ISSN 2634-1379

The Herpetological Journal

Volume 33, Number 4
October 2023

Published by the British Herpetological Society

British Herpetological Society

The Herpetological Journal is published quarterly by the British Herpetological Society and is issued free to members. Articles are listed in Current Awareness in Biological Sciences, Current Contents, Science Citation Index and Zoological Record. Applications to purchase copies and/or for details of membership should be made to the Hon. Secretary, British Herpetological Society, The Zoological Society of London, Regent's Park, London, NW1 4RY, UK. Instructions to authors are printed inside the back cover. All contributions should be addressed to the Scientific Editor.

Front cover: Adult female big-eyed pit viper *Trimeresurus macrops* captured and measured as part of general ecology study of green pit vipers at the Sakaerat Biosphere Reserve in Thailand. See article on page 88. (© Curt H. Barnes)

Herpetological Journal

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https://doi.org/10.33256/33.4.8896

Published by the British Herpetological Society

Bayesian insights into Green Pit Viper *Trimeresurus* (*Cryptelytrops*) *macrops* sexual dimorphism with respect to influence of gravidity and habitat disturbance

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Sexual dimorphism is expressed in many taxa as a form of resource partitioning and as a response to unique challenges faced by male and female organisms. We investigated this phenomenon for an arboreal green pit viper species, *Trimeresurus macrops*, previously suggested to display obvious sexual dimorphism. We evaluated seven external characters and scaled mass index (SMI) of 73 vipers (N = 32 males, N = 41 females) obtained during night surveys and opportunistic searches in north-east Thailand from January 2015–January 2017. We utilised Bayesian inference to investigate the influence of sex, gravidity, and rural habitat disturbance on sexual dimorphism of *T. macrops*, and we provide 95 % highest density intervals (HDI) for the difference in means (posterior distribution). We also present ecologically relevant probabilities of observing dimorphism for each external character and SMI by sex, gravidity (gravid or not), and habitat type (rural or forest). Females were larger than males in both forested and rural habitat, females in rural habitat were larger than forest females, and all males displayed a conspicuous white eye-stripe. When gravid females were excluded from analyses, probability of observing dimorphism drastically decreased. Knowledge of niche partitioning and the specific challenges encountered by males and females, gravid and non-gravid females, and individuals persisting in rural habitats will prove valuable for conservation and snakebite mitigation of *T. macrops*.

Keywords: Difference of body size, south-east Asia, Bayesian inference, arboreal viper

ความแตกต่างระหว่างเพศ (sexual dimorphism) ถูกปรากฏในอนุกรมวิธานสัตว์ว่าเป็นรูปแบบหนึ่งของการแบ่งปันทรัพยากร (resource partitioning) และการท้าทายจำเพาะจากสิ่งมีชีวิตเพศผู้และเพศเมีย เราศึกษาปรากฏการณ์นี้ในกลุ่มงูเขียวหางไห้ทีมีถินอาศัยอยู่บนต้นไม้ ชนิดพันธุ์ งูเขียวหางไหม้ตาโต *Trimeresurus macrops* ซึงได้รับการเสนอในการศึกษาก่อนหน้าว่ามีการแสดงออกของความแตกต่างระหว่างเพศอย่างชัดเจน นักวิจัยทำการประเมินลักษณะภายนอกทั้ง 7 ลักษณะและScaled mass index (SMI) ของงูเขียวหางไหม้จำนวน 73 ตัว (ตัวผู้ 32 ตัว, ตัวเมีย 41 ตัว) จากการสำรวจตอนกลางคืนและการสำรวจตามโอกาสในพื้นทีภาคตะวันออกเฉียงเหนือของประเทศไทยตั้งแต่เดือนมกราคม พ.ศ. 2558 - มกราคม พ.ศ. 2560 เราใช้การอนุมานแบบเบย์เพือตรวจสอบอิทธิพลของเพศ การตั้งครรภ์ และการรบกวนถินอาศัยในพื้นทีเกษตรกรรมต่อความแตกต่างระหว่างเพศของงูเขียวหางไหม้ตาโต *T. macrops* และเราให้ช่วง HDI อยู่ที 95% สำหรับความแตกต่างของค่าเฉลีย (การแจกแจงหลัง) นอกจากนี้ เรานำเสนอความน่าจะเป็นทีเกียวข้องกับระบบนิเวศในการสังเกตความแตกต่างของลักษณะภายนอกและ SMI ตามเพศ การตั้งครรภ์ (มีหรือไม่มี) และประเภททีอยู่อาศัย (พื้นทีเกษตรกรรมหรือปา) การศึกษาพบว่า งูเขียวหางไหม้ตัวเมียมีขนาดใหญ่กว่าตัวผู้ทั้งทีอาศัยในปาและพื้นทีเกษตรกรรม โดยงูเขียวหางไหม้ตัวเมียในทีอยู่อาศัยพื้นทีเกษตรกรรมจะมีขนาดใหญ่กว่าทีพบในปา และตัวผู้ทุกตัวมีแถบตาสีขาวทีสังเกตเห็นชัดเจน ทั้งนี้เมืองูเขียวหางไหม้เพศเมียทีตั้งครรภ์ถูกแยกออกจากการวิเคราะห์ ค่าความน่าจะเป็นในการสังเกตความแตกต่างระหว่างเพศถูกลดทอนลงเป็นอย่างมาก โดยความรู้เกียวกับการแบ่งกลุ่มเฉพาะและความท้าทายจำเพาะทีพบโดยงูเขียวหางไหม้เพศผู้และเพศเมีย งูเพศเมียทีมีการตั้งครรภ์ และตัวอย่างอืน ๆ ทีอาศัยอยู่ในเกษตรกรรมจะพิสูจน์ได้ว่ามีคุณค่าสำหรับการอนุรักษ์และการลดปัญหาการถูกงูกัดของงูเขียวหางไหม้ตาโต *T. macrops* สืบไป

INTRODUCTION

Sexual dimorphism has independently evolved in many groups of animals (Williams & Carroll, 2009). Colouration, body size and body shape are some of the phenotypic differences which can be expressed by male and female organisms (Mori et al., 2017). Sexual dimorphism is thought to have evolved due to unique challenges faced by each of the two sexes and as a form of resource partitioning. In general, when the male is larger than the female, sexual size dimorphism (SSD) increases with body size, but decreases with body size in groups in which the male is smaller than the female ("Rensch's rule; Rensch, 1950; Fairbairn, 1997). Vipers (family Viperidae) express SSD in many forms, which provides an interesting study system for this phenomenon.

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Many viper species display male-biased SSD (males express larger morphological characters), particularly New World representatives (such as the genera *Crotalus* and *Agkistrodon*). Male biased SSD is particularly prevalent for terrestrial species which engage in ritualised male-male combat behaviour, which selectively pressures for larger body mass and length (Shine, 1978; Bonnet et al., 1998). Females of some species of viper are larger than males, despite males of those species engaging in male-male combat (e.g. *Calloselasma rhodostoma* and *Vipera berus*; York, 1984; Madsen, 1988; Strine et al., 2015).

Phylogenetic study of Neotropical viperids suggest that SSD patterns are driven primarily through sexual selection on males rather than females, with the notable exception being arboreal species, which favour female biased SSD (Hendry et al., 2014). Arboreal Old World vipers may follow similar trends with most arboreal vipers displaying female biased SSD (as proposed for the genus *Tropidolaemus*, Kuch et al., 2007). Some arboreal species such as the insular *Gloydius shedaoensis* do not display obvious sexual dimorphism (Shine et al., 2002).

Male-biased SSD may enable individual males to outcompete other males and provide increased access to females (Shine, 1978). Alternatively, female-biased SSD may promote greater gravidity (King, 2000), larger clutch sizes (Seigel & Ford, 1987; Shine, 1993; Manjarrez et al., 2014) and larger offspring with better body condition at birth for larger females (Bronikowski, 2000; Kissner & Weatherhead, 2005; Manjarrez et al., 2014). Selection for large female body size due to increased reproductivity (Darwin, 1874; more recently termed "fecundity-advantage hypothesis", Trivers, 1972) may be associated with smaller and more mobile males (Trivers, 1972; Ghiselin, 1974; termed the "small male hypothesis" by Zamudio, 1998).

Snakes in human-disturbed habitats may also display SSD, similar to their natural habitat counterparts (Fearn et al., 2001). Significant direct mortality of snakes by humans can skew size distributions, however (Sasaki et al., 2008). Different expression of morphology may allow females and males to occupy separate ecological niches, particularly influencing food requirements and reducing intersexual resource competition (Shine, 1991; 1993; Houston & Shine, 1993; Pearson et al., 2002; Manjarrez et al., 2014). Reduction of intraspecific resource competition may be particularly crucial in tropical regions, where niche breadth is considerably narrower than temperate regions (Krasnov et al., 2008).

Green pit vipers (usually *Trimeresurus* spp.) are a tropical and sub-tropical group of pit vipers (sub-family Crotalinae), which have previously been suggested to exhibit significant sexual dimorphism and geographic variation of morphological characters. Female *T. vogeli* from Laos and Cambodia expressed significantly shorter tails, larger heads and reached larger overall body lengths than conspecific males (Malhotra et al., 2004). Female *T. albolabris* were also observed to be significantly heavier than males in captivity by Herrel et al. (2011), but no differences in head shape were found. Subsequent study of wild *T. albolabris* by Devan-Song et al. (2017) in Hong Kong further revealed significant dimorphism of size and colouration by sex. Interestingly, initial studies suggested *T. stejnegeri stejnegeri* to be sexually dimorphic in northern Taiwan (Tsai & Tu, 1998), although more recent studies of the species have not subsequently observed significant size differences between sexes (Tu et al., 2000; Creer et al., 2002).

