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Social and physiological drivers of rapid colour change in a tropical toad

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ABSTRACT

Dynamic sexual dichromatism occurs when males and females differ in colouration for a limited time. Although this trait has been primarily studied in cephalopods, chameleons, and fishes, recent analyses suggest that dynamic dichromatism is prevalent among anurans and may be mediated through sexual selection and sex recognition. Yellow toads, *Incilius luetkenii*, exhibit dynamic dichromatism during explosive breeding events at the onset of the rainy season: males change from a cryptic brown to a bright yellow and back again during the brief mating event. We tested the hypothesis that dynamic dichromatism in yellow toads is influenced by conspecific interactions and mediated through sex hormones and stress hormones. We placed male toads into one of four social treatments (with three other males, one male, one female, or no other toads). Immediately before and after each one-hour treatment, we quantified male colour with a reflectance spectrometer and we collected a blood sample to assess plasma concentrations of both testosterone and corticosterone. We found that males held with conspecific animals showed the brightest yellow colour and showed little or no change in their corticosterone levels. Across treatments, toads with duller yellow colour had higher levels of corticosterone. Male colour showed no association with testosterone. Interestingly, males showed substantial temporal variation in colour and corticosterone: toads were duller yellow and exhibited greater levels of corticosterone post-treatment across subsequent days at the onset of the rainy season. Our findings reveal that both conspecific interactions and corticosterone are involved in the dynamic colour change of yellow toads.

1. Introduction

Animals routinely use elaborate visual signals for both intraspecific and interspecific communication (Bradbury and Vehrencamp, 1998). These signals are often energetically expensive to produce and maintain, and their presence may increase the signaller's risk of predation (Endler, 1980; Andersson, 1994). In some species, these elaborate visual traits are expressed only temporarily, minimizing the costs and risks associated with displaying that trait over longer time periods (Zahavi, 1975; Booth, 1990; Endler, 1992). By studying these dynamic signals, we gain a deeper understanding of the costs and benefits of elaborate visual traits.

Elaborate visual signals are often associated with sexual dimorphism (i.e. phenotypic differences between sexes within a species; Darwin, 1871). Sexual dichromatism is one prevalent form of sexual dimorphism wherein the sexes differ in colour or pattern, often resulting in one sex being more conspicuous than the other (Andersson, 1994). Sexually dichromatic signals are understood to have evolved through both sexual selection (i.e. advertisement in the conspicuous sex), and natural selection (i.e. concealment in the cryptic sex; Darwin, 1871). Sexually dichromatic signals commonly arise via permanent morphological colour changes, typically at the onset of sexual maturity. Morphological sexual dichromatism exhibits trait flexibility in some species through seasonal colour changes, such as the shedding of fur or feathers to bring about a new coat or plumage (Hamilton and Barth, 1962; Bartlett and Light, 2017). These seasonal signals cannot be easily manipulated in real time because they are morphological and occur within dead tissue. In some instances, however, sexually dichromatic signals are expressed through temporary physiological colour changes by altering chromatophores present in the skin. Dynamic sexual dichromatism is a unique form of physiological sexual dichromatism wherein one sex undergoes a drastic and rapid change in colouration, occurring within hours (e.g. anurans; Doucet and Mennill, 2010; Bell and Zamudio, 2012), minutes (e.g. fishes; Nilsson Sköld et al., 2016), or even seconds (e.g. chameleons; Stuart-Fox and Moussalli, 2008). The fact that both sexual dichromatism and dynamic sexual dichromatism appear to be more common in anurans than previously recognized has led to increased research on the mechanisms and functions of visual

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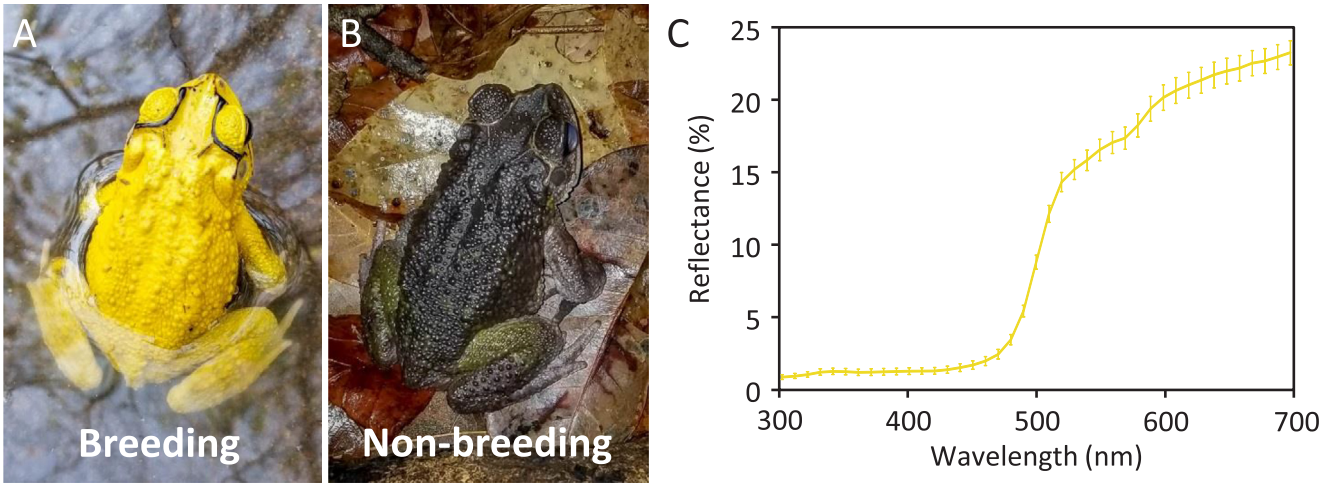

Fig. 1. Male yellow toads, *Incilius luetkenii*, exhibit dynamic colour change: (A) a male photographed early in the day during a breeding event, and (B) a different male photographed later that day. (C) Average reflectance spectrum of all the males in this study as measured prior to experimental treatment (n = 81). Vertical bars show the standard error of measurements summarized in 10 nm bins.

signalling in this group (Doucet and Mennill, 2010; Bell and Zamudio, 2012; Kindermann et al., 2013, 2014; Tang et al., 2014; Rehberg-Besler et al., 2015; Kindermann and Hero 2016a,b; Bell et al., 2017).

