the GSI. We analysed whether GSI in Stage 4 fish was different from GSI in breeding males using a two-way analysis of covariance (ANCOVA) with population (SC or CT) and age (Stage 4 or adult) as fixed effects and body length as a covariate. We used power analysis for ANCOVA to assess the robustness of the results using G*Power (Faul *et al.*, 2007) with Power calculated as 1 – (Type II error). All means are presented with their standard deviation and all analyses were performed with the software R v.3.3.1.

We used Fulton's condition factor as a metric of male quality, computed as $100 \times (W/L^3)$, where W = wet mass at week 12 and L = standard length at week 12. To compare body condition among the different developmental stages and mature males, we used a two-way ANCOVA with population (SC or CT) and developmental stage as fixed effects.

RESULTS

We found that development could be divided into a sequence of five distinct stages (Fig. 1a). Juvenile males initially had no iridescent blue coloration (Stage 0, S0). Secondary sexual coloration began with two bright iridescent blue dots behind the eyes on the dorsal surface (Stage 1, S1). These extended into parallel lines as more blue dots developed (Stage 2, S2). The dorsal surface between the two lines then began to fill in (Stage 3, S3) until the bright iridescent blue colour became fully developed (Stage 4, S4). The transition from one stage to the next took approximately one week.

By 7 weeks post-hatching, males from both populations began to develop iridescent blue coloration on their dorsal surface (Fig. 1). In week 8 post-hatching, 98.1% and 97.9% of males in the SC and CT population respectively showed two bright iridescent blue dots behind the eyes (Stage 1). In week 9, 70.3% and 72.0% of SC and CT males respectively developed more dots that extended into two parallel lines (Stage 2). In week 10, a filling in of the blue coloration was seen in 78.5% and 71.4% of SC and CT males respectively (Stage 3). And by week 11, 71.6% and 70.7% of SC and CT males respectively had fully developed iridescent blue coloration on the dorsal surface (Stage 4).

There was a positive correlation between GSI and the development of iridescent blue coloration (Polyserial correlation: SC, $r = 0.869$, $P = 0.030$; CT, $r = 0.914$, $P = 0.021$; Fig. 2a). Population had no effect on these positive correlations (Fisher *R*-to-*Z* transformation: $Z = 1.17$, $P = 0.242$, Power = 0.945). GSI in Stage 4 males was indistinguishable from that in breeding males (ANCOVA: $F_{1,35} = 0.359$, $P = 0.553$, Power = 0.892; Table 1). The GSI of each developmental stage was positively correlated with body length, wet mass, and body condition (Table 2, Figs. 3 and 4).

In week 12 when males became mature, mean body condition was 1.35 ± 0.11 in SC males ($n = 43$) and 1.37 ± 0.07 in CT males ($n = 34$). Body condition among developmental stages in same-age males was significantly different (effect of developmental state: $F_{4,67} = 11.556$,

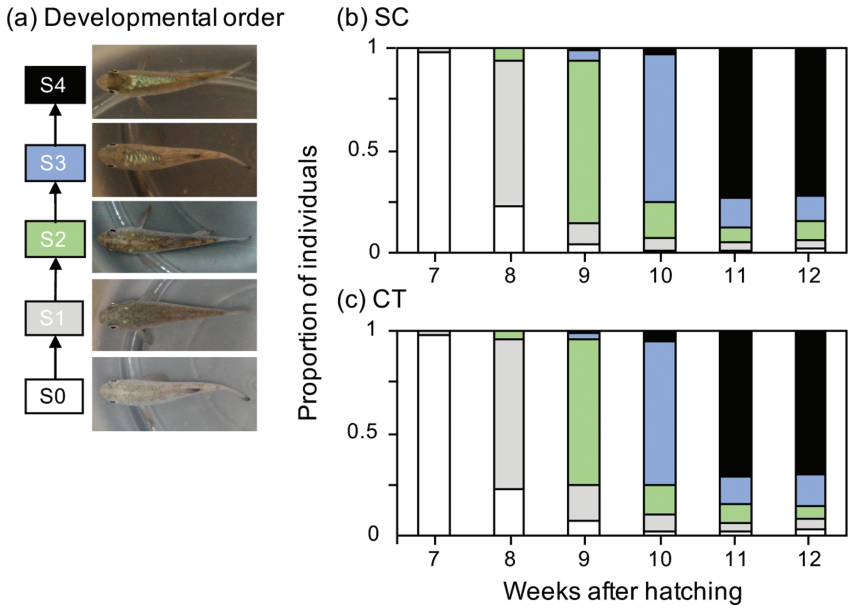

Fig. 1. (a) Developmental order of iridescent blue coloration (the secondary sexual trait) in male sheepshead minnows: S0 = no iridescent blue coloration (white bar), S1 = development of two blue iridescent dots (light grey bar), S2 = development into two parallel lines (light green bar), S3 = filling in of the dorsal surface (light blue bar), and S4 = fully developed blue coloration (black bar). (b, c) Proportions of fish from South Carolina (b) and Connecticut (c) showing the different stages of iridescent blue coloration.

