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Susanne Stückler BSc MSc MSc

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Betreut von | Supervisor:

Univ.-Prof. Mag. Dr. Thomas Bugnyar
ao. Univ.-Prof. i.R. Mag. Dr. Walter Hödl

Mitbetreut von | Co-Supervisor:

Dr. Doris Preininger



unimpressed frog by Matsumoto (CC BY-NC)

to all frogs and toads everywhere

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General Introduction

The topics of animal colouration and adaptive colour behaviour have attracted considerable attention from the research community, going back to Alfred Russel Wallace and Henry Walter Bates (Bates 1862; Wallace 1877). Both conducted extensive research on animal colouration, however, many functional questions which they posed remain unanswered until today (Caro 2017).

Body colouration is an important feature directly connecting an animal to its environment (e.g., perception of conspecifics or predators, thermo- and osmoregulation, protection from UV radiation) (Cuthill et al. 2017) and are integrated in **visual communication systems** subject to evolutionary transitions guided by natural and sexual selection mechanisms (Andersson 1994).

Several signals provide organisms with traits that improve their chances of survival in the complex dynamics of predator-prey interaction (Freeberg et al. 2017). For example, animals integrate body shapes, colourations, and behaviours to blend into their environment, or use different visual antipredator strategies at different times of day, lighting conditions, distances, during movement or when motionless (Stevens 2015; Caro and Koneru 2021). On the other hand, sexual communication signals not only help individuals attract mates and/or compete for opportunities to breed, but they also ensure that individuals recognize their own species and potential mating partners to reproduce (Andersson 1994).

In **anuran amphibians** (frogs and toads) acoustic signals were historically regarded as the primary communication mode subject to natural and sexual selection (Wells 2007; Coleman 2009). However, visual communication in anurans is nothing less than spectacular, with diverse species using an incredible range of elaborate colours to court mates, compete with rivals or evade predators (Hödl and Amézquita 2001). For example, male Moor frogs (*Rana arvalis*) turn from brown to blue during their short reproductive period to avoid mismatching (Sztatecsny et al. 2012) (Fig. 1A), or male Krefft's river frogs (*Phrynobatrachus krefftii*) predominantly use bright yellow vocal sac displays without acoustic signals during male-male interactions (Hirschmann and Hödl 2006) (Fig. 1B).

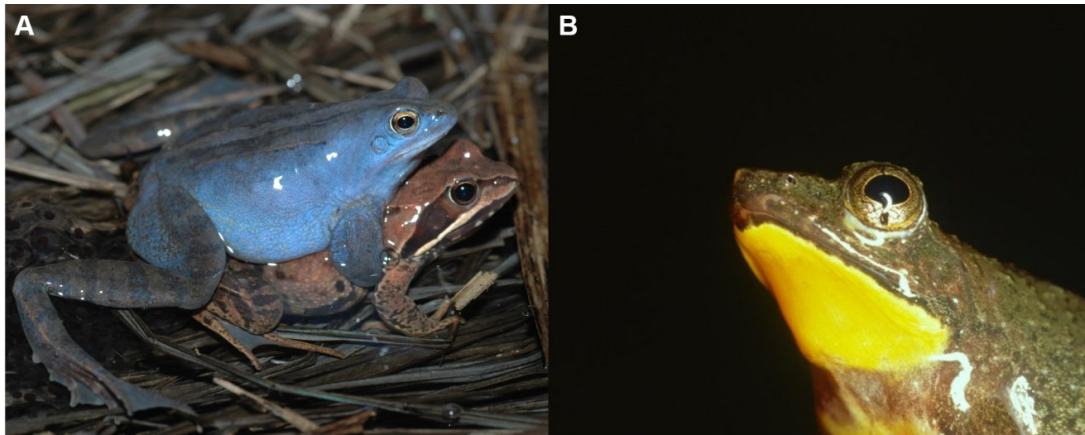


Figure 1. (A) A blue male Moor frog (*Rana arvalis*) in amplexus with a brown female. (B) The yellow vocal sac of *Phrynobatrachus krefftii* (Krefft's river frog). Pictures provided by Marc Sztatecsny (A) and Walter Hödl (B).

Exploring the significance of adaptive body colourations involves investigating both the proximate causes (mechanisms) and ultimate causes (functions) aiming to understand the interplay of different selection pressures shaping animal colouration (Booth 1990; Caro et al. 2016; Cuthill et al. 2017). Thereby, asking how an animal changes body colouration include the physiology of an animal, such as hormonal control of pigment response (aggregation vs. dispersion), pigment production, pigment uptake (e.g., carotenoids), food availability, or exposure to sunlight (fading of colours) (Booth 1990; Sköld et al. 2013). On the other hand, when asking why an animal changes its body colouration, one must consider several factors that influence an animal's life including changes in body size, vulnerability, reproductive status, habitat, predators, metabolism or environmental factors. Additionally, various situations such as courtship, mating, or predation may also play a role (Booth 1990; Sköld et al. 2013). Understanding the pressures acting on colour adaptations provide insights into the diversity of visual signalling strategies, influencing crucial aspects of **survival** and **reproduction** in different anuran species (Hernández et al. 2021; Postema et al. 2023).

This thesis investigates functions and mechanisms of two completely different visual communication strategies: first, the slow ontogenetic colour change from conspicuous red with white spots to emerald green body colouration in the Wallace's flying frog, *Rhacophorus nigropalmatus* (Fig. 1A,B), and second, the rapid dynamic colour change from brown to yellow in the Asian common toad, *Duttaphrynus melanostictus* (Fig. 1C,D). We explored the visual significance of adaptive body colourations in the context of **survival** and **reproduction**, integrating physiology, ecology and behaviour. The studies also emphasize how zoo-based research (captive settings) in combination with field experiments can help to study visual signal behaviour and their production in different species.



Figure 2. Juvenile (A) and adult (B) Wallace's flying frog, *Rhacophorus nigropalmatus*, and a brown non-breeding (C) and yellow breeding (D) male Asian common toad, *Duttaphrynus melanostictus*. Picture (A) provided by Samantha Cloer, picture (B) by Daniel Zupanc.

Colour change used for survival: Ontogenetic colour shifts in antipredator camouflage in Wallace's flying frogs

Ontogenetic colour change between life stages where juveniles have completely different colourations than adults commonly take several weeks to months and result in a permanent change of body colouration (Booth 1990). Ontogenetic colour change is mostly triggered by a long-term influence of hormones which change morphology, amount and distribution of pigments during ontogenesis (Leclercq et al. 2009). Today, 92 anuran species are reported to exhibit colour differences during ontogeny (Bell and Zamudio 2012), serving as adaptations to changing ecological factors and habitats (e.g., *Hyla regilla* (Wente and Phillips 2003)), as static sexual signal for conspecifics (e.g., at sexual maturity in several Bufonidae and Hylinae (Bell and Zamudio 2012)) and/or as antipredator strategies (e.g., *Oreophryne ezra* (Bulbert et al. 2018)).

To understand how changing colour adaptations are applied as antipredator mechanisms, we investigated underlying mechanisms (**Chapter 1**), and how they effectively reduce predation risks (**Chapter 2**) in the Wallace's flying frog (Fig. 2A,B).

Chapter 1

Carotenoids are often part of (direct and indirect) honest signals, used as visual indicator for health (reviewed in Svensson and Wong 2011). Carotenoid supplements have positive effects on general health as well as ornamentation in several anuran species (Dugas et al. 2013; Umbers et al. 2016). Carotenoids can only be absorbed by diet (Ziegler 2003; Feltl et al. 2005), but are assumed to be rare in nature (Olson and Owens 1996). A lack in carotenoids result in several nutritional diseases, which bear negative effects on immune modulation or reproductive success, but also colour signal or cue production modulating sexual and natural selection (Ogilvy et al. 2012; Ferrie et al. 2014).

In the **first chapter** of this thesis, we investigated the influence of dietary carotenoid pigments on colour production and colour changes. To this end, we tested the influence of dietary carotenoids on body colouration of the Wallace's flying frog – a species with a striking ontogenetic colour change from red juveniles to green adults. However, adults raised in captivity often exhibit lighter and/or turquoise body colourations compared to wild individuals (Ready 2009). We predicted that the intake of carotenoids during post-metamorphic stage aid in storing and accumulating carotenoid pigments and establishing the wildtype colouration of adults.

Chapter 2

Effective **antipredator** colourations are challenged by conflicting selection pressures such as conspecific communication, variation in transmission environment, ontogenetic changes, or abiotic/physiological factors amongst others (Cuthill et al. 2017; Postema et al. 2023). In turn, animals developed different strategies to cope with these selection pressures, for example to use plastic colour strategies (changing colour over time) (reviewed in Postema et al. 2023). To the best of our knowledge, research on adaptive defence colourations in response to changing predators, habitats, or body sizes has only been carried out in invertebrates such as moths and butterflies (Valkonen et al. 2014; Gaitonde et al. 2018; Postema 2022) or spiders (Yu et al. 2022a; Yu et al. 2022b). Plastic antipredator strategies allow animals to adapt their appearance to their environment, to signal their toxicity or to be camouflaged as something inedible – all while growing and moving through different habitats (Booth 1990).

In the **second chapter** we aimed to reveal the function of ontogenetic colour changes as antipredator strategies in the Wallace's flying frog. The bright red juveniles develop several white spots on their back one month after metamorphosis that almost vanish when they turn green as adults. We hypothesized that Wallace's flying frogs use two distinct defence colouration strategies: **crypsis** and **masquerade**. Cryptic animals are hard

to detect, thereby working on the sensory level of the predator, in that the predator does not detect the prey in the first place. Contrary, masquerade works on the cognitive level of the predator – the predator detects the object, but misclassifies it as something inedible (Skelhorn et al. 2010a; Skelhorn et al. 2010b; Skelhorn and Ruxton 2010; Skelhorn 2015). In anurans crypsis such as background matching, is a common antipredator strategy, which reflects that many species are brown, green, or grey (Wells 2007; Rojas 2016) and thereby cryptic in their respective habitats. To date, we have only very little evidence of anurans using masquerading strategy to evade predators (Toledo and Haddad 2009; Rojas 2016). Here, we explored whether the juvenile colouration and the white spots contribute to predator defence in frogs and compared their efficacy in predator avoidance with that of cryptic green colour of adult frogs.

Colour change used for reproduction: Dynamic colour change and mate recognition in the explosive breeding Asian common toad

Anurans use diverse visual signals in their reproductive behaviours, influencing successful reproduction, for example during courtship (Reichert 2012) or for mate choice (Gomez et al. 2009). The nature of these signals might vary based on their mating system (Wells 2007; Bell and Zamudio 2012). Anuran mating systems are described along a continuum from prolonged to explosive breeding strategies. In **prolonged breeders**, breeding period lasts over a longer time span, often females compare and select their mating partners in extensive courtships using acoustic, tactile, chemical, and visual signals. Contrary, in **explosive breeders**, all sexually receptive individuals arrive synchronously at a spawning site, and breeding takes place over a period of a few days. High male-male competition and males searching and scrambling for females result in a low reproductive success rate for males and limited female choice (Wells 2007). During such encounters, some species exhibit a dynamic change of body colouration.

During this **dynamic sexual dichromatism** – a particular type of dynamic colour change – species dynamically develop differing body colouration from the other sex (Bell and Zamudio 2012). Generally, 178 anuran species are known to express dynamic colour change (Bell et al. 2017). In anurans, dynamic colour changes can occur within minutes to hours, or within days to weeks (e.g., Kindermann et al. 2013). Interestingly, the majority of (tropical) anurans exhibiting dynamic colour change turn from brown to yellow (Bell et al. 2017), whereas one (temperate) explosive breeder turns from brown to blue (male Moor frogs, *Rana arvalis*) (Brehm 1893).

In the following chapters, we explored the hormonal regulation of dynamic colour change (**Chapter 3**), and the significance of dynamic visual signals (**Chapter 4**) in the explosive breeding Asian common toad (Fig. 2C,D).

Chapter 3

From a mechanistic perspective, dynamic colour change in anurans likely works through modulation of pigment aggregations or pigment dispersion. Likewise, dynamic colour change may also occur by changing the angles of light reflecting crystals in the skin. All these processes can take from seconds to days (Figon and Casas 2018). Compared to fish and cephalopods, dynamic colour change via aggregation and dispersion is slower in amphibians, likely because chromatophores in the latter group are regulated by hormones and lack neuronal innervation (Aspengren et al. 2008; Aspengren et al. 2009). We hypothesize that the onset of sudden, strong rain showers, a drop in temperature and/or a drop in air pressure shortly before the explosive breeding event, as well as a highly competitive breeding aggregation act as environmental and social stressors triggering stress hormone release.

In the **third chapter**, we studied the hormonal basis and explored the role of stress hormones (catecholamines) in the activation of rapid dynamic colour change. Stress hormones mediating fight or flight response are highly conserved and known to influence skin colour changes, e.g., dispersion (Burgers et al. 1953) or aggregation (Wright and Lerner 1960) of pigments. Environmental stressors certainly influence endocrine systems and physiology (Zaffaroni-Caorsi et al. 2023). We thereby assume that the onset of monsoon coupled with high competition and a short breeding period elicits a stress response. Dynamic sexual dichromatism could be a by-product of this response which was eventually co-opted and adapted as a visual signal.

Chapter 4

In dynamic and complex breeding environments, discriminating and detecting signals is challenging (Bee and Micheyl 2008). Species that reproduce in large and explosive aggregations are confronted with a high occurrence of frequency overlap of concurrently vocalizing conspecifics, which could impede signal recognition and assessment by females and thus might reduce individual male reproductive success. Acoustic signals therefore likely serve as long-range localization signals (Bee 2007), whereas sexual selection integrates visual signals into the reproductive display to help facilitate the detection of individuals within dense groups (Gomez et al. 2011; Taylor et al. 2011a; Taylor et al. 2011b). This suggests that dynamic sexual dichromatism is particularly

advantageous in large choruses (Sztatecsny et al. 2010) and might influence mate preference and mate success (Taylor and Ryan 2013; Stange et al. 2016). There are two, non-mutually exclusive ways that dynamic sexual dichromatism could act as a visual signal in intraspecific communication in explosive breeding anurans. First, male colouration could function as preference trait for females to choose a breeding partner according a certain quality (i.e., body size, sperm quality), or as a signal during male-male interactions to facilitate efficient mate selection and prevent mismatch.

In the **fourth chapter**, we characterized and studied the function of dynamic colour change in the Asian common toad. Males change body colouration from brown to bright yellow during their short explosive breeding period. We tested the hypothesis that male colouration functions as a visual male-male signal facilitating rapid mate recognition to increase mating opportunities in highly competitive breeding aggregations. Furthermore, we test whether male body colouration, morphology, and body condition explain mating success.

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Synopsis of Publications

Chapter 1: Carotenoid intake during early life mediates ontogenetic colour shifts and dynamic colour change during adulthood

This chapter has been published in the peer-review journal *Animal Behaviour*.

Author contribution: conceptualisation: SS, DP; methodology: SS, SC, DP; analysis: SS, DP; investigation: SS, SC, DP; resources: SS, DP; writing—original draft: SS, DP; writing—review and editing: SS, SC, WH, DP; supervision: DP; project administration: DP; funding acquisition: SS, DP.

Chapter 2: From masquerading to blending in: ontogenetic shifts in antipredator camouflage in Wallace's flying frogs

This chapter has been published in the peer-review journal *Behavioral Ecology and Sociobiology*.

Author contribution: conceptualisation: SS, MJF, DP; methodology: SS, DP; analysis: SS, DP; investigation: XID, MJF, DP; resources: SS, XID, MJF, DP; writing—original draft: SS, MJF, DP; writing—review and editing: SS, XID, MJF, DP; supervision: MJF, DP; project administration: DP; funding acquisition: SS, MJF, DP.

Chapter 3: Evidence that catecholaminergic systems mediate dynamic colour change during explosive breeding events in toads

This chapter has been published in the peer-review journal *Biology Letters*.

Author contribution: conceptualisation: MJF, DP; methodology: MJF, DP; analysis: SS, DP; investigation: SS, DP; resources: DP; writing—original draft: SS, MJF, DP; writing—review and editing: SS, MJF, DP; supervision: DP; project administration: DP; funding acquisition: SS, DP.

Chapter 4: Dynamic colour change promotes rapid mate recognition in an explosive breeding toad

This chapter is a developed manuscript ready for submission.

Author contribution: conceptualisation: SS, GKV, DP; data curation: SS, DP; formal analysis: SS, DE, DP; funding acquisition: SS, DP; methodology: SS, WT, DP; investigation: SS, HG, WT, DP; software: SS; visualization: SS; validation: SS, DP; project administration: DP; resources: SS, MJF, HG, DP; supervision: DP; writing—original draft: SS, DP; writing—review and editing: SS, MJF, DP.

Chapter 1

Carotenoid intake during early life mediates ontogenetic colour shifts and dynamic colour change during adulthood

This chapter has been published in the peer-review journal **Animal Behaviour**.

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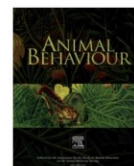
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Carotenoid intake during early life mediates ontogenetic colour shifts and dynamic colour change during adulthood

Susanne Stücker^{a,*}, Samantha Cloer^b, Walter Hödl^c, Doris Preiningner^{b,c}^a Department of Cognitive Biology, University of Vienna, Vienna, Austria^b Vienna Zoo, Vienna, Austria^c Department of Evolutionary Biology, University of Vienna, Vienna, Austria

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Carotenoids play an important role as one of the most prevalent pigments in animals. Carotenoid-based coloration accounts for striking sexually and naturally selected colour adaptations. Several anurans (frogs and toads) change body coloration either slowly and permanently between life stages (ontogenetic colour change), or rapidly and temporarily within minutes or hours (dynamic colour change). We investigated ontogenetic colour change from orange to green morphs in the Wallace's flying frog, *Rhacophorus nigropalmatus*, and tested the influence of dietary carotenoids on colour change during post-metamorphic development. At the age of 9 months, while all individuals still possessed orange-red body coloration, a 20-week-long feeding experiment was performed by supplying the frogs with either no carotenoid supplements or dietary carotenoids once or four times per week. A high carotenoid diet resulted in a faster increase in green colour chroma as well as higher levels of green and carotenoid chroma of back coloration. Less or no carotenoid supplementation led to an increase in UV-blue chroma, contributing to a dull turquoise appearance often observed in captive-bred and captive-raised anurans. In addition, we showed for the first time that Wallace's flying frogs also perform dynamic colour changes. We tested dynamic changes triggered either by 2 min tactile handling or varying 1 h dark and light conditions. Our results demonstrate that a high carotenoid diet facilitates rapid and reversible change of body coloration in response to a tactile stressor, an adaptation absent in frogs receiving no carotenoids. Dynamic colour changes were likewise observed in response to changing light conditions presumably camouflaging individuals and providing protection from UV irradiation. The ontogenetic and dynamic pigmentation changes are discussed in relation to mechanism and as a likely strategy to avoid predation both at different life stages and in different environments.

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Carotenoids are one of the predominant pigments used in animal coloration. All carotenoids have a chromophore in the centre of the molecule that absorbs light from 400–500 nm and is responsible for the appearance of yellow, red and orange colours (Bjørnland, 1997). Carotenoid-based coloration has received most attention in the context of sexually selected signals (Blount & McGraw, 2008; Svensson & Wong, 2011), such as visual signals during male–male competition (Hamilton et al., 2013), species recognition (Blount & McGraw, 2008; Macedonia & Stamps, 1994) and courtship displays (Hill, 1991; Maoka, 2011). However, several carotenoid-based colours and patterns have evolved under natural

selection because they promote predator avoidance strategies, for example aposematism (Bezzarides et al., 2007) and crypsis (Blount & McGraw, 2008; Delhey et al., 2010). Certain defence colorations may only be effective in specific habitats (Endler, 1978; Endler, 1993b; Merilaita et al., 2001; Price et al., 2019) or against particular predators (Barnett et al., 2018; Higginson & Ruxton, 2009). These relationships might change with age and/or across habitats (Endler, 1978; Rojas et al., 2018; Toledo & Haddad, 2009), for example, caterpillars of the genus *Saucrobotys* change from crypsis to aposematism as a response to shifting foraging demands during ontogenetic development (Grant, 2007). Natural and/or sexual selection for certain colour traits at differing life stages may result in ontogenetically changing pigmentation as species use different behavioural strategies to enhance survival and reproduction. Little is known about the underlying mechanism influencing this

* Corresponding author.

E-mail addresses: susanne.stueckler@univie.ac.at (S. Stücker), s.cloer@zoovienna.at (S. Cloer), walter.hoedl@univie.ac.at (W. Hödl), d.preiningner@zoovienna.at (D. Preiningner).

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process; however, carotenoids play an important role as one of the most prevalent pigments guiding coloration. They also function as important antioxidants, modulating a range of immune effects and physiological functions (Martin et al., 1999; Surai et al., 2001). Accordingly, they influence the development, phenotype, reproductive success, growth, immune system and parasite load (Ogilvy et al., 2012; Svensson & Wong, 2011).

Amphibians can tell us a great deal about carotenoid-based coloration used as defence mechanisms and during intraspecific communication at different life stages (Bajger, 1980; Ferreira et al., 2019; Starnberger et al., 2014; Toledo & Haddad, 2009). Frogs and toads can change colour coloration either slowly and permanently across life stages (ontogenetic colour change) or rapidly and temporarily (dynamic colour change) in response to specific social or anti-social encounters (Bell et al., 2017; Bell & Zamudio, 2012; Booth, 1990; Rojas, 2017; Rudh & Qvarnstrom, 2013). Ontogenetic colour change commonly takes several weeks to months to occur, and it results in a permanent change of body coloration mostly via pigment production/destruction during ontogenesis (Booth, 1990; Duarte et al., 2017). By contrast, dynamic colour change or polyphenism rapidly and reversibly generates new phenotypes within minutes to hours by dispersion and aggregation of pigments in the chromatophores and/or the angles of the light-reflecting crystals (Bell & Zamudio, 2012; Toledo & Haddad, 2009). These colorations and colour changes are guided by three pigment cell types: melanophores, reflecting iridophores and nonreflecting xanthophores/erythrophores. Melanophores contain the pigment melanin and are responsible for dark coloration; iridophores contain guanine and are responsible for blue and green coloration; xanthophores/erythrophores contain carotenoids and pteridines and are responsible for yellow, orange, red and, in combination with iridophores, also green coloration (Bell & Zamudio, 2012; Duarte et al., 2017; Figon & Casas, 2018).

Carotenoids are biosynthesized only by photosynthetic organisms and some fungi and bacteria (Goodwin, 1984); hence, animals cannot produce carotenoids de novo, but instead must obtain them by ingestion (Feltl et al., 2005; Olson & Owens, 1996; Ziegler, 2003). Limited carotenoid resources might lead to a trade-off of competing physiological and coloration demands. Dull or miscalorations resulting from restricted carotenoid availability are observed in captive-bred and captive-raised, but also wild species, for example ornamental fish (Sathiyaruban et al., 2021), birds (Sumasgutner et al., 2018) and anurans (Frost & Robinson, 1984; Ogilvy et al., 2012). However, husbandry reports demonstrate successful reproductive events of miscoloured rhacophorids (Dunce, 2016). In such conditions, limited carotenoid resources might be primarily used for health and reproductive demands, for example egg production (Svensson & Wong, 2011), and are no longer available in sufficient amounts to invest in pigmentation. Duller or degraded colorations could be a first indication of nutritional problems and carotenoid deficiency. In the false tomato frog, *Dyscophus guineti*, dietary carotenoids increased both orange body coloration and circulating retinol concentrations, suggesting improved health (Brenes-Soto & Dierenfeld, 2014). Commercially available carotenoid supplements provide an important dietary component that could counteract health issues and promote correct pigmentation. Some studies in anurans have revealed positive effects of dietary carotenoid supplements on general health and/or coloration (Brenes-Soto & Dierenfeld, 2014; Dugas et al., 2013; Umbers et al., 2016) and suggest a critical effective period for their uptake during post-metamorphic development (Ogilvy et al., 2012).

Over recent decades, knowledge about nutritional requirements in husbandry, zoo-based amphibian research and conservation initiatives have become more important for a wide range of amphibian species due to rapidly declining habitats (Browne et al., 2011; Jacken et al., 2020; McFadden et al., 2008). Optimal

husbandry conditions are, however, difficult to determine (Livingston et al., 2014), highlighting the immense difference between what animals can tolerate to survive and what they need for successful reproduction. Despite the clear importance of mimicking natural habitat conditions (temperature, humidity, light), there is still a lack of knowledge regarding feeding regimes in captivity compared to resources utilized in the individuals' natural habitat.

Wallace's flying frog, *Rhacophorus nigropalmatus*, undergoes a striking and irreversible colour change from orange-red juvenile to emerald-green adult morph (Ready, 2009). In the current study we investigated the ontogenetic colour change during the first 9 months and tested the influence of carotenoids on colour change which, despite its conspicuousness, has not been explored in detail. Captive-raised adults often exhibit lighter and turquoise body colorations (Ready, 2009) compared to wild conspecifics. We tested the hypothesis that carotenoid pigments in chromatophores supplied during postmetamorphic development affect body colour. We predicted that carotenoid resources during this life stage facilitate storage and accumulation of carotenoid pigments in the skin to establish the species' wildtype coloration as adults. Additionally, we explored the species' ability to dynamically change colour, an adaptive behaviour only briefly described for a related species, *Rhacophorus smaragdinus*, after handling an individual (Deepak et al., 2019). Based on the assumption that tactile stress effects dynamic colour changes we tested the behaviour and its dependency on previous carotenoid diets, predicting that carotenoids support sufficient accumulation and rearrangement of pigments for rapid changes. In a final step, we investigated a yet undescribed colour change of the frogs' complete and partial body in response to environmental light. We hypothesized that dynamic body coloration changes could be a beneficial camouflage strategy in fluctuating light environments of rainforests and predicted rapid and reversible changes when exposing the frogs to varying light and dark conditions.

METHODS

Study Animals and Site

Rhacophorus nigropalmatus is a large nocturnal treefrog (males 79–89 mm; females 89–100 mm) that is native to the rainforest of South East Asia (Inger, 1966; Inger & Stuebing, 2005). These frogs are able to glide and manoeuvre easily through the understory of rainforests (Emerson & Koehl, 1990), which suggests that they live in the canopy of primary forests. During breeding, small groups have been observed in vegetation overhanging forest pools, in which females lay eggs protected by foam nests. Adults have green backs, white bellies and vocal sacs, yellow flanks and prominent black and yellow webbing between the toes (Inger & Stuebing, 2005). Juveniles look very different from adults, displaying an orange-red body coloration (Haas et al., 2021).

The study was conducted with a captive population of *Rh. nigropalmatus* at the Vienna Zoo, Austria. The Rainforest House in the Zoo houses adult frogs raised in captivity with an average snout–vent length of 85 mm (mean $\pm$ SD = 85.28 $\pm$ 3.65 mm, $N = 9$) and body mass of 37 g (mean $\pm$ SD = 36.65 $\pm$ 6.91 g, $N = 9$). Forty-eight *Rh. nigropalmatus* juveniles were bred and raised for the first time at the Zoo.