The big-eyed pit viper (*T. macrops*) is a common and widespread species of arboreal green pit viper (found in central and northern Thailand, Cambodia and Laos; Stuart et al., 2012) which has previously been suggested to exhibit sexual size dimorphism (Strine et al., 2015). With total body length between 520–660 mm as adults (maximum 720 mm; Das, 2010; Cox et al., 2012) and mass between 36–108 g (Cox et al., 2012), *T. macrops* is a relatively small species. However, *T. macrops* accounts for a large percentage of venomous snake bites throughout Thailand (about 30 %; Viravan et al., 1992; Soogarun et al., 2006) and other south-east Asia countries.

Dimorphism can be an indicator of niche partitioning and intraspecific diet (Temeles et al., 2010) and inclusive understanding can be useful for snake conservation and ecological knowledge in natural and human disturbed habitats. We further describe sexual dimorphism of *T. macrops* at the Sakaerat Biosphere Reserve in northern Thailand, blending replication (refer to Fraser et al., 2020 and Marshall et al., 2021, for discussion and benefits of replication and reproducibility) with additional novel findings of influence of gravidity and habitat. We hypothesise 1) that gravidity will influence perception of SSD by investigators and 2) estimates and SSD will differ between forest and rural environments. We report (1) sexual size dimorphism (SSD) of *T. macrops* in rural and natural habitat, (2) assess the influence of female viper gravidity on dimorphism and (3) assess general morphological size differences of vipers in rural and natural habitats in northern Thailand. By approaching the SSD phenomenon from a Bayesian framework, we are able to incorporate previous knowledge and provide direct inferential conclusions (probabilities of observing males or females measurements being larger) which are ecologically meaningful and easy to interpret.

MATERIALS & METHODS

Study site

Our study was conducted in rural and forested areas of the Sakaerat Biosphere Reserve (SBR), Nakhon Ratchasima Province, Thailand (14.44–14.55° N, 101.88–101.95° E; Fig. 1). The reserve is comprised of a core (forest) area of approximately 80 km^2 and a further 360 km^2 comprising buffer and transitional (rural) areas. The protected (core, forest) area ranges in elevation from 280 to 762 m and consists of primary growth dry evergreen forest, dry dipterocarp forest and secondary reforestation (Trisurat, 2010). The buffer and transition (rural) areas of the SBR are comprised of human settlements and

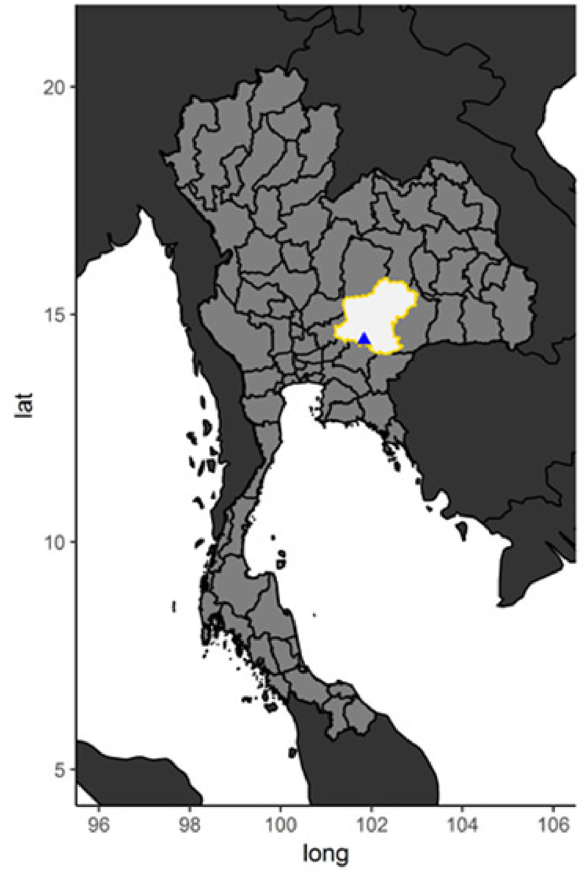

Figure 1. Map with Thailand highlighted in light grey, Nakhon Ratchasima province outlined in yellow, and study site (Sakaerat Biosphere Reserve) indicated by the blue triangle.

agriculture interspersed with small remnant patches of disturbed forest. Primary agriculture in the area includes rubber (*Hevea brasiliensis*), coconut, Eucalyptus tree (*Eucalyptus camaldulensis*), cassava and corn at various stages of cultivation.

Data collection

We followed similar methodology to Strine et al. (2015) for data collection. We captured vipers during surveys at night and opportunistic searches between January 2015–January 2017, and recorded morphometrics the following day using the acrylic tube method and isoflurane anesthesia as described by Wilkinson (2014). After complete body tone loss was achieved, we measured snout vent length (SVL) and tail length (TL) of vipers with a custom-made, semicircular measuring pipe. Snout vent length was defined as the distance from the tip of the snout to the posterior tip of the anal scale. Tail length was defined as the distance from the anterior tip of the first sub-caudal scale to the tip of the tail. Total body length (TBL) was then obtained by adding SVL and TL. Girth was measured at 25 %, 50 % and 75 % intervals of SVL, starting from the head, using digital callipers. Head length (HL) and head width (HW) were determined through the use of digital callipers also, from the widest part of the side of the head (HW) and from the notch behind the head which separates the head and neck (HL). We determined sex of each viper by inserting a probe into the cloaca and gently easing it into one of the cavities at the base of the tail, recording the distance the probe travelled in number of sub-caudal scales. For females it was possible to pass the probe only as far as the musk glands, which lie beneath the first one or two sub-caudal scales. The probe passed to at least the ninth sub-caudal scale for males. We further assessed body condition for *T. macrops* using a scaled mass index (SMI, Peig & Green, 2009) with previous *T. macrops* captured at the Sakaerat Biosphere Reserve as a comparison population (Strine et al., 2015). Finally, each *T. macrops* captured was uniquely marked with a Bovie® cauterising unit for future identification following Winne et al. (2006).

All measurements presented in our work are similar to Strine et al. (2015), except for girth which was not collected during that study period. We also determined female gravidity (gravid or not- indicated by presence or absence of ova) through light palpation while vipers were under anesthesia, which was not presented in Strine et al. (2015). We were unable to confidently determine gravidity for a small subsample of females (< 10 individuals). These vipers were still included in general analyses but not gravidity-specific analyses. After measurements were recorded, we photographed each individual viper's body, head, ventral and sub-caudal scales, tail, brand and other identifying characteristics such as scars. All vipers were returned to their location of capture within 72 hours of capture.

For recaptured snakes, we only used measurements obtained from the most recent capture. Juveniles and neonates were excluded from analyses. We considered individuals of both sexes greater than 450 mm TBL to be sexually mature, based on the smallest gravid female captured in SBR previously (Strine et al., 2015). Although males less than 450 mm TBL may be sexually mature, we selected our mature size conservatively (similar to Strine et al., 2015). No comprehensive investigation of reproductive ecology has yet been conducted for *T. macrops*, and the life history of the species remains understudied.

Statistical analyses

We used Bayesian methods in the R program package "wiqid" (version 0.1.5, Meredith, 2018) to estimate morphometric variables of male and female *T. macrops* in rural and forest habitats of SBR. Within the wiqid package, we used the function "Bnormal" which generates a Bayesian estimation of center (μ) and scale (σ) of a Gaussian ("normal") distribution based on 100,000 Metropolis-Hastings Markov chain Monte Carlo (MCMC) samples (with a 10,000 sample burn-in, thinning rate of 1 and 4 chains) to achieve > 10,000 effective samples obtained from a Gibbs sampler and our dataset. Well mixed traceplots and appropriate Gelman and Rubin statistics ($\hat{r} < 1.01$, Gelman & Rubin, 1992) suggested MCMC chains to have completely traversed all features of posterior distributions, resulting in achievement of stationary distributions. Flat (uniform distribution, reflecting lack of previous data) priors were initially applied for both rural and forest vipers collected during our study period. Our girth methodology has not been widely utilised (but could provide an additional tool for assessing body condition and dimorphism), so we only applied flat priors to these measurements.

For comparison to Strine et al. (2015) and to better understand the ecological implications of head size, we applied Bayesian linear regression models to these dependent (head width and head length) variables with SVL and SMI as predictor variables. We tested each variable for normality, homoscedasticity and independence. Linear regression models were run with the "brm" function in the "brms" package (version 2.9.0, Buerkner, 2017) of R, with flat priors and 100,000 Hamiltonian MCMC samples (with a 10,000 sample burn-in, thinning rate of 1 and 4 chains) to achieve > 10,000 effective samples obtained from a No-U-Turn Sampler (NUTS) and our dataset. Well mixed traceplots and appropriate Gelman and Rubin statistics ($\hat{r} < 1.01$) suggested regression MCMC chains to have completely traversed all features of posterior distributions, resulting in achievement of stationary distributions. We report model fit with widely applicable information criterion (WAIC; Gelman et al., 2014; McElreath, 2016) and leave-one-out cross-validation (LOO; Vehtari et al., 2016a; 2016b) metrics.

While uniform (flat) priors are the most frequently prior information utilised in ecological Bayesian models, they have recently been criticised for not actually being "non-informative," for simply providing the same results as frequentist methods, and perhaps most importantly potentially suffering the high Type I and Type M error rates as frequentist methods (summarised in Lemione, 2019). Weakly informative priors present one solution, incorporating basic biological sense-regularising data through constraining inferences (stabilising priors) to a feasible and reasonable range (Lemione, 2019). Weakly informative priors were compiled through review of previous literature (Malhotra et al., 2011; Cox et al., 2012; Strine et al., 2015) and this current work, specifically looking at minimum, maximum, standard deviation and mean values. We provide weakly informative priors with wide (but still biologically realistic) standard deviations as a reasonable constraint for all measurements (not distinguishing between sex, study area, or gravidity, Supplementary Table 1).

