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Effect of enrichment on gamete production, gamete quality, and spawning coloration in hormonally induced redside dace *Clinostomus elongatus*

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ABSTRACT: Due to the alarming rates of freshwater fish extinctions, urgent action is needed to develop captive breeding programs for imperiled species and enhance existing practices to improve reproductive outcomes. Here, we investigated the effects of enrichment on gamete production, quality, and spawning coloration following hormone injection (i.e. carp pituitary, gonadotropin) in the endangered redside dace *Clinostomus elongatus*, a sexually dimorphic, presumably obligate nest parasite. *C. elongatus* were reared in either a non-enriched environment (i.e. barren) or an enriched environment (i.e. substrate, plants, and spawning nest-building hosts) for 1 yr prior to hormone induction. We found no differences in the proportion of free-flowing gamete expression between male and female *C. elongatus* in the non-enriched and enriched environments. However, males reared in enriched environments had higher sperm motility, while among females, there was no significant difference in egg diameter. Furthermore, enrichment was found to influence spawning coloration, with males and females reared in enriched environments displaying redder hues compared to those reared in non-enriched environments prior to hormone induction. However, post hormone injection, no significant differences in red coloration were observed between non-enriched and enriched males and females, indicating that hormone induction improved coloration in non-enriched fish. This study highlights the effect of enrichment on gamete production, quality, and spawning coloration and provides information for captive breeding *C. elongatus*. These findings provide valuable insights into the potential of enrichment and induction techniques to enhance reproductive outcomes when captively breeding endangered species of fishes.

KEY WORDS: Enrichment · Induction · Endangered species · Captive breeding · Secondary sexual character · Gamete quality · Fish

1. INTRODUCTION

In response to the dramatic decline in global freshwater biodiversity, and with freshwater fishes having some of the highest extinction rates worldwide, there has been a pressing need, in recent years, to establish effective captive breeding programs for species at risk (Burkhead 2012, Harrison et al. 2018, Lamothe & Drake 2019, Lamothe et al. 2019). Traditionally, captive breeding has been employed to enhance and augment wild game fish populations with captive-reared individuals (Stickney & Treece 2012). More recently, however, captive breeding programs have become a common tool to help reduce the probability of extirpation or extinction of imperiled non-game species (Donaldson et al. 2019, Rytwinski et al. 2021). In order to establish a captive-bred population, it is necessary (among other steps) to be able to manipulate reproductive pro-

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cesses and collect high-quality gametes from individuals in order to produce viable offspring (Mylonas et al. 2010). Typically, this is achieved by adjusting abiotic and biotic conditions in the captive environment before breeding, including photoperiod, water temperature, flow, and diet. These modifications are designed to induce 'natural' reproductive signals and behaviors (e.g. spawning coloration, mate choice, courtship, and territoriality) and cue gamete production. However, even with the implementation of abiotic and biotic measures in captivity, individuals often still fail to produce gametes naturally, thereby emphasizing the need to exert greater control over reproductive processes (Mylonas et al. 2010). In such instances, hormone induction techniques can be an effective solution (e.g. Caille et al. 2006, Maria et al. 2012).

Reproductive hormones have been extensively studied for their ability to induce spawning signals, behaviors, and gametogenesis (the process of gamete formation and development) in captive reared fish (Mañanos et al. 2008, Mylonas et al. 2010, Zohar 2021). Synthetic analogues of gonadotropin-releasing hormone and luteinizing hormone-releasing hormone are commonly employed to promote gamete maturation (Zohar & Mylonas 2001). Additionally, human chorionic gonadotropin, as well as pituitary homogenates from salmon or carp, have proven effective in inducing ovulation and sperm release in captive-reared fish (Mylonas et al. 2010). In hatchery settings, commercial pituitary homogenates are a common choice for induction, because these preparations often contain gonadotropin hormones, which bypass the hypothalamus–pituitary link, acting directly on the ovaries and testes (Rottmann et al. 1991). This direct action leads to a significant increase in blood gonadotropin levels, a phenomenon that typically precedes spawning (Rottmann et al. 1991). Furthermore, hormones present in pituitary homogenates have been associated with the ability to influence various physiological processes, including the regulation of coloration expression (reviewed in Fujii 2000). In line with this, many studies have demonstrated that pituitary homogenates can successfully induce spawning signals (i.e. coloration), behaviors, and gametogenesis in a variety of freshwater species, including sterlet sturgeon *Acipenser ruthenus* (Williot et al. 2005), summer flounder *Paralichthys dentatus* (Berlinsky et al. 1997), surubim do paraíba *Steindachneridion parahybae* (Caneppele et al. 2009), peppered chub *Macrhybopsis tetranema* (Urbanczyk et al. 2019), sharpnose shiner *Notropis oxyrhynchus* (Urbanczyk et al. 2019), and smalleye shiner *N. buccula* (Urbanczyk et al. 2019). However, the effectiveness of such homogenates can vary and are often influenced by factors such as administration method, dosage, and environmental variables (Billard et al. 1984). Consequently, difficulties in achieving high-quality gametes (i.e. highly motile sperm and large eggs capable of successfully fertilizing into viable offspring) often persist in captivity and it may therefore be necessary to introduce supplementary measures of environmental enrichment (i.e. environmental stimuli [motor or sensor stimulation] to help captive fish meet their physiological, behavioral, and psychological needs; Arechavala-Lopez et al. 2021) to further enhance reproductive outcomes (Williams et al. 2009, Arechavala-Lopez et al. 2021).