Husbandry

Frogs were obtained as juveniles and were 7 years old at the start of the study. Adults were fed three times per week with crickets, *Acheta domesticus*, and Turkestan cockroaches, *Shelfordella lateralis*. Feeder insects were dusted with either Amphib (Herpetal,

Keweloh Animal Health GmbH & Co KG, Neuenkirchen, Germany), cuttlefish bone or Cal Plus LoD (Repashy Ventures Inc., Oceanside, CA, U.S.A.) once a week and were fed with each of the (multi-) carotenoid supplements Superload (Repashy Ventures Inc.), Superpig (Repashy Ventures Inc.) and spirulina algae (Blattner Heimtiefutter, Kempten, Germany) once per week. Carotenoid food supplementation of Superload and Superpig started 6 months before the reproductive event described below and adult individuals received no additional dietary carotenoids during earlier development.

On 12 August 2019 a brief but heavy thunderstorm in Vienna caused a drop in air pressure. This was a possible stimulator of breeding behaviour and, on the following day, a foam nest was discovered in the breeding terrarium. We immediately removed the two adult males and one female from the tank. Twenty-four hours later, the first tadpoles could be observed moving inside the nest and after 3 days 95 tadpoles had dropped out of the nest into the 20 cm deep water below (temperature 25–26 °C). The tadpoles were transferred to five separate aquaria (60 × 30 cm and 30 cm deep) equipped with air pumps as well as stones and clay pots as shelter. Water temperature was 25–26 °C. Tadpoles received a diverse diet of Pleco tablets (Tetra GmbH, Melle, Germany), Sera Vipachips, Sera Catfish Chips, Sera Vipan Staple Food and Sera spirulina flakes (Sera GmbH, Immenhausen, Germany). In addition, over the 2-month larval stage, algae were offered once, blanched stinging nettle, *Urtica dioica*, twice and pollen granulate (Blattner Heimtiefutter) four times. The survival rate was 97.9%.

The first froglets were found 37 days after tadpole hatching and, within 2 weeks, the majority had metamorphosed. Forty-eight juveniles successfully metamorphosed and survived the first 2 weeks of development. As information about nutritional requirements is limited for most amphibian species and only general qualitative and quantitative guidelines are available (Ferrie et al., 2014; Pramuk & Gagliardo, 2012), the diet of the juveniles up until 9 months of age consisted of daily feedings alternating between fruit flies, *Drosophila melanogaster*, crickets, Turkestan cockroaches, silverside, *Lepisma saccharina*, and waxmoths, *Galleria mellonella*. Once a week Superpig and spirulina algae were fed to feeder insects 24 h prior to feeding. In addition, feeder insects were dusted three times a week with Amphib, cuttlefish bone, Vitamin D3 or Cal Plus LoD alternating every 2 days.

Data Collection

Ontogenetic colour change

To determine coloration changes of *Rh. nigropalmatus*, a spectrometer (JAZ series; Ocean Optics, Dunedin, FL, U.S.A.) was used to measure the spectral reflectance of the back, flank and throat of a randomly selected group of 12 juveniles during the first 9 months of development. The spectrometer had an integrated pulsed xenon light source (Jaz-PX) with a spectral response of 190–1100 nm. The reflectance data were collected for 300–700 nm and expressed as the percentage of reflectance relative to a white standard (WS-1 Diffuse Reflectance Standard, Ocean Optics). To reduce specular reflection, we used a customized probe holder to keep the reflection probe at a distance of 5 mm and at an angle of 45° to the surface of the frog's skin. The probe holder touched the frog's skin, preventing stray light from entering. For each measurement on the back, flank or throat three reflectance scans were averaged. Measurements were performed with the same group of individuals every 2 months starting 1 month after metamorphosis. We measured snout–vent length (SVL; ± 0.1 mm) and body mass (± 0.1 g) of each individual. Additionally, we took dorsal pictures of individuals immediately after metamorphosis ($N = 7$) until 14

months of age ($N = 26$) on millimetre-paper to quantify white spots (>1 mm) on the individual's back.

Feeding experiment

To experimentally test the impact of a carotenoid-supplemented diet on the coloration of *Rh. nigropalmatus*, we randomly assigned 26 individuals at the age of 9.5 months to one of three experimental groups: group 1 ($N = 8$, G1) did not receive carotenoid supplements; group 2 ($N = 9$, G2) received carotenoid supplements via gut-loaded crickets once a week; and group 3 ($N = 9$, G3) received carotenoid supplements via gut-loaded crickets four times a week. Gut-loaded crickets were fed with cucumbers covered with the carotenoid supplement Superpig (0.7 g) containing the carotenoids astaxanthin, capsanthin, capsorubin, beta carotene, alpha carotene, beta cryptoxanthin, zeaxanthin, neoxanthin, cucurbitaxanthin, violaxanthin, lutein, echinenone, canthaxanthin and lycopene. This comprehensive carotenoid supply was chosen as no knowledge of the frogs' natural carotenoid profile is available and several carotenoids might contribute to pigmentation. Additional ingredients were *Phaffia* yeast, paprika, marigold flower, algae meal (*Chlorella*), rosehips, hibiscus flower, dried watermelon and turmeric. The general diet apart from the carotenoid supplement remained the same as described above.

The feeding experiment started after a baseline reflectance measurement of the back, flank and throat of the 26 test individuals. Reflectance and morphometric (SVL and body mass) measurements were performed every 2 weeks after the start of group diets for a period of 20 weeks until 14 months of age. One measurement had to be excluded due to incorrect data (errors in the reflection measurement). Reflectance measurements of back, flank and throat were also collected from nine 8-year-old adult individuals maintaining their regular diet with carotenoid supplements once per week for later comparison.

Dynamic colour change

Following the feeding experiment, we tested reversible and rapid colour change triggered by tactile handling in 14-month-old subadult *Rh. nigropalmatus*. The spectral reflectance of the back, flank and throat of eight adults and the 26 subadults, previously assigned to three different supplementary diets, were measured in three steps: (1) the baseline coloration was measured immediately after taking the individual out of the terrarium (handling time less than 30 s); (2) the next measurement was made after a 2 min handling period (touching, holding and placing the individual on a scale); and (3) the last measurement was recorded after a 30 min recovery period of resting on a plant in the terrarium.

In a further experiment, we tested whether different light conditions elicit dynamic colour change in two set-ups. In set-up 1, adults ($N = 6$) and subadults from feeding group G3 ($N = 6$) were placed in four empty terraria (three individuals per terrarium) during daytime (the natural resting period for the frogs). After a 1 h acclimatization period with regular daytime light conditions (Lucky Reptile Bright Sun UV 35W Model #BSJ-35), two terraria (one containing three adults, the other three subadults) were shaded with a black board allowing only ambient room light (Osram Lumilux Cool Daylight, L36W/865, 325 LM) to enter the front of the terraria. The other two terraria remained under daytime light conditions. Reflectance measurements of the backs of all 12 individuals were taken after 1 h without handling the individuals by placing the probe of the spectrometer on the back of the individuals while they were at rest. Subsequently the conditions were switched by removing the black boards and placing them on the previously lit terraria. The formerly shaded terraria now received regular daytime light conditions. Reflectance measurements were repeated after 1 h for all 12 individuals.

In the second set-up, we shaded parts of the individuals' body by loosely taping a leaf to the terrarium wall, covering the side or back of the frogs without touching them. We tested five adults and five subadults (G3) under regular daytime light conditions. After 1 h, reflectance measurements were collected from the light-exposed and shaded backs of all individuals.

Data Analysis

Three overall parameters of coloration were extracted from the reflectance spectra using Avicol software v6 (Gomez, 2006): brightness, hue, and chroma. Brightness corresponds to the total reflectance, calculated as the surface area under the spectral curve. Hue corresponds to the notion of colour and we calculated it as maximal reflectance searched in the range 300–700 nm and/or the wavelength of the maximal slope because several reflectance measurements lacked distinct peaks at specific wavelengths (Endler, 1990; Gomez, 2006; Gomez et al., 2009). Chroma is a measure of saturation of colour judged as a proportion of the brightness of a similarly illuminated area, or colour signal intensity describing the concentration of the reflectance around a wavelength. We analysed different parameters depending on the evaluated body part (Andersson et al., 2002). To describe the frogs' back reflectance and colour alterations due to diet we analysed UV-blue and green chroma as well as maximal average yellow chroma (Blévin, 2018; Gomez, 2006), referred to below as carotenoid chroma. Carotenoid chroma is a measure linked to the incorporated level of carotenoids as they maximally absorb around 450 nm (Andersson et al., 2002; Andersson et al., 2007; Isaksson et al., 2008). UV-blue chroma is expressed as the difference between the reflectance of 300–450 nm divided by the total reflectance ($R_{(300-450\text{ nm})}/R_{(300-700\text{ nm})}$). Green chroma is computed as the proportion of the total reflectance that occurs over the range of 500–560 nm ($R_{(500-560\text{ nm})}/R_{(300-700\text{ nm})}$). Carotenoid chroma is the difference of the maximal reflectance between 500 and 700 nm and the reflectance at 450 nm divided by the average reflectance from 300 to 700 nm ($(R_{\max(500-700\text{ nm})} - R_{450})/R_{\text{AV}}$) and reported as absolute value. From flank measurements we extracted green chroma and carotenoid chroma. To express ontogenetic changes from orange-red to bright white throat coloration we analysed UV chroma, the chroma over the range 300–400 nm ($R_{(300-400\text{ nm})}/R_{(300-700\text{ nm})}$) and carotenoid chroma. In addition, we analysed chroma in the range 400–500 nm ($R_{(400-500\text{ nm})}/R_{(300-700\text{ nm})}$) to measure all wavelengths as reflected in white coloration.

To test differences in colour parameters during the ontogenetic development, the feeding experiment and the dynamic colour change experiment, we used Kruskal–Wallis tests to compare test groups and generalized linear mixed models (GLMMs) to compare parameters of the same test groups at differing measurement points. The GLMMs allow repeated measurements of the same individual to be fitted in the model as random variables. We used a GLMM with a normal distribution and identity link function. For post hoc tests, we used Student's *t* statistic with sequential Bonferroni correction for alpha. The statistical assumptions for GLMM analysis were met (Kolmogorov–Smirnov test). For statistical analysis we used the program R (v3.4.2; R Core Team 2021) and SPSS version 26 (IBM SPSS Statistics, Armonk, NY, U.S.A.).

To describe colour change over the first 9 months of development we compared brightness, hue and chroma colour parameters of juveniles with GLMMs. The colour parameters were entered as dependent variables, with measurement point in time as predictor variables. We entered the identities of measurement point (individuals) as nested random variable to correct for repeated measurements of the same individuals. Similarly, we compared the

number of dorsal spots during ontogenetic development. The numbers of spots were entered as dependent variables, with measurement point as predictor variable. The identities of measurement point (individuals) were entered as nested random variables.

To investigate the influence of carotenoid supplements we tested colour parameters of back, flank and throat between the three test groups and additionally compared coloration of adults. We used Kruskal–Wallis tests with Bonferroni-corrected pairwise comparisons to analyse group differences in the first baseline measurement and the last measurement after 20 weeks. Similarly, we compared size and weight of the three test groups. To test differences and similarities in colour change during the feeding experiment within each test group, we used GLMMs entering colour parameters as dependent variables, with the point of measurement as predictor variable. We entered the identities of measurement point (individuals) as nested random variables to correct for repeated measurements of the individuals.

Dynamic changes in colour parameters were compared between the treatments with GLMMs: baseline, handling and 30 min resting for each of the three feeding groups and the adults. For each test group and the adults, we entered the respective colour parameters as dependent variables, with treatment as predictor variable. We entered the identities of treatment (individuals) as nested random variables.

Further GLMMs were used to test dynamic colour change of shaded or lit terraria or body parts of adults and subadults. We entered the colour parameters as dependent variables, with the treatment (dark/light) as predictor variable. We entered the identities of treatment (terraria (individuals)) as nested random variables.

Ethical Note

Frogs were handled carefully and handling time was kept to a minimum (≤ 2 min). In between reflectance and morphometric measurements, frogs were able to move freely around their terraria, feed ad libitum and otherwise behave normally without interference from the experimenters. The study was designed to improve the welfare of captive-bred anurans and investigates the importance of carotenoids as dietary component during development. All experiments reported in this article complied with the current laws of the country in which they were performed (Austria) and required no institutional or governmental reviews. We followed the ASAB/ABS Guidelines for the treatment of animals in research.

RESULTS

Ontogenetic Colour Change

Within 9 months, the appearance of the frogs changed slightly from orange-red to orange-brown (Fig. 1a, b, Table A1). The maximal reflectance of the juveniles' backs decreased from 700 nm to 672 nm in the first 5 months (GLMM: $F_{4,45} = 2.927$, $P = 0.031$; pairwise comparison, 1–5 months: $\beta = -27.889$, $SE = 8.940$, $t = -3.120$, $P = 0.032$), but subsequently increased and no difference was found between 1 and 9 months ($P > 0.05$; Fig. A1). No differences were found in brightness or chroma (GLMMs: $P > 0.05$). The flank coloration of juveniles increased in carotenoid chroma (GLMM: $F_{4,45} = 4.327$, $P = 0.005$; Fig. 2a) until the age of 9 months. The throats became brighter during ontogenetic development (GLMM: $F_{4,45} = 6.085$, $P = 0.001$; Fig. 2b) and UV colour chroma decreased (GLMM: $F_{4,45} = 9.466$, $P < 0.001$; Fig. 2c).

The juveniles developed several whitish dorsal spots 1 month after metamorphosis (Figs. 1b, 3). At 7 months, juveniles exhibited



Figure 1. (a) Freshly metamorphosed juvenile; (b) 14-month-old subadult; (c) 7-year-old adult *Rhacophorus nigropalmatus*. The white scale bars correspond to 1 cm.

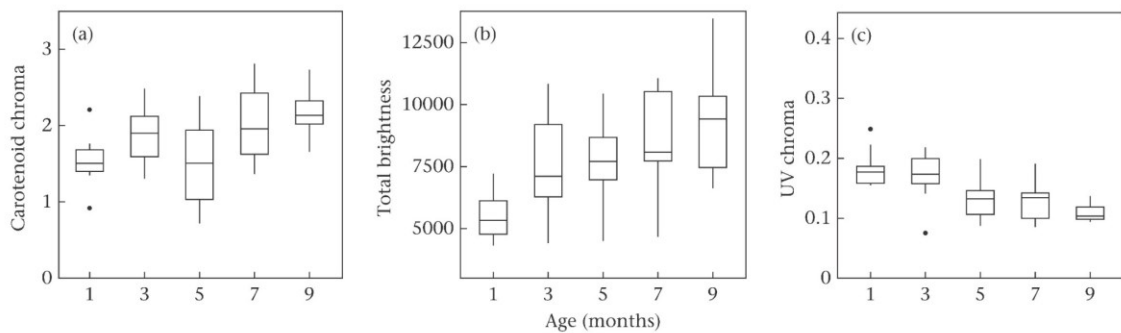


Figure 2. Colour parameter changes (GLMMs: $P < 0.05$) of *Rhacophorus nigropalmatus* juveniles ($N = 12$) observed during the first 9 months of ontogenetic development. Colour parameter comparisons of (a) carotenoid chroma of flanks, (b) total brightness of throats and (c) UV chroma of throats. Box plots show the median (centre horizontal line), the 25th to the 75th percentile (box), and the 5th and 95th percentiles (whiskers); dots denote outliers.

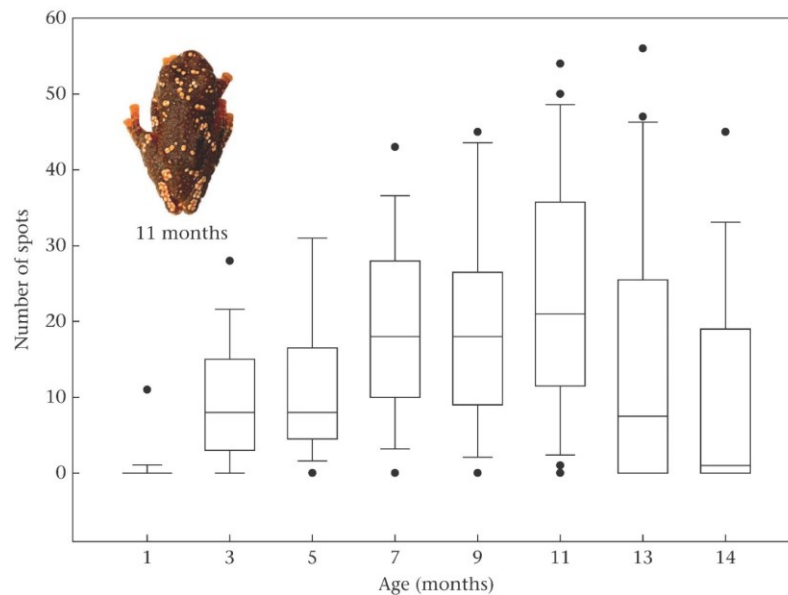


Figure 3. Comparison of number of dorsal spots during ontogenetic development of *Rhacophorus nigropalmatus* (1 month: $N = 7$; 3–9 months: $N = 17$; 11–14 months: $N = 26$). Box plots represent the median (centre horizontal line), the 25th to the 75th percentile (box), and the 5th and 95th percentiles (whiskers) of absolute measurements; dots denote outliers.

more spots than at 1 month (pairwise comparison, 1–7 months: $\beta = 18.330$, $SE = 4.412$, $t = 4.155$, $P = 0.001$). However, the number of spots decreased from age 11 to 14 months (pairwise comparison, 11–14 months: $\beta = -14.000$, $SE = 3.618$, $t = -3.870$, $P = 0.004$).

Feeding Experiment

At the beginning of the feeding experiment, 9.5-month-old juveniles had orange-red body coloration and across-group comparisons of back, flank and throat colour parameters showed no differences (Kruskal–Wallis tests: $P > 0.05$). By the end of the experiment, at the age of 14 months, the subadults had developed their adult body coloration (green backs, yellow flanks, white throats; Table 1, Fig. 4). The body size and weight were similar between the groups at the beginning and end of the experiment (Kruskal–Wallis tests: $P > 0.5$) and was thereby unaffected by supplementary carotenoids. No difference in whitish dorsal spot number was found between the groups during the feeding experiment from 9.5 to 14 months (Kruskal–Wallis tests: $P > 0.5$).

Back colour changes

Comparisons of back reflectance across the test groups after the feeding experiment showed no differences in brightness, hue (maximal reflectance) or UV-blue chroma (Kruskal–Wallis tests: $P > 0.5$). Test group G3 had higher green chroma than G2 (Kruskal–Wallis test: $H_2 = 6.6$, $P = 0.037$; pairwise comparison, G3 versus G2: $P = 0.043$), and carotenoid chroma was higher in G3 and G2 than G1 (Kruskal–Wallis test: $H_2 = 12.306$, $P = 0.002$; pairwise comparison, G1 versus G3: $P = 0.005$; G1 versus G2: $P = 0.024$; Table 1, Fig. 5).

Within test group comparisons showed that the hue of the frogs' backs before and after 20 weeks of food treatment decreased similarly in G1 (GLMM: $F_{9,70} = 2.484$, $P = 0.016$), G2 ($F_{9,80} = 3.518$, $P = 0.001$) and G3 ($F_{9,80} = 3.403$, $P = 0.001$). Back brightness varied during the experiment, but no differences between the beginning and end of the experiment were found in any test group (GLMM: $P > 0.05$ for all groups).

Changes in back colour chroma comprised a decrease in carotenoid chroma (GLMM: G1: $F_{9,70} = 5.078$, $P < 0.001$; G2: $F_{9,80} = 7.138$, $P < 0.001$; G3: $F_{9,80} = 5.991$, $P < 0.001$), an increase in green chroma (GLMM: G1: $F_{9,70} = 7.330$, $P < 0.001$; G2: $F_{9,80} = 12.741$, $P < 0.001$; G3: $F_{9,80} = 12.056$, $P < 0.001$) and UV-blue chroma (GLMM: G1: $F_{9,70} = 5.399$, $P < 0.001$; G2: $F_{9,80} = 7.369$, $P < 0.001$; G3: $F_{9,80} = 5.877$, $P < 0.001$) in all three test groups. Green chroma increased from the start to the end of the experiment from 16 to 26% in G1 (pairwise comparison: $\beta = 0.1$, $SE = 0.017$, $t = 6.07$, $P < 0.001$), from 14 to 25% in G2 (pairwise comparison: $\beta = 0.117$, $SE = 0.015$, $t = 7.864$, $P < 0.001$) and from 14 to 29% in G3 (pairwise comparison: $\beta = 0.142$, $SE = 0.016$, $t = 8.942$, $P < 0.001$). There was no change in UV-blue chroma in G3 (pairwise comparison: $\beta = 0.048$, $SE = 0.018$, $t = 2.617$, $P = 0.361$), but this increased in G2 (pairwise comparison: $\beta = 0.088$, $SE = 0.018$, $t = 4.842$, $P < 0.001$) and G1 (pairwise comparison: $\beta = 0.080$, $SE = 0.021$, $t = 3.746$, $P = 0.014$) and contributed to a bluish appearance (Fig. 4a).

A high carotenoid diet in G3 resulted in a faster increase in green chroma (pairwise comparison, 1–4 weeks: $\beta = 0.055$, $SE = 0.016$, $t = 3.483$, $P = 0.023$), whereas less or no carotenoid supplementation led to a slower increase in green chroma (G2: pairwise comparison, 1–6 weeks: $\beta = 0.062$, $SE = 0.015$, $t = 4.134$, $P = 0.003$; G1: pairwise comparison, 1–10 weeks: $\beta = 0.063$, $SE = 0.017$, $t = 3.827$, $P = 0.011$).

Flank colour changes

Across-group comparisons of flank reflectance after the feeding experiment showed differences in carotenoid chroma

(Kruskal–Wallis test: $H_2 = 6.95$, $P = 0.030$). Group G3 had higher carotenoid chroma than G1 (pairwise comparison, G3 versus G1: $P = 0.046$), but G2 did not differ from G3 or G1 ($P > 0.05$). During the experiment juveniles developed their yellow flank pattern and, correspondingly, hue (maximal slope) decreased in G1 (GLMM: $F_{9,70} = 2.636$, $P = 0.011$), G2 (GLMM: $F_{9,80} = 3.337$, $P = 0.002$) and G3 (GLMM: $F_{9,80} = 2.471$, $P = 0.015$) after 20 treatment weeks (Table 1).

Comparisons of flank coloration before and after the experiment within the test groups indicated that brightness increased in G2 (GLMM: $F_{9,80} = 7.162$, $P < 0.001$; pairwise comparison: $\beta = 3749.938$, $SE = 660.064$, $t = 5.681$, $P < 0.001$) and in G3 (GLMM: $F_{9,80} = 4.864$, $P < 0.001$; pairwise comparison: $\beta = 3326.033$, $SE = 914.986$, $t = 3.635$, $P = 0.02$), but not in G1 (GLMM: $F_{9,70} = 2.961$, $P = 0.005$; pairwise comparison: $\beta = 2834.22$, $SE = 1004.537$, $t = 2.821$, $P = 0.261$). The flank of all groups showed a similar increase in green chroma (GLMM: $P < 0.001$) during the feeding experiment.

The high carotenoid diet resulted in a faster increase in green chroma (G3: pairwise comparison, 1–6 weeks: $\beta = 0.039$, $SE = 0.011$, $t = 3.616$, $P = 0.018$), whereas low or no carotenoid supplements led to a slower increase (G1: pairwise comparison, 1–10 weeks: $\beta = 0.063$, $SE = 0.015$, $t = 4.241$, $P = 0.003$; G2: pairwise comparison, 1–10 weeks: $\beta = 0.045$, $SE = 0.012$, $t = 3.84$, $P = 0.008$).

Throat colour changes

Comparison between test groups showed no differences in throat colour parameters after the feeding experiment (Kruskal–Wallis tests: $P > 0.05$; Table 1). Consistent with the white appearance of throats at the age of 14 months, we found similar colour changes within all groups: maximal slope and carotenoid chroma decreased (GLMMs: $P < 0.001$), UV chroma and chroma 400–500 nm increased (GLMMs: $P < 0.001$), whereas brightness did not change (GLMMs: $P > 0.05$).

Comparison with adults

The hue (maximal reflectance), brightness and green chroma of the back of adults were similar to all three test groups at the end of the feeding experiment (Kruskal–Wallis tests: $P > 0.05$; Fig. A2). Back coloration of adults showed similarities in UV-blue and carotenoid chroma to G1 and differed from G2 and G3 (Kruskal–Wallis test: UV-blue chroma: $H_3 = 15.005$, $P = 0.002$; pairwise comparison, adults versus G1: $P = 0.447$; adults versus G2: $P = 0.005$; adults versus G3: $P = 0.002$; carotenoid chroma: $H_3 = 18.633$, $P < 0.001$; pairwise comparison, adults versus G1: $P = 1$; adults versus G2: $P = 0.024$; adults versus G3: $P = 0.003$; Table 1, Fig. A3a).

The flanks of adult individuals also showed no differences in brightness or green chroma (Kruskal–Wallis tests: $P > 0.05$) compared to the three test groups. However, the carotenoid chroma of the flanks of adults was higher than that of G1 and G2 and similar to G3 (Kruskal–Wallis test: $H_3 = 16.608$, $P = 0.001$; pairwise comparison, adults versus G1: $P = 0.001$; adults versus G2: $P = 0.017$; adults versus G3: $P = 0.462$; Table 1, Fig. A3b).

The adults had brighter throats than all three test groups (Kruskal–Wallis test: $P < 0.001$). The hue of adults' throats was similar to that of G1, but differed from G2 and G3 (Kruskal–Wallis test: maximal slope: $H_3 = 17.681$, $P < 0.001$; pairwise comparison, adults versus G1: $P = 0.093$; adults versus G2: $P = 0.011$; adults versus G3: $P = 0.003$; Table 1, Fig. A3c). No differences were found in carotenoid chroma, chroma 400–500 nm and UV chroma (Kruskal–Wallis tests: pairwise comparisons: $P > 0.05$).