Mean and standard deviation measurements from Strine et al. (2015) were incorporated as informed priors for a subset of our data-forest snakes which included both gravid and non-gravid females as well as males (Supplementary Table 1). The Strine et al. (2015) study specifically investigated male and female *T. macrops* in dry evergreen and mixed deciduous forest of SBR, so we did not utilise them as a prior for rural vipers in our study. Additionally, we did not use Strine et al. (2015) results as a prior for non-gravid female and male comparative analyses because the previous study did not distinguish between female gravidity.

We provide 95 % highest density intervals (HDI) for the difference in means (posterior distribution) of viper sex in the forest and rural areas for each morphometric variable estimated, calculated with the "wiqid" R package. For each measurement, we also attempt to provide an ecologically relevant probability of observing a given difference between sexes (i.e. one sex being 15 g heavier) calculated through the posterior distribution.

RESULTS

We made 93 captures of 85 different vipers (8 recaptures) between January 2015–January 2017. After removing measurements from the first captures of recaptured snakes, juvenile and neonate snakes (N = 12), sample size for our analyses was 73 individuals. Similar to Strine et al. (2015), we also observed a postocular eye stripe present for all males analysed (N = 32), which was absent in females (N = 41). Final analyses included 41 individuals in forest habitat (N = 14 male and N = 27 females; N = 14 gravid, N = 7 non-gravid and N = 6 unknown gravidity females) and 32 individuals in rural habitat (N = 18 male and N = 14 females; N = 6 gravid, N = 6 non-gravid and N = 2 unknown gravidity females).

Initial analysis of male and female vipers (regardless of gravidity or capture location) followed a similar trend to Strine et al. (2015). All measurements showed obvious dimorphism between the sexes (Supplementary Table 2; 80–100 % probability of observing differences, Supplementary Table 3) with head length, head width and SMI exhibiting lowest dimorphism. Females were larger than males for all measurements, except for tail length (males longer than females, > 99 % probability).

We observed similar relationships between head size and SVL and SMI (Figs. 2 & 3, summarised in Supplementary Table 4) as Strine et al. (2015). Head size was positively correlated between SVL for both males and females (Fig. 2). Head size was positively correlated between SMI for females, but negatively for males (Fig. 3). There was a relatively weak relationship between head size and SMI for gravid and non-gravid females (Fig. 4).

Sexual dimorphism was strongly predicted for *T. macrops* in rural habitat (Supplementary Table 5; > 90 % probability of observing differences, Supplementary Table 6), with females estimated to be larger than males for all measurements other than TL, for which there was strong support (> 95 %) that males were longer than females. However, SMI was similar between the sexes (about 60 % probability of observing a difference) in rural habitat. Support for sexual dimorphism for forest vipers was strong (> 90 %) for SVL, TL, mass and girth measurements but not for total body length, head length and head width (about 54–80 %, Supplementary Table 6). Forest female viper SMI was clearly larger than male (Supplementary Table 5; > 95 % probability, Supplementary Table 6).

Closer inspection of sexual dimorphism with respect to gravidity of females yielded contrasting results to general differences observed between sexes in our study

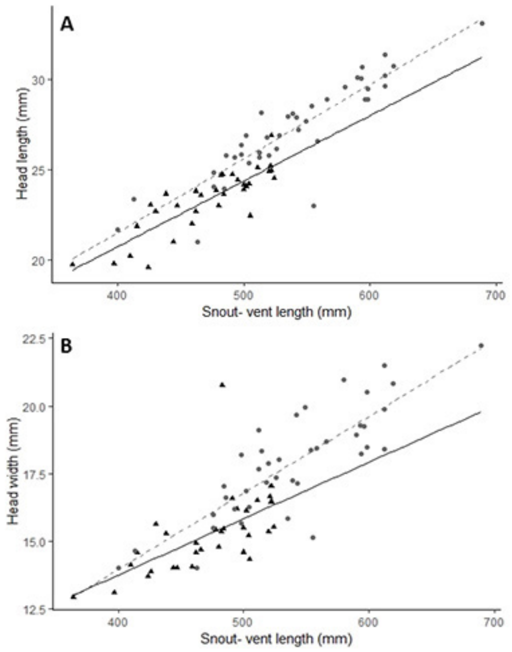

Figure 2. The relationship between **(A)** snout- vent length (SVL) and head length (HL) of male (N = 32) and female (N = 41), and between **(B)** SVL and HW of male (N = 32) and female (N = 41) *T. macrops*. Model summaries are presented in Supplementary Table 4.

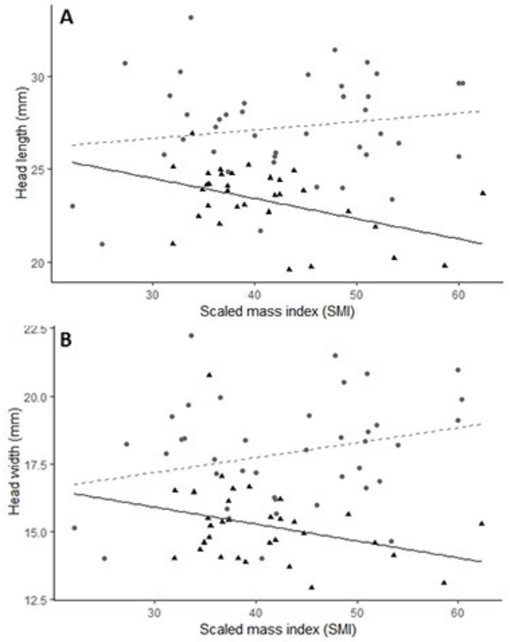

Figure 3. The relationship between **(A)** scaled mass index (SMI) and head length (HL) of male (N = 32) and female (N = 41), and between **(B)** SMI and HW of male (N = 32) and female (N = 41) *T. macrops*. Model summaries are presented in Supplementary Table 4.

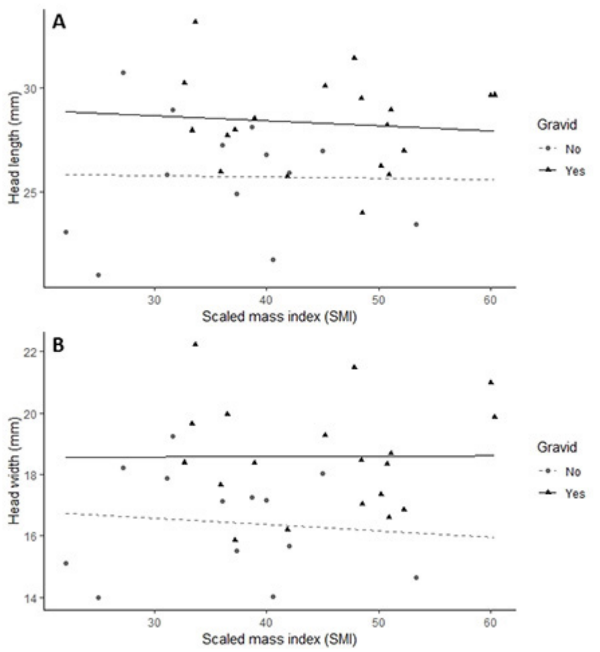

Figure 4. The relationship between **(A)** scaled mass index (SMI) and head length (HL) of gravid (N = 20) and non-gravid (N = 13), and between **(B)** SMI and HW of gravid (N = 20) and non- gravid (N = 13) *T. macrops*. Model summaries are presented in Supplementary Table 4.

and previous study (Supplementary Table 7). Probability that females were 15 g heavier than males dropped from > 90 % in the forest area when gravid and non-gravid females were included in analyses down to just 8 % when only non-gravid females were gauged against males (Supplementary Table 8). Sexual dimorphism was even less pronounced for rural areas, when gravid females were excluded there was only about 2 % probability that non-gravid females were 15 g larger than males (compared to about 94 % with both gravid and non-gravid females analysed, Supplementary Table 8). Girth as a function of dimorphism also decreased with exclusion of gravid females, although markedly less so (Supplementary Table 8). Male body condition (SMI) was higher than non-gravid females in both study areas (Supplementary Tables 7 & 8).

Males in rural habitat were not markedly larger or smaller than male forest vipers for most measurements, except for mass and SMI (Supplementary Tables 9 & 10). Measurements of male SVL, TBL and girth (except at 50 % of the body) were particularly similar for both habitat types. Rural females were larger than forest females for all measurements, however, with the exception of TL (Supplementary Tables 9 & 10).

DISCUSSION

We observed similar trends of sexual dimorphism as previous study of *T. macrops* (Strine et al., 2015) and other green pit viper species such as *T. albolabris* (Devan-Song et al., 2017) and *T. vogeli* (Malhotra et al., 2004), with females attaining larger sizes than males (except for tail length). Similar to Strine et al. (2015), there was also pronounced colouration difference (eye stripe present for males, but not females) for *T. macrops* in our study. Sexual size dimorphism was clearly evident in both rural and natural forested habitat for *T. macrops* in our study, although to different degrees. Sexual dimorphism was greatly diminished when gravid females were excluded from analyses, however. Interestingly, we observed that males were similar in size in rural and forested habitats but females were larger (except for TL) in rural habitats.