Elaborate sexual signals are commonplace in animals that engage in explosive breeding, wherein animals gather in large temporary mating aggregations and one sex physically competes for access to the other (Apollonio et al., 2000). These conspicuous signals allow animals to distinguish themselves when attempting to attract a mate or deter a rival. A recent comparative study investigated the evolution of dynamic sexual dichromatism and explosive breeding in a group of anurans, revealing that the evolution of explosive breeding preceded the evolution of dynamic sexual dichromatism (Bell et al., 2017). In this context, dynamic sexual dichromatism is likely to have evolved in the same manner as traditional, non-dynamic sexual dichromatism, in addition to being shaped by the ecological and temporal pressures of explosive breeding in some species. The ephemeral nature of dynamic sexual dichromatism raises many questions regarding the function, mechanism, and expression of this trait. In this study, we investigate the social and physiological mechanisms driving the evolution of a dynamic sexual signal in an explosively breeding Neotropical toad.

Recent research suggests that dynamic sexual dichromatism may serve diverse behavioural functions across species (Bell and Zamudio, 2012; Hutton et al., 2015). African dwarf chameleons (*Bradypodion* spp.), for example, alter body colouration to signal dominance or submissiveness during intrasexual contests and intersexual courtship rituals (Stuart-Fox and Moussalli, 2008). Male eastern fence lizards (*Sceloporus undulatus*) change body colouration in response to abiotic influences, such as temperature, which likely aids in thermoregulation and may signal a male's ability to perform important biological functions in suboptimal environments (Langkilde and Boronow, 2012). In anurans, dynamic colour change is most commonly observed in males, occurring primarily in the context of mating events (Bell and Zamudio, 2012), and therefore sexual selection is hypothesized to be the mechanism driving the expression of this trait (Doucet and Mennill, 2010; Bell and Zamudio, 2012). In the moor frog (*Rana arvalis*), the stony creek frog (*Litoria wilcoxii*), and the yellow toad (*Incilius luetkenii)*, colour change appears to facilitate sex recognition within explosive breeding aggregations (Sztatecsny et al., 2012; Rehberg-Besler et al., 2015; Kindermann and Hero, 2016b). Alternative explanations for the function of male colour change in frogs and toads have received limited investigation: researchers found no relationship between male nuptial colouration and paternity success in the moor frog (Rausch et al., 2014), nor was there evidence to support yellow colouration functioning as an aposematic signal in the stony creek frog (Kindermann and Hero, 2016a,b).

The physiological mechanism driving dynamic colour change in anurans has been experimentally investigated in two species to date. In both the strong stream frog (*Buergeria robusta*) and the stony creek frog (*Litoria wilcoxii*), testosterone has been hypothesized to influence dynamic sexual dichromatism due to colour change coinciding with breeding events (Kindermann et al., 2014; Tang et al., 2014). When frogs of both species were experimentally injected with testosterone, a colour change was induced in only the strong stream frog. Additionally, researchers have hypothesized that stress hormones may be responsible for colour changes in dynamic sexually dichromatic anurans. In both the yellow toad (Doucet and Mennill, 2010) and the stony creek frog (Kindermann et al., 2013), males were observed to change colour when engaged in presumably stressful situations (i.e. confinement and toe-clipping, respectively). Although the physiological mechanism has yet to be experimentally investigated in the former species, the stony creek frog was not shown to change colour in response to an injection of adrenocorticotropic hormone (a precursor hormone released in response to stress; Kindermann et al., 2013); however, this species was shown to undergo a change of colour after a presumably moderate stressor was introduced (toe-clipping). These contrasting results reveal that the physiological mechanisms of colour change may not be uniform across species in this group and may be mediated through multiple mechanisms.

In this study, we investigate the influence of social environment and hormones on rapid colour change in male yellow toads. Yellow toads breed in Neotropical Dry Forests, where they emerge from aestivation at the start of the rainy season (Savage, 2002) and immediately form dense mating aggregations in ponds and streambeds (Doucet and Mennill, 2010). They engage in scramble competition polygyny (Wells, 2007; Rehberg-Besler et al., 2015), where males actively compete with conspecific males to access a breeding partner. At the start of their breeding event, males are a conspicuous lemon-yellow colour and females are a cryptic brown colour. Once a male has entered amplexus with a breeding partner, he gradually shifts back to a cryptic brown, resembling a female, during the period of amplexus and fertilization. Males then remain brown for the rest of the year (Fig. 1). A recent model presentation experiment supports the hypothesis that bright yellow colouration serves as an intrasexual signal used by males for the purpose of sex recognition in this species (Rehberg-Besler et al., 2015). However, there is considerable variation in the brightness of male yellow colour, and no study has investigated whether this variation may serve a dual function in male-male communication or male-female communication. Furthermore, nothing is known about the social or

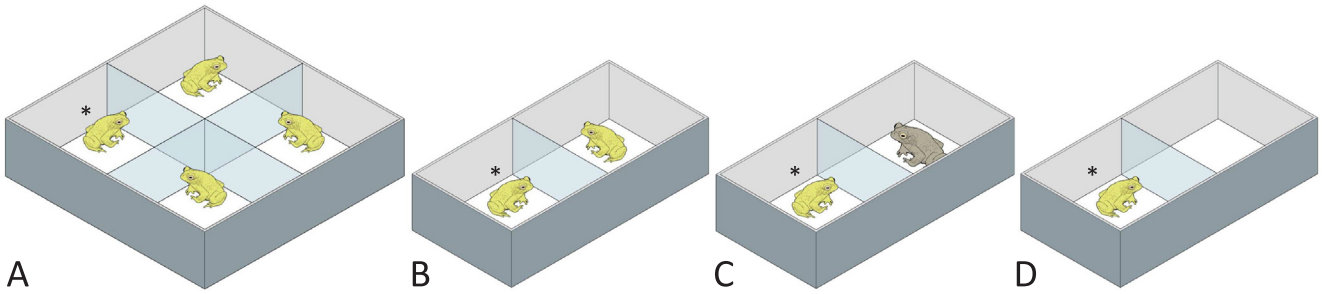

Fig. 2. Male yellow toads, *Incilius luetkenii,* were exposed to one of four social treatments to determine the influence of social environment on hormones and colour change in this species. An asterisk (*) indicates the subject from which we collected colour measurements and blood samples. Subjects were placed into an arena with (A) three conspecific males, (B) a single male, (C) a single female, or (D) into an arena alone. Each chamber within the arenas measured 30 × 30 × 30 cm. The perimeter of the arena was opaque and the chambers were separated by transparent partitions so that conspecific animals were visible to each other, but unable to physically interact.

physiological mechanisms responsible for driving this dramatic change in male colour.