While the manipulation of photoperiod, temperature, flow, and diet (reviewed in: Mañanos et al. 2008, Oliveira & Sánchez-Vázquez 2010) have been widely recognized for their influence on freshwater fish reproduction, other forms of environmental enrichment can also be implemented in a captive setting. These include physical enrichment (i.e. introducing structures, objects, or structural modifications to increase the environment's complexity), sensorial enrichment (i.e. introducing visual, auditory, chemical, hydromechanical, or electrical stimuli to increase sensory cues in captivity), and social enrichment (i.e. taking into account both the density of conspecific and heterospecific species and providing ample space for interactions or the ability to avoid others) (Arechavala-Lopez et al. 2021). Previous studies have shown that incorporating various forms of environmental enrichment, beyond just abiotic enrichment, can contribute to preparing individuals to reproduce (e.g. Cacho et al. 1999, Delicio et al. 2006, Galhardo et al. 2009). For instance, Galhardo et al. (2009) demonstrated that male Mozambique tilapia *Oreochromis mossambicus* showed a clear preference for physical enrichment when engaging in territorial reproductive behaviors compared to barren environments in captivity. Furthermore, environmental enrichment has been linked to coloration changes in fish, driven by alterations in the morphology and density of chromatophores (i.e. specialized cells responsible for controlling the color and pattern of skin; Fujii 2000). Although the exact mechanisms are not fully understood, there is a growing body of evidence supporting the idea that enrichment types such as sensory stimuli (e.g. visual) can serve as cues for inducing coloration changes (Sugimoto 2002). For example, a study conducted by Yaripour et al. (2020) provided evidence that physical and sensory enrichment, specifically brown-grey gravel compared to a barren environment, contributed to the development of skin coloration in adult brown trout *Salmo trutta* L. that

closely resembled wild counterparts. Despite this, little research has been conducted on how environmental enrichment in addition to induction hormones affects fish reproduction in captivity. Therefore, it is favorable to investigate the potential benefits of environmental enrichment on reproductive signals (i.e. spawning coloration) and gamete production and quality, particularly in endangered species with complex life histories.

Redside dace *Clinostomus elongatus* is a small endangered leuciscid found across much of northeastern North America (SARA 2002). In the wild, *C. elongatus* typically engage in spawning activities from May to early June, coinciding with the accumulation of thermal energy in the environment — a measure often expressed as growing degree days (GDD). For *C. elongatus*, spawning is expected to commence when the cumulative GDD reaches 288°C d (Watt et al. 2023). *C. elongatus* is presumed to be an obligate nest parasite (providing no parental care to offspring), relying on gravel nests built by larger-bodied minnows such as creek chub *Semotilus atromaculatus* and common shiner *N. cornutus* (Koster 1939). However, the extent of its host dependence and specificity remains poorly understood (Rakes et al. 2013). Observations of their spawning behavior indicate a polygynandrous mating system, with individuals spawning both independently and in large groups in nests situated near fast-flowing riffles and pools (Watt et al. 2023). Throughout much of the year, *C. elongatus* maintains a dull coloration, but during the breeding season, both sexes develop a dramatic red pigmentation spanning from the opercula to just below the dorsal fin, presumably derived from carotenoids (Davidson 2011, Beausoleil et al. 2012). Previous research has shown that the red coloration is more saturated, darker, and larger on males than it is on females, suggesting subtle dimorphism between the sexes (Beausoleil et al. 2012). Examination of the red coloration has also found that males with relatively more red coloration had greater sperm velocity (Beausoleil et al. 2012), and as sperm velocity correlates with fertility in many species (e.g. Gage et al. 2004), the area of red coloration may be an important index of fertility to explore.

Because *C. elongatus* is endangered throughout most of its range, a better understanding of inducing gamete production for the development of a captive breeding program has been identified as a critical next step in its recovery strategy (Redside Dace Recovery Team 2010, Lamothe et al. 2019). However, because *C. elongatus* is difficult to breed in captivity, measures must be taken to develop enrichment practices and suitable hormone induction techniques that enhance reproduction. This study tested the effect of environmental enrichment on gamete production, gamete quality, and spawning coloration in hormonally induced *C. elongatus* to help inform captive breeding practices. To investigate this, we first compared the production and quality of gametes between hormonally induced males and females reared in non-enriched (i.e. control) and enriched environments, hypothesizing that environmental enrichment could enhance gamete production and quality. We predicted that in enriched environments, hormonally induced males and females would exhibit increased gamete production, with the gametes being higher quality compared to those produced by individuals in non-enriched environments. Next, we examined the influence of enrichment and hormone induction on spawning coloration by examining differences in red coloration (i.e. hue and area) between enriched and non-enriched *C. elongatus* before and after hormone induction. First, we hypothesized that environmental enrichment influences spawning coloration prior to hormone induction. We predicted that hormonally induced *C. elongatus* reared in enriched environments will exhibit differences in red coloration (i.e. redder hue and larger areas of red coloration) compared to hormonally induced *C. elongatus* in non-enriched environments. Second, we hypothesized that hormone induction affects spawning coloration. We predicted that both non-enriched and enriched *C. elongatus* would exhibit more pronounced coloration (i.e. redder hue and larger areas of red coloration) following hormone induction. Taken together, this study provides information for captive breeding endangered *C. elongatus* and answers important questions about environmental enrichment and its ability to influence reproduction in captivity.

2. MATERIALS AND METHODS

2.1. Animal collection and housing

On 2 occasions in 2019 (27–29 August and 4–8 November), adult redside dace *Clinostomus elongatus* were collected from the northern branch of the Kokosing River, Morrow County, Ohio, USA (40.545909° N, 82.654234° W) via seining. Fish collection permits were issued by the Ohio Department of Natural Resources Division of Wildlife. On each occasion, captured fish (n = ~150 individuals) were transported to the Freshwater Restoration Ecology Centre (University of Windsor), Lasalle, Ontario, Canada. Fish were initially housed in 2 recirculating 850 l round fiberglass tanks at temperatures ranging

between 10 and 12°C and pH ranging between 7.1 and 7.8 (Turko et al. 2020). Recirculating flow rate was kept constant at ~0.3 m s^{-1}. A light cycle of 12 h light: 12 h dark (with lights on at 06:00 h) was maintained by overhead fluorescent lights. Fish were fed frozen red bloodworms daily and supplemented with commercial fish flakes multiple times a week. All experiments were approved by the Animal Care Committee at the University of Windsor.