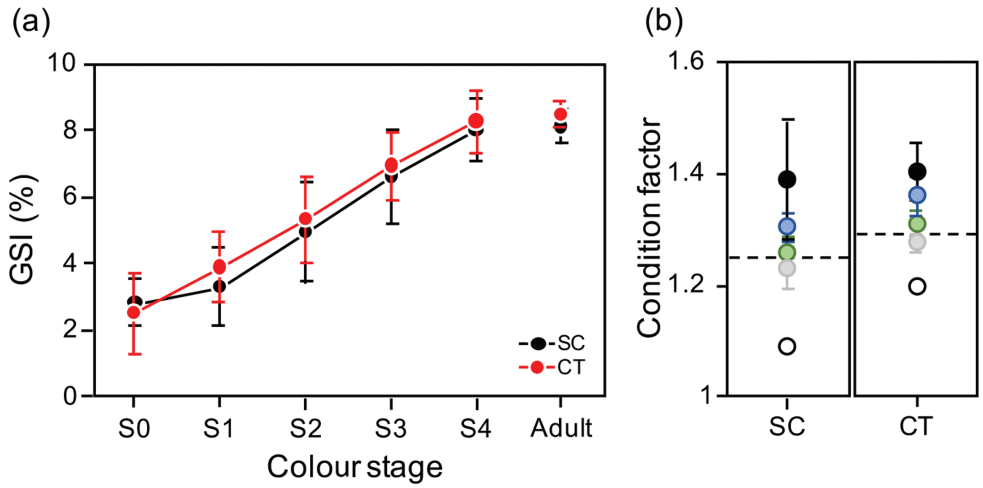

Fig. 2. (a) Demonstration that iridescent blue coloration (the secondary sexual trait) is an accurate predictor of the gonadosomatic index (GSI, %) between the South Carolina (black dot and solid line) and Connecticut (red dot and solid line) populations, and mean (±SD) GSI in adult males from the two populations. (b) Body condition when males became mature (week 12): S0 = white dot, S1 = light grey dot, S2 = light green dot, S3 = light blue dot, S4 = black dot. Dashed line in (b) represents mean body condition in each of the two populations.

Table 1. Results of two-way ANCOVA for gonadosomatic index (GSI) of Stage 4 versus that of fully mature males with population (SC or CT) and age (Stage 4 or adult) as fixed effects and body length as a covariate

Source	d.f.	Mean square	*F*	*P*
Population	1	2.431	2.006	0.166
Age	1	0.435	0.359	0.553
Body length	1	0.348	0.287	0.595
Population × Age	1	0.045	0.037	0.858
Error	35	1.212		
Total	39			

Table 2. Results of two-way ANCOVA for log-transformed gonadosomatic index (GSI) with developmental stage (Stages 0 to 4) as a fixed effect and log-transformed length and log-transformed mass as covariates

Source	d.f.	Mean square	*F*	*P*
Stage	4	0.542	215.80	<0.001
Log(length)	1	0.150	58.44	<0.001
Log(mass)	1	0.031	12.23	<0.001
Stage × log(length)	4	0.019	1.87	0.122
Stage × log(mass)	4	0.016	1.55	0.194
Log(length) × log(mass)	1	0.006	2.34	0.129
Stage × log(length) × log(mass)	4	0.002	0.21	0.934
Error	100	0.251		
Total	119			

$P < 0.001$): Stage 4 males in week 12 had the best body condition whereas Stage 0 males in week 12 were in the poorest condition. There was no difference between populations (effect of population: $F_{1,67} = 2.568$, $P = 0.114$, Power = 0.730).

DISCUSSION

We conclude that the secondary sexual coloration in sheepshead minnows (*Cyprinodon variegatus*) is an honest signal of male reproductive status: it develops through five stages and is positively related to testes growth in males. In addition, developmental stage was positively correlated with body condition. Since the timing and order of development was not different between populations, we suggest that this positive correlation is a general phenomenon in sheepshead minnows. Sexual blue coloration began at around 7 weeks post-hatching (roughly 60% into the growth period).

The observed parallel development of sexual coloration and gonad mass might be associated with maturation timing through allocation of resources to growth and reproduction. A developing gonad requires energy reserves prior to the timing of the investment decision.