We tested the hypothesis that dynamic colour change in yellow toads is influenced by conspecific interactions (Leclercq et al., 2010) and mediated through sex hormones and stress hormones (Bell and Zamudio, 2012). We captured free-living toads and temporarily placed them in arenas designed to simulate four different social environments (Fig. 2): (1) in the presence of three other males; (2) in the presence of one other male; (3) in the presence of one female; and (4) in isolation of conspecific animals. If social environment influences conspicuous male colouration in this species, we predicted that males held in the presence of conspecific individuals would be brighter yellow than males held in the absence of conspecific individuals. Furthermore, given the overwhelmingly male-biased sex ratios of yellow toad mating aggregations and observations of intense male-male competition (Doucet and Mennill, 2010; Rehberg-Besler et al., 2015), we predicted that intrasexual selection may be the driving force behind dynamic colour change in this species, and that subjects placed in the presence of one or three other males would develop the brightest colour signals. Group size, however, can influence the expression of secondary sexual characteristics in some species (e.g. horn length in bovids is positively correlated with breeding group size; Bro-jorgensen, 2007). If intrasexual selection is the driving force of colouration in yellow toads and if group size influences colour, we predicted that subjects held with three rival males would develop brighter colouration than subjects held with just one rival male. It is also possible that the presence of females could be the primary stimulus for male colour change, in which case we predicted that males in the presence of females would be brighter than subjects held with males and solitary males.

To evaluate the mechanism driving dynamic colour change in yellow toads, we examined two hormones hypothesized to be associated with colour change in other dynamically dichromatic anuran species: testosterone (a sex hormone) and corticosterone (a stress hormone). We predicted that bright male colouration could be driven by testosterone, given that male colour change coincides with their mating event (Tang et al., 2014), and in this scenario we predicted that testosterone levels would be highest in the brightest toads. We also predicted that bright male colouration could be facilitated by moderately elevated levels of corticosterone, which have been shown to mobilize energy stores and enable energetically expensive reproductive efforts such as competitive interactions with rival males or courtship with females (Husak and Moore, 2008). Therefore, we predicted that subjects exposed to conspecifics would be the brightest yellow and retain moderate levels of plasma corticosterone whereas toads held in isolation would become duller yellow and show decreased levels of corticosterone. Moreover, because corticosterone is also a cyclical hormone associated with circadian rhythms (Gamble et al., 2014), we investigated the influence of time of day and time of year on colouration and corticosterone.

2. Methods

2.1. General methods

We studied yellow toads in late April through early May of 2017 immediately following the first heavy rains of the wet season, when toads emerge from aestivation and exhibit bright yellow skin colouration (Doucet and Mennill, 2010). Our field site in Sector Santa Rosa, Area de Conservación Guanacaste (10°52′ N, 85°36′ W) is a Neotropical dry forest which exhibits a pronounced dry season, from December to April or May, followed by a season of abundant rainfall, from April or May to November (Campos and Fedigan, 2013). In 2017, some toads emerged from aestivation after an early rainfall on April 28, and then again after the next sustained rainfalls on May 5, May 7, and May 13. We sampled a total of 81 focal male toads between the hours of 4:50am and 11:19am over the six days when yellow toads were observed at our study site (April 28, 29; May 5, 7, 13, 14). The aggregation moved to newly formed ponds along the streambed over this period. Capturing toads by hand, we collected unpaired (unmated) male subjects, and immediately (within 5 min of capture; see below) obtained a blood sample and colour measurement to establish baseline hormone and reflectance measurements.

We placed male yellow toads into one of four arenas (Fig. 2): (1) an arena with three other males; (2) an arena with one other male; (3) an arena with a single female; or (4) an arena with no other toads. After approximately one hour, we removed the subject from the arena and immediately collected a second blood and colour measurement to quantify the change in hormones and reflectance. We also measured each subject's mass (to the nearest g) and snout-vent-length (to the nearest mm) before release. Each trial included new subjects and stimulus toads; animals were not reused across treatments. We placed one toad into each treatment type simultaneously and we sampled a similar number of toads per treatment each day. Arenas were thoroughly wiped with a damp towel between trials to control for olfactory cues (Fischer et al., 2001; Phillips et al., 2002). This study was approved by the University of Windsor Animal Care Committee under AUPP 16-10 and the government of Costa Rica (MINAE).

3. Experimental arenas and social stimuli

We constructed 16 arenas (4 replicates of the 4 arenas depicted in Fig. 2). The perimeter of each arena was opaque so that outside visual stimuli could not distract focal males. The inside of each arena was partitioned into chambers of equal size with clear acrylic sheets so that focal males were able to view the social stimuli, but were unable to make direct physical contact (aggressive or mating interactions). To simulate the natural conditions of the breeding aggregation, the first treatment was designed so that several males were in close proximity to the focal male. The second treatment, a single focal male and single

stimulus male, was designed to test for potential confounds of a group size effect in the first treatment. These two treatments were intended to simulate a competitive environment. The third treatment, a single focal male with a single stimulus female, was intended to simulate an instance of mate choice. Finally, the single focal male kept in isolation of conspecifics was designed to remove any sort of social stimulus. The size of the arena for the final treatment was the same as for the second and third treatments to control for an effect of arena size; the second compartment of the arena was left empty (see Fig. 2).

3.1. Colour measurements

To objectively quantify colour change in male yellow toads, we used a field portable spectrometer with a pulsed xenon light source and a bifurcated fibre-optic probe (model: JAZ-COMBO; Ocean Optics, Dunedin, FL). We encased the tip of the probe in a black rubber probe holder, which allowed us to maintain a consistent fixed distance of approximately 5 mm perpendicular to the measurement surface while also blocking external light (Doucet et al., 2007). We measured reflectance relative to a white standard (Ocean Optics WS-1). We collected three reflectance measurements from the dorsum of each focal male (all taken between the scapulae), which we later averaged together. Using the program CLR 1.05 (Montgomerie, 2008), we summarized reflectance values into 1 nm bins and calculated the mean brightness (sum of relative reflectance from 300 to 700 nm divided by the number of wavelength intervals) per individual (Doucet and Montgomerie, 2003; Montgomerie, 2008). We chose to focus on brightness of the dorsum because this variable captured the most variation in colour change in yellow toads; the coefficient of variation in brightness (51%) was much larger than the coefficient of variation in chroma (5%) and hue (12%).