2.2. Manipulation of physical and biotic enrichment

Approximately 1 yr prior to experimentation, fish were haphazardly sorted into 2 non-enriched tanks and 2 enriched general population tanks. Non-enriched tanks were empty of substrate and plants, and enriched tanks included physical/structural enrichment with a layer of loose multi-colored (brown, grey, white) pebble gravel (3/8 inch [0.95 cm]) at the bottom of each tank (~80% of the base of the tank, allowing for nest depressions to be made) (Wentworth 1922). Vertical floating plastic plants (~30 cm tall) were also added to each enriched tank to mimic a natural habitat. Enriched tanks were also outfitted with a programmable light system (Ecotech Radion), designed to simulate the natural daylight cycle found in the wild. This light system consisted of LED lights that emitted a wide spectrum of wavelengths within the visible spectrum (395–665 nm). The light system was installed inside the lid of each tank to provide sensorial enrichment. Enriched tanks also contained 2 creek chub *Semotilus atromaculatus* (with breeding tubercules present), providing both sensorial (i.e. visual and chemical) and social (i.e. heterospecific) enrichment for 1 yr prior to experimentation. In both the non-enriched and enriched tanks, the fish were exposed to the same abiotic enrichment protocol, which involved the use of recirculating tanks and accounted for factors such as photoperiod, temperature, and flow (as described in Section 2.1; temperature = 10–12°C, pH = 7.1–7.8, flow rate = 0.3 m s^{-1}, photoperiod = 12 h light: 12 h dark). Additionally, the diet provided to the fish was kept consistent across both treatment groups (frozen red bloodworms and commercial fish flakes).

2.3. Thermal regime

To induce spawning in *C. elongatus*, we focused on replicating their predicted spawning temperature (Watt et al. 2023). We initiated this process 3 mo before induction by implementing a thermal regime schedule designed to mimic the natural increase in water temperature during springtime. This schedule was based on prior research, which indicated a 95% likelihood of *C. elongatus* spawning when the cumulative thermal energy in the environment reached 288°C d (Watt et al. 2023). To estimate the timing of spawning in captivity, we used this critical value and available water temperature data from known *C. elongatus* streams. To recreate the temperature conditions of their natural streams, we began by lowering the water temperature to 10°C beginning 1 January 2022. Water temperature was held at 10°C until 13 March 2022. Starting 14 March 2022, we gradually increased the water temperature by ~1°C wk^{-1} until 11 April 2022, when water temperature across all tanks reached 15°C and coincided with the 288°C d threshold (thus reaching the cumulative thermal energy predicted to trigger spawning).

2.4. Carp pituitary extract protocol and phenotypic measurements

On 13 April 2022 (~1 yr of being exposed to enrichment), all males (in non-enriched and enriched tanks) received an intraperitoneal injection using an insulin syringe (BD, 8 mm, 0.3 ml volume) of 1.0 mg kg^{-1} body weight of carp pituitary extract (CPE) (i.e. aqueous suspension of acetone-dried CPE) just below the pelvic fin. All females were given a priming dose (25%) and a resolving dose (75%) of 4.0 mg kg^{-1} body weight of CPE ~15 h apart beginning 13 April 2022. Hormone dosages were based on previous studies in small-bodied fishes (Dorafshan & Heyrati 2006, Urbanczyk et al. 2019).

Fish from the non-enriched tanks (n = 70; n = 39 males, n = 31 females) and fish from the enriched tanks (n = 105; n = 72 males, n = 33 females) were removed from holding and anesthetized in a buffered (0.15 g l^{-1} MS-222 and 0.2 g l^{-1} sodium bicarbonate) aerated bath (Beausoleil et al. 2012). Under anesthesia, all fish were immediately photographed for pre-hormone coloration alongside a ruler for scale using a digital camera (Nikon D60) and weighed to document standard length (±0.1 cm), body mass (±0.001 g), condition (Fulton's condition factor), body area (cm^2), and the area of red spawning coloration area (cm^2; i.e. area of red pigmentation) (Beausoleil et al. 2012, Turko et al. 2020). Fish were photographed inside a foldable photobox (16 × 16 inch [40.6 × 40.6 cm]; KHS-TECH) lined with a white background (Parolini et al. 2018). To

the left side of the ruler, a color checker card (Datacolor, Spyder Checkr 24) was placed to correct for subtle differences in lighting or exposure. To ensure consistency between photographs, the color checker card did not move throughout the duration of the study. On top of the photobox, the camera was mounted at a standard distance of ~40 cm from the fish (Parolini et al. 2018). Next, fish were checked for the presence of free-flowing gametes (i.e. sperm or eggs) by gently applying pressure to the abdomen using a stripping motion (i.e. beginning at the anterior end of the abdomen and gently moving toward the urogenital pore) (Rothbard 1981). If fish were expressing free-flowing gametes prior to hormone induction, they were removed from the study. For fish where gametes were not expressed, their sex was inferred using physical characteristics previously described by Beausoleil et al. (2012) in their investigation of dimorphism in *C. elongatus.* These characteristics encompassed various aspects, including body size and shape, as males were found to exhibit a more streamlined body shape than females (Beausoleil et al. 2012). Additionally, the fins were also examined, as males have been found to possess more elongated and pointed dorsal and anal fins than females, particularly during the breeding season. In cases where sex could not be confidently guessed based on these physical characteristics, coloration was used as a secondary measure. Beausoleil et al. (2012) reported that males typically display a larger area of saturated red coloration compared to females.