Dynamic Colour Change

Mild handling stress in adults and subadults after 20 weeks of different carotenoid supplementations caused rapid coloration

Table 1Comparison between adult *Rhacophorus nigropalmatus* and juveniles after a 20-week carotenoid supplement feeding experiment for back, flank and throat colour parameters

Colour parameter		Test group	Median	SE	Min	Max		
Back								
Brightness	Total reflectance	G1 _a	5377	832	3620	10728		
		G2 _a	5103	521	2800	8138		
		G3 _a	4748	414	2925	6414		
		Adult _a	4722	504	3037	7911		
Hue	Maximal slope (nm)	G1 _{a,b}	505	22	503	679		
		G2 _a	507	29	504	679		
		G3 _a	507	19	505	674		
		Adult _b	503	20	500	679		
		G1 _a	539	27	533	700		
	Maximal reflectance (nm)	G2 _a	545	28	536	700		
		G3 _a	542	18	535	700		
		Adult _a	538	18	529	700		
		Chroma	Carotenoid chroma	G1 _a	0.95	0.08	0.73	1.4
				G2 _b	1.43	0.07	0.96	1.58
G3 _b	1.58			0.13	1.2	2.3		
Adult _a	0.99			0.05	0.79	1.27		
UV-blue chroma (%)	G1 _{a,b}	19	1	13	21			
	G2 _a	18	0	16	19			
	G3 _a	15	1	9	19			
	Adult _b	20	0	18	23			
	Green chroma (%)	G1 _{a,b,c}	25	1	24	29		
G2 _{b,c}		26	1	23	29			
G3 _{a,c}		28	1	24	33			
Adult _c		27	1	21	30			
Flank								
Brightness	Total reflectance	G1 _a	7114	1037	3310	12074		
		G2 _a	7637	551	4881	10368		
		G3 _a	7884	1036	4832	13251		
		Adult _a	10064	595	6888	11802		
Hue	Maximal slope (nm)	G1 _a	507	26	325	510		
		G2 _a	509	0.2	508	510		
		G3 _a	510	22	327	554		
		Adult _a	511	1	507	513		
	Maximal reflectance (nm)	G1 _a	601	12	553	663		
		G2 _a	608	9	557	624		
		G3 _a	612	8	558	634		
		Adult _a	611	7	565	619		
Chroma	Carotenoid chroma	G1 _a	0.94	0.06	0.53	1.08		
		G2 _{a,b}	1.16	0.06	0.83	1.37		
		G3 _{b,c}	1.24	0.1	0.66	1.62		
		Adult _c	1.38	0.06	1.07	1.72		
	Green chroma (%)	G1 _a	21	1	17	23		
		G2 _a	20	1	18	23		
		G3 _a	19	1	16	24		
		Adult _a	21	0	20	22		
Throat								
Brightness	Total reflectance	G1 _a	11885	674	9526	15530		
		G2 _a	13549	820	9888	18167		
		G3 _a	12816	1039	8983	17847		
		Adult _b	18280	394	15332	19286		
Hue	Maximal slope (nm)	G1 _{a,b}	327	0.4	325	328		
		G2 _a	326	0.4	324	327		
		G3 _a	325	0.4	324	327		
		Adult _b	328	0.3	327	329		
	Maximal reflectance (nm)	G1 _a	494	52	353	656		
		G2 _a	629	5	618	663		
		G3 _a	622	38	353	654		
		Adult _a	622	6	618	676		
Chroma	Carotenoid chroma	G1 _a	0.05	0.05	0.01	0.35		
		G2 _a	0.3	0.06	0.07	0.64		
		G3 _a	0.27	0.06	0.06	0.52		
		Adult _a	0.2	0.04	0.17	0.56		
	UV chroma (%)	G1 _a	21	0	16	27		
		G2 _a	18	0	16	20		
		G3 _a	21	0	17	27		
		Adult _a	18	0	15	21		
	Chroma 400–500 nm (%)	G1 _a	26	1	23	27		
		G2 _a	24	2	21	27		
		G3 _a	24	2	21	27		
		Adult _a	26	2	23	26		

Group 1 received no food supplements (G1), group 2 received a carotenoid supplement once per week (G2) and group 3 a carotenoid supplement four times per week (G3). The table reports medians, SE and range of absolute measurements. Values without the same subscript letter (a, b, c) differ significantly at $P < 0.05$ (Kruskal–Wallis test). Total reflectance and carotenoid chroma are reported as absolute values.

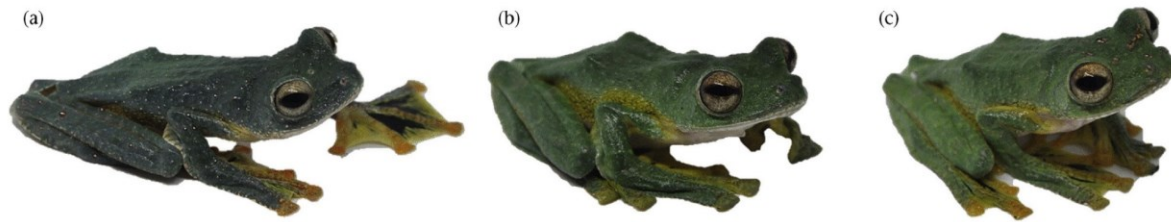


Figure 4. Photo images of representative *Rhacophorus nigropalmatus* individuals of each test group after a 20-week carotenoid feeding experiment: (a) G1: no supplement; (b) G2: low supplement; (c) G3: high supplement.

changes in all subadult frogs that previously received dietary carotenoids. The backs of the adults also changed in brightness (GLMM: $F_{2,21} = 6.593$, $P = 0.006$). However, body reflectance in adults brightened during the resting period (pairwise comparison, handling versus resting: $\beta = 2445.963$, $SE = 673.761$, $t = 3.63$, $P = 0.005$), but did not change after handling (pairwise comparison, baseline versus handling: $\beta = -1171.717$, $SE = 673.761$, $t = -1.739$, $P = 0.145$).

Group G3 changed in brightness (GLMM: $F_{2,24} = 10.625$, $P < 0.001$). The back darkened after handling (pairwise comparison, baseline versus handling: $\beta = 2190.6$, $SE = 475.26$, $t = 4.609$, $P < 0.001$) and tended to recover after a 30 min resting period (pairwise comparison, handling versus resting: $\beta = -1123.41$, $SE = 475.26$, $t = -2.364$, $P = 0.053$; baseline versus resting: $\beta = -1067.191$, $SE = 475.26$, $t = -4.609$, $P = 0.053$). All other colour parameters remained the same in G3 during the experiment. The back of G2 tended to darken after handling (GLMM: brightness: $F_{2,24} = 3.386$, $P = 0.051$; pairwise comparison, baseline versus handling: $\beta = -1735$, $SE = 667.272$, $t = -2.6$, $P = 0.047$) and remained dark in the resting period (GLMM: pairwise comparison, handling versus resting: $\beta = 927.732$, $SE = 667.272$, $t = 1.39$, $P = 0.354$). Interestingly, UV-blue chroma (GLMM: $F_{2,24} = 4.982$, $P = 0.015$) and green chroma (GLMM: $F_{2,24} = 9.061$, $P = 0.001$) also changed during the experiment. Handling elevated UV-blue chroma (GLMM: pairwise comparison, baseline versus handling: $\beta = 0.032$, $SE = 0.012$, $t = 2.699$, $P = 0.032$) whereas resting reduced UV-blue chroma (GLMM: pairwise comparison, handling versus resting: $\beta = -0.033$, $SE = 0.012$, $t = -2.767$, $P = 0.032$) leading to full reversal (GLMM: pairwise comparison, baseline versus resting: $\beta = 0.001$, $SE = 0.012$, $t = 0.069$, $P = 0.946$).

Contrarily, green chroma decreased after handling (pairwise comparison, baseline versus handling: $\beta = -0.025$, $SE = 0.009$, $t = -2.840$, $P = 0.018$) and increased after resting (pairwise comparison, handling versus resting: $\beta = 0.037$, $SE = 0.009$, $t = 4.166$, $P = 0.001$) resulting in green chroma comparable to the baseline (pairwise comparison, baseline versus resting: $\beta = 0.012$, $SE = 0.009$, $t = 1.326$, $P = 0.197$). No colour change was observed in G1 between baseline, handling or recovery period (GLMM: $P > 0.05$).

Additionally, dynamic colour adaptations were tested depending on differing light condition in subadults receiving a high carotenoid diet (G3) and adults. Exposure to 1 h of light and shade rapidly influenced body coloration (Fig. 6a). The adults were brighter in shaded than lit conditions (GLMM: $F_{1,10} = 18.364$, $P = 0.002$; pairwise comparison: $\beta = 3086.408$, $SE = 720.227$, $t = 4.285$, $P = 0.002$) and hue changed (GLMM: maximal reflectance: $F_{1,10} = 12.87$, $P = 0.005$) from 538 nm in the shade to 651 nm under light (pairwise comparison: $\beta = 112.333$, $SE = 31.313$, $t = 3.587$, $P = 0.005$). The adults' green chroma decreased (GLMM: $F_{1,10} = 14.864$, $P = 0.003$) from 27% colour chroma in the shade to 20% under light (pairwise comparison: $\beta = -0.071$, $SE = 0.019$, $t = -3.855$, $P = 0.003$). Back brightness also changed dynamically in the subadult group (GLMM: $F_{1,10} = 6.239$, $P = 0.032$). Like adults, subadults had brighter backs in the shade than under light (pairwise comparison: $\beta = 2154.588$, $SE = 862.613$, $t = 2.498$, $P = 0.032$). In contrast to adults, subadults did not show changes in hue and chroma (GLMMs: $P > 0.05$).

During partial shading of the body (Fig. 6b) the back of the adults increased in brightness (GLMM: $F_{1,8} = 5.111$, $P = 0.054$) on shaded body parts compared to those exposed to light. Like our

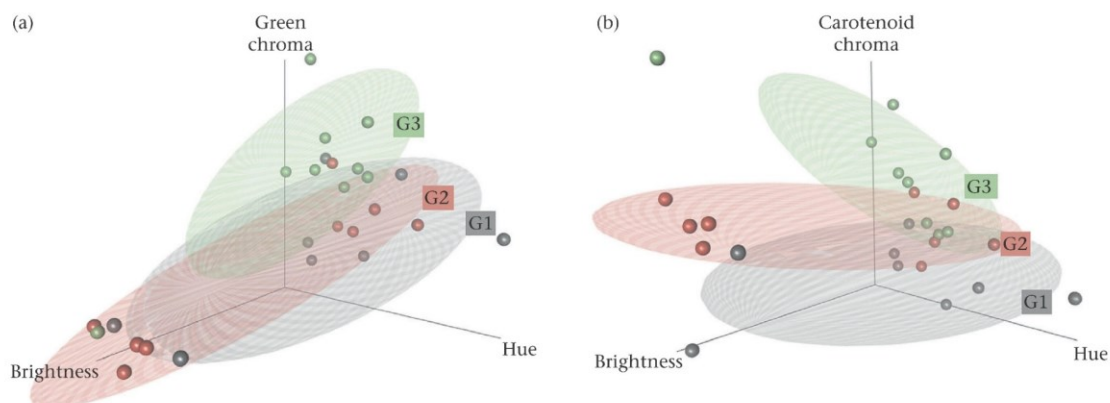


Figure 5. Back colorations of *Rhacophorus nigropalmatus* juveniles after a 20-week carotenoid supplement feeding experiment. Group 1 received no supplements (G1, grey, $N = 8$), group 2 received carotenoid supplements once per week (G2, red, $N = 9$) and group 3 received carotenoid supplements four times per week (G3, green, $N = 9$). Colour space graphs show the chroma parameters (a) green chroma and (b) carotenoid chroma on the y-axis. Circles show individual data points and the boundary of the ellipsoids represents the mean $\pm$ 95% confidence interval of the colour parameters along the three axes.

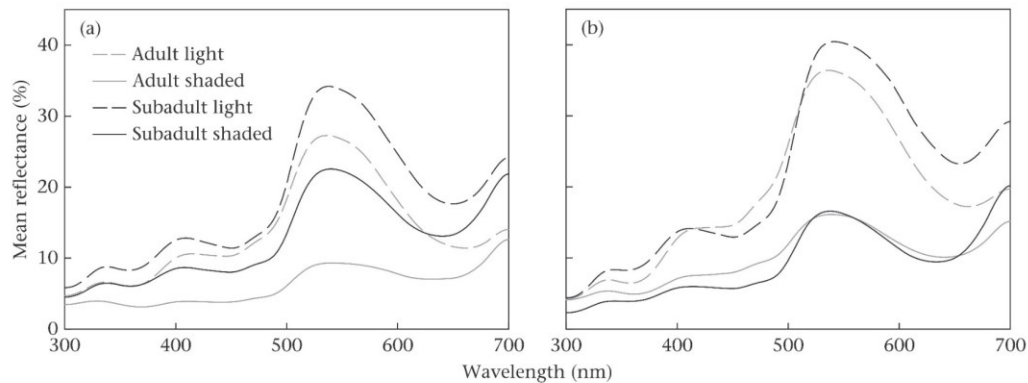


Figure 6. Spectral reflectance of the back of *Rhacophorus nigropalmatus* adults ($N = 6$) and subadults ($N = 6$) during shaded or lit conditions (a) in the entire housing terraria or (b) on the frogs' body when partially covered with a leaf.

findings above, the hue also differed between the two body parts (GLMM: $F_{1,8} = 16.655$, $P = 0.004$). Maximal reflectance in the shade averaged at 538 nm and at 668 nm when exposed to light (pairwise comparison, shade versus exposed: $\beta = 130$, $SE = 31.854$, $t = 4.081$, $P = 0.004$). Shaded body parts showed higher green chroma than those exposed to light (GLMM: $F_{1,8} = 12.565$, $P = 0.008$; pairwise comparison: $\beta = 0.047$, $SE = 0.013$, $t = 3.545$, $P = 0.008$). We also found a dynamic change in brightness (GLMM: $F_{1,8} = 56.013$, $P < 0.001$) in subadults, which is consistent with the light green appearance of individuals when sitting under leaves (Fig. 7). Shaded body parts were brighter than light-exposed body parts (pairwise comparison: $\beta = -4940.764$, $SE = 660.158$, $t = -7.484$, $P < 0.001$), but did not differ in hue or chroma (GLMMs: $P > 0.05$).

DISCUSSION

Rhacophorus nigropalmatus has a striking orange-red coloration in postmetamorphic development. In addition to the conspicuous coloration, several whitish spots form on the juvenile's back. A high carotenoid diet during postmetamorphic development resulted in a faster increase in green colour chroma and higher levels of green and carotenoid chroma of the back coloration (Fig. 5). Less or no carotenoid supplementation led to an increase in UV-blue chroma, contributing to a dull turquoise appearance often observed in captive-bred and captive-raised anurans. The frogs additionally were able to perform dynamic colour changes in response to tactile stress and changing light conditions. Our results demonstrate that a high carotenoid diet facilitates rapid and reversible change in body coloration, an adaptation absent in frogs receiving no carotenoids.

Insects, birds and Old World anthropoids have the ability to see red coloration (Bennett et al., 1994; Gerl & Morris, 2008; Surridge et al., 2003). Therefore, juvenile coloration in *Rh. nigropalmatus* can be perceived by predators and cannot be considered to contribute to camouflage like the cryptic back patterns and stripes observed in other rhacophorids (Biju et al., 2013). Natural selection for crypsis in the dappled light of the understory of primary rainforests should favour dark colours whereas the canopy promotes green body coloration (Endler, 1993a; Théry, 2001). We suggest juvenile coloration could resemble a defence strategy by masquerading as a conspicuous, inanimate part of the environment (Ferreira et al., 2019; Toledo & Haddad, 2009). Several fruit-eating bats, as well as the Asian glossy starling, *Aplonis panayensis*, produce orange-red droppings. We propose that conspicuously coloured juvenile frogs mimic these animal droppings (Toledo & Haddad, 2009), which acts as an antipredator strategy mainly

described for adults of some anurans such as *Theloderma asperum* (Jablonski & Hegner, 2015) and *Dendropsophus marmoratus* (Toledo & Haddad, 2009). White spots in *Rh. nigropalmatus*, only maintained during the early life stage, change the appearance of the frog's body which hence resembles the Asian crab spiders (*Phrynarachne* spp.) commonly known as 'bird dropping crab spiders' (Koh & Bay, 2019). The diurnal, immotile sleeping behaviour of the frogs on leaves and the indistinct body shape with tightly folded extremities reinforces the efficacy of masquerading as animal droppings. Morphological features, such as full webbing and accessory skin flaps, allowing the frogs to glide away and hence survive a predation attempt (Emerson & Koehl, 1990), develop throughout the first year of ontogenesis and are not present in juveniles.

During the carotenoid feeding experiment, all juveniles changed from orange-red to a body coloration similar to that of adults with green backs, yellow flanks and white throats, regardless of the test group (Figs. 1, 4). Carotenoids influenced the temporal occurrence



Figure 7. *Rhacophorus nigropalmatus* subadult after partial shading. Bright-green body part was shaded with a leaf for 1 h; dark-green body part was light exposed.

of colour change and amounts of green and carotenoid chroma in the test groups. A high-carotenoid diet in G3 resulted in a faster increase in green chroma and higher levels of green and carotenoid chroma of the back coloration, whereas less or no carotenoid supplementation led to an increase in UV-blue chroma, contributing to the dull turquoise appearance of G1 (Fig. 4a). Similarly, the yellow flank stripes of G3 showed higher carotenoid chroma than G1. Overall, carotenoids contribute to the green body coloration in anurans (Michaels et al., 2015; Ogilvy et al., 2012) and effectively absorb light at wavelengths of 400–500 nm (blue-green). Green coloration results when light hits the amphibian skin and the iridophores reflect blue wavelengths, which passes through the overlying carotenoid-containing yellow/red xanthophores (Bagnara et al., 1968; Bagnara et al., 1969). Xanthophores also contain pteridines, which are produced by the chromatophores. Together with carotenoids, they are the main factors producing yellow body coloration (Duellman & Trueb, 1986; Steffen & McGraw, 2009; Umbers et al., 2016; Weiss et al., 2012). Our results are consistent with previous studies demonstrating that dietary carotenoids do not affect morphometrics, but increase yellow (e.g. false tomato frog) and green (e.g. red-eyed tree frog) pigmentation in anurans (Brenes-Soto & Dierenfeld, 2014; Ogilvy et al., 2012). Comparisons of the adults' back coloration showed similarities to that of G1 in UV-blue and carotenoid chroma. The adults at the Vienna Zoo were not raised on a high-carotenoid diet and never had the emerald-green appearance observed in wild populations in Borneo (D. Preininger, personal observation). Additional carotenoid supplementation in the adult population started 6 months prior to the reproductive event. This result suggests a paramount importance of carotenoid availability during post-metamorphic development and the likely inability to fully restore ontogenetic deficits in later life stages. Flank colorations are more difficult to interpret in this manner, as the adults' yellow coloration was similar to that of G3. The yellow flanks might constitute a sexually selected colour signal and/or could enhance close distance visual detection and recognition of conspecifics under low light conditions (Gomez et al., 2009; Robertson & Greene, 2017; Taylor et al., 2011). Several species demonstrate preferences for carotenoid pigments (McGraw & Ardia, 2003; Saino et al., 2000) conveying potential information about signaller quality and thereby influencing receivers. Limited carotenoid resources need to be balanced between competing coloration and physiological demands creating a trade-off (Svensson & Wong, 2011). Building on these assumptions, adults displaying degraded body colours would experience predation risks due to camouflage mismatch as a major cost from a limited carotenoid supply, while presumably sexually selected flanks would still favour investment in carotenoid-based signals. While predation risk is negligible in captivity, determining nutritional requirements remains a key element in successful breeding management especially in light of the rising number of ex situ conservation programmes (Jacken et al., 2020). Carotenoid availability in postmetamorphic frogs directly influenced skin colorations and we suggest a carotenoid-based diet contributes to the amphibian's welfare and might further prevent potential fitness consequences of captivity.

The behavioural relevance of dietary carotenoids was evident when we exposed the frogs to a mild stressor. Our results suggest that early life acquisition of carotenoids is essential to mediate the robustness of the adaptive behaviour of rapidly and reversibly changing body coloration in later life stages. The high carotenoid supplement group darkened significantly after handling and tended to recover after a resting period, unlike the other groups. A similar rapid colour change was observed in *R. smaragdinus* changing from green to brown in a few minutes, and reversing colour change after 25 min, a suggested camouflage strategy

observed while handling the individual to obtain photo images (Deepak et al., 2019). Rapid colour changes have also been described in leaf frogs (Phyllomedusidae) and were suggested to act as a defence strategy, by increasing the contrast of the back coloration to the aposematic coloration of the flanks (Machado et al., 2015). In some species of the genus *Hyla*, stress leads to an aggregation of xanthophore pigments, a dispersion of melanin and a decrease in lightness, but epinephrine induces the opposite colour reactions (Nielsen & Dyck, 1978). Melanin and carotenoids are the two pigments responsible for most animal coloration serving several functions including crypsis and protection from sunlight (Alton & Franklin, 2017; Blount & McGraw, 2008; Byron, 1982; Licht & Grant, 1997). While melanin is synthesized within the body from amino acids, carotenoids need to be obtained by ingestion (Felt et al., 2005; Ziegler, 2003). We propose that dietary carotenoids in *Rh. nigropalmatus* are necessary for accumulation and rearrangement of pigments inside skin chromatophores to mediate adaptive dynamic colour changes. Stress responses prompting quick colour change after surviving a predator attack while actively switching light environments by gliding through the forest understory might greatly improve crypsis and predation avoidance.

Further support for dynamic camouflage comes from experimental exposure to 1 h of light and shade conditions which reversibly influenced body coloration of *Rh. nigropalmatus* adults and subadults (Fig. 6). All individuals exhibited brighter backs in shaded conditions compared to lit conditions. Rapid changes in pigmentation in response to environmental light cues suggest that frogs can assess the brightness differences with skin and/or eye pigment cells, to achieve full or partial body colour changes in heterogeneous environments. In this case, light could act as a stimulus for the pituitary to release melanocyte-stimulating hormone, resulting in the dispersion of melanin and thereby elicit dynamic coloration changes (Camargo et al., 1999). In contrast, exact delimitations between coloration differences on the frog's back in response to partial shading suggest that light-sensitive pigment cells in frog skin such as melanopsin control pigmentation (Provencio et al., 1998). Rapid and reversible changes resulting from minor temperature variations are questionable; slight temperature changes could have arisen in our set-up, but the experimental room was maintained at a fairly constant temperature of 25 °C during experiments and thermoregulation has less importance in tropical environments (Duellman, 1999). Pigmentation could also depend on the light regime of the frog's habitats (e.g. understory versus canopy) and environmental conditions, such as temperature or UV radiation (Endler, 1993a; Kang et al., 2017; Théry, 2001). Chromatophores expand and accumulate in response to UV radiation and provide protection from the damaging effects of UV radiation, which is especially beneficial in the canopy (Blaustein & Belden, 2003; Garcia et al., 2004). Skin darkening has also been suggested to result in an increased absorption of UV radiation by the skin and a decreased transmission of UV radiation to deeper tissues (Blaustein & Belden, 2003; Coohill et al., 1970; Coohill & Fingerman, 1975). Studies in several animal taxa have documented the efficiency of a carotenoid-rich diet in photoprotection via the filtering of harmful radiation (Britton, 2008), but experimental studies in anurans addressing this question are scarce.

We suggest that accumulation of skin carotenoids and dispersal of melanin support rapid and reversible skin coloration and act as both dermal defence against UV radiation and camouflage strategy. Dynamic colorations allowed individuals to change their appearance based on differing light conditions and in response to tactile stress. The cellular and/or endocrine control and mechanisms have yet to be explored in detail. Colour-changing species also provide an opportunity to assess how changes in individual

appearance influence predation risk. We should be cautious about drawing conclusions on wild populations from a captive population, but the observed adaptations warrant future studies. Investigations of predation events and visual environments, coupled with measurements of background matching, dynamic colour change and masquerading in the natural habitat could help to explain how differing strategies benefit predator avoidance and might challenge our current view of the adaptive function of coloration in amphibians.

Conclusion

Across several animal taxa, carotenoids are the main components of colour production depending on the individuals' nutrition. Successful husbandry and breeding of amphibians in conservation and research programmes requires knowledge of nutritional demands, which is still scarce and often species specific. Carotenoids are fundamental dietary components and their exclusion in the diet of frogs had effects on natural coloration and the stress response. Pigmentation changes likely contribute to predator avoidance, which is certainly inconsequential in captive populations. Nevertheless, we suggest dull body colorations are the first indication of nutritional deficiencies that could subsequently lead to poor fitness in captive populations. Effectively compensating for a lack of carotenoids during postmetamorphic development might be difficult at later life stages. In our experiments, commercially available carotenoids supplied via gut-loaded feeder insects provided a sufficient diet for frogs to invest in skin pigmentation. However, it remains questionable what can be considered a deficient, adequate or excessive quantity of carotenoids. High amounts of supplements might be harmful for individuals (Svensson & Wong, 2011) or even toxic (Britton, 1995; Burton & Ingold, 1984). There are certainly consequences at both ends of the continuum, and it remains immensely important to understand gradual variations in dietary supplements to reveal what animals need in contrast to what they can tolerate. Such conclusions can only be drawn when investigating dietary components in natural habitats. Considering the current alarmingly rapid species and environmental declines, this research is desperately needed.

Author Contributions

S.S. and D.P. planned the experiments. S.S., S.C. and D.P. carried out the experiments. S.S. and D.P. analysed the results. All authors contributed to the interpretation of the results. S.S. and D.P. wrote the manuscript. All authors provided critical feedback and helped improve the manuscript.

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Appendix

Table A1

Colour parameter comparison between the first and ninth months of ontogenetic development of *Rhacophorus nigropalmatus* ($N = 12$)

Colour parameter		Age	Median	SE	Min	Max	P
Back							
Brightness	Total reflectance	1	3755.37	184.14	2670.49	5280.28	0.46
		9	4880.88	344.56	2888.47	5736.39	
Hue	Maximal slope (nm)	1	584	1	575	588	<0.05
		9	586	14	578	664	
	Maximal reflectance (nm)	1	700	0	700	700	1
		9	700	2	682	700	
Chroma	Carotenoid chroma	1	2.31	0.06	2.06	2.69	1
		9	2.29	0.15	1.72	3.13	
	UV-blue chroma (%)	1	11	0	9	14	1
		9	8	2	4	17	
	Green chroma (%)	1	11	0	8	13	0.33
		9	13	1	9	18	
Flank							
Brightness	Total reflectance	1	4218.34	398.92	3419.03	5626.5	0.09
		9	5159.61	270.81	4492.48	6654.09	
Hue	Maximal slope (nm)	1	560	21	320	589	1
		9	576	4	558	598	
	Maximal reflectance (nm)	1	700	7	621	700	0.11
		9	625	13	621	700	
Chroma	Carotenoid chroma	1	1.5	0.09	0.92	2.21	<0.05
		9	2.13	0.11	1.65	2.73	
	Green chroma (%)	1	13	0	10	17	0.06
		9	11	0.01	8	15	
Throat							
Brightness	Total reflectance	1	5335.34	286.01	4322.09	7213.66	<0.05
		9	9417.51	830.89	6629.8	13467.6	
Hue	Maximal slope (nm)	1	580	34	316	586	0.7
		9	578	2	577	595	
Chroma	Carotenoid chroma	1	1.37	0.07	0.63	1.6	0.44
		9	1.17	0.13	1.09	2.29	
	UV chroma (%)	1	18	1	15	25	<0.05
		9	10	0	9	14	
	Chroma 400–500 nm (%)	1	13	1	11	19	1
		9	12	1	9	19	

The table reports medians, SE and range of absolute measurements. Significance levels (*P*) represent post hoc pairwise comparisons of GLMMs. Values in bold indicate significant differences at $P < 0.05$. Total reflectance and carotenoid chroma are reported as absolute values.