3.2. Hormone measurement

We collected ~20 μL of blood immediately before and after each trial, approximately 3.7 ± 0.1 min (mean ± SE; range: 0.7–5.5 min) after first capturing a toad and 75.5 ± 1.6 min (mean ± SE; range: 49–111 min) later, after the conclusion of the behavioural trials. Using a 28-gauge needle and 0.5 mL syringe, we collected blood via a vessel behind the knee and preserved it in heparinized tubes stored on ice for approximately 5.29 ± 0.11 hrs (mean ± SE; range: 2.27–8.08 h). We later centrifuged the blood, collected the plasma, stored the plasma at approximately −18 °C for 6 weeks in Costa Rica, before freezing at −80 °C upon return to Canada, until plasma concentrations of both testosterone and corticosterone were quantified using double antibody radioimmunoassays. We quantified testosterone and corticosterone using double-antibody ^{125}I RIA kits (ImmuChem 07-189102; and ImmucChem 07-120103 respectively, MP Biomedicals, Orangeburg, NY).

We performed validations for both testosterone and corticosterone using a plasma pool generated from yellow toad blood collected in this experiment. For each assay we serially diluted plasma and compared the slope of the dilution curve to the standard curve using ANCOVA to test for parallelism. There was no significant interaction between the standard curve and the serial dilution curve, indicating that the slopes of the dilution curves are similar (Supplementary material Fig. S1; Testosterone: $F_1 = 0.09$, $p = 0.77$; Corticosterone: $F_1 = 3.03$, $p = 0.12$; Chard, 1995; Newman et al., 2008). For testosterone, we measured samples in duplicate after diluting plasma 1:3500 (5 μL plasma first diluted in 495 μL assay buffer, then 5 μL of that solution diluted in 170 μL assay buffer for an end result of 0.05 μL plasma per 500uL solution). The intra-assay coefficient of variation was 10.42% for the low control (0.312 pg/tube) and 17.01% for the high control (250 pg/tube). The total intra-assay coefficient of variation was 14.92%. We trimmed testosterone data to only include those in which replicates were within 15% of each other (pre-treatment: $n = 57$; post-treatment: $n = 51$); immunoassay kits are subject to the effects of inexact calibration or interfering compounds (Vesper et al., 2014; Handelsman et al., 2015) and we felt it was appropriate to increase the integrity of the data by focusing only on points where the replicates were within 15%. Testosterone was not measured at the same time as corticosterone, therefore multiple freeze-thaw cycles may have contributed to the variation. Testosterone levels of all samples fell within the range of the standard curve (0.312–250 pg/mL). For corticosterone, we measured samples in duplicate after diluting plasma 1:20 (2.5 μL plasma plus 47.5 μL assay buffer). The intra-assay coefficient of variation was 1.25% for the low control (3.12 pg/tube) and 0.79% for the high control (250 pg/tube). The total intra-assay coefficient of variation was 5.11%. Corticosterone levels of all samples fell within the range of the standard curve (3.12–250 pg/mL).

3.3. Statistical analysis

Our total sample size was 81 toads exposed to the four social treatments: $n = 19$ males in an arena with three other males; $n = 21$ males in an arena with one other male; $n = 21$ males in an arena with a single female; and $n = 20$ males in an arena with no other toads. Of these toads, we collected both pre- and post-treatment colour data for $n = 68$ toads (for some toads, we were unable to collect post-treatment data due to the spectrometer overheating), pre- and post-treatment corticosterone data for $n = 71$ toads (for some toads, we were unable to collect a blood sample within 5 min of capture), and pre-and-post treatment testosterone data for $n = 42$ toads (for some toads, testosterone data was discarded due to high variation between replicates in our assay; see above).

Our approach to statistical analysis was to investigate the influence of experimental treatment on toad colour, corticosterone level, and testosterone level using General Linear Models (GLMs, in JMP version 14; JMP, 1989–2019) and then to conduct planned comparisons to test the predictions stated in the Introduction. For toad brightness, we conducted a GLM, followed by an information-theoretic model averaging approach (in the R package MuMIn; Barton, 2013; RStudio version 1.1.463; R Core Team, 2018) that allowed us to maximize our sample size ($n = 66$; see the following paragraph). For toad hormone levels we conducted two GLMs, with one model for $n = 71$ toads in our corticosterone analysis, and another for $n = 42$ toads in our testosterone analysis (model averaging did not allow us to increase our sample size in the testosterone analysis due to testosterone being the limiting variable; see above). When comparing pre- and post-treatment colour, we had pre-treatment colour data for $n = 81$ toads and post-treatment colour data for $n = 68$ toads. When comparing pre- and post-treatment hormone levels, we had pre- and post-treatment corticosterone data for $n = 71$ toads, we had pre-treatment testosterone data for $n = 57$ toads, and we had post-treatment testosterone data for $n = 51$ toads.

To assess the influence of social environment and hormones on yellow toad brightness, we used a GLM with a model-averaging approach. We first created a GLM (response variable: toad brightness after treatment; fixed effects: treatment type, both corticosterone and testosterone levels before and after treatment, sampling day, trial start time, total trial time, hormone sampling time before and after treatment, and pre-treatment toad brightness) to analyze a subset of our data for which we had complete hormone and colour data ($n = 38$ toads). Using the Akaike weights from that model (and all subsequent models) we calculated a summed Akaike weight, or the relative importance, for each variable, and because relative importance values can potentially be misleading on their own (Galipaud et al., 2014) we created a final model where we excluded fixed effects with a relative importance of less than 0.5 (Simpson and McGraw, 2018, 2019; see Table 1). Most notably, this approach led to the exclusion of testosterone as a fixed effect in the model. We included the fixed effects variables deemed important via our model averaging approach (i.e. sampling day, time of

Table 1
General Linear Model (GLM) analysis ($n = 66$) of variation in the brightness of male yellow toads, *Incilius luetkenii*, in response to four social treatments: (1) in the presence of three other males; (2) in the presence of one other male; (3) in the presence of one female; and (4) in isolation of conspecific animals, coupled with an information-theoretic model-averaging approach to evaluate a larger subset of our data. We created an initial model containing all of the fixed effects and from that model, and all subsequent nested models, we calculated a summed Akaike weight of relative importance for each variable and excluded fixed effects with a summed weight of less than 0.5 from our final GLM. Significant effects are shown in bold.