Thirty hours post-hormone injection, males were checked for the presence of sperm (a subset of fish were periodically checked at 12 and 24 h post-injection but were not expressing free-flowing sperm) (Urbanczyk et al. 2019). Males were removed from their holding tanks and anesthetized (see details above). Males expressing sperm were stripped using a dry technique, where excess water was gently dried with a paper towel to prevent sperm activation (Rothbard 1981). Sperm was collected using a pipette and stored in an Eppendorf tube (0.5 ml) and placed into a cooler with ice and cardboard until being analyzed (non-enriched: n = 6 of the 39 males expressed sperm, enriched: n = 7 of the 72 males expressed sperm). Approximately 30 h after the resolving dose, females were removed from their tanks (a subset of fish were checked at 12 and 24 h post-resolving dose but were not expressing free-flowing eggs), anesthetized, and checked for the presence of free-flowing eggs. Females were held over a Petri dish and gentle pressure was applied to the abdomen using a stripping motion. Eggs were collected and placed in a cooler with ice and cardboard until being analyzed. Following gamete collection (~30 h after induction), male and female *C. elongatus* were photographed (see details above) to document possible color change post-CPE injection. Following hormone induction, we identified the sexes of the fish using the same dimorphic characteristics as stated above. Additionally, we used the release of seminal plasma as an indicator of male sex (non-enriched: n = 27/39 males with seminal plasma release; enriched: n = 43/72 males with seminal plasma release). When physical cues were not clear, we considered color as a supplementary indicator (Beausoleil et al. 2012).

2.5. Sperm quality measurements

Within 1 h of collection, sperm collected from each male was analyzed (data was able to be collected from n = 4 non-enriched and n = 6 enriched males). Sperm (< 0.1 µl) was pipetted onto the chamber of a 2X-CEL glass microscope slide (Hamilton Thorne) and covered with a glass coverslip (22 × 22 mm). Sperm were activated with 10 µl of 15°C water taken from a holding tank. Within 5 s of activation, sperm were video-recorded using a 10× objective lens (see Pitcher et al. 2009). Video recordings were analyzed using HTM-CEROS sperm-tracking software (CEROS version 12, Hamilton Thorne Research) (Kime et al. 2001). The following recording parameters were used: number of frames captured in sequence = 60, minimum contrast = 11, minimum cell size = 8 pixels. Sperm velocity was measured as (1) curvilinear velocity (i.e. the average velocity on the actual point-to-point path followed by the cell) at 5 s post-activation, (2) straight-line velocity (i.e. the average velocity along a straight line from a starting point to an ending point on the path), and (3) average path velocity (i.e. the average velocity along a smooth cell path) (Pitcher et al. 2009, Beausoleil et al. 2012). The sperm-tracking software determined the sperm velocity for each individual sperm cell and generated an average of these sperm cells (mean ± SD) from each video recording.

Sperm motility was defined by the percentage of sperm that exhibited forward movement. Sperm longevity was determined as the time from activation until ~95% of all spermatozoa within the field of view were no longer motile (i.e. no forward movement) (Gage et al. 2004). Sperm density was determined using a Neubauer hemocytometer (Pitcher 2007). A mixture containing 1.5 µl sperm and 500 µl saline was created, and 10 µl of this mixture was pipetted onto the hemocytometer. Density estimates came from counting the number of sperm cells visualized under

400× magnification in 5 of the 25 larger squares. The mean number of sperm from the 5 squares was then multiplied by 25 (to approximate the number of sperm in all squares), then by 10 (to account for the depth of the hemocytometer chamber in µm), and again by 10 (for the original sample volume in µl). The estimated densities were reported as the number of sperm ml^{-1} ($\times 10^6$).

Sperm morphology was assessed by taking a subsample of sperm (1.5 µl) and mixing it with saline solution and 2.5% glutaraldehyde (Leach & Montgomerie 2000, Pitcher et al. 2009). Approximately 1 µl of the sperm solution was placed onto a glass microscope slide. Sperm were observed at 100× magnification under oil immersion using an Olympus BX52 microscope. Intact sperm head (including the midpiece; Gage et al. 2002, Pitcher et al. 2009) and flagellum images were taken using an Olympus DP72 digital camera and lengths were measured using an ImageJ plug-in. Twenty sperm per male were measured for head length (L_H), head width (W_H), flagellum length (L_F), and total length (L_T) (see Pitcher et al. 2009 for definitions). The mean of the 20 measurements taken were used in all analyses.

2.6. Egg diameter measurements

Eggs collected from each female (non-enriched: n = 9 of the 31, enriched: n = 8 of the 33 females) were photographed using a digital microscope (DCorn 7 inch [17.8 cm] display, 1200×) and a reference scale. Approximately 15 eggs (mean = 0.22 mm, range = 0.13–0.38 mm) haphazardly selected from each female were measured for egg diameter (mm) using ImageJ software.

2.7. Red coloration analysis

Body photographs of males and females (taken prior to and ~30 h post-induction) were objectively measured for numerical estimates of hue, saturation, and brightness to estimate the intensity of red coloration using Adobe Photoshop® software (Skarstein & Folstad 1996, Pitcher et al. 2007). Hue, saturation, and brightness were measured to examine possible changes in red coloration pre- and post-induction. Hue refers to the wavelength of a color, which is typically identified by its name, such as red. Adobe Photoshop® measures hue using an angle (between 0 and 360°) on a color wheel (true red = 0°). The saturation of a color shows its strength or purity and is a measure of how much grey is in proportion to the hue, expressed as a percentage from 0% (grey) to 100% (full saturation). Thus, an individual would be highly ornamented if it exhibited a redder hue (i.e. a hue closer to 0°) and greater saturation (closer to 100%). Brightness is a measure of how light or dark an image appears, ranging from 0% (black) to 100% (white).