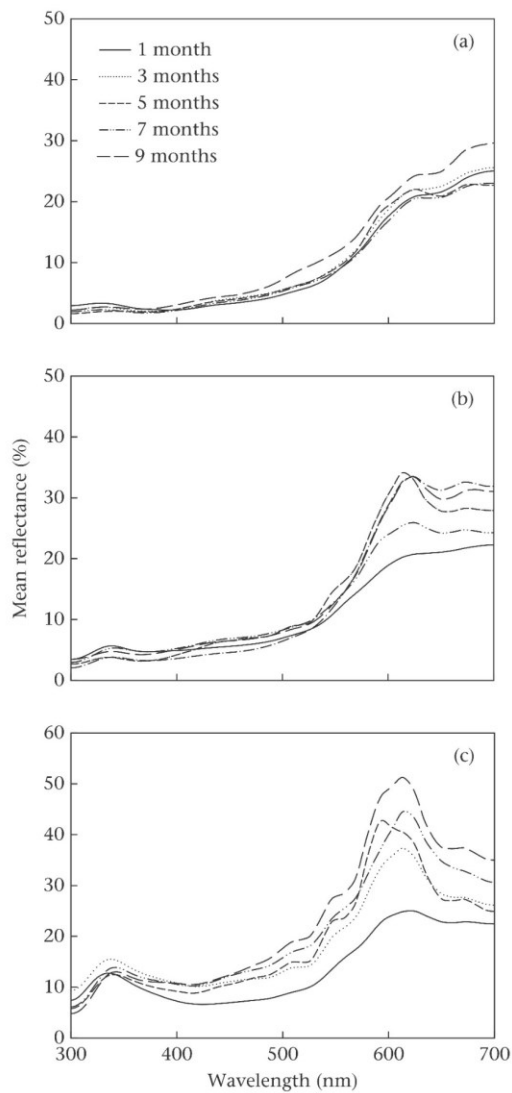


Figure A1. Spectral reflectance comparisons of (a) back, (b) flank and (c) throat of *Rhacophorus nigropalmatus* juveniles at the age of 1, 3, 5, 7 and 9 months.

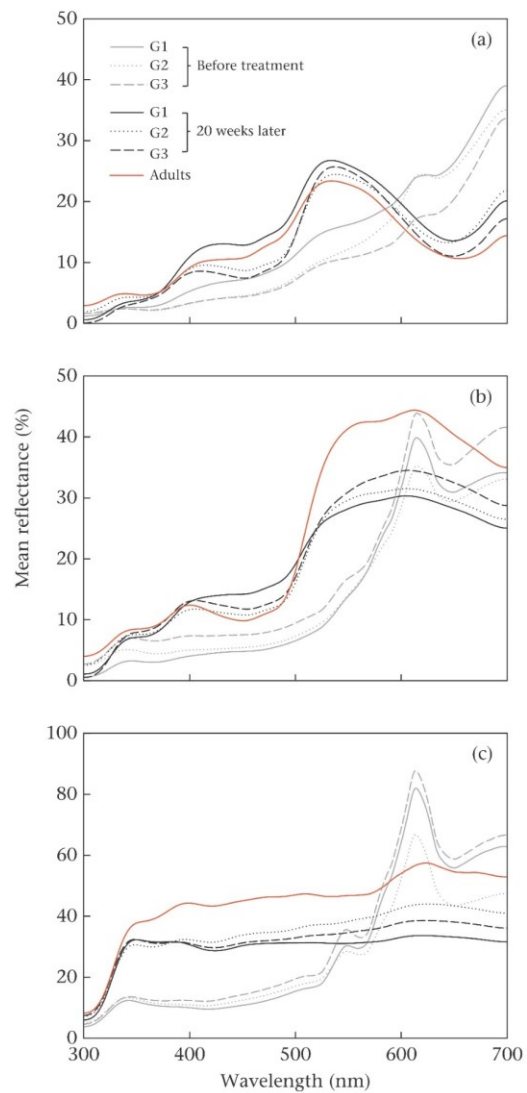


Figure A2. Spectral reflectance of (a) back, (b) flank and (c) throat of *Rhacophorus nigropalmatus* adults and juveniles before and after a 20-week carotenoid supplement feeding experiment. Group 1 received no food supplements (G1, solid line, $N = 8$), group 2 received a carotenoid supplement once per week (G2, dotted line, $N = 9$) and group 3 received a carotenoid supplement four times per week (G3, dashed line, $N = 9$).

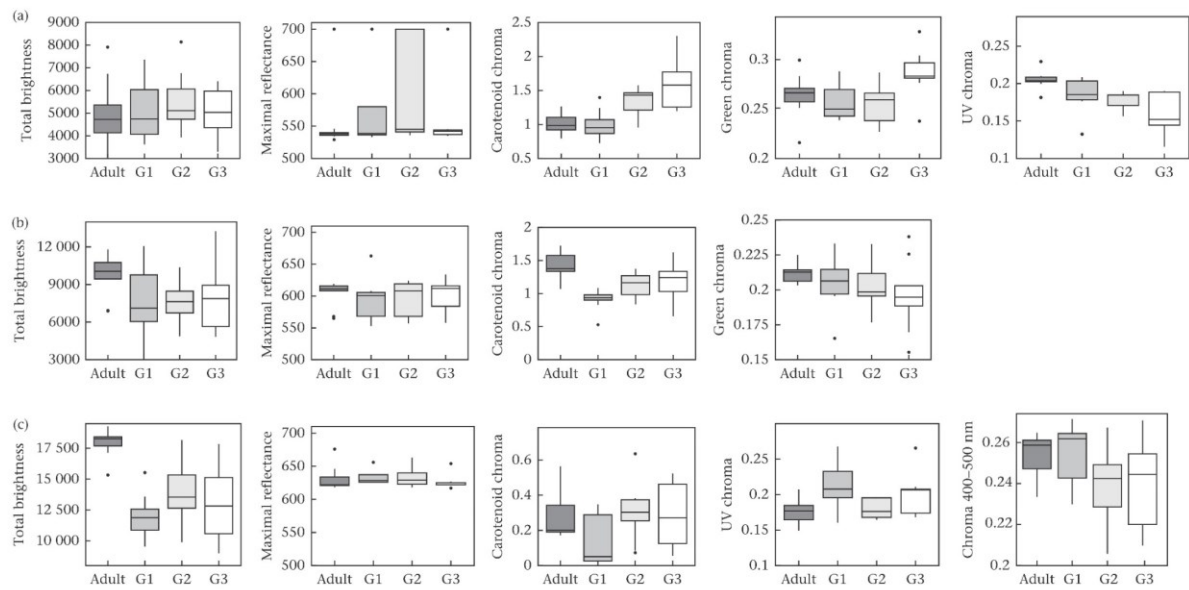


Figure A3. Comparisons of colour parameters of (a) back, (b) flank and (c) throat between *Rhacophorus nigropalmatus* adults and juveniles after a 20-week carotenoid supplement feeding experiment. Group 1 received no supplements (G1, $N = 8$), group 2 received a carotenoid supplement once per week (G2, $N = 9$) and group 3 received a carotenoid supplement four times per week (G3, $N = 9$). Box plots represent median (centre horizontal line), the 25th to the 75th percentile (box), and the 5th and 95th percentiles (whiskers); dots denote outliers (also see Table 1).

Chapter 2

From masquerading to blending in: ontogenetic shifts in antipredator camouflage in Wallace's flying frogs

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From masquerading to blending in: ontogenetic shifts in antipredator camouflage in Wallace's flying frogs

Susanne Stücker¹ · Xavier I. Dawkins² · Matthew J. Fuxjager² · Doris Preininger^{3,4}

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Abstract

A diversity of defence colourations that shift over time provides protection against natural enemies. Adaptations for camouflage depend on an organism's interactions with the natural environment (predators, habitat), which can change ontogenetically. Wallace's flying frogs (*Rhacophorus nigropalmatus*) are cryptic emerald green in their adult life stage, but juveniles are bright red and develop white spots on their back 1 month after metamorphosis. This latter conspicuous visual appearance might function as antipredator strategy, where frogs masquerade as bird or bat droppings so that predators misidentified them as inedible objects. To test this idea, we created different paraffin wax frog models—red with white spots, red without white spots, green, and unpainted—and placed them in equal numbers within a > 800 m² rainforest house at the Vienna Zoo. This environment closely resembles the Bornean rainforest and includes several free-living avian predators of frogs. We observed an overall hit rate of 15.5%. A visual model showed that the contrast of red, green and control models against the background colouration could be discriminated by avian predators, whereas green models had less chromatic difference than red morphs. The attack rate was significantly greater for red but was reduced by half when red models had white spots. The data therefore supports the hypothesis that the juvenile colouration likely acts as a masquerade strategy, disguising frogs as animal droppings which provides similar protection as the cryptic green adult colour. We discuss the ontogenetic colour change as a possible antipredator strategy in relation to the different habitats used at different life stages.

Significance statement

Predation pressure and the evolution of antipredator strategies sit at the cornerstone of animal-behaviour research. Effective antipredator strategies can change in response to different habitats that animals use during different life stages. We study ontogenetic shifts in colour change as dynamic antipredator strategy in juvenile and adult Wallace's flying frogs. We show that the unusual colour pattern of juveniles (bright red with small white spots) likely functions as a masquerade of animal droppings. Specifically, we show that white dotting, which can be associated with animal faeces, acts as the main visual feature that turns an otherwise highly conspicuous individual into a surprisingly camouflaged one. To our knowledge, this is the first experimental exploration of a vertebrate masquerading as animal droppings.

Keywords Avian predator · Background matching · Bird-dropping frog · Ontogenetic colour change · *Rhacophorus nigropalmatus* · Visual model

Introduction

Animal colour patterns are nothing less than spectacular, evolving in diverse ways across the tree of life. To better understand why such colouration arises in the first place, many look to the functions of animal colouration. Some

species, for example, evolve unique combinations of colour that help individuals avoid being eaten by predators (Caro et al. 2016; Cuthill 2019; Postema et al. 2023). The process by which natural selection through predation drives the evolution of these colour traits can be a complex, in that it often involves a host of factors related to other (sometimes opposing) forces of selection (Cuthill et al. 2017; Postema et al. 2023). An important aspect in this regard is an animal's life cycle with different requirements depending on the animals age, size and/or habitat (Endler 1978, 1993).

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Truly effective antipredator strategies may therefore also need to be adapted in light of changing factors during different life stages (Wilson et al. 2007). However, this phenomenon is infrequently studied, particularly in taxa that adopt extraordinary antipredator defence strategies related to colouration (Caro et al. 2016; Duarte et al. 2017; Caro and Koneru 2020).

Camouflage is one of the most common predator defence strategies in the animal kingdom. It refers to instances in which animals adopt morphological and/or behavioural traits that help individuals resemble part of the environment or background, especially when these individuals are most vulnerable to predation (Rojas et al. 2018; Stevens and Ruxton 2018). Most camouflage strategies involve forms of mimesis, or mimicking objects in the environment and altering movements to better appear as those objects. As such, cryptic animals avoid detection from predators by either generally resembling certain objects and/or by background matching a landscape composed of multiple objects (Endler 1981, 1984; Cuthill 2019). For example, many animals have colours and patterns that help individuals look like dominant objects in their environment, or the substrate they use, such as leaves or moss (Toledo and Haddad 2009). This strategy works on the sensory level of the predator, hampering its ability to detect prey items (Skelhorn 2015). Alternatively, other animals have colour patterns that help them look like objects in the environment that are rarer to find, such as bird droppings, or isolated stones (Toledo and Haddad 2009). This form of camouflage is called a masquerade (Endler 1981; Allen and Cooper 1985; Skelhorn et al. 2010a), and sits in contrast to the above outlined background matching strategy, because an animal appears like an inanimate environmental object that is highly conspicuous. The key, however, is that this object is inedible to a predator (Skelhorn et al. 2010b; Skelhorn 2015). A masquerade therefore works at the cognitive level of the predator—in other words, while the predator clearly detects the prey item, it routinely misclassifies the prey as something it cannot (or should not) eat because the object is likely unpalatable or even harmful (Skelhorn 2015). Both camouflage strategies—background matching and masquerade—involve significant decreases in physical movement, with species often sitting perfectly still or move less to maintain the deception (Stevens and Ruxton 2018).

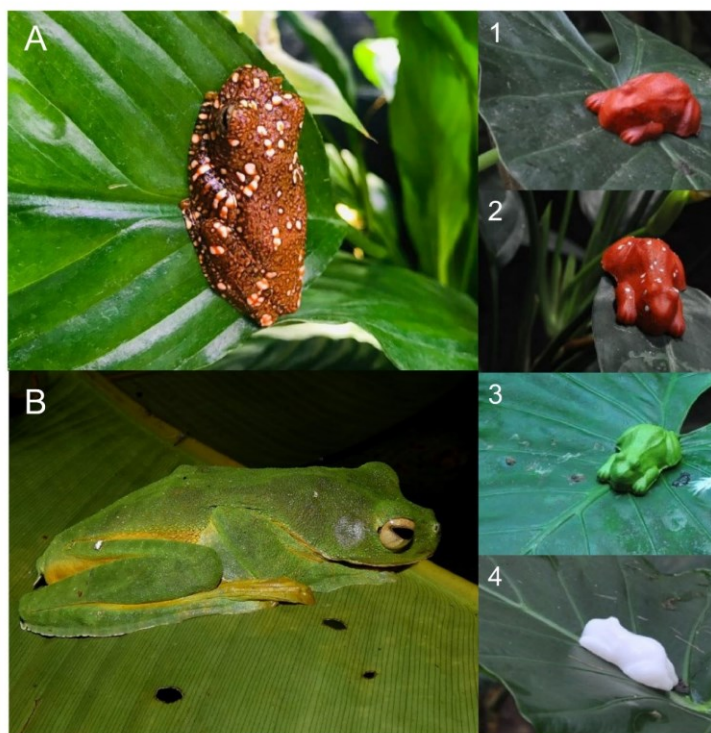
One of the main challenges for camouflage strategies used as predator defence is that they have to meet different colour-matching requirements to provide protection. For instance, certain colour patterns may only be effective antipredator strategies in particular habitats (Endler 1978; Merilaita et al. 2001) or against specific predators (Higginson and Ruxton 2009; Barnett et al. 2018), both of which can change as animals age, grow, and/or inhabit different environments (Endler 1978; Toledo and Haddad 2009). Evolutionarily,

one solution to this challenge is that animals ontogenetically change their camouflage strategies (Booth 1990). Crab spiders (*Phrynarachne ceylonica*), for example, adopt a cryptic strategy when they are young, but then transition into a particular masquerade strategy—mimicking bird droppings—when they age, as the latter strategy is considered more effective for individuals with a larger body size (Yu et al. 2022a, b). Likewise, swallowtail larvae (*Papilio* sp.) switch from a bird-dropping masquerade to a cryptic green appearance with eyespots. In this case, it is hypothesized that body size is crucial for this transition (Postema 2022). To our knowledge, all experimentally tested examples come from invertebrate species (Valkonen et al. 2014; Gaitonde et al. 2018; Postema 2022; Yu et al. 2022a).

Here, we address this gap in the Wallace's flying frog, *Rhacophorus nigropalmatus*. Many Old World tree frogs (Rhacophoridae) change their colouration during ontogenesis, with juveniles typically displaying cryptic back patterns or stripes and adults possessing cryptic green colouration (Biju et al. 2013). In this case, even though there is a change in colouration, the general antipredator strategy to cryptically match the background is the same. By contrast, the body colouration of juvenile Wallace's flying frogs are bright red/orange, with conspicuous white spots that formed on the back during the first weeks after metamorphosis (Fig. 1A). These spots disappear after one year, when individuals mature into adults and develop their iconic emerald green colour (Fig. 1B) (Stückler et al. 2022). For juveniles, the striking colouration is suggested to resemble bird or bat droppings. Indeed, most taxa that mimic bird droppings have white spots, which facilitate imitation and possibly help change the appearance of the animal's body shape (Liu et al. 2014; Valkonen et al. 2014; Yu et al. 2022a, b). All reported species masquerading animal droppings have white spots in common, but to our knowledge studies testing white spots as a common feature to show unpalatability are scarce.

In the current study, we test whether white spots contribute to antipredator defence in frogs living in a rainforest environment. We hypothesize that such spots, which are present only during the conspicuous red juvenile stage, help frogs masquerade as unpalatable objects for predators. Accordingly, we predict that juvenile colour patterns provide camouflage against predators that is similarly effective as the cryptic green colour seen in adult flying frogs. We predict that the models painted in this manner will receive less attacks than the plain red models. Furthermore, because both conspicuous and cryptic camouflage colouration depend on the *i*) spectral environments of the mimic's habitats and *ii*) the visual capability of their predators (Endler and Théry 1996), we also investigated the potential predation risk of different colour signals under semi-natural conditions in the rainforest where we worked. In other words, we modelled

Fig. 1 Resting juvenile (**A**) and adult (**B**) *Rhacophorus nigropalmatus* individuals. Experimental set-up of the wax models (1–4), placed on leaves in the rainforest house of the Vienna Zoo: (1) red model, (2) red model with white spots, (3) green model and (4) unpainted control. Photo (**B**) provided by Kai Squires



how well potential predators could see the different colour morphs presented in our study. We conducted this work in the large rainforest house of the Vienna Zoo, which contains a diverse community of avian predators that are well known to use colour vision to detect frogs as prey (Jacobs 1981; Endler and Théry 1996; Jones et al. 2007).

Methods

Study animals and study site

Rhacophorus nigropalmatus is a relatively large tree frog (males 79–89 mm; females 89–100 mm) distributed in primary lowland rainforests of South East Asia (Inger 1966; Inger and Stuebing 2005). The size of juveniles at the age of 9–11 months ranges from 42 to 52 mm (SS, unpublished data). Adults have full toe webbings and skin flaps on arms and legs, which they use to glide through rainforests (Emerson and Koehl 1990). This species performs a drastic ontogenetic colour change from bright orange-red juveniles to adults with green dorsal colouration (Inger and Stuebing 2005). Additional white spots form on the frog's back 1 month after metamorphosis and reduce 10–13 months later when the frogs change to their adult green colouration (Stückler et al. 2022). Adults live high

up in the canopy (Haas et al. 2022), whereas the juveniles' habitat is so far unknown. However, early life stages likely inhabit the forest understory, which is where foam nests are laid and tadpoles develop in forest pools and puddles (Ready 2009; DP personal observation). Literature about predators of *R. nigropalmatus* is limited, but birds are known predators of other Rhacophoridae species (e.g. *R. pseudomalabaricus* (Vasudevan and Dutta 2000) and *Polypedates otilophus* (O. Konopik personal observation)). We also observed several bird species in the rainforest house attacking frogs (S. Cloer, MJF and DP personal observation). We conducted this study in the > 800 m² large rainforest house at the Vienna Zoo. This enclosure has three levels, with a large cascading waterfall. The rainforest house also contains over 15 bird species (> 150 individuals) endemic to the South-East Asian rainforest (see supplementary material) and that overlap with habitat that includes Wallace's flying frogs. Individuals in the avian community are able to freely move around in the rainforest house at all parts of the day. The rainforest house also inhabits several free ranging Southeast Asian frog species: *Duttaphrynus melanostictus* (Bufonidae), *Ingerophrynus divergens* (Bufonidae), *Polypedates otilophus* (Rhacophoridae), *P. leucomystax* (Rhacophoridae) and *Staurois parvus* (Ranidae). The diet of the birds consisted of daily feedings of fruits (apples, pears, oranges, kiwi, melon,

berries, papaya, mango, figs, grapes etc.), animal products (curd, insects, boiled eggs) and vegetables (lettuce, carrot, cucumber, fennel, celery, paprika etc.).

Experimental design

We tested the idea that the frog's juvenile and adult colourations provide a sufficient antipredator strategy using wax models that were painted to resemble juvenile red colouration with or without spots, adult green colouration, and white (unpainted) controls. Birds in the rainforest house rarely have contact with pure white objects, especially in the area where wax models were placed. Overall, we produced 640 models of identical size (SVL 70 mm) and shape that looked like sleeping/resting flying frogs. These models had an intermediate size between juvenile and adult Wallace's tree frogs, and they were made with paraffin wax (Stafil, Bozen, Italy) casted in silicon moulds (webake, Essex, Great Britain). We painted each model (except for controls) with non-toxic, water-based acrylic colour paint (Daler-Rowney Ltd., Bracknell, United Kingdom), and we carefully mixed the acrylic colours to closely match the available spectral reflectance of juveniles and adult individuals (Stückler et al. 2022) (Figs. 1 and 2). Notably, few studies use spectral measurements of their colour models to verify that they match the right objects/animals (e.g. Postema (2022)); however, the work that does conduct such validation measurements appears to report a colour match between the model and animal that is similar to what we show herein (Dreher et al. 2015).

Data collection

Our experiment ran over the course of 8 days. At the beginning of each day (≈ 0700 h), we attached 80 models (20 models of each colour, see above) to leaves and branches

throughout the rainforest house. On the lowest floor of the facility (which contains a 280 m² patch of forest), we placed 48 models (5.8 m²/model). On the middle floor (which contains a 227 m² patch of forest), we placed 32 models (7.1 m²/model). The different levels of the rainforest house did not influence the attack rates on the different models (LMM: lower level: $1s$ mean = 0.151, SE = 0.055; upper level: $1s$ mean = 0.16, SE = 0.056; $p > 0.05$); therefore, we did not include the levels in further analysis. During our experiment, both levels were closed off to zoo visitors, providing an interference-free study location. Importantly, we first assigned colour models to a given location at random, ensuring only that equal numbers of colour models were represented at a given location at the same time. We collected each model after 24 h in the rainforest house, and we replaced the model with another model of a different colour. In this way, all the different models used in our experiment were displayed in each location. Replacement models were fixed in a nearby location to avoid habituation effects (within 0.5 m of the previous model).

Once the models were collected for the day, we carefully inspected each one for evidence of an attack. If an attack was found, we photographed it and recorded the event as a predation attempt. Bird attacks were identifiable by marks in the wax (Fig. 3) and they were quite common in our experiment. We did not use blind methods but to minimize observer bias, attacks were verified independently by three people each day after collecting the models. Participants in the study routinely noticed birds attempting to attack models, pecking them vigorously on leaves and even the ground. To avoid classifying cockroach bites as predator attacks in our data collection, two wax models were placed for 24 h in a fauna box with different cockroaches (*Periplaneta americana* and *P. australasiae*). The insect marks had the appearance of scratches and could be clearly differentiated from bird attacks.

Fig. 2 Mean spectral reflectance and standard error of 9-month-old juvenile body colouration (orange line, $n=12$), adult body colouration (bright green line, $n=9$) and the corresponding models (red ($n=5$) and green ($n=5$))

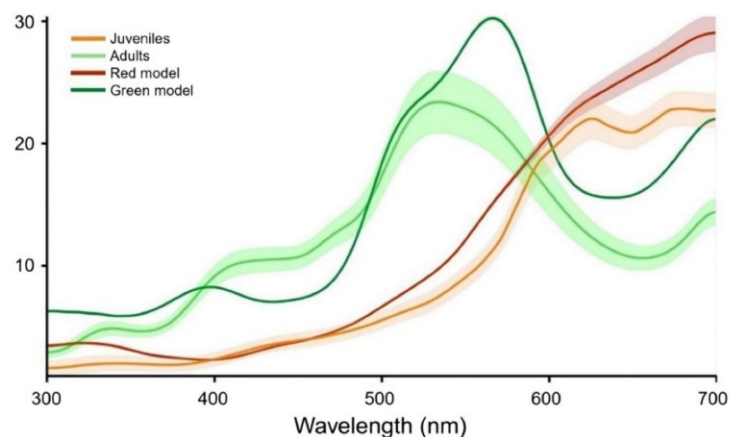


Fig. 3 Examples of observed bird attacks on wax models. Black arrows indicate attack sites



Colour measurements and visual model

To investigate differences in discriminability between different colour models and their background (leaves and branches) for potential predators, we quantified spectral reflectance of models, as well as background and calculated a colour vision model. Background and model colourations were measured with a spectrometer (JAZ series; Ocean Optics, Dunedin, FL, USA), according a standardized protocol (Szatecsny et al. 2010; Stücker et al. 2022). We took five measurements of each colour morph model, 25 measurements of various leaves and 10 measurements of branches that were used as resting positions for models. Colour reflectance of models and background were extracted for 300–700 nm and expressed as percentage relative to a white standard (WS-1 Diffuse Reflectance Standard, Ocean Optics) with the programme Avicol (Gomez 2006). Attack marks on wax models suggested birds as potential predators of *R. nigropalmatus* in the rainforest house; thus, we examined the perceptual capability of birds to discriminate among red, green and white (control) models relative to the leaf or branch background.

Next, we used the programme R (package PAVO) to model a bird's visual system (Maia et al. 2019). As the receptor sensitivity of bird species inhabiting the rainforest house are unknown, we used the visual system of the Eurasian Blue Tit, *Cyanistes caeruleus*—available in PAVO. Note that past work suggests that resulting small error in the estimation of receptor sensitivities do not seriously affect model results (Lind and Kelber 2009; Bitton et al. 2017). For the analysis, we used the function 'vismodel' to calculate quantum catches of each photoreceptor for the visual system with the following arguments (Guidi et al. 2021): visual=bluetit, illum=forestshade, trans=bluetit, scale=1, relative=false; the remaining arguments were set to the default setting.

To calculate chromatic discriminability between colouration differences, we used a well-established receptor-noise model that calculates colour differences or estimates of discriminability expressed as just-noticeable-differences (chromatic JND: $JND_{(ds)}$). These estimates are based on the relative

photoreceptor densities, and thus account for receiver (predator) sensory capabilities (Vorobyev and Osorio 1998). JND values of 1 represent the threshold of predictably noticeable discriminability between two colour patches. JNDs higher than 1 provide increased distance in the colour space, and thus proportionally increased discernibility and discrimination (Igic et al. 2012; Chaves-Acuña et al. 2020). JND values < 1 indicate that two colours remain visually indistinguishable (Vorobyev et al. 2001; Siddiqi et al. 2004). We calculated JNDs with the function 'coldist' of the R package PAVO comparing the models' colouration against the background colouration with the following arguments (Guidi et al. 2021): subset=null, achromatic=false, $n=1, 1.9, 2.7, 2.7$ (Hart et al. 2000), weber=0.1, weber.ref=longest, weber.achro=false; the remaining arguments were set to default settings.

Data analysis

Statistical analyses were conducted in R (R Studio Team 2021). First, we classified each frog model as either "hit" or "not hit", with several predation attempts on one model were counted as one attack. Then, to test how rates of attack varied among the colour models, we compared hits or no-hits between colour morphs with Fisher's exact test and a generalized linear model ('glm'), using a binomial error distribution and log-link function. We used 'lsmeans' package to further evaluate differences in attack rates between models, implementing least-square means pairwise comparisons alongside Tukey p -value adjustment for multiple comparisons (Lenth 2016). Hit or no-hit were entered as the dependent variable, whereas colour morphs were entered as the predictor variable.

To test the discriminability differences of the model morphs against the background, we performed a generalized linear mixed model (GLMM) on JNDs. Chromatic differences were entered as the dependent variables, colour morph as the predictor variable. Multiple measurements of models (red, green and control) and background (leaves and branches) were entered as random variables. For post-hoc tests, we used Student's t tests, with sequential Bonferroni correction for alpha.