Previous study of SSD for *T. macrops* by Strine et al. (2015) proposed morphological differences between males and females of this species to be due to several factors. Smaller size of males was suggested to increase mobility for finding and tracking females, decrease predation risk and the ability to utilise higher perches and more distal edges of vegetation. Explanation provided by Strine et al. (2015) for longer TL was that longer tails potentially contained longer hemipenes, thus leading to higher mating success of males. Increased perch availability was anecdotally supported by that study through several observations of predation of *T. macrops* by king cobras (*Ophiophagus hannah*). Support for increased mobility (compared to females) has somewhat been supported by subsequent Strine et al. (2018) and Barnes et al. (2017) radiotelemetric studies of *T. macrops* spatial ecology. This system might be female-biased due to reproduction costs of females for this viviparous species.

Recent publication of several observations of *T. macrops* mating at the Sakaerat Biosphere Reserve (Strine et al., 2018) provides mixed insight into sexual size selection of *T. macrops*. Females (larger in all observations) were observed pulling males, which suggests size may be important (except tail length, following the female reproductive output hypothesis, King, 1989) for this sex with regards to strength and subsequently being able to select microhabitat with reduced predation risk during copulation- which was observed to last as long as nearly 13 hours in duration. A second male was observed aggressively attempting to disrupt (wrestling and biting) a copulating male in one of the observations, although this larger intruder was unable to disrupt his competitor and ultimately failed to copulate with the female in the observation. The hemipenes of the copulating male were visibly stretched outside of the body while combat ensued, supporting selection for increased tail length of males (following the morphological constraint and male mating ability hypotheses, King, 1989; Shine et al., 1999). The failure of the larger male to disrupt the copulation may provide evidence for limited or lack of selection pressure for larger males from a reproductive context.

Our results suggest non-gravid females to be similar morphologically to males (particularly in rural areas), with much of the observed intraspecific dimorphism resulting from gravid individuals. Gravidity incurs unique challenges to viviparous snakes, particularly locomotion (movement and home range; Seigel & Ford, 1987; Johnson, 2000). Spatial ecology of *T. macrops* in rural areas of Sakaerat Biosphere Reserve by Barnes et al. (2017) previously suggested non-gravid females to exhibit increased median movement and larger median home ranges than gravid females, although these results were not statistically significant.

In addition to sexual size dimorphism, Malhotra et al. (2011) also suggested geographic variation between populations of *T. macrops*. Our results suggest habitat and human disturbance may influence external morphology expression. Rural female *T. macrops* in our study were larger morphologically than forest individuals (mean 553.1 cm, 534.2 cm SVL, respectively). Mean home range sizes (minimum convex polygon estimation method) of female vipers from rural areas of SBR from Barnes et al. (2017) were somewhat smaller than female vipers from more protected forested areas in the Strine et al. (2018) study (0.099 ha and 0.175 ha, respectively). Reduced habitat complexity and availability of suitable habitat (reflected by smaller home range size compared to forest females, which are smaller morphologically), thus increasing intrasexual selection, may provide a possible explanation for this observation. Additionally, rural environments pose unique challenges to venomous snakes, and we have observed both mortality (snake) and human-snake conflict for other species at our study site in northeast Thailand (Knierim et al., 2017; Marshall et al., 2018). However, gravid rural females in our study appeared to skew sexual dimorphism results more significantly than forest snakes (potentially influencing inclusive comparison of females in the two habitat types), which requires further investigation. Male *T. macrops*, however, were similar in size morphologically between forest and rural habitat. How males utilise habitat and space is more difficult to adequately address due to limitations of technology (current radio transmitter size and corresponding battery life restrictions) and significantly smaller body size of this sex.

Future study to better understand the role of SSD for *T. macrops* and other green pit viper species requires an increase in ecological and natural history study. Radiotelemetry has traditionally been the go-to method for addressing such questions for snakes, however, green pit viper size (particularly males) makes this a costly and potentially unethical endeavour due to multiple transmitter replacements and size of transmitters compared to mass and girth. Harmonic tracking may provide a suitable alternative for spatial, movement and habitat selection investigation of green pit vipers due to smaller tag size and nature of the technology (tags reflect, rather than transmit signals; methodology presented specifically for small snakes by Engelstoft et al., 1999). Furthermore, fixed camera technology has shown remarkable promise for prey selection, activity period, conspecific interactions and general behaviour investigation of green pit viper species at SBR (Barnes et al., 2018; Barnes & Knierim, 2019; Barnes et al., 2020).

Long-term investigations are required for better understanding of green pit viper basic biology and ecology. Interestingly, our longest individual (689 mm SVL, 829 mm TBL and 88.9 g), a gravid female, was captured in the forested study area and was longer than sizes previously reported by Cox et al. (1998; 720 mm TBL), Das (2010, 720 mm TBL), Malhotra et al. (2011; 629 mm SVL), Cox et al. (2012; 660 mm TBL), Chanhome et al. (2011, 600 mm TBL) and Strine et al. (2015; 663 mm

SVL, 791 mm TBL). This individual was initially captured as an adult on 1 June 2014 (thus, was part of both our and Strine et al. 2015 study; 663 cm SVL, 791 cm TBL) and then again on 2 May 2015 (674 cm SVL, 806 cm TBL) before the last/final capture (17 September 2016) which was used for our analyses. Furthermore, this individual supported previous small home range hypotheses for the species nicely (< 0.5 ha minimum convex polygon method; Barnes et al., 2017; Strine et al., 2018, Barnes et al., 2019)- all captures were < 150 m of each other (or an area of 0.3 ha when the points are connected). Thus, multi-year morphological investigations such as ours (or longer) can provide valuable insight into survival and growth, as well as dimorphism and other ecological variables of interest. For green pit viper species (or other snake taxa) which are threatened with imminent extinction (such as the Kanburi pit viper, *Trimeresurus kanburiensis*), photographic image processing programs (such as ImageJ) can provide less invasive alternatives (refer to Astley et al., 2017 for comparison; and Herrel et al., 2011 for use with another green pit viper) to traditional measuring methods utilised for morphological investigations.

Despite being one of the more commonly encountered snake species at SBR, *T. macrops* can be relatively difficult to locate during quantified searches at this site (9.1 surveyor hours to find one at all study locations, total 228.7 surveyor hours surveyed during our study period). Although logistically challenging and requiring much thought into study design, comprehensive natural history and ecology work would prove invaluable to understanding green pit viper sexual size dimorphism and general biology. Conservation and snakebite management may also benefit from further SSD study, such as diet and habitat differences between males and females (niche partitioning), particularly through application of Bayesian frameworks which have the potential to incorporate previous knowledge and the benefit of providing intuitive and useful probabilities (crucial for lay-people and managers with limited statistical background) rather than arbitrary p–values (refer to Ellison, 2004 for benefits, caveats, comparison to frequentist methodology and application of Bayesian inference to date in the field of ecology).

DATA & CODE AVAILABILITY

Supplementary Table 1 contains previous knowledge incorporated into priors utilised for this study; Supplementary Tables 2–10 contain summary inferential information including summaries of mean differences, probabilities of observing differences and posterior summaries and convergence statistics; Supplementary Table 11 contains basic data (month of capture, whether a capture was in the forest or rural site, viper sex and if a viper was gravid or not) of vipers included in analyses; and example R code to run “wiqid” models are available at https://osf.io/rgf4y and “brms” model code is available at https://osf.io/gbzw5.

ACKNOWLEDGEMENTS

We thank Suranaree University of Technology (SUT) for supporting this project. All methods were carried out under the guidelines and approval of the SUT animal use and ethics committee. The National Research Council of Thailand (NRCT), Thailand Institute of Scientific and Technological Research (TISTR) and Sakaerat Environmental Research Station (SERS) provided funding, site permission and logistical and technical support throughout the project. Our work was possible because of the invaluable assistance provided by many field assistants, fellow SUT graduate students and Sakaerat Environmental Research Station staff.

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Accepted: 27 February 2023

Please note that the Supplementary Material for this article is available online via the Herpetological Journal website: https://thebhs.org/publications/the-herpetological-journal/volume-33-number-4-october-2023

Herpetological Journal

FULL PAPER

https://doi.org/10.33256/33.4.97102

Published by the British Herpetological Society

The plasticity of metamorphic traits in the Chinese brown frog tadpoles fails to obey Richards' hypothesis

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According to Richards' hypothesis, algae or cells in the intestinal tract has been considered the cellular inhibitory factor, when they fall off and hide in the faeces, excreted together with the faeces. If the animals feed on these faeces with algae or cells, and bring them into their systems, then the cellular inhibitory factor would play the main responsibility for growth inhibition. Here, we surveyed the effects of different combinations of faeces and food level on growth rates, survivorship, larval age and mass, and SVL at metamorphosis of the Chinese brown frog *Rana chensinensis*. Our results showed that food level can influence the length of the larval period of Chinese brown frog tadpoles, suggesting that delayed metamorphosis is caused by low food supply, indicative of a function of effective energy. Our data also clearly indicated that tadpoles in the presence of faeces were on average larger in body mass than those in the absence of faeces, which failed to obey Richards' hypothesis. Moreover, our results found evidence that faeces have a positive effect on the growth rate of tadpoles. Thus, there is no evidence for Richards' hypothesis, suggesting that this novel mechanism is selected for where coprophagy is likely to prove profitable, irrespective of the abundance of alternative food.

Keywords: Rana chensinensis, Richards' hypothesis, faecal material, mass at metamorphosis, growth rate

INTRODUCTION

In animals with complex life cycles, such as anurans, size and age at metamorphosis have a strong relationship with individual fitness (Arnold & Wassersug, 1978; Wilbur, 1980). Larval amphibians are more likely to experience variation in food availability because of the variety of spawning sites (Morey & Reznick, 2004; Skelly, 2004). In addition to energy uptake, food availability can be generally regarded as a major proximal cause of variation in metamorphic size and timing of metamorphosis (Newman, 1998; reviewed by Álvarez & Nicieza, 2002; Castano et al., 2010).