Adobe Photoshop® was used first to measure the brightness of the color checker card to ensure uniform lighting conditions between images. A mean value for hue, saturation, and brightness was then calculated for each red spot along the middle of the fish based on 3 measurements of red coloration. Hue, saturation, and brightness were averaged for each male and female separately. The area of red spawning coloration (cm^2) and the total body area (cm^2) were measured on the left side of each fish per group using ImageJ software (http://imagej.nih.gov/ij/) to calculate the relative area covered by the red spawning coloration before and after injection (Pitcher et al. 2003, Beausoleil et al. 2012).

2.8. Statistical analyses

To investigate variations in the proportion of individuals producing gametes, we employed 2 analytical approaches. First, we conducted chi-squared goodness of fit tests to determine whether the proportion of males and females producing sperm and eggs was equal between non-enriched and enriched environments. Second, in order to account for a broader array of variables that may influence gamete production, we employed a generalized linear model with a binomial error distribution (GLM; with logit link function). A GLM was chosen because data can be handled in a variety of ways with different statistical properties (Venables & Ripley 2002). We created a full model which included our response variable 'gametes' (gamete absence = 0, gamete presence = 1) against our predictor variables: environment (2 levels; non-enriched or enriched), sex (2 levels; male or female), tank number (4 levels; 1–4), body condition, and their interactions. To select the most parsimonious model, a 'backwards stepwise' model-selection approach was used. The statistic used to select the final model was the Akaike information criteria (AIC).

Next, to investigate potential differences in gamete quality between fish reared in non-enriched and enriched environments, a series of 2-way comparisons were performed. To reduce the number of variables and emphasize variables of biological signifi-

cance, we assessed differences in sperm quality (i.e. average path velocity, motility, longevity, density, and total length) between non-enriched and enriched environments using 2-sample independent *t*-tests and Mann-Whitney *U*-tests. Sperm motility and total length were measured using a Mann-Whitney *U*-test, while average path velocity, longevity, and density were assessed using a *t*-test. For egg quality, because no correlation was found between female body condition and egg diameter across environment (Pearson correlation: $r_p = -0.18$, df = 15, p = 0.501), a 2-sample independent *t*-test was used to examine whether there was a difference in egg diameter between non-enriched and enriched environments.

Lastly, because red coloration could not be examined at the individual level (to avoid mortality and limit stress), we investigated possible variations in red coloration (i.e. hue and area) between environments (i.e. non-enriched vs. enriched) and treatment (i.e. before and after hormone induction). To streamline our analysis and prioritize biologically important variables of color, we employed statistical tests (separately for males and females), including 2-sample independent *t*-tests and Mann-Whitney *U*-tests, with a significance threshold (alpha) set at 0.05.

In males, pre-CPE red coloration (hue and area) was measured using a *t*-test. Post-CPE, hue was measured using a Mann-Whitney *U*-test, while area was assessed using a *t*-test. For females, pre-CPE red coloration (hue and area) was measured using a *t*-test. Post-CPE, hue was measured using a Mann-Whitney *U*-test, while area was assessed using a *t*-test. Because males' area of red spawning coloration was a function of their body area (non-enriched: $r_P = 0.56$, n = 39, p = 0.0002; enriched: $r_P = 0.6$, n = 72, $p < 0.001$), the residual area of red spawning coloration was used to determine how much red spawning coloration each male possessed (Pitcher et al. 2007, Beausoleil et al. 2012). The residual area of red spawning coloration was estimated from the least-squares residuals of the regression between red coloration area and body area. Hue was not related to body area (all $p > 0.05$). Similarly, as females' area of red spawning coloration was found to be a function of their body area (non-enriched: $r_S = 0.58$, n = 31, p = 0.0006; enriched: $r_P = 0.42$, n = 33, $p < 0.02$), the residual area of red spawning coloration was used to determine how colorful females were. Hue was not related to body area (all $p > 0.05$). All analyses were conducted in R version 3.6.1 (R Development Core Team 2013). Data were checked for normality prior to all analyses using visual assessment techniques (i.e. histogram and qq-plots) and Shapiro-Wilk tests.

3. RESULTS

3.1. Gamete production and quality

The proportion of males producing sperm and females producing eggs did not differ significantly by environment (males: $\chi^2 = 0.4$, df = 1, p = 0.53; females: $\chi^2 = 0.06$, df = 1, p = 0.81). For GLM, the model with the best 'fit' used 2 variables and their interaction: (1) environment, (2) sex, and (3) environment × sex. The AIC value for this model was 169.65. Environment ($z = -0.43$, p = 0.666), sex ($z = -1.36$, p = 0.173), and their interaction ($z = 0.006$, p = 0.996) were not significant against the response variable 'gametes' (gamete absence = 0, gamete presence = 1) in the model.

Viable sperm samples were obtained from 10 of 111 (9%) male redside dace *Clinostomus elongatus* (non-enriched: n = 4 of the 39; enriched: n = 6 of the 72 males). Sperm motility was found to differ significantly between non-enriched and enriched-reared males (Fig. 1), while the sperm traits of average path velocity, longevity, density, and total length did not differ significantly between non-enriched and enriched-reared males (all p-values > 0.05; Table 1). Eggs were collected from 17 of 64 (26.5%) female *C. elongatus* (non-enriched: n = 9 of the 31; enriched: n = 8 of the 33 females). There was no significant dif-