Results

Avian predators detected and attacked the wax models resembling resting Wallace's flying frogs. Of the 640 models that we placed into the rainforest house, we found that 99 were attacked. This resulted in an overall attack rate of 15.5%, which is on par or higher than attack rates reported in studies that use a similar approach (Hegna et al. 2011; Preißler and Pröhl 2017). The higher attack rate compared to other frog model studies (Stuart et al. 2012; Lawrence et al. 2019) can be explained by predator density of the study site, with over 150 free ranging birds in an area of 800 m².

Our analyses showed that the odds of attack differed significantly among the colour models (Fisher's exact test; $p < 0.001$; GLM: $p < 0.01$; Supplementary Table 1). Post-hoc analyses revealed that red models received the highest attack rates, compared to all other colours (ls means: red vs. red-spotted: $p = 0.001$; red vs. green: $p = 0.003$, red vs. control: $p < 0.001$; Fig. 4A; Supplementary Table 2). Interestingly, there were no differences in the odds of attack between red models with white spots and emerald green models (ls means: red-spotted vs. green: $p > 0.05$). White control models received the lowest number of attacks compared to all other groups (ls means: control vs. red: $p < 0.001$; control vs. red-spotted: $p = 0.026$; control vs. green: $p = 0.01$; Fig. 4A; Supplementary Table 2, 3).

Visual model

Our analyses of how models are perceived in the rainforest also showed significant effects. Specifically, the contrast of red, green and control models against the background colouration—as viewed by potential bird predators—differed significantly (GLMM: $F_{5,444} = 127.945$, $p < 0.001$; Fig. 4B). Although all colour morphs could be discriminated against the background by bird predators (i.e. JND values were above 1.0; results reported as mean $\pm$ SE), green models (leaves: $dS = 9.004 \pm 1.022$; branches: $dS = 5.434 \pm 1.23$) showed less chromatic difference than either red models (leaves: $dS = 17.511 \pm 1.022$; GLMM pairwise comparison: $\beta = -8.507$, $SE = 0.376$, $t = -22.628$, $p < 0.001$; branches: $dS = 13.046 \pm 1.23$; GLMM pairwise comparison: $\beta = -7.613$, $SE = 0.464$, $t = -16.397$, $p < 0.001$) or white control models (leaves: $dS = 11.363 \pm 1.022$; GLMM pairwise comparison: $\beta = -2.359$, $SE = 0.376$, $t = -6.273$, $p < 0.001$; branches: $dS = 9.989 \pm 1.23$; GLMM pairwise comparison: $\beta = -4.555$, $SE = 0.464$, $t = -9.812$, $p < 0.001$). Green models were similarly discriminable against both backgrounds (leaves and branches) (GLMM: pairwise comparisons: $p > 0.05$). Moreover, we found that red models had a higher discernibility against the

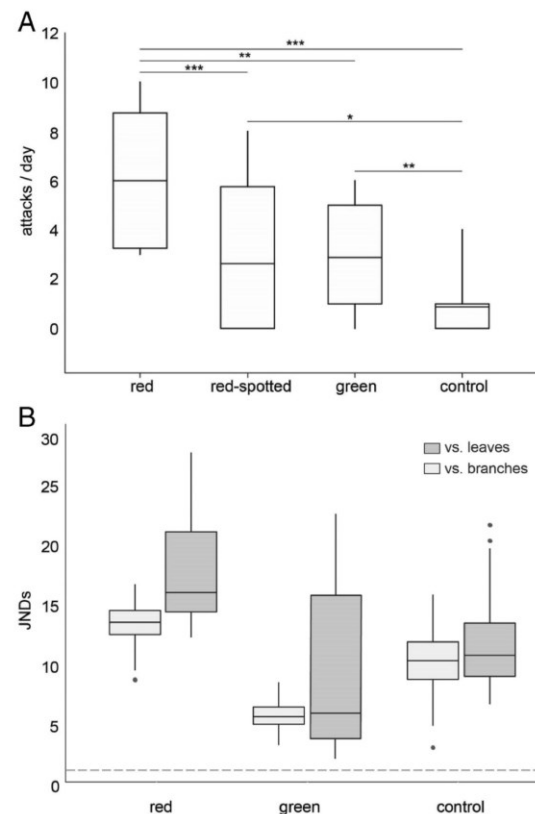


Fig. 4 Comparisons of **A** bird attacks on four different frog models (red, red-spotted, green and control) and **B** chromatic contrast (expressed as just-noticeable differences (JNDs)) of red ($n = 5$), green ($n = 5$) and white (control, $n = 5$) models against background (leaves: $n = 25$; branches: $n = 10$), as perceived by birds (spectral sensitivity of Eurasian Blue Tit, *Cyanistes caeruleus*). Boxplots show the median, the 25th to the 75th percentile, and the 5th and 95th percentiles. Asterisk indicate significant differences between models from GLM pairwise comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Supplementary Table 3). Dashed line = minimum threshold of discrimination (JND = 1)

respective background compared to white control models (leaves: GLMM pairwise comparison: $\beta = 6.149$, $SE = 0.376$, $t = 16.355$, $p < 0.001$; branches: GLMM pairwise comparison: $\beta = 3.057$, $SE = 0.464$, $t = 6.585$, $p < 0.001$; Fig. 4B).

Discussion

Here, we show that the unusual colour pattern of juvenile Wallace's tree frogs—bright red with small white spots—likely functions as an antipredator strategy. Frog models painted in this manner are attacked significantly less often

than identical red models without spots. In fact, attack rates on the red-spotted models were as low as those for the emerald green models, which corresponds to the colour that allows adult males to cryptically blend into the upper rainforest canopy (Endler 1993; Gomez and Théry 2007). Importantly, we verified that avian predators likely see all the models in our study (Fig. 4B). Hence, predators were able to detect all colour models against the background on which they perched, suggesting that the white spots may have mediated a misclassification of the prey as a potentially inedible object. Moreover, it seems that red models are especially conspicuous to avian predators, as the JND values exceed such values associated with both emerald green models and white control models. If anything, this implies that red juveniles (with or without white spots) likely stand out to potential predators. Interestingly, the white control models received the lowest number of attacks. The birds do not have contact with plain white objects in the rainforest house. The avoidance of white models could reflect a general neophobia towards an unfamiliar new object, or that white colour is commonly perceived as a warning colour (Lecuelle et al. 2011; Cibulková et al. 2014). Together, our findings therefore suggest that *i*) red colouration with white spots confers antipredator benefits to juvenile Wallace's flying frogs and that *ii*) the effectiveness of this strategy is on par with crypsis camouflage strategies in adults. We are therefore seeing evidence for two distinct—but equally successful—antipredator defence strategies that unfold ontogenetically as frogs mature after metamorphosis.

A masquerade for antipredator defence

We hypothesize that juvenile frogs appear bright red with white spots because this colour pattern functions as a masquerade for antipredator defence. Specifically, we suspect that the combination of colour and its aesthetic appearance make young frogs closely resemble bird droppings. There are several reasons for this conclusion. The first is that white spots are a common characteristic of supposed masquerade strategies, in which individuals are thought to appear as animal droppings. Examples include orb web spiders (*Cyclosa ginnaga*), which decorate their webs with white shapes (Liu et al. 2014), and the moth *Acronicta alni*, which displays white body parts during its early larval stage (Valkonen et al. 2014). The second reason for our conclusion is that many birds and bats defecate faeces that are red with white spots, particularly fruit eating animals (Vander Wall et al. 2005) (Supplementary Fig. 1). It therefore makes sense that other birds would avoid eating food items that resemble such faeces, because the latter material would likely be unpalatable.

With these considerations in mind, it is worth pointing out that our study is the first experimental exploration of a bird/animal-dropping masquerade in anurans, although the

validation in the field is missing. To this end, relatively few studies (Liu et al. 2014; Suzuki and Sakurai 2015; Yu et al. 2022a), rigorously test how conspicuous colour patterns function as animal dropping masquerade. We specifically tested the importance of the white spots on the frogs' conspicuous red body, and we find that these seemingly subtle white markings reduce the rate of attack from predators by roughly 50%. Thus, this innocuous white patterning associated with animal faeces acts as the main visual feature that turns an otherwise highly conspicuous individual into something perceived as unpalatable.

We suggest red juvenile colours with white spots might not rely on the predator's detection abilities (vision), but rather how it centrally processes its search image. In other words, the white spots may cause predators to interpret or classify the frog as an inedible item, as the spots are associated with animal faeces. The predator's search image may neglect certain prey or features to improve foraging efficiency and effectiveness. Such prey need not resemble their 'model' accurately to create an effective masquerade, since predators more often misclassify masquerading prey when the learning template is rare or when the masquerade is ubiquitous (Skelhorn and Ruxton 2010; Skelhorn 2015). For adults, green colouration likely helps them to match the background of their surroundings in the rainforest canopy, one of many other tactics for antipredator defence. For example, adult Wallace's tree frogs also have accessory skin flaps on arms and legs, large webbings between toes and a bent arm and leg position, which they use to glide (or 'fly') from the canopy to avoid being attacked by a predator (Emerson and Koehl 1990; Brodie et al. 1991). This behaviour also improves migration (Emerson and Koehl 1990) and is energetically less costly than hopping down the trees (Stewart 1985).

We recognize that other aspects of the colour phenotype may influence its ability to match the environment. For example, it is possible that the white spots of juveniles show some degree of UV reflectance to mirror animal droppings more closely; additionally adult frogs are able to dynamically change their green colouration depending on light conditions (Stückler et al. 2022). Differences in body size between juveniles and adults could also potentially enhance or limit the predation risk. We do not test these ideas in our current study, but it is something that could be looked at in future studies.

Ontogenetic shifts in antipredator strategies

Our study strongly suggests that Wallace's flying frogs experience a major developmental shift in antipredator camouflage strategy. Studies revealing such dynamism in antipredator strategy in frogs are scarce. Even if we look to other nocturnal tree frogs that inhabit the South and Southeast

Asian rainforest, there is little evidence of masquerading as a major antipredator defence strategy. Indeed, species of the genus *Theloderma* sp. are often cryptic, except for *T. asperum*, which is commonly known as ‘bird poop frog’ (but no studies tested this hypothesis). This observations is consistent with broader work in anurans that finds that most nocturnal tree frogs similarly rely on crypsis as the primary way to hide from predators (Toledo and Haddad 2009; Rojas 2016). To this end, it is also worth highlighting that the ontogenetic transition in camouflage we discover is exceptionally uncommon. Most shifts in camouflage occur in the direction of crypsis to conspicuousness, with the latter state being closely associated with aposematism (Grant 2007; Valkonen et al. 2014). This specific order of events is likely the result of normal growth and its negative effect on crypsis (i.e. it is more difficult to stay hidden in plain sites when you are bigger). To our knowledge, there are very few species like the Wallace’s tree frog, which transition from a conspicuous form of camouflage to a cryptic one. Examples only come from a species of cross frog (*Oreophryne ezra*), where juveniles are suggested to use a chemical defence strategy or Batesian mimicry before turning cryptic later in life (Bulbert et al. 2018).

Why would flying frogs change their camouflage strategy as they mature? None of the behavioural and morphological adaptations of the adults are present in the juvenile stage and only slowly develop during the first year of development. We assume that until flying frogs change their colouration to a dynamic cryptic green and develop morphological adaptations for gliding, they spend their early life stage in the forest understory. In this habitat, masquerading as bird droppings might be a reliable way of gaining protection from predators, as bird droppings are probably more common in the lower areas of rainforests. However, little information is available about where juvenile flying frogs live during their first year after metamorphosis once they leave the forest pools, and we assume the frog’s habitat changes considerably during their transition to adulthood (Ready 2009). Certainly, adults are known to inhabit the canopy of primary forests (Inger and Stuebing 2005; Haas et al. 2022), even if they periodically use the understory for foraging and reproduction. A common feature of all life stages of the nocturnal flying frogs is an immobile sleeping behaviour on leaves during the day and an indistinct shape with appendages closely folded to the body, a behavioural trait that might further contribute to both forms of camouflage—masquerading and crypsis—during ontogeny. We suggest that ontogenetic shifts in morphology, behaviour and colouration might be driven by environmental and natural selection factors the species experiences in different habitats they utilize at different life stages. Future studies exploring the habitats and predators of juveniles and adults are necessary to investigate the main drivers of the ontogenetic colour change in the Wallace’s flying frog.

Conclusions

Here, we suggest that the Wallace’s flying frogs undergo a major ontogenetic shift in camouflage strategy—as juveniles they likely masquerade as red bird droppings by appearing bright red with white spots, but then they transition to an emerald green colour to background match with foliage high in the forest canopy. Importantly, we show that the white spots during this early life stage are a critical component to the camouflage strategy, turning what is a highly conspicuous body colour (bright red) into a disguise. There are few examples of ontogenetic changes to camouflage, particularly in the order we show (masquerade to crypsis). These findings therefore make the Wallace’s flying frog a unique example of how animals might change their antipredator defences as they age and move to new or different environments.

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Data availability The dataset is available from the Dryad Digital Repository: <https://datadryad.org/stash/share/-FtwruOZwIkklspC0Kxzy45EnPrmVW8MAOPlzTP3ICQ>.

Declarations

Ethics approval All experiments reported in this article comply with the current laws of the country in which they were performed.

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Susanne Stückler¹  · Xavier I. Dawkins²  · Matthew J. Fuxjager²  · Doris Preininger^{3,4} 

✉ Susanne Stückler
susanne.stueckler@univie.ac.at

¹ Department of Behavioral and Cognitive Biology, University of Vienna, Vienna, Austria

² Department of Ecology, Evolution and Organismal Biology, Brown University, Providence, RI, USA

³ Vienna Zoo, Vienna, Austria

⁴ Department of Evolutionary Biology, University of Vienna, Vienna, Austria

Supplementary material

Supplementary methods

Free-ranging bird species in the rainforest house

White-breasted woodswallow (*Artamus leucorhynchus*), Nicobar pigeon (*Caloenas nicobarica*), Orange-bellied leafbird (*Chloropsis hardwickii*), Pied imperial pigeon (*Ducula bicolor*), Black bulbul (*Hypsipetes leucocephalus*), White-rumped Shama (*Kittacincla malabarica*), Blue-crowned hanging parrot (*Loriculus galgulus*), Pheasant pigeon (*Otidiphaps nobilis*), Java sparrow (*Padda oryzivora*), Blue-winged pitta (*Pitta moluccensis*), Beautiful fruit dove (*Ptilinopus pulchellus*), Black-capped bulbul (*Pynconotus melanicterus*), Crested partridge (*Rollulus rouloul*), Spotted dove (*Streptopelia chinensis*), Indian white-eye (*Zosterops palpebrosus*).

Supplementary results

Supplementary Table 1 Generalized Linear Model differences of number of bird attacks on different model frogs

	Estimate	SE	z	p
Intercept	-3.085	0.387	-7.982	< 0.001 ***
Green	1.3	0.447	2.906	0.0037 **
Red	2.237	0.423	5.286	< 0.001 ***
Red-spotted	1.195	0.452	2.644	0.0082 **

Supplementary Table 2 Pairwise comparisons between attacks on colour models from Tukey adjusted least squares means

contrast	Estimate	SE	z	p
control vs green	-0.1	0.0321	-3.115	0.0099
control vs red	-0.2562	0.0397	-6.459	< 0.001
control vs red-spotted	-0.0875	0.0312	-2.804	0.026
green vs red	-0.1562	0.0456	-3.425	0.0034
green vs red-spotted	0.0125	0.0385	0.325	0.9882
red vs red-spotted	0.1688	0.045	3.75	0.001

Supplementary Table 3 Least squares means (lsmeans) of attacks on coloured model frogs; lsmeans and standard error (SE) was back-transformed

	lsmean	SE	Lower CI	Upper CI
Control	0.0437	0.0162	0.0121	0.0754
Green	0.1437	0.0277	0.0894	0.1981
Red	0.3	0.0362	0.229	0.371
Red-spotted	0.1313	0.0267	0.0789	0.1836



Supplementary figure 1 Animal droppings in the rainforest house of the Vienna Zoo.

Chapter 3

Evidence that catecholaminergic systems mediate dynamic colour change during explosive breeding events in toads

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Author for correspondence:

Susanne Stückler

e-mail: susanne.stueckler@univie.ac.at

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Animal behaviour

Evidence that catecholaminergic systems mediate dynamic colour change during explosive breeding events in toads

Susanne Stückler¹, Matthew J. Fuxjager² and Doris Preininger^{3,4}

¹Department of Cognitive Biology, University of Vienna, Austria

²Department of Ecology, Evolution and Organismal Biology, Brown University, Providence, RI, USA

³Department of Evolutionary Biology, University of Vienna, Austria

⁴Vienna Zoo, 1130 Vienna, Austria

SS, 0000-0003-1903-3171; MJF, 0000-0003-0591-6854

Many animals communicate by rapidly (within minutes or seconds) changing their body coloration; however, we know little about the physiology of this behaviour. Here we study how catecholaminergic hormones regulate rapid colour change in explosive breeding toads (*Duttaphrynus melanostictus*), where large groups of males gather and quickly change their colour from brown to bright yellow during reproduction. We find that both epinephrine (EP) and/or norepinephrine (NE) cause the toads' skin to become yellow in minutes, even in the absence of social and environmental cues associated with explosive breeding. We hypothesize that natural selection drives the evolution of rapid colour change by co-opting the functional effects of catecholaminergic action. If so, then hormones involved in 'fight or flight' responses may mechanistically facilitate the emergence of dynamic visual signals that mediate communication in a sexual context.

1. Introduction

Many species rapidly and reversibly change their body colour [1–4]. Iconic examples include cephalopods and chameleons [4], which alter their colours within seconds to provide instant camouflage, signal conspecifics [5], court mates [6] or display aggressive motivation [7]. Yet, functions of rapid colour change are often better studied than the physiological mechanisms that support it [8]. While past work implicates a variety of hormonal and/or neuronal systems that help mediate quick changes in body coloration patterns [1], the precise nature of these systems remains unclear.

In at least 178 anurans, males use rapid colour change (dynamic dichromatism) to mediate socio-sexual interactions during the breeding season [9]. This behaviour is especially important for species that engage in explosive breeding, where all sexually receptive individuals arrive synchronously at a spawning site, and breeding takes place over a period of a few days. Explosive breeding events are characterized by male-biased sex ratios, with intense competition among males, who then scramble to access female mates. Males consequently experience low rates of reproductive success, whereas females experience relatively high rates of mortality [10]. When dynamic colour change precedes explosive breeding, it therefore likely regulates sexual recognition by helping males more easily distinguish each other from females [11–13]. In this way, we might consider sexual dichromatism that occurs prior to explosive breeding events an adaptive strategy that facilitates the speedy acquisition (and subsequent loss) of a signal for intense male–male competition [9]. If this is true, however, then mechanisms must evolve to facilitate colour change in anticipation of reproduction.

For most species, sudden and dramatic changes to the environment trigger explosive breeding, including the onset of monsoon rains and fluctuations in temperature, humidity, or barometric pressure [14]. These environmental perturbations—particularly when paired with increases in social stress—can engage animal stress systems [15–17], including the highly conserved stress systems that mediate adaptive ‘fight or flight’ responses [18]. This latter system involves the release of the catecholamine hormones epinephrine (EP) and norepinephrine (NE) [19], which both act to modify an organism’s physiology to better contend with stressors. Importantly, catecholamines also influence skin colour, as these hormones’ receptors are present on most chromatophores [20]. Such effects can occur within minutes of catecholamine release [21,22], resulting in either skin lighting [23,24] or darkening [25,26]. We hypothesize that natural selection drives the evolution of rapid colour change during explosive breeding events by co-opting the functional effects of catecholaminergic hormone action, which originally arose to mediate stress responsiveness. We, therefore, expect that (1) colour change is a by-product or direct effect of a stress response triggered by cues associated with the onset of explosive breeding events, and (2) stress-related EP and/or NE pathways help activate rapid colour change even in the absence of social stimulation (which might be required later on to maintain colour change). We examine this idea in Asian common toads (*Duttaphrynus melanostictus*), where males change from an inconspicuous brown (similar to females) to bright yellow immediately prior to the formation of explosive breeding aggregations. This behaviour is triggered by monsoon rains, and causes greater than 200 individuals to arrive synchronously at spawning sites during the daytime. We, therefore, test for the effects of EP and NE on toad body coloration over a 12-h period at the Vienna Zoo, as well as whether these colour changes are sufficient for receiver discrimination.

2. Methods

The Vienna Zoo houses semi-free ranging Asian common toads in the rainforest house (greater than 1000 m²). We caught 25 males from this population and placed them in four terraria (100 × 50 × 50 cm) equipped with hiding structures, water bowls, and humid and dry zones (mean temperature: 25 ± 1°C; mean humidity 74 ± 17%). Toads were housed under a 12-h light/dark cycle, and were fed with vitaminized crickets every second day.

On three separate experimental sessions, we examined the effect of EP and NE treatment on skin coloration (from December 2020 to February 2021). In each session, we randomly assigned 15 males to three different treatment groups (EP, NE or control; $n = 5$ per group). Body size (mean/s.d. = 49.05 ± 0.83 mm, range = 40.19–58.42 mm, $n = 25$) and weight (mean/s.d. = 17.31 ± 0.91 g, range = 11.17–29.05 g, $n = 25$) were similar among each treatment group of each session (Kruskal–Wallis: size: $K = 14.351$, $p = 0.0731$; weight: $K = 10.402$, $p = 0.238$), suggesting similar age. We allowed four weeks to pass between sessions, as this time exceeds an adequate period that ensures all catecholamines have cleared from the toads’ circulation [27–29].

Each experimental session consisted of two parts: (1) priming with human chorionic gonadotropin (hCG) and (2) treatment with different catecholamines or saline solution (control). Before part 1, we measured the toads’ size (SVL, mm), weight (g) and back reflectance (baseline 1). Subsequently, we primed each toad with a single injection of 30 µl hCG (Sigma-Aldrich CG10-1VL; 20 IU g⁻¹ bodyweight in saline solution) and placed them in their

respective fauna box (20 × 19 × 20 cm) for a period of 5 h. hCG is a common exogenous reproductive hormone, widely used to induce reproduction and breeding behaviour in amphibians [30,31]. To verify the impact of hCG injections on body coloration, we visually checked the individuals body coloration hourly. Pigmentation changes between the toad’s general brown and the conspicuous nuptial bright yellow coloration are highly noticeable to human observers and easy to assess. After 5 h, we again performed reflectance measurements of all individuals (baseline 2) followed by the respective EP, NE or saline treatments via 50 µl intraperitoneal injection in the dorsal lymph sac. The test groups were administered a dose of (1) 50 µl EP (Sigma-Aldrich E4250; 1.8 µg g⁻¹ bodyweight), (2) 50 µl NE (Sigma-Aldrich A7256; 0.4 µg g⁻¹ bodyweight), or (3) 50 µl saline (0.9% NaCl) as a control. Drug dosages were chosen according to the weight of the species based on prior studies in fish and amphibians [21,22, 32,33]. In all experiments, we minimized handling time to less than 5 min to reduce unintended stress effects on circulating levels of catecholamines.

Back coloration was measured 30, 60, 120, 180 min and 12 h after treatment (electronic supplementary material, figure S1) with a spectrometer (JAZ series; Ocean Optics, Dunedin, USA) according a standardized protocol. We used Avicol v6 software [34] to extract brightness, hue and chroma measures—the essential parameters to describe coloration from reflectance spectra (see electronic supplementary material) [35].

Significant effects of our treatments on the colour variables do not necessarily imply that these effects can be perceived by conspecific rivals or mating partners. Thus, we additionally quantified whether spectral differences could be detectable by toads using a colour vision model with the package ‘pavo’ [36]. We thereby examined the ability of toads to discriminate among untreated females ($n = 15$), control group males, EP and NE treated males, 30 min after the treatment (see electronic supplementary material).

Statistical analyses were performed with the program R (v. 3.4.2; R Studio Team 2021) and SPSS 26 (IBM SPSS Statistics, USA). To test the impact of hCG on body coloration, we compared colour parameters of baseline 1 and 2 using generalized linear mixed models (GLMMs) with normal distribution, identity link function and Student’s t statistic with sequential Bonferroni correction for *post-hoc* tests. The colour parameters were entered as dependent variables, with measurement point (baseline 1 or 2) as predictor variables and the identities of male and session as random variables to correct for repeated measurements. Next, we visually determined colour change from brown to yellow (yes/no) and used Fisher’s exact test (with sequential Bonferroni correction) to test for differences in the odds individuals change colour among treatment groups. To understand what colour parameters (brightness, hue or chroma) determine yellow coloration, we selected measurements of males that were affected by respective hormone treatments (EP, NE) and compared them to the control with GLMMs. The colour parameters were entered as dependent variables, with treatment, measurement point (30, 60, 90, 120 min, 12 h) and treatment × measurement point as predictor variables and male and session as random variables. Finally, to test conspecific discriminability we performed a GLMM on just-noticeable differences (JNDs) 30 min after treatment. Chromatic and achromatic differences were entered as dependent variables, treatment as predictor variable and identities of males and females as random variables.

3. Results

Priming males with hCG had no effect on coloration (GLMM (B1 versus B2): brightness: $F_{1,88} = 1.084$, $p = 0.301$; hue: $F_{1,88} = 0.512$, $p = 0.476$; chroma: $F_{1,88} = 0.665$, $p = 0.417$; electronic

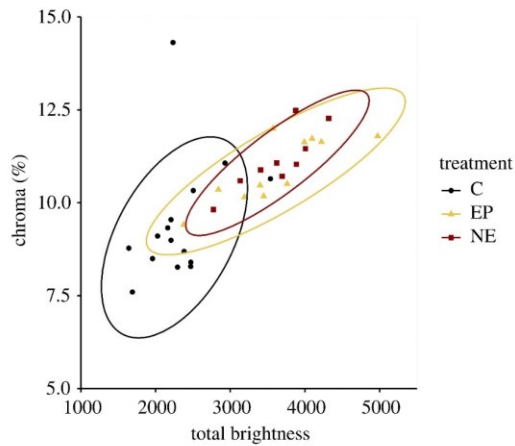


Figure 1. Back coloration of males 30 min after EP/NE and control treatment. Compared are colour parameters of the control ($n = 15$), EP ($n = 11$) and NE ($n = 9$). Symbols denote individual data points, while ellipsoid boundaries represent mean $\pm$ 95% confidence interval of colour parameters.

supplementary material, figure S2). By contrast, catecholamine administration triggered significant changes in colour (Fisher's exact test: $p < 0.001$), with the most visible changes occurring 1.5–2 cm around the injection site. Compared to controls, toads receiving EP and NE were brighter yellow (GLMM: $F_{2,160} = 20.428$, $p < 0.001$) and had higher yellow saturation (GLMM: $F_{2,160} = 34.802$, $p < 0.001$; figure 1, electronic supplementary material, table S1). EP/NE had no effect on hue (GLMM: $F_{2,160} = 1.552$, $p = 0.215$; electronic supplementary material, figure S3, S4). Notably, this colour change is similar to that which we see in wild populations of Asian common toads, as individuals migrate to breeding sites.