Brightness				
General Linear Model averaging	Parameter estimate ± SE	Akaike weight		
Treatment		0.11		
Post-treatment corticosterone	−0.10 ± 0.09	**0.65**		
Pre-treatment corticosterone	−0.05 ± 0.08	0.44		
Post-treatment testosterone	0.0007 ± 0.03	0.18		
Pre-treatment testosterone	−0.004 ± 0.04	0.18		
Sampling day		**1.00**		
Time of day	−0.002 ± 0.001	**0.87**		
Total trial time	−0.002 ± 0.005	0.35		
Post-treatment hormone sampling time	−0.0005 ± 0.0009	0.39		
Pre-treatment hormone sampling time	−1.9e−5 ± 0.0006	0.19		
Pre-treatment brightness	−0.03 ± 0.11	0.24		
Final GLM analysis	Parameter estimate ± SE	*df*	*F*-ratio	*P*
Treatment		3,54	2.52	0.07
LSM contrast solitary vs. three male/single male/female	−0.34 ± 0.13	154	6.94	**0.01**
LSM contrast three male/single male vs. female/solitary	0.14 ± 0.11	1,54	1.52	0.22
LSM contrast female vs. three male/single male/solitary	0.16 ± 0.13	1,54	1.52	0.22
Post-treatment corticosterone	−0.13 ± 0.05	1,54	6.65	**0.01**
Sampling day		5,54	5.76	**0.0003**
Day 2–1	−0.09 ± 0.19	1,54	0.20	0.66
Day 3–2	−0.60 ± 0.20	1,54	9.29	0.004
Day 4–3	−0.14 ± 0.19	1,54	0.51	0.48
Day 5–4	0.34 ± 0.25	1,54	1.88	0.18
Day 6–5	−0.26 ± 0.25	1,54	1.08	0.30
Time of day	−4.87e−5 ± 1.52e−5	1,54	10.22	**0.002**
Pre-treatment brightness	0.05 ± 0.15	1,54	0.13	0.72

day, and post-treatment corticosterone) in a final GLM performed with our entire dataset to determine how treatment influenced brightness in our experiment. We had testosterone data for $n = 42$ toads, but the final non-testosterone GLM had a larger sample size ($n = 66$ toads for which we had both colour data and corticosterone data).

To assess the influence of experimental treatment on both corticosterone and testosterone, we used two GLMs. In each model, our response variable was hormone concentration after treatment; our fixed effects were treatment type, sampling day, trial start time, total trial time, hormone sampling time before and after treatment, and hormone concentration before treatment.

We performed planned comparisons to explore the predictions stated in the Introduction using Least Squares Means (LSM) contrast tests on the fixed effect of treatment within models. We also used *post hoc* testing to explore additional unplanned comparisons. Furthermore, we compared the relationship between pre- and post-treatment colour, corticosterone, and testosterone, as well as corticosterone and testosterone with Pearson correlations. To ensure that model residuals met the assumptions of normality and homoscedasticity, we performed ln-transformations of the variables brightness, corticosterone, and testosterone.

4. Results

4.1. Colour and social context

We tested whether our experimental treatments influenced male yellow toad brightness using a GLM with a model-averaging approach (final model; response variable: toad brightness after treatment; fixed effects: treatment type, sampling day, time of day, post-treatment corticosterone, and pre-treatment brightness; n = 66) followed by planned comparisons of different experimental treatments to test our predictions. To test the prediction that dynamic colour change in yellow toads is influenced by interactions with conspecific animals, we compared the brightness of males in the solitary treatment with that of males in the three male, single male, and female treatment groups. We found that toads held in the absence of conspecifics became significantly darker than toads held with conspecifics (Fig. 3A; LSM contrast for solitary versus three male/single male/female: Table 1). To test the prediction that dynamic colour change in yellow toads is an intrasexually selected trait for the purpose of signalling to other males, we compared toads placed with three other males or with a single male with males held with a female or kept in isolation of conspecifics. We found that males in these groups did not differ significantly in brightness (LSM contrast for three male/single male vs. female/solitary: Table 1). To test our alternative prediction that females may be the primary stimulus for male colour change, we compared toads placed with a female with those placed with three other males, a single male, or solitarily. We found that males in these groups also did not differ significantly in brightness (LSM contrast for female vs. three male/single male/solitary: Table 1). Post-treatment corticosterone levels were associated with male colour; toads with elevated corticosterone levels had the dullest yellow colouration (Fig. 3B; Table 1). We also found that male colour faded from bright yellow to duller yellow across subsequent sampling days (Fig. 3C; Table 1); colour was approximately 28% duller, on average, on the sixth day of sampling compared to the first day. Additionally, males were duller when trial start times were later in the day (Fig. 3D; Table 1). Toad brightness before treatment was included to account for potential differences among toads prior to experimentation, and was not significantly correlated with male brightness after treatment in this analysis (Table 1).