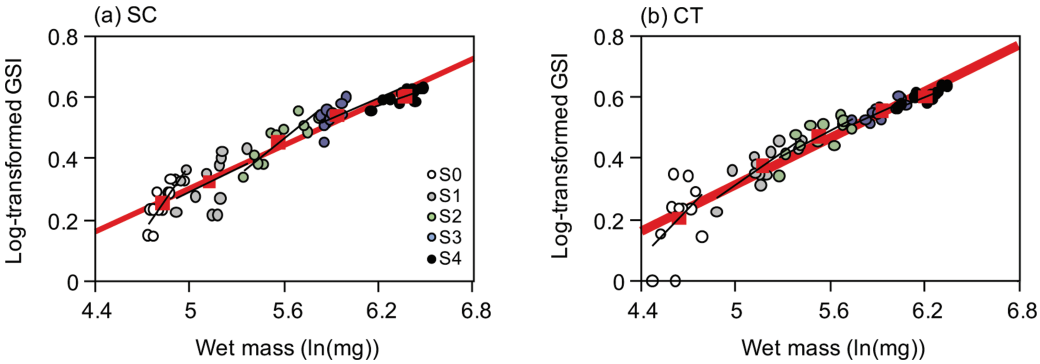

Fig. 3. Relationship between wet mass (ln(mg)) and log-transformed GSI between (a) the South Carolina and (b) Connecticut populations. Large red squares and bold red lines denote developmental order mean values and between-developmental order regression lines from the estimates of the correlation coefficients. Within-developmental order regression lines (thin black lines) are categorized by developmental order of blue coloration. See Table 2 for statistical analysis.

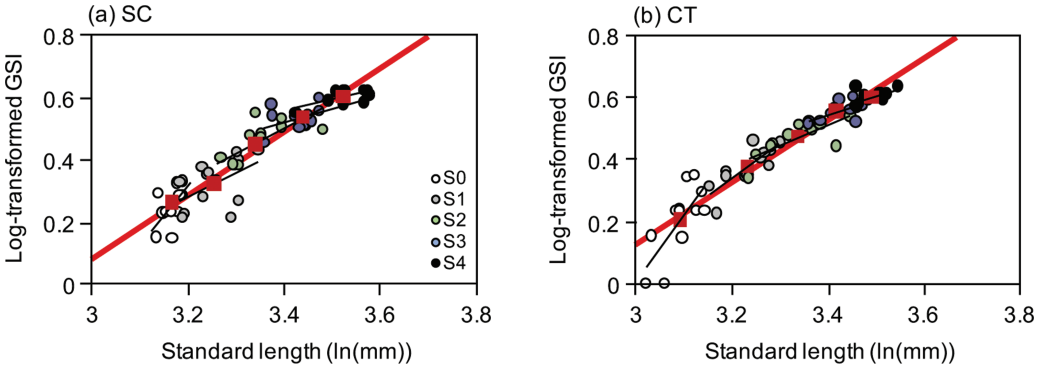

Fig. 4. Relationship between standard length (ln(mm)) and log-transformed GSI between (a) the South Carolina and (b) Connecticut populations. Large red squares and bold red lines denote developmental order mean values and between-developmental order regression lines from the estimates of the correlation coefficients. Within-developmental order regression lines (thin black thin lines) are categorized by developmental order of blue coloration. See Table 2 for statistical analysis.

Sexual coloration, commonly influenced by sex hormones (Helfman *et al.*, 2009), is an excellent candidate to explain such physiological mechanisms. For example, sexual hormones (e.g. prolactin and melatonin) were positively related to sexual coloration during courtship in two-spotted gobies (*Gobiusculus flavescens*) (Sköld *et al.*, 2008). Although body condition could affect brightness and size of sexual coloration (Gonçalves and Oliveira, 2011), the timing of emerging sexual coloration might depend on investment decisions rather than body condition.

The tight relationship between a well-defined set of stages of sexual coloration and gonadal development may be a signal to conspecifics. Sheepshead minnows are quasi-lek breeders, with males maintaining and defending territories while groups of females visit these territories (Raney *et al.*, 1953; Kodric-Brown, 1998). Variation in the strength of secondary sexual traits (e.g. brightness or size) correlated with body condition represents information useful

to fish of either gender when assessing the competitive and reproductive capabilities of a male. Alternatively, the sequence of coloration stages may be a signal from juveniles to mature individuals that an aggressive response is unnecessary, allowing immature males to pass by unscathed. Further study is needed to examine how fish of both sexes respond to males of different colour stages in sheepshead minnows.

CONCLUSION

In conclusion, we have shown that the sexual blue coloration in sheepshead minnows develops both in a consistent fashion and in parallel with gonadal growth. Thus, coloration is an accurate signal that may allow sheepshead minnows to accurately assess male maturation status, thereby informing mate choice and intrasexual competition decisions. Our work thus lays the foundation for how this pattern of colour development is used for sexual selection in both males (as competitor) and females (as mate) and to understand what mechanisms regulate sexual coloration and gonadal growth simultaneously.

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