We next assessed contrast among females, brown control males, and hormone-treated yellow males, as viewed by conspecifics (figure 2). Dynamic yellow coloration shows high discernibility in contrast to brown control males ($F_{2,521} = 7.895$, $p < 0.001$; EP versus control: $p = 0.003$; NE versus control: $p < 0.001$). When looking at JND values (GLMM estimated means $\pm$ SE), yellow provides sufficient contrast for discrimination between brown control males (EP: $dS = 3.022 \pm 0.621$; $dL = 11.552 \pm 1.965$; NE: $dS = 3.328 \pm 0.622$; $dL = 11.998 \pm 1.967$) and females (EP: $dS = 3.768 \pm 0.494$; $dL = 8.833 \pm 1.474$; NE: $dS = 4.196 \pm 0.494$; $dL = 8.812 \pm 1.477$) in chromatic and achromatic conditions.

4. Discussion

Using Asian common toads, we show that catecholaminergic hormones EP and NE help regulate rapid colour change. Reproductively primed males given either hormone increase their skin brightness and yellow colour saturation for at least 1 h, whereas reproductive priming itself (via hCG) has no such effect. This indicates that catecholamine action is sufficient to trigger changes in skin colour from dull brown to bright yellow, which likely mediates socio-sexual communication during explosive breeding events [11,37].

Work in other anuran species similarly shows that catecholamines induce rapid colour change [21,23–26]. However, these studies show that such changes occur *after* individuals engage

in sociosexual interactions (e.g. amplexus), suggesting that conspecific encounters are important for hormonal effects on colour to manifest. Our results indicate that this is not the case in Asian common toads; rather, EP and NE change male colour in the absence of any sociosexual interaction. The difference in these results is critical because it implies that stimuli unrelated to male–male and/or male–female conspecific encounters can start off the process of rapid colour change. Our results, therefore, point to the idea that sexual selection exploited (or co-opted) systems that govern adaptive catecholaminergic responses to environmental changes [38–40] to help facilitate evolution of rapid colour change as a display phenomenon. Such responses may have stemmed from abiotic stressors associated with the onset of explosive breeding, such as monsoon rains and/or sudden drops in barometric pressure. Factors, like social stimuli from male–male encounters, are also likely involved (see below). Our captive samples provide first directions, but further experiments—in free-living toads—are needed to more thoroughly pinpoint precise combinations of factors that elicit dynamic colour and underlie its evolution.

Equally importantly is that our results suggest that conspecifics can perceive EP- and NE-mediated changes to toad skin colour. This conclusion comes from results showing that colour difference between treated (yellow) and control (brown) animals are sufficient for conspecific discrimination. Indeed, toads visual systems are capable of colour discrimination through trichromatic colour vision [41], even under low light conditions [42]. Work will be needed to verify this finding in field settings to better establish how individuals navigate complex breeding environments.

Colour change in *D. melanostictus* works exclusively via large melanophores in the sub-epidermis, as individuals have no other chromatophores (xanthophores, erythrophores) [43]. In other anurans, dispersion of yellow pigments in xanthophores and aggregation of dark pigments in melanophores underlies yellow and orange colour [21,22,44]. But without xanthophores, yellow coloration in our toads can only be produced by pigment movement in melanophores. Thus, catecholamines potentially mediate colour change by promoting the aggregation of melanin pigments [8]. This idea is consistent with our findings that EP and NE have no effects on hue, since yellow and brown are virtually the same in this regard [45]. We should, however, acknowledge that our treated individuals only displayed colour changes around the injection site, and some individuals did not change body coloration. As indicated above, this suggests that other factors are likely involved in mediating rapid colour change in our study species; for instance, endocrine regulators such as corticosterone [46], melanophore-stimulating hormone [47,48], and androgenic steroids [44,49] may play a role. This latter hormone is especially intriguing, given that it underlies ‘male-typical’ reproductive behaviour [50] and influence colour in several anurans [44,49]. Similarly, physical and social cues associated with explosive breeding events may interact with catecholamine action to induce a more complete change in male colour.

In sum, our results show that EP and NE help mediate the production of bright yellow coloration in Asian common toads. This finding supports the idea that selection can co-opt catecholaminergic hormone systems to help individuals quickly adopt a discernible colour to optimize behavioural interactions. Mechanisms of stress responsivity may therefore be exploited by evolutionary forces for effective

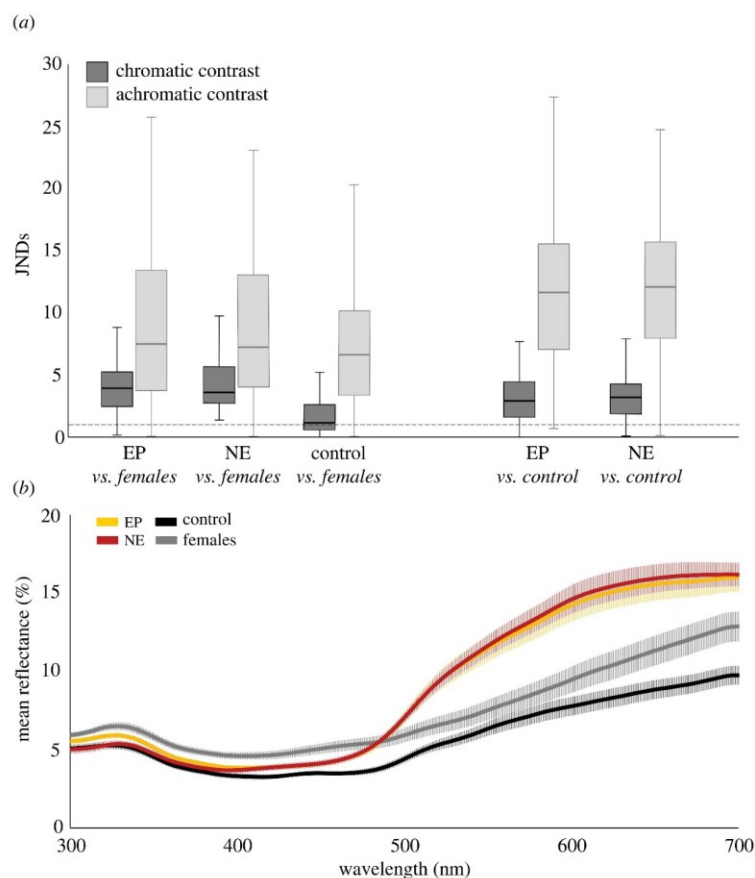


Figure 2. Colour differences of male (30-min post-treatment) and female toads. (a) Chromatic and achromatic contrast (expressed as just-noticeable differences (JNDs) of males [control, epinephrine (EP) and norepinephrine groups (NE)] against female body coloration, and contrast of males (EP and NE group) against control group coloration, as perceived by toads (spectral sensitivity of *Bufo bufo*). Dashed line = minimum threshold of discrimination (JND = 1). (b) Mean spectral reflectance (including standard error) of back coloration of male (control: $n = 15$; EP: $n = 11$; NE: $n = 9$) and female toads ($n = 15$).

communication in reproductive situations characterized by intense male–male competition for mates.

Ethics. The study was approved by the Austrian animal experimentation act (TVG 2012, BMBWF, 2020-0.547.761), and it complies with all current laws of the country in which it was performed (Austria). We followed the guidelines of the Association for the Study of Animal Behaviour [41]. Study animals originated from a population bred and raised in the rainforest house of the Vienna Zoo (Zoological Information Management System, GAN: MLJ20-02606).

Data accessibility. The dataset is available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.dbrv15f4b> [51].

The data are provided in the electronic supplementary material [52].

Authors' contributions. S.S.: data curation, formal analysis, funding acquisition, investigation, software, visualization, writing—original draft, writing—review and editing; M.J.F.: conceptualization, methodology,

validation, writing—original draft, writing—review and editing; D.P.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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Supplementary material

Supplementary methods

Colour analysis

We used Avicol v6 software (Gomez 2006) to extract brightness, hue, and chroma measures – the essential parameters or attributes to describe colouration from reflectance spectra. Hue corresponds to the everyday meaning of colour and was calculated as maximal reflectance searched in the range 300–700 nm (Endler 1990; Gomez 2006; Gomez et al. 2009). Brightness corresponds to colour lightness or darkness (total reflectance) and is calculated as the surface area under the spectral curve. Chroma is the concentration of the reflectance around a wavelength, and it corresponds to colour saturation, or colourfulness. To describe colour changes from brown to yellow, we analysed chroma in the wavelength range for the spectral location of yellow, which we computed as the proportion of the total reflectance ranging from 560–590 nm ($R_{(560-590\text{nm})}/R_{(300-700\text{nm})}$) (Dimmick and Hubbard 1939; Gomez 2006; Hall et al. 2010; Long et al. 2010; Sétamou et al. 2014).

Visual modelling and colour discrimination

Significant effects of our treatments on the colour variables do not necessarily imply that these effects can be perceived by conspecific rivals or mating partners. Thus, we additionally quantified whether spectral differences could be detectable by toads using a colour vision model and then calculating the discriminability between different body colourations. We thereby examined the ability of toads to discriminate among untreated females (n=15), control group males (n=15), and EP (n=11) and NE (n=9) treated males. As spectral sensitivities are not available for our study species, we modelled the toads' visual system by using a user-defined model with the spectral sensitivity of the Common toad (*Bufo bufo*) (Yovanovich et al. 2017; Yovanovich et al. 2019). Resulting small errors in the estimation of receptor sensitivities do not seriously affect model results (Lind and Kelber 2009; Bitton et al. 2017). For the analysis, we used the function 'vismodel' of the R package PAVO to calculate quantum catches of each photoreceptor for the visual system (Maia et al. 2013). To compute the contrast in the toads' visual space for daylight, the lighting conditions were defined as 'D65', which corresponds to standard daylight conditions (Siddiqi et al. 2004; Green et al. 2019; Chaves-Acuña et al. 2020). We considered the longest photoreceptor (LWS cone) being responsible for achromatic vision (Siddiqi et al. 2004; Gödert et al. 2021).

To calculate chromatic and achromatic discriminability between colouration differences, we used a well-established receptor-noise model which calculates colour

differences or estimates of discriminability expressed as just-noticeable-differences (JND; chromatic: $JND_{(dS)}$; achromatic: $JND_{(dL)}$) based on the relative photoreceptor densities (Vorobyev and Osorio 1998). JND values of 1 represent the threshold of predictably noticeable discriminability between the toad's body colours. JNDs higher than 1 provide increased distance in the colour space and proportionally increased discernibility and discrimination (Igic et al. 2012; Chaves-Acuña et al. 2020). JND values < 1 provide not noticeable contrast and colour of conspecifics remain visually indistinguishable (Vorobyev et al. 2001; Siddiqi et al. 2004). We calculated JNDs with the function 'coldist' of the R package PAVO comparing females against control males, EP- and NE-treated males, and control males against EP- and NE-treated males measured 30 min post-administration. For the chromatic and achromatic calculations, the photoreceptor proportion was set to 3 : 17 for the two cone types, derived from the frog species *Xenopus laevis*, which have circa 15% BS and 85% RS cones (Wilhelm and Gábel 1999); the Weber fraction was set to 0.05 following Siddiqi et al. 2004, which assume this threshold for frogs as an intermediate value between humans and birds (Vorobyev and Osorio 1998; Siddiqi et al. 2004).

Supplementary results

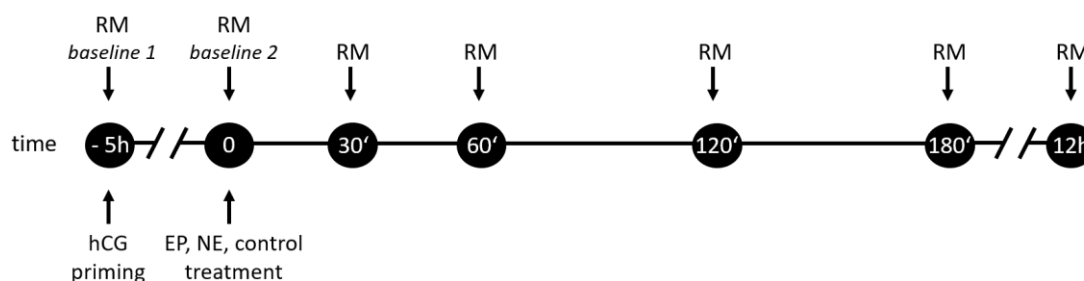


Figure S1. Schematic timeline of experimental sessions, indicating human Chorionic Gonadotropin (hCG) priming, epinephrine (EP), norepinephrine (NE) and control treatment and back reflectance measurements (RM).

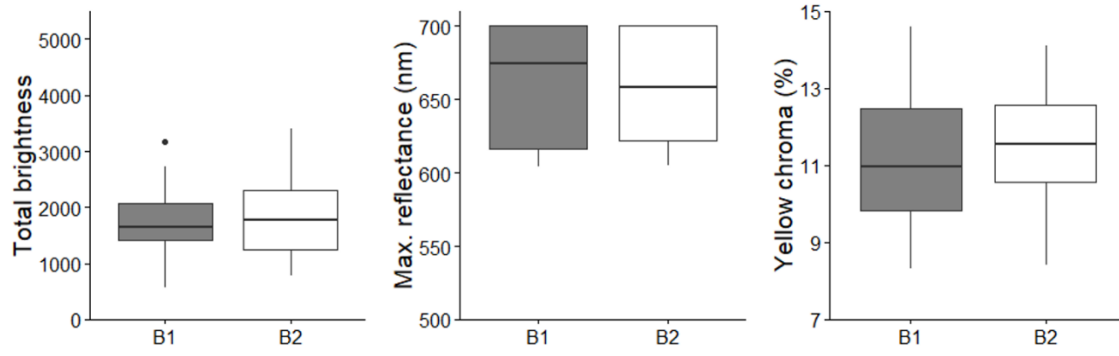


Figure S2. Comparisons of colour parameters before [baseline 1 (B1), n=45] and after [baseline 2 (B2), n=45] priming with human Chorionic Gonadotropin (hCG) of *Duttaphrynus melanostictus*. The individuals received a single injection of 30 μ l saline and hCG (20 IU/g bodyweight). Boxplots represent median and percentiles of absolute measurements; dots denote outliers. Total brightness is given in absolute values, maximal reflectance in nm and yellow chroma in %.

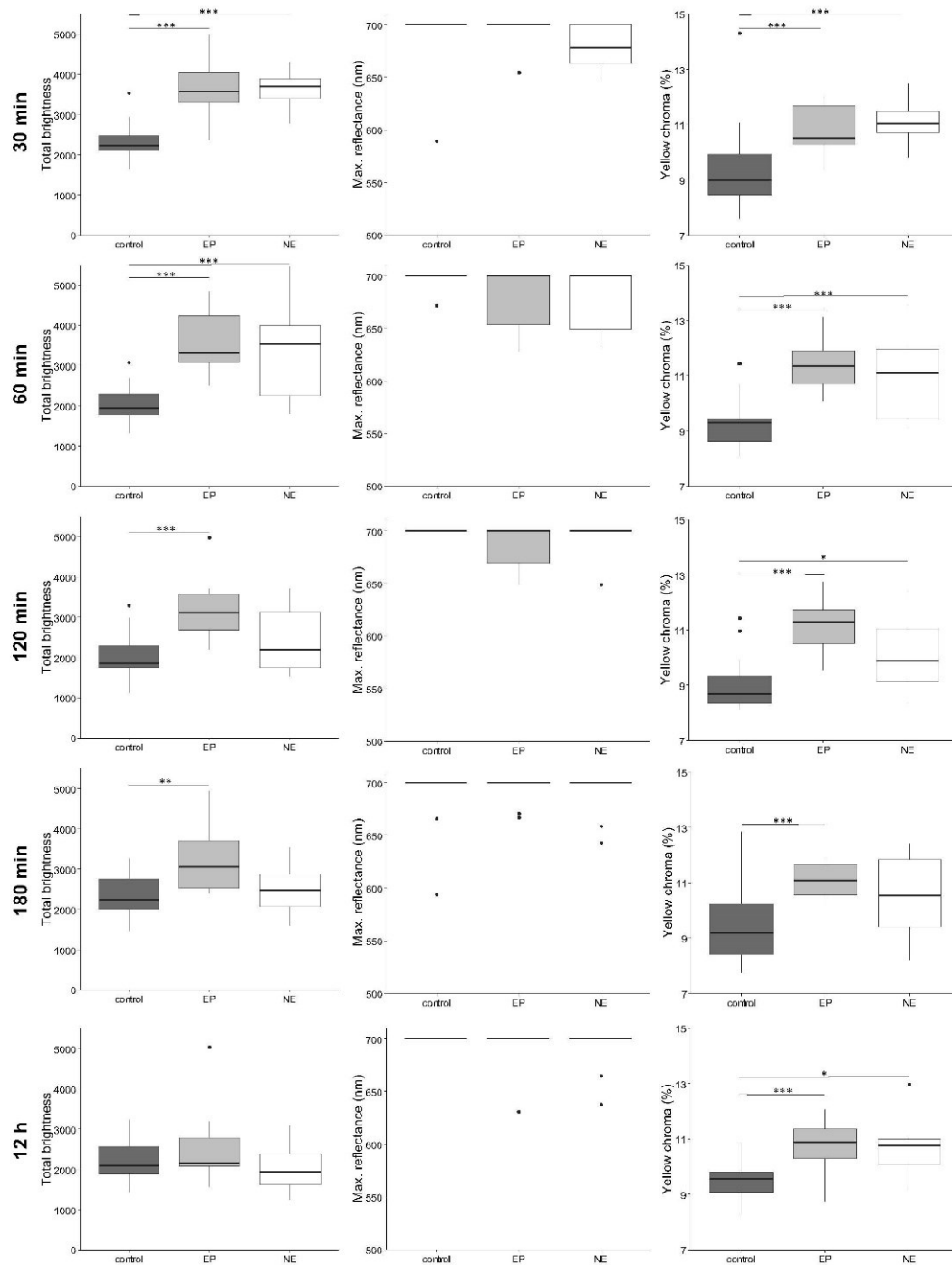


Figure S3. Comparisons between respective colour parameters of *Duttaphrynus melanostictus* males 30 min, 60 min, 120 min, 180 min and 12 hours after treatment. Test groups received either saline solution (control, $n=15$), epinephrine (EP, $n=11$) or norepinephrine (NE, $n=9$). Boxplots represent means and percentiles of absolute measurements; dots denote outliers (also see supplementary material, table S1). Asterisk indicate significant differences between test groups from GLMMs (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

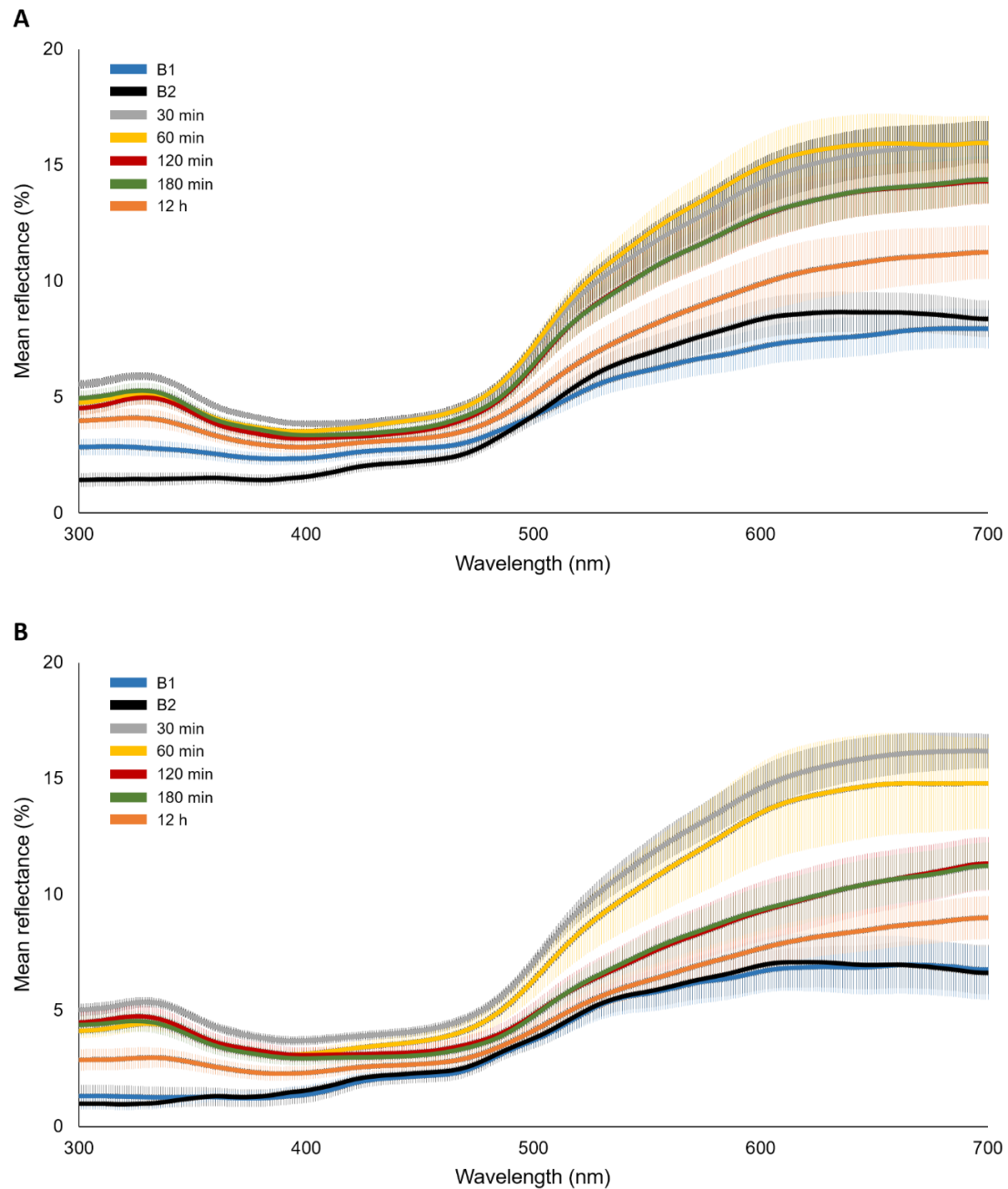


Figure S4. Mean reflectance spectra including standard error of male *Duttaphrynus melanostictus* back colouration before hormone treatment [baseline 1 (B1)], after the human Chorionic Gonadotropin (hCG) treatment [baseline 2 (B2)], and after 30, 60, 120, 180 min and 12 hours after catecholamine treatment [panel A – epinephrine (EP), n=11; panel B – norepinephrine (NE), n=9].

Table S1. Comparison of body colouration of *Duttaphrynus melanostictus* males 30, 60, 120, 180 min and 12 hours after hormone [epinephrine (EP) and norepinephrine (NE)] or control treatment (saline solution). Reported are means and range. Total brightness results are reported as absolute values.

30 min	Total brightness	Max. reflectance (nm)	Yellow chroma (%)
EP	3624 (2375–4974)	692 (654–700)	11 (9–12)
NE	3635 (2774–4319)	678 (646–700)	11 (10–12)
Control	2313 (1642–3538)	693 (589–700)	9 (8–14)
60 min	Total brightness	Max. reflectance (nm)	Yellow chroma (%)
EP	3636 (2511–4847)	680 (628–700)	11 (10–13)
NE	3273 (1804–5470)	675 (632–700)	11 (9 – 14)
Control	2041 (1322–3081)	696 (671–700)	9 (8–11)
120 min	Total brightness	Max. reflectance (nm)	Yellow chroma (%)
EP	3215 (2205–4978)	685 (649–700)	11 (10–13)
NE	2526 (1527–3705)	694 (649–700)	10 (8–12)
Control	2076 (1123–3297)	700 (700–700)	9 (8–11)
180 min	Total brightness	Max. reflectance (nm)	Yellow chroma (%)
EP	3255 (2396–4944)	694 (667–700)	11 (10–12)
NE	2498 (1587–3527)	689 (643–700)	10 (8–12)
Control	2398 (1475–3267)	691 (594–700)	9 (8–13)
12 h	Total brightness	Max. reflectance (nm)	Yellow chroma (%)
EP	2572 (1558–5037)	694 (631–700)	11 (9–12)
NE	2024 (1253–3089)	689 (638–700)	11 (9–13)
Control	2197 (1443–3238)	700 (700–700)	9 (8–11)

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Chapter 4

Dynamic colour change promotes rapid mate recognition in an explosive breeding toad

This chapter is a developed manuscript ready for submission.

Citation:

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Dynamic colour change promotes rapid mate recognition in an explosive breeding toad

Susanne Stücker¹, Evan Donnachie², Matthew J. Fuxjager², KV Gururaja³, Gopalkrishna Hegde⁴, Thomas Wampula⁵, Doris Preininger^{5,6}

¹ Department of Behavioral & Cognitive Biology, University of Vienna, Djerassiplatz 1, 1030 Vienna

² Department of Ecology, Evolution and Organismal Biology, Brown University, Providence, RI, 02912

³ Srishti Manipal Institute of Art, Design and Technology, Yelahanka, Bengalore 560 064, India

⁴ no affiliation. Honnepam, Bare Post-581 337, Karnataka, India

⁵ Vienna Zoo, Maxingstraße 13b, 1130 Vienna

⁶ Department of Evolutionary Biology, University of Vienna, Djerassiplatz 1, 1030 Vienna

Abstract

In some anuran species (frogs and toads), males rapidly change their body colour during brief mating aggregations. In such explosive breeding events, many males actively search and compete for a limited number of females. Male Asian common toads (*Duttaphrynus melanostictus*) change body colouration from brown to a conspicuous bright yellow and from a mechanistic perspective, we know that stress hormones are able to elicit this rapid colour change. We hypothesize that male yellow colour acts as visual signal facilitating mate recognition in dense breeding aggregations. First, we explored whether colour differences are sufficient to be perceived and distinguished by the toads using a colour vision model. In behavioural experiments, we tested the interactions of male toads with a yellow 3D model (resembling a breeding male) and a brown 3D model (resembling a female). Then we compared male body colour, morphological parameters and body condition of mated and non-mated males to investigate if colouration explains mating success in their natural habitat during breeding. Our results show that dynamic yellow colouration displayed by males during mating can be easily discriminated against brown female conspecifics from a toad's point of view. During model experiments, males had twice as much physical contact with brown models and clasped them 40 times more often than yellow models. Colouration and several morphological traits did not differ between mated and non-mated males, hence mating success was not influenced by variations in colour or morphology. We discuss that dynamic sexual dichromatism in the Asian Common toad has evolved as a visual signal mediating fast mate recognition and solves a common problem in time-limited, dense breeding aggregations by minimizing male mismatch and optimizing mate finding.

Key words: amphibians; Asian common toad; *Duttaphrynus melanostictus*; sexual dichromatism; visual signal; 3D model experiment

Introduction

Rapid, dynamic colour change is widespread across several animal taxa, including cephalopods (Mäthger et al. 2009), fish (Sköld et al. 2013), reptiles (Ligon and McGraw 2018) and amphibians (Kindermann and Hero 2016). It can be described as a quick and reversible adaption of body colouration for thermoregulation, UV protection, predator avoidance, or sexual communication (Hutton et al. 2015). In some species, only one sex rapidly changes body colouration. This phenomenon is known as dynamic sexual dichromatism, and in these cases colour change is mostly used as visual signal to communicate with conspecifics (Bell and Zamudio 2012), as for example male panther chameleons (*Furcifer pardalis*) during courtship interactions (Herrel et al. 2020). This specific type of sexual dichromatism is rare, mostly observed in cephalopods (Hall and Hanlon 2002) and anurans (frogs and toads) (Doucet and Mennill 2009). In the latter, dynamic sexual dichromatism mainly occurs in explosive breeding species (Bell et al. 2017), e.g., *Hoplobatrachus tigerinus* (Dicroglossidae), *Rhinella granulosa* (Bufonidae), *Scinax ruber* (Hylidae). During short breeding events, males display bright conspicuous body colours compared to the dull brown they exhibit around the year (Bell et al. 2017).