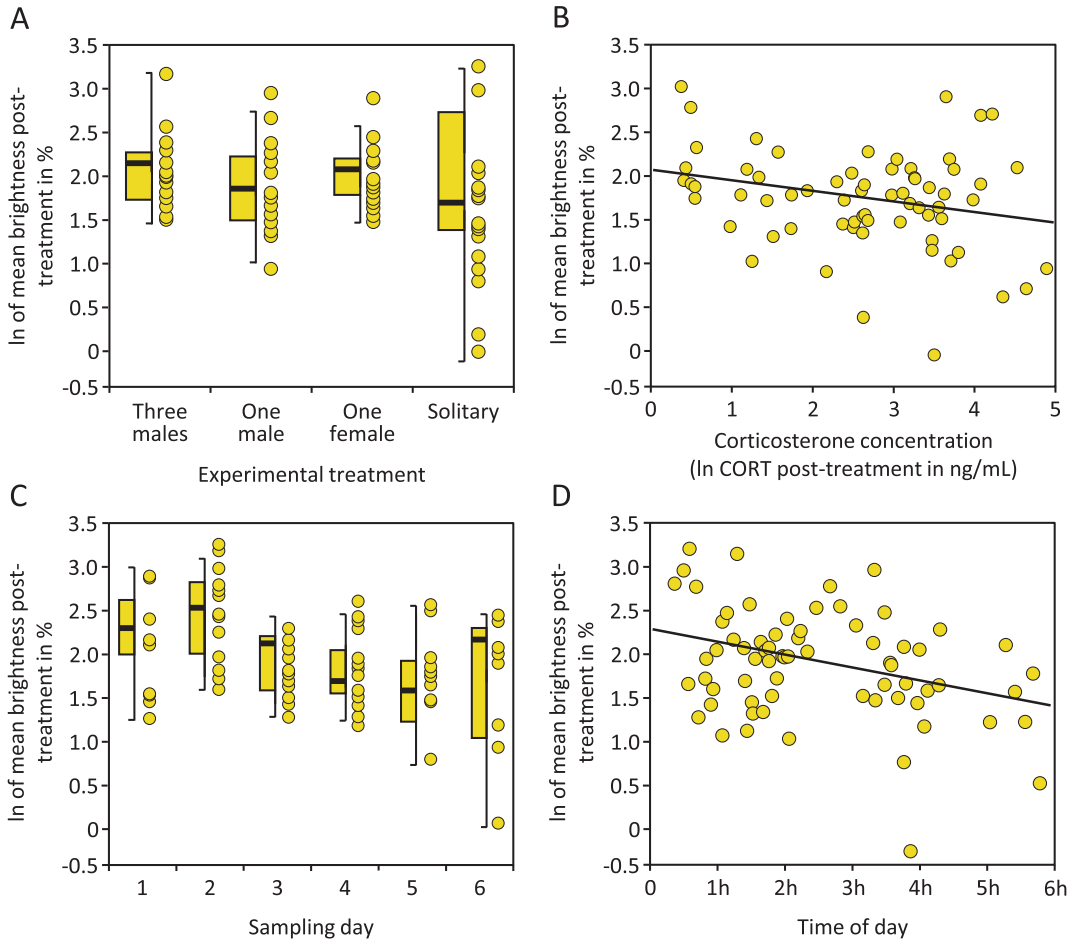

Fig. 3. Multiple variables predict male brightness in yellow toads, *Incilius luetkenii*, placed in different social environments. (A) Distribution of male colour across the four groups. (B) Duller yellow males had significantly higher levels of plasma corticosterone. (C) Male colour changed from bright yellow to dull yellow across the breeding season. (D) Male colour changed from bright yellow to dull yellow across the course of a day. Corticosterone and brightness values were ln-transformed to ensure normality and homoscedasticity. Figures depict raw data, but significance of relationships is based on the General Linear Model (see Results). Boxplots show the median (thick black bar), 1st and 3rd quartiles, and the point value of each individual.

4.2. Hormones and social context

We tested whether our experimental treatments influenced male yellow toad corticosterone levels using a GLM (response variable: toad corticosterone levels after treatment; fixed effects: treatment type, sampling day, trial start time, total trial time, hormone sampling time before and after treatment, and pre-treatment corticosterone levels; $n = 71$). We again performed a planned comparison within this model to test our specific prediction. To test the prediction that toads exposed to conspecific animals would retain moderate levels of plasma corticosterone, and toads held in the absence of conspecifics would experience diminished levels of this hormone, we compared the corticosterone levels of males in the solitary treatment with that of males in the three male, single male, and female treatment groups. Toads in the solitary treatment did significantly differ in corticosterone levels from toads held in the presence of conspecifics (LSM contrast for solitary vs. three male/single male/female: Fig. 4; Table 2). Corticosterone levels were correlated with total trial time; toads exhibited greater levels of corticosterone as total trial time increased (Table 2). Sampling day also influenced corticosterone in our experiment; overall, toads showed increased corticosterone across subsequent sampling days (Table 2). Trial start time and hormone sampling time did not influence the change in corticosterone levels within this experiment (Table 2). In a similar GLM with testosterone levels after treatment as the response variable, no significant patterns were observed (Supplementary material Table S1; all tests $p > 0.05$).

Initial analyses suggested that toads held with a single male underwent a different change in corticosterone levels compared to toads held in isolation. We therefore performed an additional *post hoc* analysis to further investigate the influence of treatment on hormone concentrations among the groups. This analysis revealed that toads held with just a single male underwent a significant change in corticosterone levels compared to solitary males; toads held with a single male showed increased corticosterone levels in comparison to toads held in a three male group or with a female, who showed only a moderate change in corticosterone, and solitary males, who showed a decrease in corticosterone levels (Tukey-Kramer HSD; Table 2).

4.3. Pre- and post-treatment correlations

Toad colour pre-treatment was positively correlated with toad colour post-treatment ($r = 0.05$, $n = 68$, $p = 0.03$) and pre-treatment corticosterone levels were positively correlated with post-treatment corticosterone levels ($r = 0.19$, $n = 71$, $p < 0.0001$); however, testosterone levels pre- and post-treatment were not correlated ($r = 0.03$, $n = 42$, $p = 0.15$). Corticosterone and testosterone levels were unrelated (pre-treatment: $r = -0.004$, $n = 57$, $p = 0.39$; post-treatment: $r = -0.02$, $n = 51$, $p = 0.97$). Handling time did not influence corticosterone or testosterone levels (pre-treatment corticosterone: $r = 0.008$, $n = 71$, $p = 0.21$; pre-treatment T: $r = -0.02$, $n = 57$, $p = 0.95$; post-treatment corticosterone: $r = -0.002$, $n = 71$, $p = 0.35$; post-treatment T: $r = 0.02$, $n = 51$, $p = 0.16$).