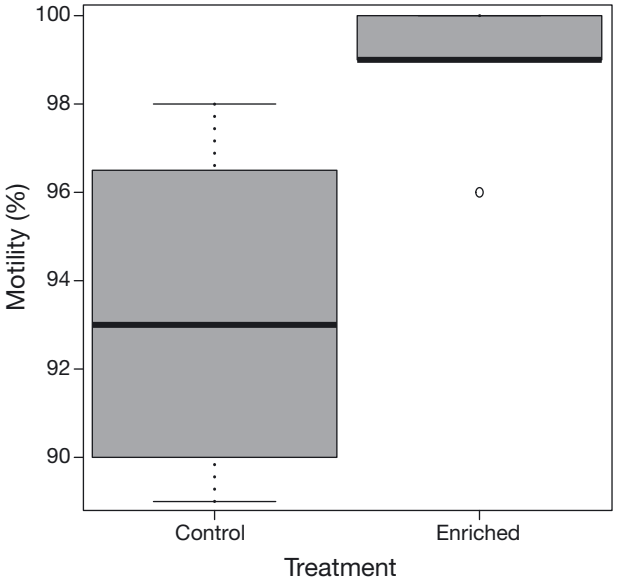

Fig. 1. Boxplots showing the significant difference in sperm motility between non-enriched (i.e. control) (n = 4) and enriched (n = 6) hormonally induced male redside dace *Clinostomus elongatus* ($w = 1.0$, p = 0.023). Bold horizontal line: median; top and bottom boundaries of the box: first and third quartiles; whiskers: highest and lowest values within the interquartile range; circles: outliers

Table 1. Sperm quality differences between non-enriched (n = 6) and enriched (n = 7) reared male redside dace *Clinostomus elongatus*. *t/w* represents either *t*: *t*-test statistic; or *w*: Mann-Whitney *U*-test statistic. Alpha: 0.05

Variable	Non-enriched		Enriched		Test statistics		
	Mean ± SD	Range	Mean ± SD	Range	*t/w* statistic	df	p
Curvilinear velocity (μm s^{-1})	152.3 ± 6.6	142.5–156.9	140.5 ± 9.0	129.7–151.2	2.37	7.8	0.046
Straight-line velocity (μm s^{-1})	77.8 ± 24.9	42.5–100.9	63.0 ± 11.1	51.6–80.8	1.11	3.8	0.330
Average path velocity (μm s^{-1})	118.7 ± 11.9	101.3–127.5	107.2 ± 7.3	99.9–118.7	1.72	4.5	0.152
Sperm motility (%)	93.3 ± 4.0	89.0–98.0	98.8 ± 1.5	96.0–100.0	1.0	–	0.023
Sperm longevity (s)	40.5 ± 7.6	33.0–48.0	30.0 ± 3.4	26.0–34.0	2.59	3.8	0.063
Density (×10^6 ml^{-1})	12.8 ± 4.6	5.1–19.1	14.6 ± 8.8	4.8–28.3	−0.49	9.3	0.638
Head length (μm)	2.8 ± 0.1	2.7–2.9	3.0 ± 0.4	2.7–3.9	12	–	0.225
Head width (μm)	2.5 ± 0.1	2.4–2.6	2.5 ± 0.1	2.3–2.7	0.59	10.5	0.568
Flagellum length (μm)	37.8 ± 3.4	34.5–44.5	35.7 ± 1.4	34.0–37.2	28	–	0.353
Total length (μm)	40.6 ± 3.4	37.2–47.2	38.7 ±1.4	36.9–40.3	28	–	0.353

ference found between egg diameter and enrichment (t = −2.096, df = 10.72, p = 0.061).

3.2. Red coloration and treatment

Prior to induction, we observed significant differences in hue between non-enriched and enriched individuals (Table 2). Comparing means, enriched males exhibited a noticeably redder hue compared to non-enriched males. Similarly, enriched females also had a redder hue compared to non-enriched females. However, there were no significant differences in the area of red coloration between non-enriched and enriched males and females (evaluated separately by sex). Following induction, hue and the area of red coloration did not differ significantly between non-enriched and enriched males and females (Table 3).

4. DISCUSSION

In this study, we examined if long-term exposure to enrichment influenced gamete production and quality and nuptial coloration in redside dace *Clinostomus elongatus*. Further, we tested if any differences persisted or were gained by hormonally induced spawning. We found no significant effect of enrichment on the proportion of gamete-producing hormonally induced males or females. There were, however, significant differences found between non-enriched and enriched reared males in terms of sperm motility, a proxy for male reproductive quality (reviewed in Gallego & Asturiano 2019). Furthermore, no differences in egg diameter (a proxy for female reproductive quality; Stuart et al. 2020) were found between non-enriched and enriched reared females. Additionally, when exploring potential differences in red coloration between non-enriched and enriched

Table 2. Differences in red spawning coloration between non-enriched (n = 70) and enriched (n = 105) reared redside dace *Clinostomus elongatus* pre-hormone induction (i.e. carp pituitary extract injection, see Section 2 for details). Residual red spawning coloration area is shown here as percentage of the body covered in red for ease of interpretation; however, in all analyses, we used residuals derived from the regression of area of red spawning coloration on overall body surface area (see Section 2 for details). *t/w* represents either *t*: *t*-test statistic; *w*: Mann-Whitney *U*-test statistic. Alpha: 0.05

Variable	Non-enriched		Enriched		Test statistics		
	Mean ± SD	Range	Mean ± SD	Range	*t/w* statistic	df	p
Males							
Hue (0–360°)	16.5 ± 7.3	6.3–41.3	12.2 ± 4.5	3.7–24.7	3.5	73.8	0.0007
Saturation (0–100%)	45.1 ± 15.4	9.3–75.0	67.4 ± 15.5	40.3–98.0	−7.3	78.3	<0.001
Brightness (0–100%)	58.7 ± 7.7	40.3–80.0	56.8 ± 6.6	33.7–70.7	1.7	68.4	0.098
Residual red spawning	14.3 ± 3.5	7.0–21.1	16.9 ± 3.0	10.5–26.7	−0.02	65.7	0.978
Females							
Hue (0–360°)	16.9 ± 4.4	4.3–24.3	12.5 ± 4.5	5.0–22.7	3.9	61.8	0.0002
Saturation (0–100%)	49.6 ± 11.7	27.3–80.0	69.6 ± 14.1	43.3–95.7	−6.2	61.0	<0.001
Brightness (0–100%)	57.3 ± 6.6	43.3–70.0	57.4 ± 7.1	40.7–70.3	−0.05	62.0	0.957
Residual red spawning	14.1 ± 2.7	7.0–19.4	16.6 ± 3.2	10.9–23.1	0.002	61.6	0.998