In explosive mating systems, individuals of one or several species aggregate to breed during limited and ecologically preferable time windows. In aggregate breeders, hundreds of individuals come together to mate and compete; mating partners are not exclusive, and thus individuals vigorously fight for limited mating opportunities. Explosive breeding takes place synchronously over a period of a few days where males search for a limited number of females (Emlen and Oring 1977; Wells 2007). Scrambling competition for females result in a low reproductive success rate for males and limited female choice (Sullivan et al. 1995; Wells 2007). The increased individual density and limited time window to breed lead to high intrasexual competition (Wells 2007; Vojar et al. 2015). Successful reproduction during explosive breeding events becomes even more difficult when multiple species gather at the same pond and breed simultaneously (Gottsberger and Gruber 2004;

Ulloa et al. 2019). Acoustic overlap of calling males, along with both biotic and abiotic background noise (e.g., rain) confounds species and mate recognition (Gerhardt and Klump 1988; Gerhardt and Bee 2006). Under such breeding conditions, we assume that selection favours strategies which facilitate rapid mate recognition, mate acquisition, and avoid mismatch between males. For example, in some species, males displace and attack other males in amplexus and mostly larger males are more successful, also males arriving earlier at the breeding pond are more successful to secure a female (Wells 2007). To avoid mismatch, males of some species emit release calls when clasped by other males (Yu and Sharma 2012) or display a striking dynamic dichromatism (Sztatecsny et al. 2012).

Pioneering work on dynamic sexual dichromatism from Sztatecsny et al. (2012) in the Moor frog (*Rana arvalis*) and Rehberg-Besler et al. (2015) in the Neotropical yellow toad (*Incilius luetkenii*) show that males distinguish males and females based on body colouration during dense explosive breeding aggregations. In the temperate *R. arvalis*, males turn blue during their short reproductive season lasting around one week (Brehm 1893). The conspicuous blue body colouration provides instant mate recognition and improves mating opportunities, but colourations are not related to body condition or morphology (Sztatecsny et al. 2012). However, mating experiments with different sized frogs showed that larger and heavier Moor frogs were able to secure a mate (Rausch et al. 2014). Male morphology (thick forearms and large nuptial pads) also influenced male mating success in Columbia Spotted frogs (*Rana luteiventris*) and allowed securing a female in an extreme scramble mating system (Greene and Funk 2009). In the tropical *I. luetkenii* males turn yellow during breeding but fade to brown as soon as they are in amplexus with a female (Doucet and Mennill 2009). Male bright and conspicuous colours displayed only for a few days facilitate mate recognition but body condition of single males does not relate to brightness (Gardner et al. 2023) and female Neotropical Yellow toads do not choose males based on body colouration (Gardner et al. 2021). Dynamic sexual dichromatism has been documented in at least 75 species that are aggregate breeders, but only few studies investigate its behavioural function in explosive breeders (Bell et al. 2017).

Effective communication is a key aspect of reproductive success in these mating systems (Wells 2007), thus we hypothesize that rapid colour change evolved multiple times as functional colour signal, a dynamic visual solution to increase mating opportunities and rapidly find a mating partner in highly competitive explosive mating systems.

We study this idea in a population of the Asian common toad, *Duttaphrynus melanostictus* under natural conditions in Karnataka, India. The species is distributed worldwide, with only some populations exhibiting dynamic colour change. Those that display dichromatism also form explosive mating aggregations (Fig. 1). In these populations, males change body colouration from inconspicuous brown – similar to females – to bright yellow during the short breeding season (personal observations of the authors). We know that stress hormones (catecholamines) mediate the production of bright yellow colourations in *D. melanostictus* (Stückler et al. 2022); however, the functional significance of sexual selected, dynamic dichromatism in males is still unknown.

Here we examine the visual signal function of male yellow colouration during reproduction. We predict that sexual dynamic dichromatism in male Asian common toads acts as visual signal to facilitate mate recognition (similar to *R. arvalis* (Sztatecsny et al. 2012) or *I. luetkenii* (Rehberg-Besler et al. 2015)). In light of the highly competitive environment and the fact that females are amplexed shortly after or upon arriving at the breeding site, we predict that females have few options for choosing mates. Consequently, male colourations might not serve as a basis for mate assessment and, in turn, mating success may not be based on female preference. To investigate this idea we first explored, whether colour differences and similarities are sufficient to be easily perceived and distinguished by the toads. In behavioural experiment, we then tested if male yellow colouration acts as visual signal to avoid mismatch and to quickly detect and secure a brown mate. Exploring this, we set up a model experiment, testing the behavioural response of toads to different body colourations (brown and yellow) of realistic model toads. We predict that male toads respond differently towards model toads (avoiding yellow models, spending

more time with brown models). However, if male dynamic colouration indicates male quality and mating success, then we predict that we find differences in body colouration in relation to morphology, or body condition between mated and non-mated males which might influence female preference or access. To test this, we measured, compared and correlated colouration, morphometry and body condition between mated and non-mated males during an explosive breeding event.

Methods

Study species and study site

The Asian common toad, *D. melanostictus* inhabits moist places, as well as urban lowland areas and breeds in stagnant water pools in South- and Southeast Asia (Ngo and Ngo 2013). Maximum reproductive activity and development of oocytes in females coincides with the arrival of heavy rains and thereby differs throughout the Asian continent (Jørgensen et al. 1986). While toads exhibit dynamic dichromatism and gather in explosive breeding events in most populations across the Indian subcontinent (e.g., Western Ghats), they breed throughout the year in other parts of South-East Asia (Thailand, Java) and no information on dynamic colour change exists for the latter populations (Church 1960; Jørgensen et al. 1986; Köhler et al. 2022). Dynamic colour change during explosive breeding was investigated from 11–12 June 2023 (day 1 and 2) in a riverbed with rainwater-filled puddles, formed by heavy monsoon rains in the previous night, in Uttara Kannada, Karnataka, India (Fig. 1). The breeding location consisted of three large, partly connected paddles of different sizes and water depths. The riverbed adjoined a 3–4 m high laterite wall on one side and a betel nut (*Areca catechu*) plantation on the other. Yellow males and brown females migrated from both sides to the breeding event.



Figure 1. Explosive breeding event of the Asian common toad (*Duttaphrynus melanostictus*) in their natural habitat in Karnataka, India.

Visual model and colour discrimination

First, we quantified whether conspecifics could detect spectral differences in body colouration between mated males (in amplexus), non-mated males (not in amplexus) and females (all in amplexus with a male) with a colour vision model calculating the discriminability between different body colourations (using the R package PAVO) (Maia et al. 2019). For this we randomly collected mated males ($N = 27$), non-mated males ($N = 27$) and females ($N = 27$) with a net from the breeding aggregation and immediately measured body colouration of flank, head, and vocal sac/throat with a spectrometer (JAZ series; Ocean Optics, Dunedin, USA) according a standardized protocol (Stückler et al. 2022). Spectral sensitivities are not available for *D. melanostictus*, therefore we modelled the visual system of the toads using spectral sensitivities of Common toads, *Bufo bufo* (function ‘vismodel’) (Yovanovich et al. 2017; Yovanovich et al. 2019). We used standard daylight as the lighting condition (‘D65’) (Siddiqi et al. 2004; Green et al. 2019; Chaves-Acuña et al.

2020), and the longest photoreceptor (LWS cone) for achromatic vision (Siddiqi et al. 2004; Gödert et al. 2021).

We used a receptor-noise model calculating colour differences (just-noticeable-differences, JNDs) with the function 'coldist' (Siddiqi et al. 2004; Chaves-Acuña et al. 2020). We calculated chromatic ($JND_{(dS)}$) and achromatic ($JND_{(dL)}$) contrasts comparing means of mated males against non-mated males and females. The threshold for discriminating between two colours is 1. Mean contrast values below 1 are indistinguishable, while JND values ranging from 1 to 3 signify poor detectability. Values above 3 indicate detectable contrasts (Vorobyev et al. 2001; Siddiqi et al. 2004; Igic et al. 2012; Chaves-Acuña et al. 2020). Photoreceptor proportion was set equally for the chromatic and achromatic calculations (Robertson et al. 2022). Following Siddiqi et al. (2004), the Weber fraction was set to 0.05.

Model experiment

To experimentally test how male yellow colouration influences mating, we used 3D model toads to observe live male responses to differing body colours in a natural explosive breeding aggregation. We created realistic toad models by casting a preserved *D. melanostictus* toad from a specimen of the collection of the Natural History Museum Vienna. The cast was filled with Polyurethane resin. The resulting object was scanned, adjusted to size (5.5 cm SVL) and seven models were printed (3Dee GmbH & Co KG, Vienna) (Fig. 2). We painted each model with non-toxic, water-based acrylic colour paint (Daler-Rowney Ltd., Bracknell, United Kingdom). As reference we used the spectral reflectance of yellow male *Hoplobatrachus tigerinus* (unpublished data D. Preininger) and the brown colouration data of the population kept at the Vienna Zoo (Stückler et al. 2022) (Fig. 5A).

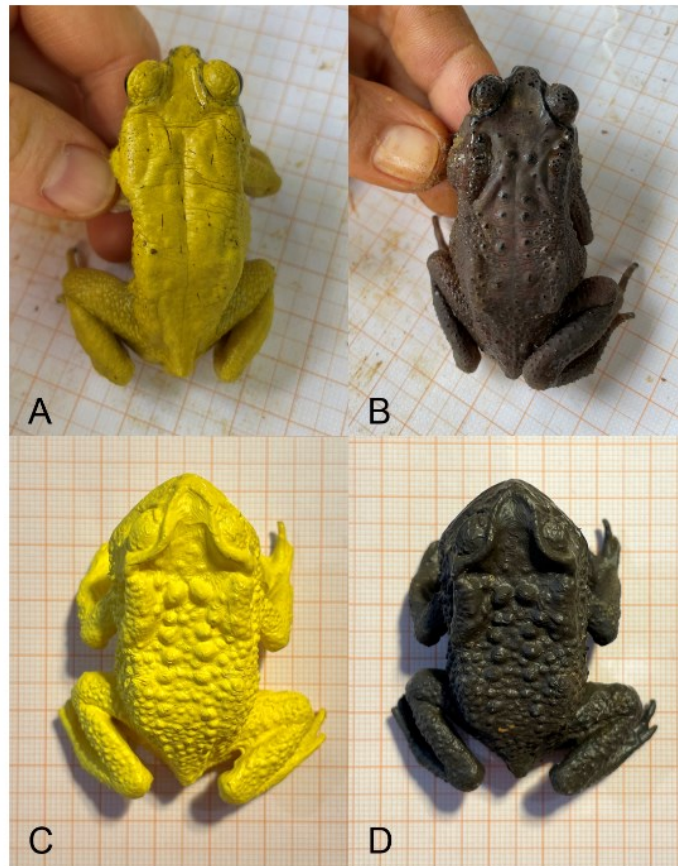


Figure 2. Comparison of live and model toads: a yellow male (A) and a brown female (B) live toad of *Duttaphrynus melanostictus*, and 3D model toads (5.5 cm SVL length) resembling a breeding yellow male (C) and a brown female model (D).

We presented a brown model, resembling a female and a yellow model mimicking a breeding competitor to a group of male *D. melanostictus* at the explosive breeding aggregation. The models were attached 70 cm apart from each other with a thin fishing line on the crossbar of a T-shaped wooden stick. The stick was 1.2 m long, allowing a distance of 1.5 m between experimenter and models as well as live toads. The experimenter lowered the models into the water and stood motionless for 2 min, so that the toads could recover from human disturbance and resume natural behaviour (habituation period). Then the experimenter moved the T-shaped stick with the model toads slightly up and down, for 5 min to simulate activity (testing period). Each trial was video recorded with a digital camera (Panasonic SDR-SW 21) on a tripod. We recorded 20 trials on day 1 of the explosive

breeding event, after each trial ended, we set up the next experiment 5 m away to avoid repeated testing of the same individuals.

Colour and morphological measurements

To investigate the relationship of colouration and male body condition, we compared body colouration of flank, head, and vocal sac of mated ($N = 27$) and non-mated males ($N = 27$). Following colour measurements, we obtained length and width of nuptial pad, forearm width and length, upper arm width and length, chest width, snout-vent length (SVL), snout-urostyle length (SUL) and weight of the same males. To calculate male body condition, we used the scaled mass index (SMI) derived from SVL and weight of males with a standard coefficient (Peig and Green 2009; Liao 2018; Landler et al. 2023). We calculated condition as $SMI = m * (L_0/L)^b$, in which m is the mass of the individual, L_0 the mean of SVL, and L the SVL of the individual. The coefficient b is calculated using a linear regression of the natural logarithm of body mass (as dependent variable) and the natural logarithm of SVL (as independent variable), following Landler et al. (2023). The coefficient (slope of the standard major axis) for our study population was 3.507916.

Colour analysis

We analysed the reflectance spectra in R Studio with the package PAVO (Maia et al. 2019). With the function 'procspec' we applied LOESS smoothing of the reflectance spectra ($opt = "smooth"$, $span = 0.35$), and set all negative values to zero ($fixneg = "zero"$) (Malpica et al. 2022; Webster et al. 2023). We analysed the colour parameters brightness, hue and yellow chroma (see supplementary material Stückler et al. (2022)) to compare male breeding colourations. Spectral analysis were performed in R Studio with the package PAVO, function 'procspec' and 'summary.rspec' (brightness: "B2"; hue: "H1"; yellow chroma: "S1Y").

Statistical analysis

Upon substantial interrater reliability agreement for video analysis (Kappa = 0.706, $P < 0.001$) (Landis and Koch 1977), we analysed the number of physical contacts and amplexus attempts per video (5 min experimental period) using Wilcoxon signed rank tests.

To compare colour measurements (flank, head and vocal sac), morphology (length and width of nuptial pad, forearm width and length, upper arm width and length, chest width, SVL, SUL and weight), and SMI between mated and non-mated males, we first tested all parameters for homogeneity by using Levene's test and Shapiro-Wilk normality test to test for normal distribution. If the parameters were normally distributed, we used a two-sample t-test; otherwise, we used a Wilcoxon rank sum test. Then we correlated colour parameters of flank, head, and vocal sac with SMI to understand if male colouration provides a cue to male quality, regardless of whether they were in amplexus or not.

Ethical statement and permits

All experiments reported in this article complied with the current laws of the country in which they were performed (India). The study was approved by the National Biodiversity Authority India (IN-KA97252580684335P). We followed the guidelines of the Association for the Study of Animal Behaviour for animals in research (ASAB 2024). Toads were handled carefully and handling time was kept to a minimum (less than 5 min for colour and morphological measurements).

Results

Description of the explosive breeding event

During five days and nights prior the explosive breeding event, we observed four male *D. melanostictus*, which displayed partial to full yellow colour change close to the eventual explosive breeding site. In these days, it rained on and off for periods of 10–30 min several

times per day and night, with heavy continuous rainfall starting in the evening of 9 June 2023. The explosive breeding event lasted two days (≈ 30 hours) and activity started on the morning of 11 June 2023 (day 1) after heavy rainfall in the previous 36 hours, followed by a drop in temperature from 28 °C to 24 °C and an increase of relative humidity from 67% to 71% (compared to the mean temperature and mean relative humidity of the week before, Fig. 3). Mean temperature on day 1 was 24.2 °C, mean relative humidity was 70.8%. On day 2, mean temperature was 26.2 °C and relative humidity was 70.9%. Sound pressure level of male chorus at the breeding site averaged 83.4 dBC (SE = 0.4, $N = 3$, distance 1 m; day 1, 14:00 h). First spawn was observed around 16:00 h of day 1. The operational sex ratio (OSR), counted in four randomly selected 1 m² plots, was 3.3:1 on day 1. During the night, we observed less activity. The following morning, the breeding event continued with an OSR of 3.3:1 counting a total of 235 males (164 single males, 71 males in amplexus) and 71 females (all in amplexus). Activity and individual number steadily decreased and ended in the early afternoon of day 2.

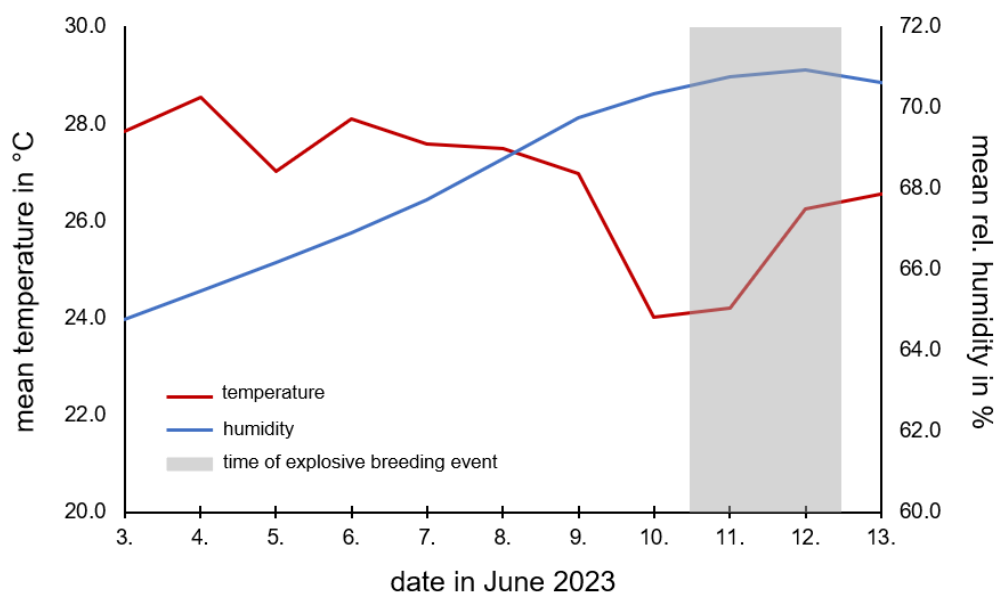


Figure 3. Mean temperature (in °C, red line) and mean relative humidity (in %, blue line) at our study site in Bare, India. The period of explosive breeding event of *Duttaphrynus melanostictus* is indicated in grey.

Colour vision model

When looking at estimates of discriminability expressed as just-noticeable-differences (JNDs), we found that comparisons of flank, head and vocal sac colourations of mated males to non-mated males showed no or poor contrast for sufficient discrimination in chromatic and achromatic conditions (flank: $dS = 0.3$, $dL = 1.1$; head: $dS = 0.8$, $dL = 1.8$; vocal sac: $dS = 1.6$, $dL = 0.3$). Whereas, contrast of yellow mated or non-mated males to females was much higher (mated males: flank: $dS = 21.3$, $dL = 26.1$; head: $dS = 17.4$, $dL = 22.6$; vocal sac/throat: $dS = 7.4$, $dL = 2.9$; non-mated males: flank: $dS = 21.6$, $dL = 25$; head: $dS = 17.9$, $dL = 20.8$; vocal sac/throat: $dS = 9.1$, $dL = 3.2$) in chromatic and achromatic conditions, showing that toads can easily discriminate a brown female from yellow breeding competitors.

Model experiment

Male Asian common toads had significantly more physical contact (Wilcoxon signed-rank test: $z = -2.277$, $N = 20$, $P < 0.05$) and amplexus attempts (Wilcoxon signed-rank test: $z = -4.514$, $N = 20$, $P < 0.001$) with brown model toads than with yellow model rivals (Fig. 4). Males had physical contact with brown models 133 times ($N = 20$ videos, mean/trial = 6.65), and 61 times with yellow models ($N = 20$ videos, mean/trial = 3.05). Males attempted amplexus 86 times with brown models resembling females ($N = 20$, mean/trial = 4.3) and only 2 times with yellow model toads ($N = 20$, mean/trial = 0.1). In the total test period of 100 min, live males spent 225 seconds (3.75 min) in amplexus with brown models and only 5 seconds with yellow models.

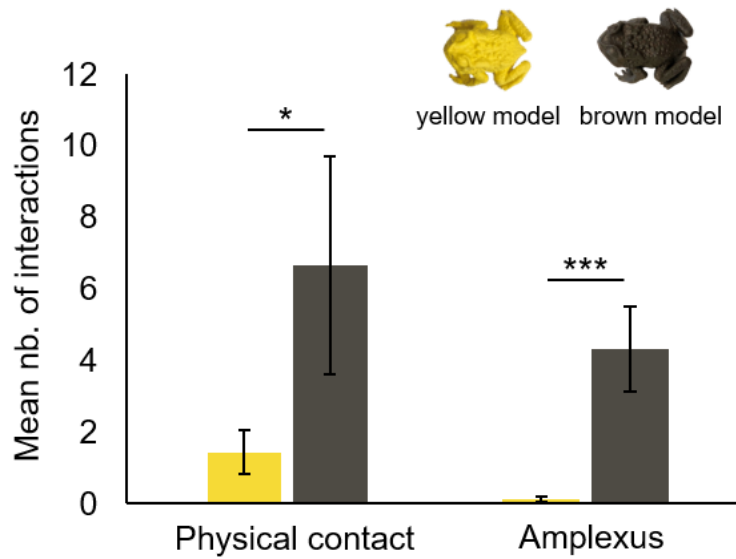


Figure 4. Mean number of interactions (active physical contact or amplexus) of male Asian common toads (*Duttaphrynus melanostictus*) with a yellow model toad (resembling a male competitor) and a brown model toad (resembling a female) during an explosive breeding event in their natural habitat ($N = 20$). Asterisk indicate significant differences of Wilcoxon signed-rank test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Comparison of mated and non-mated male traits

Mated and non-mated male toads showed no differences in the three analysed colour parameters (brightness, hue and yellow chroma) measured of flank, head, and vocal sac ($P > 0.05$, $N = 27/\text{group}$, Fig. 5), nor in length and width of nuptial pad, forearm width and length, upper arm width and length and chest width (two-sample t-test, $P > 0.05$, $N = 27/\text{group}$, Table 1), or SVL, SUL, weight and body condition (SMI) (Wilcoxon rank sum exact test: $P > 0.05$, $N = 27/\text{group}$, Table 1). Body condition of mated and non-mated male individuals did not explain yellow colour parameters (brightness, hue, yellow chroma) of flank, head, and vocal sac (spearman correlation, $P > 0.05$, $N = 54$), regardless of whether they were in the amplexus or not, e.g., males with higher SMI were not yellower or brighter or mating.

Table 1. Comparison of colour measurements (flank, head and vocal sac), morphological measurements and body condition (SMI) of mated ($N = 27$) and non-mated males ($N = 27$), providing means and standard errors.

Parameters	Mated males	Non-mated males	Z	P
Flank colouration				
Brightness	14.79 $\pm$ 0.86	13.86 $\pm$ 0.61	-1.8	0.07
Chroma (in %)	34.7 $\pm$ 0.5	34.9 $\pm$ 0.4	-0.4	0.6
Hue (nm)	644 $\pm$ 8.9	643 $\pm$ 9.9	-0.22	0.82
Head colouration				
Brightness	16.79 $\pm$ 1.28	16.00 $\pm$ 0.90	-0.18	0.86
Chroma (in %)	33.3 $\pm$ 0.5	32.8 $\pm$ 0.4	-1.06	0.29
Hue (nm)	681 $\pm$ 6.1	691 $\pm$ 2.5	-0.59	0.55
Vocal sac colouration				
Brightness	19.28 $\pm$ 1.11	19.66 $\pm$ 1.20	-0.44	0.66
Chroma (in %)	24.7 $\pm$ 0.7	25.5 $\pm$ 0.7	-0.46	0.65
Hue (nm)	682 $\pm$ 3.8	675 $\pm$ 7.1	-0.44	0.66
Morphometry				
SUL (mm)	61.89 $\pm$ 0.75	63.14 $\pm$ 0.68	-0.94	0.35
SVL (mm)	57.03 $\pm$ 1.15	59.24 $\pm$ 0.72	-1.47	0.14
Nuptial pad width (mm)	1.74 $\pm$ 0.07	1.75 $\pm$ 0.06	-0.09	0.93
Nuptial pad length (mm)	4.42 $\pm$ 0.12	4.51 $\pm$ 0.09	-0.57	0.57
Forearm width (mm)	6.47 $\pm$ 0.18	6.44 $\pm$ 0.20	-0.14	0.89
Forearm length (mm)	13.66 $\pm$ 0.28	13.10 $\pm$ 0.20	-1.58	0.11
Upper arm width (mm)	6.12 $\pm$ 0.19	6.25 $\pm$ 0.17	-0.49	0.63
Upper arm length (mm)	11.64 $\pm$ 0.34	11.39 $\pm$ 0.28	-0.58	0.57
Chest width (mm)	21.25 $\pm$ 0.40	21.73 $\pm$ 0.30	-0.94	0.35
Weight (g)	21.14 $\pm$ 0.71	22.55 $\pm$ 1.42	-0.45	0.66
Body condition				
Scales Mass Index (SMI)	22.24 $\pm$ 0.98	21.73 $\pm$ 1.20	-0.45	0.66

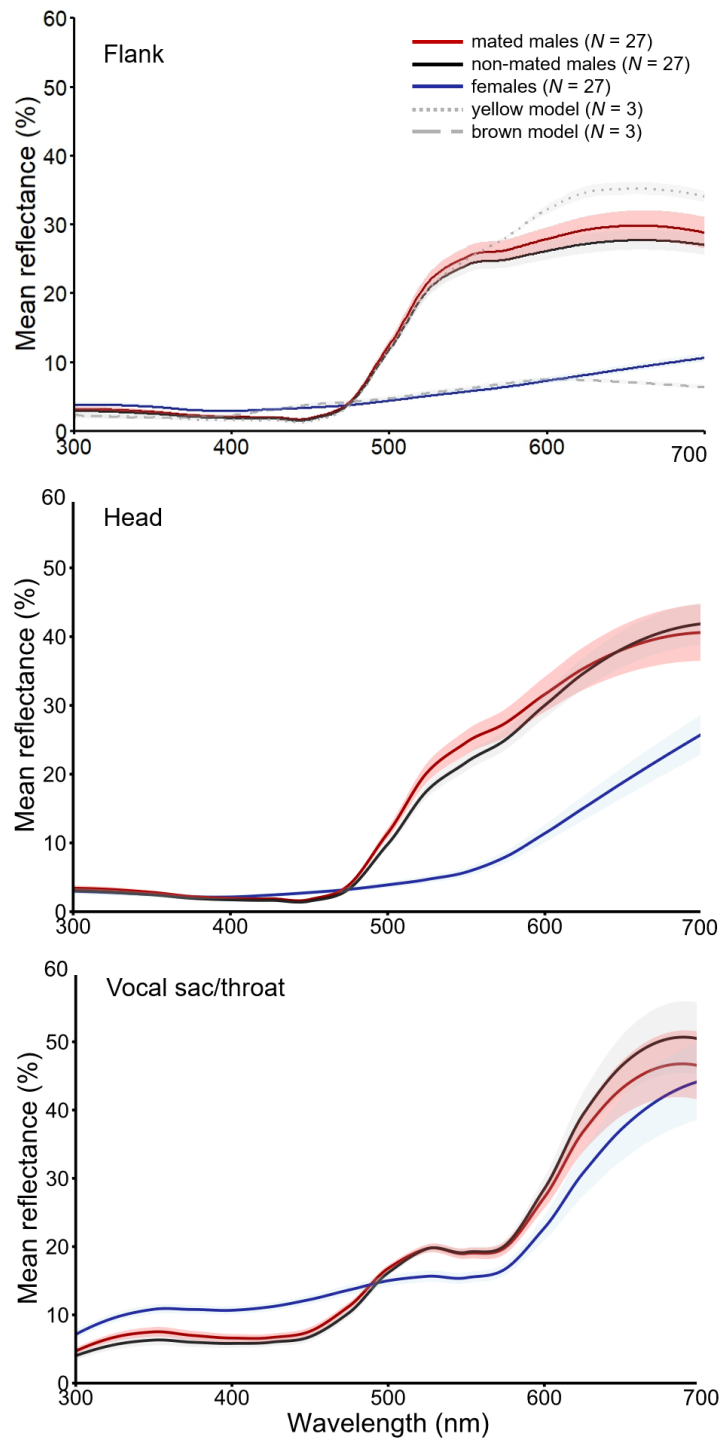


Figure 5. Mean spectral reflectance ($\pm$ SE) of flank (A), head (B), and vocal sac/throat (B) of *Duttaphrynus melanostictus* mated males (red, $N = 27$), non-mated males (black, $N = 27$), and females (blue, $N = 27$) during explosive breeding in their natural habitat. In panel A, grey dotted lines show reflectance spectra of yellow model toads, grey dashed lines of brown model toads.