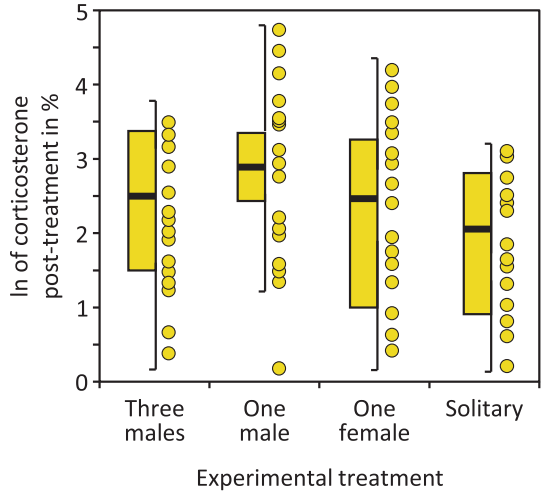

Fig. 4. Changes in corticosterone concentrations of male yellow toads, *Incilius luetkenii,* varied across experimental treatments. Males paired with three other males or with a female did not show significant changes in the concentration of plasma corticosterone. However, males paired with a single rival male showed increased levels of corticosterone post-treatment compared to males that were kept isolated from conspecifics, who showed decreased levels of corticosterone post-treatment. Figures depict raw data, but significance of relationships is based on the General Linear Model (see Results). Boxplots show the median (thick black bar), 1st and 3rd quartiles, and the point value of each individual.

5. Discussion

Male yellow toads retained their bright colouration in the presence of conspecific animals, whether it was an audience of a single female, a single male, or multiple males. However, toads held in the absence of conspecific animals underwent a significant change in colour, fading to a duller yellow, suggesting that social environment influences dynamic colour change in this species. Corticosterone levels decreased for males held in isolation from other individuals, but increased in males held with one other male, and also with total trial time. Surprisingly, yellow colour was negatively correlated with elevated levels of corticosterone, where the dullest yellow individuals overall showed significantly higher levels of plasma corticosterone. In contrast, yellow colour showed no relationship with testosterone and there was no relationship between corticosterone and testosterone. Finally, both male colour and corticosterone levels varied over time, where males captured earlier in the day and earlier in the breeding season exhibited the brightest colouration and toads exhibited greater levels of corticosterone as the season progressed. Our findings suggest that dynamic sexual dichromatism in yellow toads is influenced by the presence of conspecific toads, and by the hormone corticosterone. These findings reveal that both social environment and physiology drive dynamic sexual signals.

We found that male yellow toads remained bright yellow in the presence of conspecifics and became duller yellow when conspecifics were absent. Our results suggest that the presence of conspecific animals is one stimulus influencing colour change in this species. It is noteworthy, however, that the majority of males remained yellow to some degree after the experimental treatment, even in the no-conspecific treatment. This observation suggests that conspecific presence is not the sole driving force of this trait. Within our experimental arena all subjects were within hearing distance of the acoustic signals produced by males at a nearby pond, whereas only a portion of the subjects were exposed to the visual stimulus we presented in the arenas; our experimental design does not allow us to separate the influence of the acoustic and the visual stimuli, which may together comprise a multimodal signal. Many studies investigate the responsiveness of anurans to multi-modal stimuli (Taylor et al., 2008, 2011; de Luna et al., 2010; Gomez et al., 2011) and in many of these studies subjects exhibit diminished responsiveness to these stimuli when they are presented independently of each other. For example, in a video playback study assessing the responsiveness of gray treefrogs (*Hyla versicolor*) to combinations of visual and vocal stimuli, subjects responded more strongly to multiple sensory modalities being presented (i.e. colour, vocal sac inflation, and calling) rather than a single sensory modality being presented (i.e. only the visual signals; Reichert et al., 2014). Moreover, it is possible that abiotic stimuli, such as the environmental cues which trigger emergence from aestivation, also influence this change. Additional studies will be required to determine the proximate stimuli influencing colour change in this species. Furthermore, male yellow toads

Table 2
General Linear Model (GLM) analysis ($n = 71$) of variation in corticosterone levels of male yellow toads, *Incilius luetkenii*, in response to four social treatments: (1) in the presence of three other males; (2) in the presence of one other male; (3) in the presence of one female; and (4) in isolation of conspecific animals. Results of the Tukey-Kramer HSD *post hoc* test further investigating the difference among treatment groups listed below. Significant effects are shown in bold.

Corticosterone				
GLM	Parameter estimate ± SE	*df*	*F*-ratio	*P*
Treatment		3,57	3.37	**0.02**
LSM contrast solitary vs. three male/single male/female	−0.56 ± 0.27	1,57	4.36	**0.04**
Sampling day		5,57	2.26	**0.05**
Day 2–1	−0.08 ± 0.44	1,57	0.04	0.85
Day 3–2	−0.79 ± 0.35	1,57	5.11	0.03
Day 4–3	−0.44 ± 0.41	1,57	1.17	0.28
Day 5–4	0.86 ± 0.58	1,57	2.20	0.14
Day 6–5	0.99 ± 0.47	1,57	4.31	0.04
Time of day	−1.45e−5 ± 2.64e−5	1,57	0.30	0.58
Total trial time	−0.04 ± 0.01	1,57	8.82	**0.004**
Post-treatment hormone sampling time	−4.86e−5 ± 0.001	1,57	0.001	0.97
Pre-treatment hormone sampling time	−0.002 ± 0.002	1,57	1.21	0.28
Pre-treatment corticosterone	0.53 ± 0.11	1,57	22.01	< **.0001**
GLM *post hoc* comparison	Difference ± SE	Lower CL	Upper CL	*P*
Single male vs. solitary	0.98 ± 0.32	0.13	1.82	**0.02**
Single male vs. three male	0.67 ± 0.31	−0.17	1.50	0.16
Single male vs. female	0.59 ± 0.31	−0.21	1.40	0.22
Female vs. solitary	0.38 ± 0.33	−0.50	1.26	0.66
Three male vs. solitary	0.31 ± 0.32	−0.52	1.15	0.76
Female vs. three male	0.07 ± 0.32	−0.79	0.93	1.00

exhibit substantial variation in the brightness of the yellow colour, and therefore future studies should investigate whether yellow colouration is indicative of individual quality and whether conspecific behavioural interactions influence male colouration.