In the field or laboratory, high food availability can accelerate growth and developmental rates, thus permitting larvae either to maximise metamorphic size or minimise timing of metamorphosis (Pandian & Marian, 1985; Arendt & Hoang, 2005; Yu et al., 2015; Yu et al., 2016a; 2016b; 2016c; Yu & Han, 2020). Conversely, low food availability owing to low food supply, large population density of the larvae, or both, would also postpone metamorphosis, such that if the environmental conditions were poor in the ponds, then larvae would spend plenty of time to achieve the minimum size for metamorphosis because of slow growth rates (reviewed by Wilbur & Collins, 1973).

In addition, other factors can also affect timing of metamorphosis and growth rate, such as temperature, water volume and competitors and predators (Rose, 2005). The excretory material including something inhibitory to growth is a popular idea (Richards, 1958; 1962). Here, Richards' hypothesis predicted that the crowding effect would occur if tadpoles could eat their own excretory material and then the cellular inhibitory factor is introduced into their systems (Richards, 1958). It was later identified that a non-pigmented, unicellular alga can be detached from the faecal material of larvae which is linked with growth inhibition in British anuran tadpoles. Wong et al. (1994) proposed the name *Prototheca richardsi* sp. which belonged to the genus *Prototheca* (Beebee, 1991). The growth-inhibiting algae is not overtly parasitic, instead, its mode of action seems to rely on altering feeding behaviour. For example, large, superior tadpoles can release a large number of growth-inhibiting algae hidden in the faeces. Then, small tadpoles are attracted to feed on algae-rich faeces and are thereby diverted from other higher quality food resources, while large tadpoles do not alter feeding behaviour (reviewed by Griffiths et al., 1993). Up to now, some studies have proved that faeces are indeed inhibitory to the growth of anuran tadpoles (Griffiths et al., 1991; 1993; Baker & Beebee, 2000) and snails (Crabb, 1929). Further, high food level can ameliorate growth inhibition under crowded conditions, and the production of inhibitory cells is inversely correlated with food levels (Griffiths et al., 1993).

The question arises whether the faeces is the major factor leading to crowding effects in all organisms and

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under all conditions (Richards, 1958). However, few studies have examined how faecal material and food level can interact to affect amphibian larvae (Gromko et al., 1973). Here, we investigated the effects of food level and faecal material on size and age at metamorphosis of the Chinese brown frog *Rana chensinensis* tadpoles. Specifically, if the growth inhibition of faecal material is stronger at a low food level, and thus larger body length and mass at metamorphosis are rare, longer length of larval period might be expected. If, however, the growth inhibition of faecal material is weak at a high food level, and difference between the presence of faecal material and absence of faecal material may be undetectable.

MATERIALS & METHODS

Study species

Female frogs in *Rana chensinensis* are the larger sex and are widely distributed north of the Yangtze River in China (Yu et al., 2015). This animal belongs to a typically explosive breeder with a relatively short breeding season (8–16 days; Wells, 2007; Yu et al., 2015), and favours small and medium still-water bodies for breeding behaviour. *R. chensinensis* begins reproductive behaviour in early February when the water temperature is higher than 5 °C. The frogs usually amplexus and spawn at night, while male frogs demonstrate chorusing behaviour in warm breeding ponds. After two weeks, the tadpoles hatch in a natural pond, then feed on Spirogyra and *Potamogeton crispus* for three months (Cao et al., 2002) and finally, the metamorphosis go ashore and begin to live on land.

Field and laboratory procedures

Ten fresh egg clutches of *R. chensinensis* were collected in Xinyang (114° 06′ E, 32° 12′ N, elevation 22–100 m), Henan, the central plains of China, during 11–19 February 2021. Then, to obtain the experimental tadpoles, we selected 100 fresh eggs from each clutch and placed them in an opaque round plastic container (2000 ml, diameter = 15.8 cm) with 15 cm of fresh water to hatch. The experiment was carried out in the indoor laboratory which is 0.5 km away from the spawning site.

Experiment design

We used a 2 × 2 factorial design to analyse the influences of food level and faeces on larval growth and development. For every factorial treatment we used 30 tadpoles that were carefully chosen, ensuring that all individuals were the same size (3.55–3.65 mm) and development stage (absorption of external gills and formed spiracle, Gosner stage 26). Also, we randomly selected one tadpole from each clutch in each of the treatments (each representing a different family) housed individually in opaque round plastic bowls (500 ml) and three replicates were implemented, so that it could minimise any parental or genetic effects and intraspecific competition through the experiment. We used commercial fish food (Bieyanghong, Biological Co. Ltd., Hangzhou, China, medium protein content, MPC; 30 % protein, 10 % lipids, 18 % algae, 4 % fibre, 10 % ash) to feed the tadpoles. All tadpoles experienced the same temperature (room temperature = 17.3 ± 1.33 °C) and photoperiod (13L:11D) conditions throughout the study period. We changed the water in the containers once a week.

To assess the food level influences, we designed two treatments of feeding regime: (i) low mass-specific food level (6 % of per tadpole mass perday) and (ii) a high food level (12 % per tadpole mass per day) throughout the experiment (Zhang et al., 2007). For each food level, two different faeces regimen were chosen across the larval period: (i) presence of faeces (sucked into the faeces of other individuals where 12 or more large tadpoles were put into a litre of water within two days, diluted to 60 ml, then accelerated the movement of faeces by rotating the beaker, simultaneously sucked 1 ml of faecal liquid with a pipette gun and injected into one plastic bowl; Richards, 1958) and (ii) absence of faeces (sucked out the faeces by Pasteur pipette every four to six hours, thus there was very few or a few faeces at the bottom of the plastic bowls, Biologix 30-0138A1).

Five variables were measured: (1) age at metamorphosis [number of days from incubation until metamorphosis (the emergence of at least one forelimb, Gosner stage 42)]; (2) body length (snout-vent length, SVL), the tadpole was placed into a plastic basin of flat bottom with a caliper (error 0.05 mm) to get digital images, the SVL measure was obtained using the free computer software TpsDig 2; (3) body mass, which was measured with an electric balance (error 0.001 g); (4) growth rate, which was calculated with the following equation: mass at metamorphosis/age at metamorphosis (Laurila, 2000); (5) survivorship, which was classified two-point scales based on the number of tadpoles in a plastic bowl: 1 = one tadpole survived until metamorphosis; 0 = no tadpoles survived until metamorphosis.

Data analysis

One-Sample Kolmogorov-Smirnov Test was used to test whether data met normal distribution. Then, we analysed metamorphosis age, body length and mass at metamorphosis, growth rate and survivorship, by using a univariate two-way ANOVAs with type III mean squares and faeces, food level, and their interaction as fixed factors. If the effects of any fixed variable in univariate two-way ANOVAs were significant, the data were analysed with one-way ANOVA (four experimental treatments as factors) by using Fisher's LSD post-hoc multiple comparisons to test differences between food levels or between faeces levels (IBM SPSS Statistics 20.0, IBM Corp, Armonk, NY, USA). All probabilities were two-tailed, with values presented as means ± SE (standard error).

RESULTS

The effects of food level and faeces on age at metamorphosis

The effect of food level on age at metamorphosis was significant ($F_{1,101}$ = 7.936, p = 0.006; Table 1, Fig. 1a), revealing that age at metamorphosis with presence of faeces reared at a high food level was significantly shorter than those at a low food level (p = 0.011), but the effect of food quantity was negligible when faeces were absent (p = 0.175). However, there was not a significant effect of faeces ($F_{1,101}$ = 0.171, p = 0.680; Fig. 1a), as well as an interaction between faeces and food level ($F_{1,101}$ = 0.840, p = 0.362).