Discussion

Some populations of Asian common toads (*D. melanostictus*) breed in highly competitive, explosive aggregations, where over 200 males actively search for females. Our results show that the dynamic yellow colouration displayed by males during mating can be easily discriminated against brown female conspecifics. Males attempted mating significantly more often with brown model toads resembling females than with yellow models imitating males. We suggest that dynamic sexual dichromatism in the Asian Common toad has evolved as visual signal mediating fast mate recognition in time-limited, dense breeding aggregations. Selection strongly favours the evolution of sensory and cognitive systems that help animals to find or secure a mate (Searcy and Nowicki 2005). These abilities become even more important when mating is highly time constrained, because environmental conditions for mating and offspring survival are favourable for only a brief period during each year (Sullivan 1994). Heavy rains and a temperature drop were observed before the onset of the explosive mating event, which likely triggered a physiological response in males to change body colouration and move to the breeding site. On location, male Asian common toads were able to clearly distinguish rival males by their bright yellow colouration and likely classified them as non-mating partners and continued to search for brown females. Visual signals deployed in male competition to secure a mating partner are common in several animal taxa, e.g., fish (Candolin 1999), damselflies (Khan and Herberstein 2021) or birds (Jones et al. 2017), and some anuran species developed visual signals to avoid conspecific physical attacks by exploiting sensory rival responses (Grafe et al. 2012; Anderson et al. 2021). The majority of those signals however not only regulate agonistic interaction but rather advertise condition (male quality) and/or localization towards female observers (Summers et al. 1999; Rosenthal et al. 2004; Gomez et al. 2009).

In female preference systems, male colouration or visual displays are subject to intersexual selection e.g., in several bird species, females choose males based on plumage colouration (Hill 2006). Visual preferences also mediate mate choice in a number of anurans

(Summers et al. 1999; Sullivan and Kwiatkowski 2007), e.g., in European tree frogs (*Hyla arborea*), females choose males based on colourful vocal sacs and a distinct flank stripe (Gomez et al. 2009). Colour differences related to health and condition provide a basis for females to assess a potential mate (McGraw and Ardia 2003; Pike et al. 2010). In Asian common toads, we found no differences between mated and non-mated males in colouration, morphometry, or body condition. Yellow colour brightness, hue or saturation do not relate to male's body condition and mating success. Also, amplexant males did not show any size or morphological differences (large forearms or nuptial pads, sensu (Greene and Funk 2009) that might have allowed them to secure a mate more easily independent of colouration. It remains in question what traits provide a basis for females to choose a potentially fit male. Indeed, females might not be influenced by male body colouration (as e.g., in *I. luetkenii* ((Gardner et al. 2021)) and probably do not have the possibility for pre-copulatory choice. In such dense breeding aggregations, high individual densities and strong male-biased OSR lead to high competition between males for mating opportunities (Wells 2007). Females are constantly pursued by males and one or more males attempt to clasp a female, in some cases females die in so-called mating balls (personal observation Stückler, Wampula and Preininger) (Trauth et al. 2000; Dittrich and Rödel 2023). One way for females to elevate offspring fitness in explosive breeding aggregations is polyandry (Sztatecsny et al. 2006; Rausch et al. 2014). However, there is no information on polyandry for our study species. Females were observed leaving the mating arena immediately after egg deposition, trying to hide in holes and being chased by males while doing so. On several occasions, females were amplexed with more than one male (mating balls) and had no chance to struggle free, which likely ended fatally for many females. Closer investigations should be made to explore female choice, female survival and polyandry in the Asian common toad.

Although sexual selection by mate choice is common in anurans and surely generated an enormous diversity in this and many taxa, we argue that this study adds to previous findings showing that dynamic sexual dichromatism in the Asian common toad

evolved to exploit male responses during mate searching. Admittedly, in many anurans, males advertise and females choose a preferable mate based on spectral and temporal acoustic information (Gerhardt and Huber 2002) and there is undeniable evidence for consistent trait preferences (Ryan et al. 2019). However, also visual signals (vocal sac-, foot- and body colourations) were demonstrated to play a dominant role in anuran communication systems (Starnberger et al. 2014a; Starnberger et al. 2014b). We thereby suggest that conspicuous visual signals evolved to mediate male decisions rather than those of females and regulate agonistic or aggressive competitions as particularly the case in explosive breeders.

Most dynamic dichromatic anuran species are explosive breeders and predominantly, males change body colouration to conspicuous bright colours (Bell et al. 2017). When looking at previous studies and the current results, it clearly highlights how a visual signal can enhance reproductive success in explosive breeding species. Moreover, it suggests that these dynamic colourations might have evolved several times under similar conditions to solve comparable competitive problems. We previously showed that the yellow colouration in Asian common toads is produced by catecholamines. Signal mediation by stress hormones, the intense competition and the short period to reproduce lead us to the suggestion that sexual dichromatism was initially a by-product of a stress response (Kindermann et al. 2013; Stücker et al. 2022) triggered by environmental stimuli that prelude highly competitive and brief explosive breeding events. As such, catecholamines stimulated melatonin aggregations in the skin, enabling a bright yellow appearance (Wright and Lerner 1960; Abe et al. 1969; Goldman and Hadley 1969) that presumably benefited signaller and receiver by avoiding unwanted mismatches that protract finding a female mating partner. Since females remain brown, the sensory and cognitive search mechanism for brown phenotypes as mates is maintained. In this case, the dynamic colour trait would impact competitor response by increasing the odds of mating and likewise being selected and transformed into a visual signal. Most sexual dichromatic anuran species change body

colouration to yellow, leading to the assumption that yellow colour is either easily produced or caused by physiological limits (Bell et al. 2017). Generally, yellow colour in anurans is produced by the dispersion of yellow pigments in xanthophores (Bagnara 1998; Aspöngren et al. 2008). Nevertheless, the skin of Asian common toads contains melanophores, but chromatophores important for yellow colour production are absent (Ali and Naaz 2014). Future studies exploring how such bright yellow colour is produced are needed to also understand the physiological and morphological basis of this colour change.

The question remains why evolution favoured such adaptations in some explosive breeders but not in others. How does species- and mate recognition work in monochromatic anuran explosive breeding species? In contrast to our study species, the Moor frog, the Neotropical yellow toad and also the nocturnal European common frog (*Rana temporaria*) where male throats become brighter for presumably similar signal influence (to avoid male attacks and recognize mates) (Sztatecsny et al. 2010), the majority of explosive breeding species lack dynamic colour change. Species- and mate recognition in those species might be facilitated through other cues and/or signals, such as sexual size dimorphism or distinct release calls (Marco and Lizana 2002; Yu and Sharma 2012), while dynamic visual signals might provide an optimal solution for species which physiological mechanism support such adaptations. Explosive breeding systems therefore provide an interesting study system to understand how selection acts on colour traits and influences dynamic visual signal design.

Conclusion

Our results support previous findings demonstrating that dynamic sexual dichromatism acts as a visual signal, enabling quick mate recognition and avoiding costly mismatches in time-limited, highly competitive breeding aggregations. This visual signal strategy in explosive mating systems could provide a convergent evolved solution to similar constraints in temperate and tropical anurans, to experience less harassment and allow faster mate finding. Physiological prerequisites, underlying mechanisms and adaptive

pathways facilitating dynamic signalling yet remain unknown. However, in temperate and tropical explosive breeding anurans, dynamic, visual mate recognition signals are likely more effective and efficient to avoid costs of mismatch and attacks than signals that regulate those agonistic encounters after they occurred. A dynamic signal solution further reduces costs associated with permanent conspicuousness, such as for visual predators.

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General Discussion

The four studies exploring different aspects of colour change in anurans provide insights about the requirements and effectiveness of ontogenetically changing colourations in Wallace's flying frogs and the function and underlying physiological mechanisms of dynamic colour change in male Asian common toads. The two distinct anuran study species – an arboreal, prolonged-breeding frog and an explosive-breeding toad – indicate evolutionary dynamics shaping anuran colouration with implications for ecology and behaviour. Numerous constraints and selective pressures guide, shape or even dictate adaptations of signal evolution.

The first chapter shows how dietary pigments (carotenoids) influence ontogenetic and dynamic colour changes in the Wallace's flying frog and how dietary pigments play an important role in successful husbandry. **Food** content and availability determines if this species can blend into its habitat and dynamically react to different light environments or stressors. In the second chapter, we investigated **predation** as a key selection pressure on colour adaption, which is a natural selection process eliminating or promoting colour modulations.

In the third chapter we did not target a constraint but suggest that **environmental** and/or **social pressures** trigger hormone release of catecholamines (epinephrine and norepinephrine) eliciting dynamic colour changes. In the fourth chapter we demonstrated how **sexual selection** acts on dynamic colour changes by mediating fast mate recognition – solving a common problem in time-limited, explosive breeding aggregations.

Given or because of the above-mentioned forces, and likely several others that constrain signal evolution, both investigated species have developed ontogenetically and dynamically changing colour signals over evolutionary time. These colour signals ensure survival and reproduction, likely through pre-existing adapted pathways, albeit achieved in entirely different ways. Most importantly, both study systems demonstrate the complexity of signal evolution and show how colour adaptations must meet numerous demands or selection factors.

Ontogenetic colour shifts in antipredator camouflage in Wallace's flying frogs

Chapter 1

Wallace's flying frogs exhibit colour changes that, at least partially, depend on carotenoids. A high-carotenoid diet contributes to their green body colouration, enabling them to dynamically change colour in response to varying light conditions and tactile stressors. Frogs receiving less or no carotenoids were not able to adaptively change body colouration. Carotenoids play an important role in colour production and physiological functions, but need to be obtained by ingestion. Sufficient nutrition thereby improves crypsis in the canopy and predation avoidance in adults. This strategy, however, is not applied by juveniles, likely due to of different environmental pressures in their habitat and/or morphological constraints, such as their inability to glide. Gliding is an advantageous behavioural and morphological adaptation of adults but absent in juveniles. Both factors – different habitats and the lack of gliding ability might contribute to alternative colour adaptations in juveniles (Chapter 2). However, the function of dynamic colour change remains unknown, as does the endocrine control of both, dynamic and ontogenetic colour changes in this species.

Chapter 2

The conspicuous colouration of juveniles likely serves as an antipredator strategy. Red models with white spots received half of the attacks than the plain red models, suggesting that the white spots are the primary visual feature making the animal appear inedible. While bird colour perception is thought to be well adapted for detecting red objects (Teichmann et al. 2020), a general sensory bias (neophobia) towards white likely plays a role in the evolution of white spots on a conspicuous red body. Changes in receiver behaviour may have evolutionary implications for “signal” design; for example, if avian predators tend to prey on frogs with fewer white spots, natural selection may reinforce an increased number of white spots over time. Multiple selection pressures, including predation, microhabitat background colouration, environmental factors and nutrition, likely contribute to the ontogenetic changes in mimicry strategies from juvenile masquerading to crypsis as adult.

While providing new evidence on the demands of natural colouration and predator avoidance through ontogenetic colour changes, Chapters 1 and 2 also highlight the complex interactions of selection pressures and constraints that mediated colour adaptations to match microhabitat characteristics, deceive predators and meet trophic demands.

Dynamic colour change and mate recognition in the explosive breeding Asian common toad

Chapter 3

A different complexity is evident during the reproductive period of the Asian common toads, which starts after heavy monsoon rains following a long dry period. Environmental conditions (temperature, air pressure) drastically change within a few days and initiate an explosive breeding event that lasts only 1-2 days with rigorous competition between hundreds of males for a mate. In this short time under exceptional social conditions, males dynamically change body colourations from brown to bright yellow. It remains unclear which factors trigger colour change in the first place. We hypothesize that the onset of sudden, strong rain showers, a drop in temperature and/or a drop in air pressure are triggering yellow colour change. There is plenty of evidence that environmental stressors can trigger stress hormone release (Wingfield 2003; Blas 2015). In many amphibian species, also different social stressors (such as reproduction) lead to the rapid release of catecholamines and corticosterone (Selye 1956; Carr 2011; Harvey et al. 1997). Catecholamines are released as part of the adaptive fight or flight stress response, and in our case they – at least partly – initiate dynamic colour change. We suspect that colour changes initially were a by-product of a stress response and eventually, sexual selection co-opted this effect and created an effective mate recognition signal in a challenging explosive breeding system. However, several factors remain unclear, for example what pressures – environmental or social – trigger the hormonal response mediating colour change and how such yellow colouration maintain for up to two days.

Two main stress response systems in animals are the Sympathetic-Adrenal-Medullary system (SAM) and the Hypothalamic-pituitary-adrenal system (HPA), the latter is known as the Hypothalamic-pituitary-interrenal (HPI) axis in amphibians. SAM is a rapid pathway working via the nervous system. Thereby, the adrenal medulla gets activated and releases catecholamines (fight or flight response). Contrary, stress also activates the HPA/HPI axis but via the brain, releasing glucocorticoids, which is slower than SAM (Hu et al. 2008; Kaiser and Jaillardon 2023). In amphibians, corticosterone (CORT) is the main stress hormone released via the HPA/HPI axis (Moore and Jessop 2003). For example, in some explosively breeding species (*Incilius luetkenii*), the presence of conspecifics – even just a single male – increases CORT levels and skin colour brightness in males compared to solitary housing (Gardner et al. 2020). Also, greater reproductive intensity during short breeding events and increased energy demands require higher baseline CORT (Moore and

Jessop 2003; Madelaire and Gomes 2016). Therefore, CORT levels are likely high when dynamic colour change occurs.

Another possible physiological trigger of dynamic colour change is sex steroid hormone action, given that testosterone (T) and estradiol (E₂) play a prominent role in governing important reproductive signals. Many studies show that this effect can occur on rapid timescales (within minutes) and in response to social and/or environmental cues (Remage-Healey and Bass 2006; Mangiamele and Thompson 2012; Fuxjager et al. 2015; Moore et al. 2018). For example, there is some evidence that androgens may facilitate colour change in a few frog species by acting alone or in combination with other hormones (Aspengren et al. 2008; Kindermann et al. 2014; Tang et al. 2014). Exploring the involvement of other hormones such as T and CORT in mediating dynamic colour change would be the next step in investigating the complex interplay of different hormonal pathways.

Chapter 4

Our results suggest that dynamic sexual dichromatism has evolved as a visual signal mediating male decisions in dense breeding aggregations rather than those of females. In this case, sexual selection is most likely a stronger selection force compared to predator selection. Increased number of successful mating events increases the likelihood of passing on its genetic material, or other traits such as the ability to change body colouration. A large body of literature demonstrates how female preference effects signal selection (Ryan 1990), whereas the influence of male-male competition on sexual colour dimorphism is rarely discussed. Traditionally, anuran breeding systems are viewed through the lens of female choice, however when observing an explosive breeding aggregation, it is immediately obvious that there is no or only very limited female choice possible. The minute a female arrives at a male biased breeding pond, females get clasped by one or several males and often die during this male scramble competition (Wells 2007). Male-male competition is positively correlated with population densities and influenced by the operational sex ratio (OSR). In our study species, which is characterized by high male-male competition, male yellow colouration helps to avoid male-male fights, and – considering an enormous short breeding period – saves time and increases mating opportunities.

Conclusion of Chapters

Overall, we have demonstrated that colour change as signal strategy has developed in very different ways and under completely different selection pressures in the two anuran amphibian species under investigation. Nevertheless, we also show how adaptations and investments into mere body colourations can determine if a frog or toad survives and is able to pass on its genes.

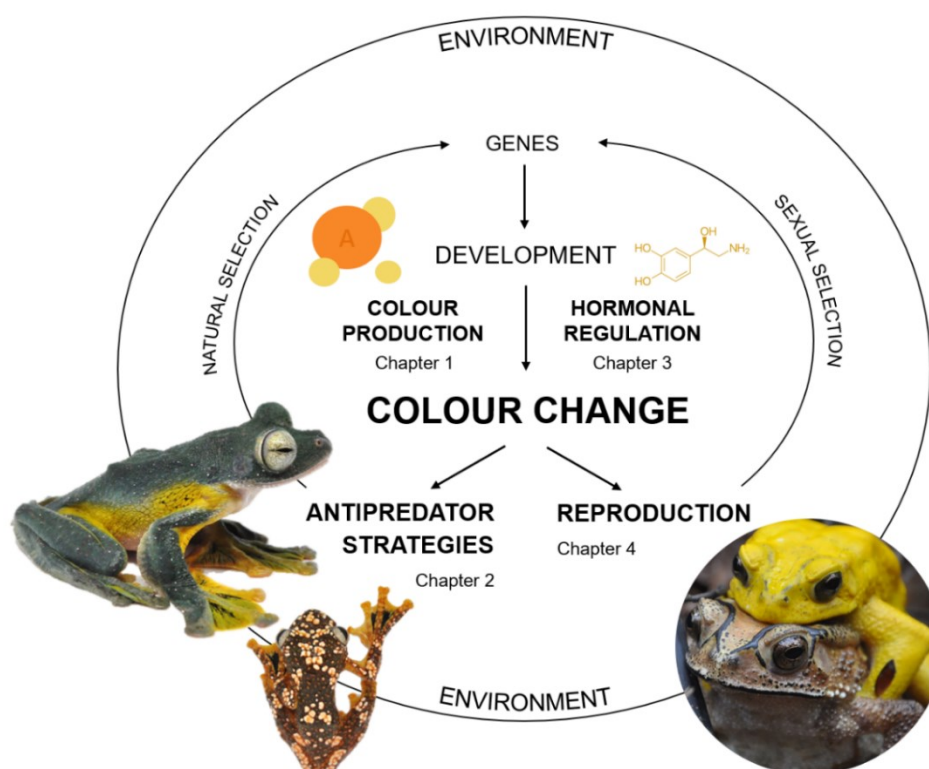


Figure 3. Thesis overview illustrating the interactions of natural and sexual selection shaping adaptive animal body colouration for enhanced survival and reproduction.

The diversity of visual signals that frogs and toads display likely reflects the diversity of evolutionary pathways that developed particular colour signals, as organisms are exposed to several selection pressures or constraints guiding (or rather confining) signal evolution. Signal function and mechanism help to understand the adaptations that we observe when we look at colour signals and the solutions that were selected to improve them. Our investigations, results and conclusions provide a basis for a number of further questions and study suggestions which at the end of this thesis we want to briefly summarize.

Future studies and challenges

Comparative approach

A comparative approach studying the proximate and ultimate causes could provide information why certain colours are selected at differing life stages, which benefits come with a diversity of colourations and why certain colours were evolutionary forced over others. For example, several *Rhacophorus* species exhibit ontogenetic colour change: *R. lateralis* and *R. malabaricus* change from yellowish juveniles with small black spots to predominantly green adults. *Rhacophorus pseudomalabaricus* juveniles are black yellow striped, transitioning to green when adult. Contrary, Wallace's flying frogs change from bright red to cryptic green adults (Biju et al. 2013). Testing functions and mechanisms, and comparing life histories of juvenile and adult colourations would certainly help to understand which ultimate and proximate causes mediate these ontogenetic colour changes.

Yellow colour production in the Asian common toad

The majority of dynamic colour changing species change colouration to yellow. In low light conditions, yellow colouration might give a high contrast, higher visibility, and a better signal detectability (Bell et al. 2017). From a physiological perspective, yellow colouration might be the “easiest” colour to produce. Yellow colour production in most anurans works via the aggregation of melanin pigments in melanophores, and a dispersion of yellow pigments in xanthophores (Hoffman and Blouin 2000; Rudh and Qvarnstrom 2013). However, the only chromatophores in the skin of Asian common toads are melanophores containing melanin pigments, which are normally known to produce dark colours (Ali and Naaz 2014). How such bright yellow colour is produced remains unanswered and the genomic mechanisms responsible for producing different colourations remain largely unknown. A transcriptomics-based approach could be helpful to understand the genetic processes influencing colour production in anurans (Stuckert et al. 2019).

Visual abilities of animals are poorly understood

One challenge in studying animal colouration is the diversity of colour vision in animals, e.g., birds and insects can perceive UV which other animals cannot (Bennett & Cuthill 1994). Animals have multiple visual pigments, photoreceptors and photoreceptor types, differences in spectral sensitivities and relative densities and therefore have different colour discrimination abilities (Kelber et al. 2003; Marshal & Arikawa 2014). Understanding the visual abilities and ecology of animals and integrating this knowledge in visual models,

is of great importance to provide information on what animals perceive visually and how this shapes their visual communication and behaviour (Kelber et al. 2003). In the past, some anuran visual systems were studied extensively, but in the last decades they have received limited attention. New investigations with modern methods and larger diversity of anuran groups are necessary to comprehend selective processes on visual pigments and photoreceptors, as well as the circuitry of retinal signals, neuronal processing, and coherent behaviours (Donner & Yovanovich 2020).

Lack of information on natural history

In-depth exploration of body colourations in anurans need a profound understanding of the natural history of the study species, encompassing fundamental aspects like diet, predators, habitats, and activity periods. Unfortunately, such information is scarce for many amphibians (Michaels et al. 2014; Candiotti et al. 2023). Descriptive field studies, observations such as predation notes, contributed by both the research community and herpetologically interested non-researchers, play a pivotal role in filling this knowledge gaps. An inclusive approach is crucial for developing a comprehensive understanding of amphibian ecology and behaviour.

Frogs and toads: model species in studying colour change

Anurans are ideal model organisms to explore function and mechanisms of colour change in both laboratory and natural conditions (Narins et al. 2003; Hirschmann and Hödl 2006; Starnberger et al. 2014). The use of models (Chapters 2 and 4) is a well-established method when studying anuran behaviour (see e.g., Sztatecsny et al. 2012; Gardner et al. 2021), allowing controlled experiments, and enabling the exploration of specific behaviours by isolating distinct traits. This approach provides standardization and enhances reproducibility in behavioural research studies. Furthermore, models are relatively affordable and easy to handle. The combination of controlled set-ups in which it is possible to manipulate different conditions and measure specific behaviours, and eventually validations and observations in the natural habitat are of great importance to get a more comprehensive overview of the functions and mechanisms of colour change. Our studies emphasize how a combination of approaches under laboratory conditions and field experiments can be selectively used to study visual signal behaviour in different species and yet give us more questions to answer.

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Abstract

Body colourations are integrated into visual communication systems and undergo evolutionary changes controlled by natural and sexual selection. This thesis investigates the visual significance of adaptive colourations in the context of survival and reproduction. In Chapters 1 and 2, we explored **ontogenetic colour change** of Wallace's flying frogs (*Rhacophorus nigropalmatus*) which are green in their adult life stage, but juveniles are red with white spots. We show that dietary carotenoids contribute to the development of green body colouration and enable dynamic colour adaptations. Furthermore, we demonstrated that the conspicuous colouration of juveniles likely serves as an antipredator strategy, assuming that the white spots of juveniles are associated with animal faeces and thus part of a masquerade protecting the animals from predators. In Chapters 3 and 4, we examined **dynamic colour change** of male Asian common toads (*Duttaphrynus melanostictus*), which become yellow during their short mating season. We could show that yellow colour is mediated by stress hormones (catecholamines). In behavioural experiments with model toads in the animals' natural habitat, males had much more contact with brown models than with yellow models. Our results indicate that male yellow colour has evolved as a sexual signal facilitating rapid mate recognition in dense breeding aggregations with intense male-male competition. Overall, we showed that colour change as a signalling strategy evolved in very different ways and under completely different conditions in the two amphibian species studied.

Zusammenfassung

Körperfärbungen sind in visuelle Kommunikationssysteme eingebunden und unterliegen evolutionären Veränderungen, die durch natürliche und sexuelle Selektionsmechanismen gesteuert werden. In der vorliegenden Arbeit wird die Bedeutung visueller, adaptiver Körperfärbungen im Zusammenhang mit Überleben und Fortpflanzung bei Anuren untersucht. In den Kapiteln 1 und 2 untersuchten wir den **ontogenetischen Farbwechsel** des Wallace Flugfrosches (*Rhacophorus nigropalmatus*). Dieser als Jungtier rote Frosch mit weißen Punkten wird als ausgewachsenes Tier grün. Wir konnten feststellen, dass über die Nahrung aufgenommene Carotinoide zur Entwicklung der grünen Körperfärbung beitragen und dynamische Farbanpassungen ermöglichen. Die Funktion der unterschiedlichen Farbmorphen wurde in einem weiteren Schritt untersucht. Wir konnten zeigen, dass die weißen Punkte, die mit Tierkot assoziiert werden können, als wichtiges visuelles Merkmal fungieren, um vor Prädatoren geschützt zu sein. In den Kapiteln 3 und 4 untersuchten wir den **dynamischen Farbwechsel** der männlichen Schwarznarbenkröten (*Duttaphrynus melanostictus*), die während der kurzen Paarungszeit leuchtend gelb werden. Wir konnten zeigen, dass die gelbe Körperfärbung durch die Stresshormone Adrenalin und Noradrenalin ausgelöst werden. In Verhaltensexperimenten mit Modellkröten im natürlichen Habitat der Tiere hatten die Männchen deutlich mehr Kontakt mit den braunen Modellen als mit den gelben Modellen. Unsere Ergebnisse zeigen, dass die Gelbfärbung der Männchen eine schnelle Partnererkennung im dichten Laichgeschehen mit viel Konkurrenz ermöglicht. Insgesamt konnten wir zeigen, dass sich der Farbwechsel als Signalstrategie auf sehr unterschiedliche Weise und unter völlig unterschiedlichen Bedingungen bei den beiden untersuchten Amphibienarten entwickelt hat.