The change in corticosterone levels also varied with social context, wherein toads held with just one other male showed an increase in plasma corticosterone, toads held with a group of males or with a female retained relatively constant moderate levels of corticosterone, and toads held in solitary showed decreased levels of this hormone. Surprisingly, we also found that overall toads exhibiting the highest levels of corticosterone were the dullest yellow. Our results imply that corticosterone is one possible mechanism involved in colour change in yellow toads. However, the extent of the colour difference was moderate compared to the variation we have observed between breeding and non-breeding male yellow toads (Doucet and Mennill, 2010), therefore corticosterone is likely not the sole mechanism driving colour change. Moderately elevated levels of stress hormones are increasingly shown to enable energetically expense mating tactics (Moore and Jessop, 2003), although extremely elevated levels of stress hormones can inhibit reproductive efforts (Husak and Moore, 2008). Therefore, stress hormones may influence yellow toad colour change in multiple, non-linear ways: (1) moderate levels may facilitate energetically expensive reproduction efforts, yet (2) elevated levels may cause an individual to be more vulnerable or react more strongly to a stressor. In yellow toads, we observed that males who began the experiment with higher corticosterone levels also had higher levels post-treatment and were significantly duller yellow. This pattern suggests that individuals with higher baseline corticosterone levels may be more reactive to subsequent stressors such as the relocation and isolation required for this experiment. In the only other study to investigate the influence of corticosterone on colour change in a dynamically sexually dichromatic anuran, the stony creek frog, Kindermann et al. (2013) found no significant relationship between colour and urinary corticosterone metabolites. A later study revealed that a neuro-endocrine stress hormone, epinephrine, does in fact elicit colour change in this species (Kindermann et al., 2014), thus it is possible that rapid catecholamine signalling may also underlie colour change in yellow toads. These results highlight the variability of mechanisms involved in colour change across and within anuran species and further reinforce the need for subsequent investigations into the physiological mechanisms responsible for this trait.

Our findings did not reveal a relationship between colour and testosterone. The role of testosterone in colour change has been experimentally investigated in only two anurans exhibiting dynamic sexual dichromatism, which revealed contrasting patterns: stony creek frogs underwent no change in colour when injected with testosterone (Kindermann et al., 2014), whereas strong stream frogs changed from brown to yellow through the actions of testosterone on yellow xanthophores (Tang et al., 2014). We had predicted that testosterone could be a driver of colour change in yellow toads because this dramatic colour change coincides with breeding events. In males, testosterone is often the predominant mechanism producing sexual traits such as colouration or inciting various reproductive behaviours such as courtship displays and vocalizations (Peters, 2007). However, testosterone is often not the only hormone regulating these sexual signals. In strong stream frogs, prolactin was shown to disperse yellow xanthophores when experimentally injected into animals, but the injection of estradiol did not appear to influence colour (Tang et al., 2014). In addition, testosterone may be metabolized into more potent androgens that we did not measure here (e.g. 5alpha-dihydrotestosterone; Grino et al., 1990). Due to the life history of yellow toads, we are not able to locate and sample toads before breeding to assess pre-breeding testosterone levels: animals are inaccessible when they aestivate in underground burrows during the dry season. It is possible that these levels increase and remain constant at the start of the brief breeding period, thus facilitating reproduction. Moving forward, investigations into the mechanisms of colour change in yellow toads should further investigate the role of testosterone and potentially other reproductive hormones that could be driving colour change in this species.

An additional finding of our study was that variation in both colour and corticosterone levels exhibited temporal variation. First, we noted that male colour changed progressively from bright yellow to dull yellow across the span of a day and across the brief breeding season and there are a few potential explanations for this pattern. Our results suggest that bright colouration may be partly influenced by conspecific stimulation. During yellow toad explosive breeding events, the first explosive breeding event of the wet season is the largest (*personal observation*). The lack of social stimulus as aggregations shrink over the span of subsequent days could be responsible for the negative correlation of colour and time that we have documented. An alternative explanation for the fading colour over time could be that colour is regulated by temporal rhythms and, therefore, hormones associated with these cyclical patterns. Melatonin is the hormone most commonly associated with the synchronization of circadian rhythms in animals (Pévet, 2000), and it has been shown to aggregate melanophores during dynamic colour change in the strong stream frog, resulting in skin pallor (Tang et al., 2014). Moreover, melatonin is generally known to aggregate melanophores in vertebrate skin (Nilsson Sköld et al., 2013) and it is possible that this hormone influences this aspect of colour change in yellow toads. Melatonin is not known to act on xanthophores to generate yellow colouration in anurans, however, so it is likely only a contributor to this phenomenon rather than the main driver of this trait. Corticosterone is also commonly studied in relation to diel, seasonal, and annual rhythms in animals (Gamble et al., 2014). Corticosterone levels in response to our experiment also increased across the start of the breeding season: as the season progressed, subjects experienced greater corticosterone levels post-treatment. These elevated corticosterone levels may have also contributed to decreasing brightness of yellow toads in breeding aggregations across time, or perhaps corticosterone is influencing colour in some sort of cyclical pattern. Future research exploring the mechanisms of colour change in yellow toads should focus on the role of cyclical regulating hormones in colour change, such as melatonin, and should further investigate corticosterone in a rhythmic context rather than solely as a stress response hormone.

Future research regarding the function of dynamic colour change in anurans should aim to investigate independently each aspect of trait selection (i.e. mate choice, competition) to definitively answer whether yellow toad colour change functions as an intersexually or intrasexually selected trait. Ongoing investigations will explore the variation in colour that exists in males of this species, even among bright yellow males, by examining male colour and other indicators of male quality such as body mass index, parasite load, etc. Research examining the potential mechanisms of colour change should continue to evaluate the role of corticosterone and testosterone in colour change to further refine our knowledge of how colour is related to these hormones and to investigate the potential cyclical regulation of colour by corticosterone. Further research that quantifies other hormones that may drive colour change would also be worthwhile, including other reproductive hormones such as prolactin and estradiol and other stress response hormones such as epinephrine, as would hormone injection experiments.

6. Conclusion

Little research has focused on the physiological and social context of visual signalling in sexually dichromatic anurans. Our findings support the hypothesis that colour change in yellow toads is a sexually selected trait influenced by conspecific animals and provide new insight into the mechanisms responsible for this unique phenomenon. Our findings suggest that corticosterone is involved in this colour change, yet further investigations are required to determine to what extent this hormone is influencing colouration. Moreover, our data reveal temporal variation

in the colouration of male yellow toads, which gives rise to new questions regarding the potential mechanisms responsible for this pattern. Our research highlights the significance of visual communication in anurans, a group in which acoustic communication is traditionally regarded as the most significant adaptation for sexual selection, and contributes to our overall understanding of the mechanisms and function of dynamic sexual dichromatism in animals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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