Table 3. Differences in red spawning coloration between non-enriched (n = 70) and enriched (n = 105) reared redside dace *Clinostomus elongatus* post-hormone induction (i.e. carp pituitary extract injection, see Section 2 for details). Residual red spawning coloration area is shown here as percentage of the body covered in red for ease of interpretation; however, in all analyses, we used residuals derived from the regression of area of red spawning coloration on overall body surface area (see Section 2 for details). *t/w* represents either *t*: *t*-test statistic; *w*: Mann-Whitney *U*-test statistic. Alpha: 0.05

Variable	Non-enriched		Enriched		Test statistics		
	Mean ± SD	Range	Mean ± SD	Range	*t/w* statistic	df	p
Males							
Hue (0–360°)	7.6 ± 4.2	3.0–20.7	8.5 ± 5.2	2.3–21.7	1336.5	–	0.679
Saturation (0–100%)	79.1 ± 21.6	24.7–99.0	78.9 ± 19.0	43.7–98.7	1424	–	0.904
Brightness (0–100%)	54.7 ± 6.0	36.3–66.7	54.6 ± 6.6	39.0–70.0	0.1	95.6	0.921
Residual red spawning coloration area (%)	19.0 ± 4.2	11.6–26.7	19.9 ± 4.7	11.4–30.3	0.02	86.6	0.979
Females							
Hue (0–360°)	10.0 ± 5.6	3.0–24.7	11.3 ± 5.8	3.3–24.7	447.5	–	0.393
Saturation (0–100%)	72.2 ± 21.1	3.0–98.0	68.2 ± 21.9	30.0–98.0	528.5	–	0.344
Brightness (0–100%)	52.9 ± 8.3	25.7–66.0	53.5 ± 9.0	20.0–68.3	467.5	–	0.559
Residual red spawning coloration area (%)	17.3 ± 3.3	10.8–24.4	16.7 ± 3.9	9.9–24.9	−0.002	61.8	0.999

reared *C. elongatus*, hue was found to be influenced by enrichment prior to hormone induction for both sexes. Males and females reared with enrichment were found to have redder hues than those reared in non-enriched environments. However, examination of red coloration following hormone induction found no differences in red coloration between non-enriched and enriched environments, suggesting that hormone induction plays a role in the development of red coloration in both sexes.

We documented no difference in the number of males and females producing gametes between non-enriched and enriched environments, suggesting that environmental enrichment does not improve the likelihood of gamete production for hormonally induced fish. Although little is known about how enrichment influences reproductive performance in freshwater fish (but see Wafer et al. 2016), a few plausible explanations for this finding may exist. First, dissimilarities in the *C. elongatus* mating system compared to other species may account for the lack of differences in gamete production between environments. For instance, *C. elongatus* does not defend territories, exhibit courtship behaviors, or have mate choice preferences, unlike other species of minnow that have a lek-like mating system (e.g. Koster 1939, Pyron 1996, Jacob et al. 2009, Beausoleil et al. 2012, Watt et al. 2023). Because of this, environmental enrichment which has been linked to the promotion of natural reproductive behaviors such as territoriality and courtship may not be required by *C. elongatus* to influence reproductive processes (Cacho et al. 1999, Galhardo et al. 2009). Second, because *C. elongatus* are seasonal spawners (with reproduction occurring in the wild following distinct seasonal cues), gamete production may simply be more closely tied to abiotic enrichment such as the manipulation of photoperiod, water temperature, and flow, which have been demonstrated consistently to influence gamete production across species (e.g. Mañanos et al. 2008). Third, because *C. elongatus* are presumably cued to spawn by sensory cues (e.g. visual and chemical signals), as well as the social interactions facilitated by their nest hosts during spawning, it is possible that the enriched tanks lacked critical elements (Koster 1939). Specifically, the enriched tanks may have had too few nest hosts, resulting in an inadequate presence of chemical cues or a poor diversity of host species. This could have ultimately led to insufficient stimuli for gamete production. Considering *C. elongatus* usually live in mixed-species shoals and spawn in the nests of larger-bodied minnows such as common shiner *Notropis c. cornutus*, future studies could explore the benefits of including a diversity of hosts and communal spawners. Fourth, because *C. elongatus* is presumed to be an obligate nest parasite, the absence of nests in the enriched tanks may have influenced gamete production. Prior studies have shown that obligate nest spawners, such as the blackside dace *Chrosomus cumberlandensis*, can adapt to using artificial nests in captivity, suggesting that wild obligate nest spawners can become facultative nest spawners in captivity (Rakes et al. 2013). Therefore, considering the host dependence and specificity of *Clinostomus elongatus* could be a valuable avenue for future investigations. Lastly, while supplementary environmental

enrichment was provided to *C. elongatus*, it is possible that the type of enrichment used, such as the in-tank light system or the presence of host species, may have induced stress, potentially limiting gamete production. Stress can lead to the shutdown of the reproductive system as energy is redirected to essential functions (Moberg 2000). While we aimed to minimize stress (i.e. programmed lights to replicate conditions in the wild and low host-species density), stress may still have been induced.