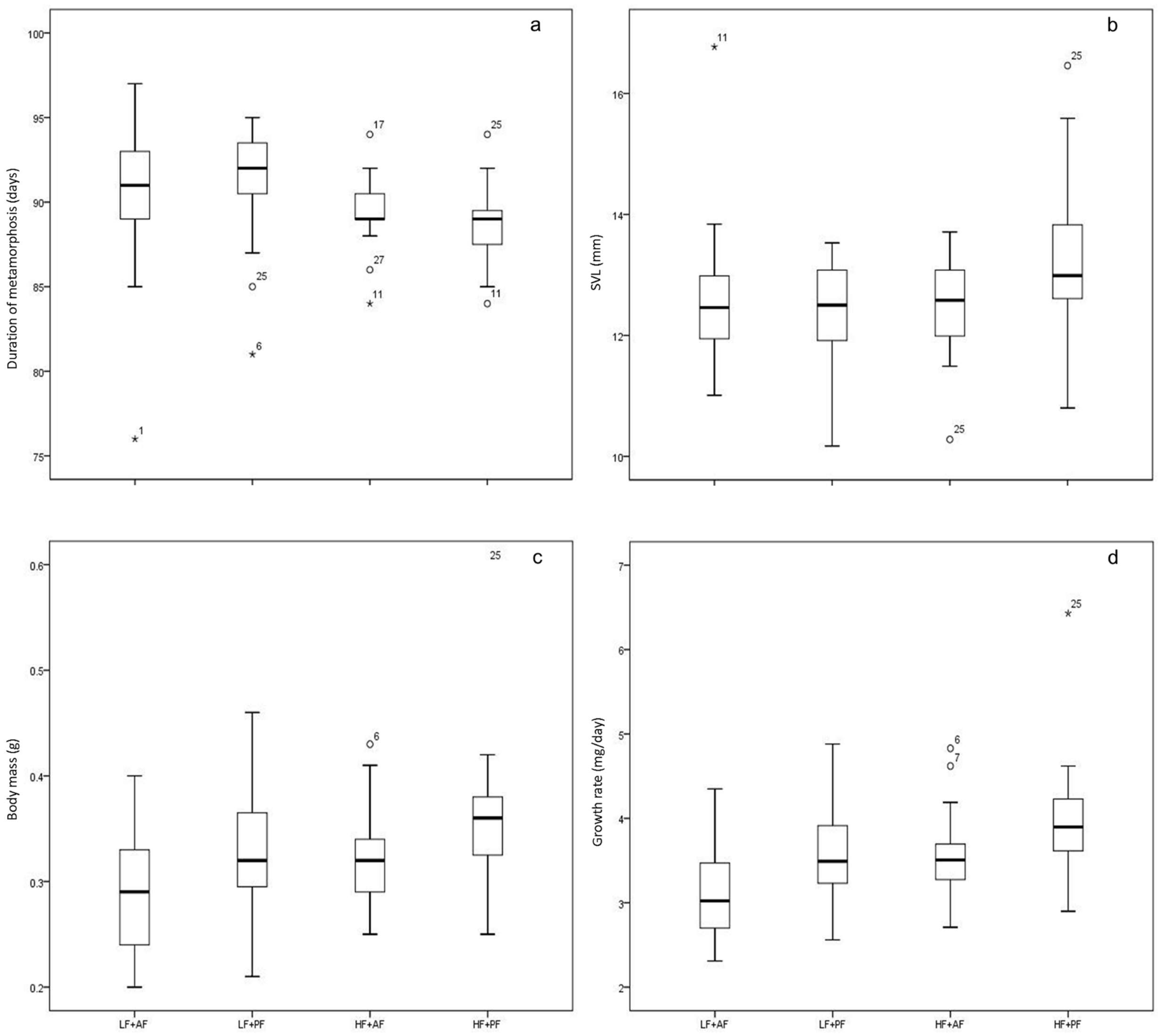

Figure 1. Influences of faecal material and food level on **(a)** age at metamorphosis, **(b)** SVL, **(c)** body mass, and **(d)** growth rate of the Chinese brown frog *Rana chensinensis* at forelimb emergence (Gosner stage 42; LF, low food level; HF, high food level; AF, absence of faecal material; PF, presence of faecal material; the box plots indicate median, 25th and 75th quartile and range).

The effects of food level and faeces on body length and mass at metamorphosis

Body length was not significantly affected by food level ($F_{1,101}$ = 3.708, p = 0.057; Table 1, Fig. 1b). The faeces was not statistically significant ($F_{1,101}$ = 1.436, p = 0.234); however, there was a tendency for the difference in body length between faeces treatments to be greater at the same food level (Fig. 1b). The interaction between faeces and food level was not significant ($F_{1,101}$ = 1.087, p = 0.300).

Mass at metamorphosis was affected by faeces ($F_{1,101}$ = 15.332, p < 0.001; Table 1, Fig. 1c) and food level ($F_{1,101}$= 9.481, p = 0.003), indicative of increasing mass at metamorphosis at a high food level (both p < 0.039) or in presence of faeces (both p < 0.008). However, there was not a significant interaction between food level and faeces ($_{F1,101}$ = 0.005, p = 0.944).

The effects of food level and faeces on growth rate and survivorship

The effects of faeces and foodlevel on growth rate were significant (faeces, $F_{1,101}$ = 17.727, p < 0.001; food level, $F_{1,101}$ = 15.694, p < 0.001; Table 1, Fig. 1d), revealed that growth rate at a high food level and presence of faeces was significantly larger than those at a low food level (both p < 0.008) and absence of faeces (both p < 0.007). However, there was not a significant interaction between food level and faeces ($F_{1,101}$ = 0.034, p = 0.854).

Survivorship was positively influenced by only food level ($F_{1,116}$ = 6.332, p = 0.013), especially in the presence of faeces (p = 0.050, Table 1, Fig. 2). The effects of faeces on

Table 1. Two-way ANOVAs table of the effects of faeces and food level on metamorphic traits in a *Rana chensinensis* population

Response variable	Source of variation	df	MS	F-value	P-value
Length of larval period	Faeces	1	1.917	0.171	0.680
	Food level	1	89.091	7.936	0.006
	Faeces × Food level	1	9.427	0.840	0.362
	Error	101	11.227		
Body mass	Faeces	1	0.045	15.332	< 0.001
	Food level	1	0.028	9.481	0.003
	Faeces × Food level	1	0.000015	0.005	0.944
	Error	101	0.003		
SVL	Faeces	1	1.758	1.436	0.234
	Food level	1	4.54	3.708	0.057
	Faeces × Food level	1	1.33	1.087	0.300
	Error	101	1.224		
Growth rate	Faeces	1	5.703	17.727	< 0.001
	Food level	1	5.049	15.694	< 0.001
	Faeces × Food level	1	0.011	0.034	0.854
	Error	101	0.322		
Survival	Faeces	1	0.075	0.704	0.403
	Food level	1	0.675	6.332	0.013
	Faeces × Food level	1	0.008	0.078	0.780
	Error	116	0.107		

survivorship was not significant ($F_{1,116}$ = 0.704, p = 0.403), revealing that presence of faeces does not increase mortality. The interaction of food level and faeces was not statistically significant ($F_{1,116}$ = 0.078, p = 0.780).

DISCUSSION

Many environmental factors including temperature, food availability, pond desiccation and predation risk may influence growth and development of larval anurans (reviewed by Laurila et al., 2001; Neptune & Bouchard, 2020; Youngquist & Boone, 2021). In most cases, the effect of enhanced growth conditions is faster development (reviewed by Álvarez & Nicieza, 2002; Rowland et al., 2016). Our results showed clearly that food level influences the length of larval period of Chinese brown frog tadpoles, suggesting that delayed metamorphosis is caused by low food level. In fact, retardation is due to low food level, indicative of a function of effective energy. Our data showed that Chinese brown frog tadpoles in the presence of faeces were on average larger in body size than those in the absence of faeces. Further, our results have found evidence for positive effect on tadpole growth rate. Thus, we found no evidence for Richards' hypothesis, but it conformed to the inverse of Richards' hypothesis.

Two possible mechanisms could explain such a faeces effect independent of food levels. First, the present study indicated the inhibited growth of faeces may be weak, which could be due to: (1) a single tadpole housed individually in relatively large beakers, indicating that the uncrowded tadpoles may be expected to produce fewer growth-inhibiting algae than the crowded ones in presence of aggressive interactions between individuals (Richards, 1958); (2) algae may suffer inhibition during early development of Chinese brown frog tadpoles because their effects on growth were correlated negatively with tadpole size (Beebee, 1991; Beebee & Wong, 1992; Baker & Beebee, 2000); or (3) single species of anuran larvae produce fewer growth-inhibiting algae of tadpole (e.g. *Prototheca* algae) in their faeces than those raised with two or more species (Griffiths et al., 1993; Bardsley & Beebee, 2001). Therefore, the Chinese brown frog tadpoles that feed their own or congener faeces probably produce a small quantity of protothecan cells in their faeces. Biesterfeld et al. (1993) suggested that Anurofeca is a natural endocommensal of tadpoles, which only inhibits larval growth when they are present in a stressful environment, such as high density or high temperatures (Hailey et al., 2007).

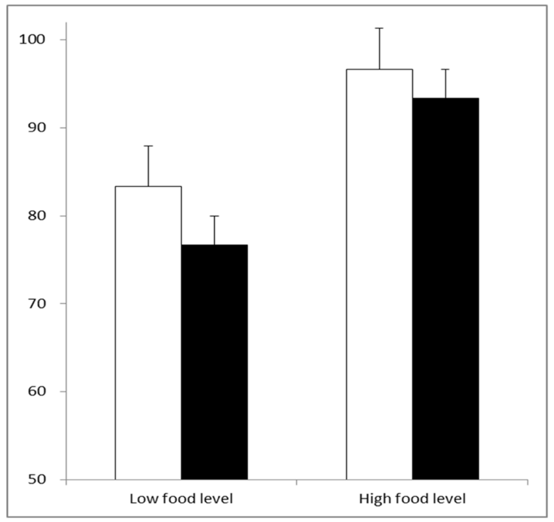

Figure 2. Influences of faecal material and food level on survival of the Chinese brown frog *Rana chensinensis* at forelimb emergence (Gosner stage 42; open columns, absence of faecal material; black columns, presence of faecal material).

A second possibility is that the results can be explained in terms of the nutritional value of the faeces. In this study, Chinese brown frog tadpoles, dawdling like micro automatic vacuum sweeps, absorb excrement with cells and food particles. In fact, compared with the wild ponds, even with the high food level in the laboratory the tadpoles cannot meet their nutritional needs because tadpoles can feed on a multitude of food sources in the wild ponds (Yu, personal observation). Here, the nutritional value of the faeces could be due to: (1) cellulose digested by organisms in the tadpole digestive tract becoming available only after the faeces have been passed, as found in some rodents (Gromko et al., 1973); or (2) it may increase intestinal microbial diversities and alter intestinal microbiota profile of larvae by means of feeding on congener faeces (Zhang et al., 2023); or (3) organisms associated with the faeces have nutritional value in themselves. Steinwascher (1978) suggested that faeces provide a ready particulate food resource for tadpoles although it seems to be a relatively low-quality food. Moreover, previous studies had demonstrated that faeces still contain a low quantity of plant material (Gromko et al., 1973). Our results showed that faeces had a significant acceleration of tadpole growth to obtain large metamorphic size. This result was consistent with Gromko et al. (1973), suggesting that it seems possible that faeces consist of a routine larval food source and are fed as a portion of the routine diet. Further, several studies revealed that there is no evidence of waterborne growth inhibitors or lack of such agents when two amphibian larvae coexist in a pond (Morin & Johnson, 1988; Petranka, 1989; Biesterfeldt et al., 1993). As a result, we suggest that faeces consist of a routine larval diet and are fed as part of the routine food source.