We determined that enrichment has a significant effect on sperm quality traits, as males reared in enriched environments exhibited higher sperm motility. However, enrichment did not influence other sperm quality metrics including velocity (average path velocity), longevity, density, or morphology (total length). These findings suggest that enrichment plays an important role in influencing sperm quality metrics (i.e. motility), which may improve reproductive outcomes in captivity (reviewed in Gallego & Asturiano 2019). While various metrics contribute to determining sperm quality in fish, sperm motility is considered the most reliable biomarker (Rurangwa et al. 2004, Gallego & Asturiano 2019). In most teleost fish, successful fertilization relies on sperm moving towards the egg and entering the micropyle (Fauvel et al. 2010). As a result, highly motile sperm, displaying greater overall movement, have the highest likelihood of successfully fertilizing the egg. Therefore, the inclusion of supplementary enrichment should be strongly considered when trying to breed captive *C. elongatus*. Furthermore, across species, correlations between sperm motility and fertilization rate have been found, including common carp *Cyprinus carpio* (Linhart et al. 2000), red seabream *Pagrus major* (Liu et al. 2007), and Atlantic halibut *Hippoglossus hippoglossus* (Ottesen et al. 2009). However, species-specific information is still needed to better understand the connection between sperm motility and fertilization success in *Clinostomus elongatus*, and future studies should explore this.

Additionally, we did not observe differences in egg diameter between females reared in non-enriched and enriched environments. Egg size, specifically egg diameter, serves as an important determinant of reproductive success in fish. This association is predicated on the idea that larger eggs are superior quality, owing to their greater metabolic reserves (Stuart et al. 2020). Contrary to our expectations, our findings indicate that supplementary enrichment does not influence egg diameter. One possible reason for this finding may be related to the type of enrichment used in this study. Previous research has demonstrated that diet, dissolved oxygen, and temperature can influence egg diameter in fish (e.g. Brooks et al. 1997, Lavens et al. 1999, Einum et al. 2002, Trippel & Neil 2004, Aryani et al. 2014). Thus, as diet, dissolved oxygen, and temperature were not manipulated and kept consistent between treatments, it may be possible that egg diameter is more strongly influenced by abiotic factors than by the type of supplementary enrichment used. To better test this hypothesis, future studies could explore the effects of abiotic factors on egg diameter and other egg quality metrics to better understand the connection between abiotic enrichment and reproductive potential.

We found a difference in red coloration between enrichment environments prior to hormone induction. Males and females reared in the enriched environment were found to have redder hues than those reared in non-enriched environments. However, a comparison of red coloration following hormone induction revealed no significant differences between non-enriched and enriched reared fish. But both non-enriched and enriched fish became redder following hormone induction compared to their pre-hormone induction condition. Here, our findings indicate that enrichment contributes to the facilitation of mating signals (i.e. spawning coloration), but that reproductive hormones such as CPE have a significant effect on the expression of coloration as well. Although the precise mechanism underlying the effect of environmental enrichment pre-hormone induction remain unclear, there may be a plausible explanation for our finding. First, it is well known that environmental enrichment can profoundly influence the growth, behavior, physiology, and overall welfare of species in captivity, including reduced stress levels (reviewed in Näslund & Johnsson 2014, Jones et al. 2021). Lowering stress levels in fish can lead to a reduced allocation of carotenoids (i.e. fat-soluble pigments) towards their antioxidant and immune defenses, thereby allowing fish to redirect more resources towards enhancing coloration (Blount et al. 2001). Thus, *C. elongatus* reared in the enriched environment may have a better physiological condition from reduced stress levels. Furthermore, our study found that hormone induction can influence the expression of red spawning coloration in *C. elongatus*. While research on spawning coloration and reproductive hormones is limited, a previous study by Harris et al. (2011) also observed clear differences in coloration after hormone injection in bigmouth sleeper *Gobiomorus dormitor*. Hormone-injected males displayed darkened skin with contrasting yellow spots and red coloration on the tips of their spines, while females exhibited

similar coloration patterns but generally appeared to be lighter in color (Harris et al. 2011). One possible reason for our finding may be that reproductive hormones such as CPE may have played a role in the maturation and associated coloration changes observed in non-enriched *C. elongatus*. This suggests that reproductive hormones may be able to overcome the effect of stressors, which are known to disrupt the normal functioning of the endocrine system (reviewed in Arcand-Hoy & Benson 1998, Schreck 2010) and influence the expression of vibrant coloration (Conte 2004). Considering our findings, our investigation highlights that enrichment can enhance spawning signals (i.e. coloration) in *C. elongatus*, but that hormone induction can also be effective in improving the fish's preparedness for reproduction. However, we acknowledge a limitation in our study, that sex determination relied solely on physical characteristics, and the impracticality of conducting post-mortem examinations on endangered fish made it impossible to verify our estimations, introducing the potential for misidentifications.

Overall, enrichment was found to not affect the probability of male and female gamete production, but it did affect sperm quality traits and spawning coloration in *C. elongatus*. As *C. elongatus* is endangered, the development of effective captive breeding techniques has been identified as vital to its recovery (Redside Dace Recovery Team 2010, Lamothe et al. 2019). Here, our study demonstrated the feasibility of obtaining gametes from *C. elongatus* in captivity, providing key information to inform an initial captive breeding protocol. Furthermore, our research addresses crucial knowledge gaps concerning supplementary enrichment practices. Moving forward, emerging captive breeding programs should continue to explore appropriate hormone dosages to yield a higher reproductive output and investigate gamete quality in relation to fertilization and hatching success to maximize offspring fitness for possible reintroductions into the wild. Additionally, it may be beneficial to investigate additional ecologically relevant enrichment measures (e.g. diversity of spawning host species, increased flow, complex substrate including rocks and logs for hiding, addition of artificial nests, light, manipulation of diet), which were not examined in this study, to assess their potential impact on gamete production. Collectively, our findings highlight the important role of environmental enrichment in enhancing natural reproductive signals and boosting sperm motility, which may ultimately improve fertilization success in captivity.

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