It had been verified that food availability plays fundamental roles in age at metamorphosis (Leips & Travis, 1994; Rowland et al., 2016). Some experimental studies have demonstrated that high food level with a large proportion of protein can generate a double action, accelerating both growth and development (Nathan & James, 1972; Steinwascher & Travis, 1983; Pandian & Marian, 1985; Yu et al., 2016a; 2016c; Yu & Han, 2020). For example, Pandian & Marian (1985) found that a richer and more nutritious food promote larval growth, resulting in shorter larval growth time and larger metamorphic size. Moreover, Wilbur & Collins (1973) model suggested that if the environmental conditions are optimal (such as adequate food), reduce the density and/or competition effects, and the larvae optimise their metamorphosis time and individual size to enter the terrestrial habitat. But limiting food before reaching the minimum size for metamorphosis will lead prolonged metamorphosis. In this study, our results revealed that *R. chensinensis* tadpoles reared at level of high food have a shorter larval developmental time and larger metamorphic size than those reared at the level of low food. Additionally, Beebee (1991) found that growth inhibition is relieved when larvae feed on high food levels.

In conclusion, we find no evidence for the suggestion that faeces are apparent growth-inhibiting factors, which failed to obey Richards' hypothesis. Interestingly, we found faeces can accelerate growth, obtain large metamorphic size and high survival of *R. chensinensis*, as well as food level, suggesting this novel mechanism is selected for where coprophagy is likely to prove profitable, irrespective of the abundance of alternative food.

ACKNOWLEDGEMENTS

We are very grateful to J. Du, Y.L. He and X.Q. Yu for their assistance with fieldwork. Handling and processing of frogs followed approved protocols from the Animal Scientifc Procedures Act 1988 by the State Department of China. All experiments were approved by the Animal Ethics Committee at Xinyang Normal University. The study was funded by Emergency Management Program of National Natural Science Foundation of China (Grant no. 31741019).

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Accepted: 4 March 2023

Herpetological Journal

FULL PAPER

https://doi.org/10.33256/33.4.103110

Published by the British Herpetological Society

Fungal pathogen infection intensity associated with reproductive mode and elevation in an afrotropical anuran community

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The effects of host ecology and environmental conditions on infection dynamics of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) have been documented in several tropical and montane regions across the globe. These interactions are often complex and unique at local scales. Data on the historical and current chytridiomycosis-amphibian system is largely lacking in Africa, especially central Africa. We conducted the first survey of *Bd* in continental Equatorial Guinea in 2019, and extensively sampled the land-bridge island, Bioko, in 2011 and 2019. Our results provide novel information on the distribution and prevalence of *Bd* in Equatorial Guinea and demonstrate the effects of host developmental mode and elevation on *Bd* infections. We found that *Bd* infection loads are positively linked with elevation and that direct-developing species have higher average *Bd* loads than aquatic breeding species at all elevations. These patterns corroborate previous findings in afrotropical amphibian assemblages. Additionally, our study confirms the presence of *Bd* in continental Equatorial Guinea, providing updated information on the distribution of *Bd* in central Africa, and confirming the need for careful monitoring of this biodiverse region.

Keywords: Bioko, Equatorial Guinea, *Bd* infection load, amphibian conservation

Los efectos de la ecología del huésped y las condiciones ambientales en la dinámica de infección del hongo quítrido anfibio *Batrachochytrium dendrobatidis* (*Bd*) se han documentado en varias regiones tropicales y montañosas de todo el mundo. Estas interacciones suelen ser complejas y únicas a escala local. Los datos sobre el sistema quitridiomicosis-anfibios histórico y actual faltan en gran medida en África, especialmente en África central. Realizamos la primera encuesta de *Bd* en Guinea Ecuatorial continental en 2019, y un muestreo extenso de la isla Bioko en 2011 y 2019. Nuestros resultados brindan información novedosa sobre la distribución y prevalencia de *Bd* en Guinea Ecuatorial y demuestran el efecto del modo de desarrollo del huésped y la elevación de las infecciones por *Bd*. Descubrimos que las cargas de infección por *Bd* están relacionadas positivamente con la elevación y que las especies en desarrollo directo tienen cargas promedio de *Bd* más altas que las especies acuáticas reproductoras a lo largo de la elevación. Estos patrones corroboran hallazgos previos en conjuntos de anfibios afrotropicales. Además, nuestro estudio confirma la presencia de *Bd* en Guinea Ecuatorial continental, proporcionando información actualizada sobre la distribución de *Bd* en África Central y confirmando la necesidad de un seguimiento cuidadoso de esta región biodiversa.

INTRODUCTION

Amphibian declines over the past three decades have impacted nearly 30 % of all amphibian species on Earth (Stuart et al., 2004). Among the leading causes for the observed amphibian declines is the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), a species of chytrid fungus that causes the infectious skin disease chytridiomycosis (Berger et al., 2005; Lips et al., 2006; Longcore et al., 1999). A generalist pathogen, *Bd* has affected amphibians in several environments and, while the effects of *Bd* on amphibian communities have been studied intensely in the New World, Europe and Australia, data on *Bd* prevalence and the impact of chytrid on African amphibian communities is sparse (Doherty-Bone et al., 2013; Hirschfeld et al., 2016; Lips, 2016; Scheele et al., 2019; Zimkus et al., 2020). *Bd* was first reported from the African continent in the early 2000s from contemporary field survey data and from

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museum-preserved specimens originally collected in the 1930s (reviewed in Zimkus et al., 2020). *Bd* has now been documented across south, central, and east Africa, as well as Madagascar and the Gulf of Guinea Islands, but has not yet been documented in the Seychelles or West Africa (Zimkus et al., 2020). At least two lineages of *Bd*, *Bd*-CAPE and *Bd*-GPL, are present in Africa, but the current ranges of these genotypes have not been fully documented (Byrne et al., 2019). Here, we performed the first survey of *Bd* in continental Equatorial Guinea and conducted extensive sampling on Bioko Island, from which only museum specimen-based surveys of *Bd* have previously been conducted (Hydeman et al., 2017).

Equatorial Guinea is situated in the Lower Guinean rainforests, which extend into the Gulf of Guinea archipelago (Bioko, Príncipe, São Tomé, and Annobón). Mainland Equatorial Guinea (also known as Río Muni) is characterised by a tropical, hot and humid climate with fairly homogeneous lowland rainforest habitats that are home to at least 66 species of amphibians (Sánchez-Vialas et al., 2020). Bioko Island (formerly known as Fernando Po) is a land bridge island composed of three volcanic peaks reaching up to over 3000 m elevation. This complex topography, along with a precipitation gradient ranging from 1930 mm annual precipitation in the north to upwards of 10,000 mm in the south, result in a patchwork of vegetation types including lowland rainforest (up to 800 m elevation), montane forest (800–1400 m), Schefflera forest/mossy forest (1400–2600 m), and montane heath (2600–3000 m; Fa, 1991). Throughout its history, Bioko has been connected to continental Africa during glacial periods and thus shares most of its 44 reported amphibian species with the adjacent mainland (Sánchez-Vialas et al., 2020). Hydeman et al. (2017) detected *Bd* in museum specimens collected on Bioko Island between 1966 and 1998; however, they did not report infection intensity – a proxy for disease severity – because the effects of preservation on estimating pathogen loads from museum specimens are poorly understood. Consequently, variation in infection intensity across species and habitats has not yet been characterised.

The anuran communities of Río Muni and Bioko Island include representatives of ten families and both aquatic breeder (AB) and terrestrial direct-developer (DD) host life histories. Studies in other amphibian communities indicate that a given individual's response to *Bd* infection can be dependent on the ecological and/or evolutionary background of host species (Valencia-Aguilar et al., 2016; Lips, 2016; Mesquita et al., 2017). For example, neotropical studies have shown significant effects of host life history in *Bd* prevalence and patterns of infection intensity (Brem & Lips, 2008; Gründler et al., 2012; Mesquita et al., 2017). Likewise, in the Neotropics, enzootic infection patterns present as increased *Bd* prevalence and infection loads at higher elevations, leading to increased risk of anuran declines at upland sites (Lips, 1999; Lips et al., 2003; Brem & Lips, 2008; Becker & Zamudio, 2011; Gründler et al., 2012). Thus, variation in both life history and habitat (largely structured by elevation) may also influence *Bd* prevalence and infection intensity in afrotropical amphibian communities.

Here, we performed the first survey of *Bd* in continental Equatorial Guinea and conducted comprehensive sampling on Bioko Island. Our field study included samples from Rio Campo Natural Reserve (sea level to ~60 m elevation) and Monté Alen National Park (~670 m elevation) on the mainland, and from sea level to over 1900 m elevation on Bioko Island. We tested whether *Bd* prevalence and infection loads were influenced by elevation and if they varied among host species identity and life histories. We hypothesised that aquatic-breeding (AB) species and upland amphibian communities would have higher *Bd* prevalence, and that infection loads would be higher in species with direct-development (DD) and in upland terrestrial environments. Our results provide novel information on *Bd* prevalence in Equatorial Guinea, support the effect of elevation and life history on *Bd* infection patterns (Brem & Lips, 2008; Gründler et al., 2012), and inform future studies of *Bd* in west and central Africa.

MATERIALS & METHODS

Geographic and taxonomic sampling

We conducted amphibian surveys during 19 August–12 September 2011 on Bioko Island (Bioko Sur province), and 11–30 April 2019 on Bioko Island (Bioko Sur) and two provinces in continental Equatorial Guinea (Centro Sur and Litoral; Fig. 1). Our sampling on Bioko Island included a range of habitats including lowland forest, agricultural fields, montane forest, and Schefflera forest/mossy forest, spanning elevations from sea level to > 1900 m. Our sampling in continental Equatorial Guinea included lowland forest and wetlands in the Rio Campo Nature Preserve, just above sea level, and agricultural fields at the edge of Monté Alen National Park ~650 m elevation. Sampling for Bioko Sur was more comprehensive, with a combined six weeks of surveys whereas the two continental sites were sampled for just two nights at each site. Our combined sampling includes a total of 455 individual amphibians (Bioko Island = 377, Centro Sur = 38, Litoral = 40) consisting of 43 species and 10 families of frogs. Very few amphibian surveys have been conducted in Equatorial Guinea and thus our understanding of amphibian species diversity is likely incomplete, but we estimate that our sampling represents ~60 % of the Bioko amphibian community and 40 % of the Río Muni amphibian community (Sánchez-Vialas et al., 2020). Species identifications were made in the field by RCB, PJM and LAS with some tentative identifications indicated by "cf.". Based on field guides and primary literature, we classified species according to their larval life histories: aquatic breeders (AB) or terrestrial direct-developers (DD). These reproductive modes are largely fixed within the genera we sampled and therefore our tentative identifications for some specimens should not affect the AB or DD classification (Portik & Blackburn, 2016; Channing & Rödel, 2019).

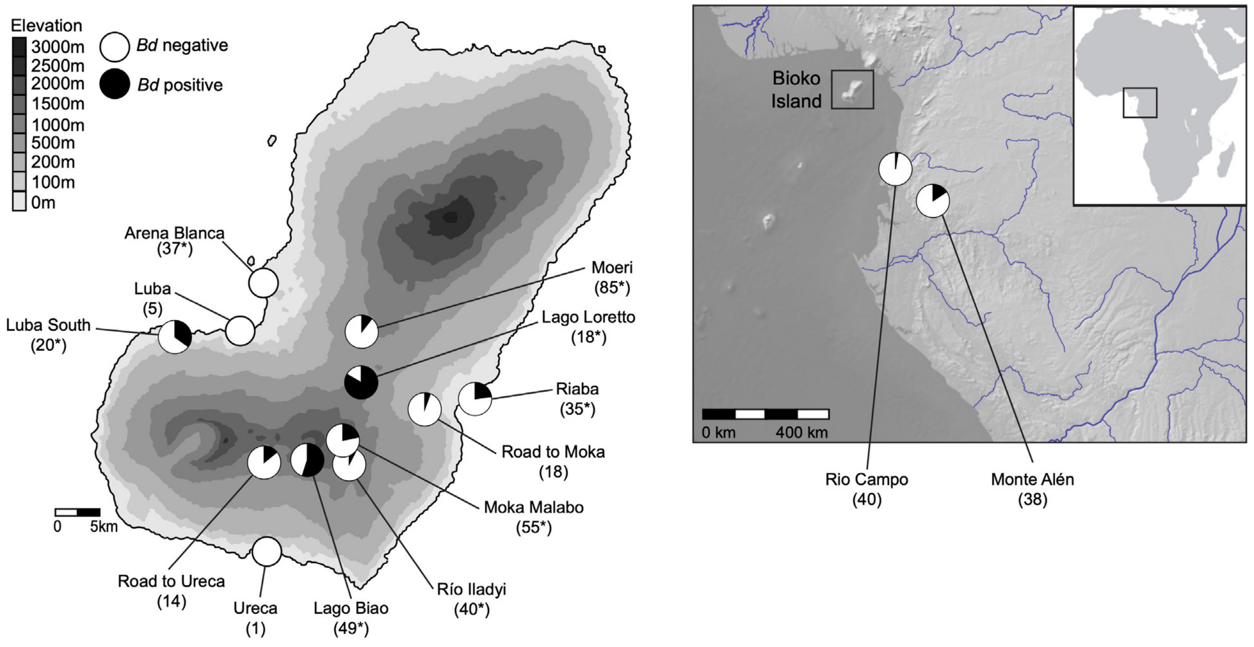

Figure 1. Sampling localities and *Bd* prevalence in Equatorial Guinea. The sample size of amphibians swabbed per site is indicated in parentheses. Asterisks denote sites sampled in both 2011 and 2019.

We conducted surveys in the evening and captured frogs by hand, placing them in individual plastic bags until processing. We collected epithelial samples from post-metamorphic individuals with sterile fine-tip swabs (Medical Wire & Equipment Co. MW113) following the methods of Hyatt et al. (2007). Swabs were stored in 95 % EtOH and kept as cool as possible in the field and then stored at -80 °C until processing. The swabbed individuals were euthanised, prepared as voucher specimens (Table S1), and deposited at the California Academy of Sciences (CAS), Cornell University Museum of Vertebrates (CUMV), North Carolina Museum of Natural Sciences (NCMS), and US National Museum of Natural History (USNM). All research was conducted with IACUC approval (Cornell University 1999-0010, Drexel University 18748, Smithsonian Institution National Museum of Natural History 2016-09).

Molecular analyses

Prior to DNA extraction, we placed the sample tubes in a SpeedVac to evaporate excess ethanol. We performed DNA extractions using the PrepMan Ultra kit (Applied Biosystems). To measure *Bd* prevalence and infection loads, we used a 1:10 dilution of the extracted DNA samples for qPCR analysis in duplicate plates. Samples collected in 2011 were analysed via zoospore genomic equivalents (GE) and ViiA 7 software (Applied Biosystems). Standard curves ranging from 0.1 to 1000 zoospores were generated from templates of known zoospore concentrations of *Bd* strain JEL427 (following Hydeman et al., 2017). Analyses of samples collected in 2019 are a measure of ITS copies based on synthetic standards, not zoospore genomic equivalents. We used primers ITS-1 and 5.8s, Taqman reagents, and synthetic standards (102 to 106 ITS copies) in the QuantStudio™ 3 system to amplify rRNA regions of *Bd* (Boyle et al., 2004).

Statistical analyses

We used Generalised Linear Models (GLMs) to test whether elevation, host species identity, and life history (aquatic breeding vs. direct-developing) were significant predictors of *Bd* prevalence (GLM with binomial distribution and logit link) and log-transformed *Bd* infection loads (GLM with normal distribution and identity link; *Bd*-positive samples only). The most parsimonious model for *Bd* prevalence and *Bd* infection loads were chosen based on AICc and we confirmed normality of residuals for each of our models using the Anderson-Darling test. Due to the different methods of qPCR quantification for the 2011 and 2019 surveys, estimates of infection intensity across years were not combined in the main reported models.

RESULTS

Of the 455 frogs sampled across 2011 and 2019, 91 individuals tested positive for *Bd*, resulting in a 20.0 % (Clopper-Pearson 95 % confidence interval [hereafter CI] = 16.58–23.92 %) global prevalence for our Equatorial Guinea samples. Of the 321 aquatic breeding individuals sampled on Bioko Island, 72 tested positive for *Bd* [prevalence = 22.4 %, CI = 18.21–27.30 %]. Of the 56 direct-developing individuals sampled on Bioko Island, 12 tested positive for *Bd* (prevalence = 21.4 %, CI = 12.71–33.82 %). Of the 75 aquatic breeding individuals sampled in Rio Muni, 6 tested positive for *Bd* (prevalence = 8.0 %) and of the 3 direct-developing individuals, 1 tested positive for *Bd* (prevalence 33.3 %). For more detailed information on 2011 Bioko Island, 2019 Bioko Island, 2019 Rio Muni (mainland) and total Equatorial Guinea prevalence and load data, see Table 1.

For the 2019 Bioko sampling season, the best AICc model pointed to a significant effect of elevation linked

Table 1. Summary table of host life history (AB - aquatic breeder, DD - direct-development), sample size, *Bd* prevalence, and *Bd* infection load data for 2011 Bioko, 2019 Bioko, 2019 Rio Muni and total Equatorial Guinea data surveys. 2011 *Bd* load quantification is a measure of zoospore genomic equivalents (GE), while 2019 *Bd* load quantification is a measure of ITS copies based on synthetic standards.

	Life history	Sample size	Prevalence (%)	Mean load (positives only)	Min, max loads (positives only)	Median load (standard deviation)
2011 Bioko		187	13.9	1.76	0.04, 7.36	1.03 (1.95)
	AB	163	14.1	1.79	0.04, 7.36	0.99 (2.07)
	DD	24	12.5	1.53	0.89, 2.41	1.29 (0.79)
2019 Bioko		190	30.5	332.10	2.34, 2368.13	35.27 (617.44)
	AB	158	31.0	281.49	2.34, 2368.13	29.17 (579.48)
	DD	32	28.1	607.67	9.18, 2005.05	41.05 (774.85)
2019 Rio Muni		78	9.0	139.52	3.13, 841.35	13.12 (311.25)
	AB	75	8.0	22.55	3.13, 96.06	9.62 (36.31)
	DD	3	33.3	841.35	841.35	841.35
All		455	20.0	222.90	0.04, 2368.13	10.42 (519.86)
	AB	396	19.9	179.09	0.04, 2368.13	7.15 (476.85)
	DD	59	22.0	485.76	0.89, 693.23	31.62 (693.23)

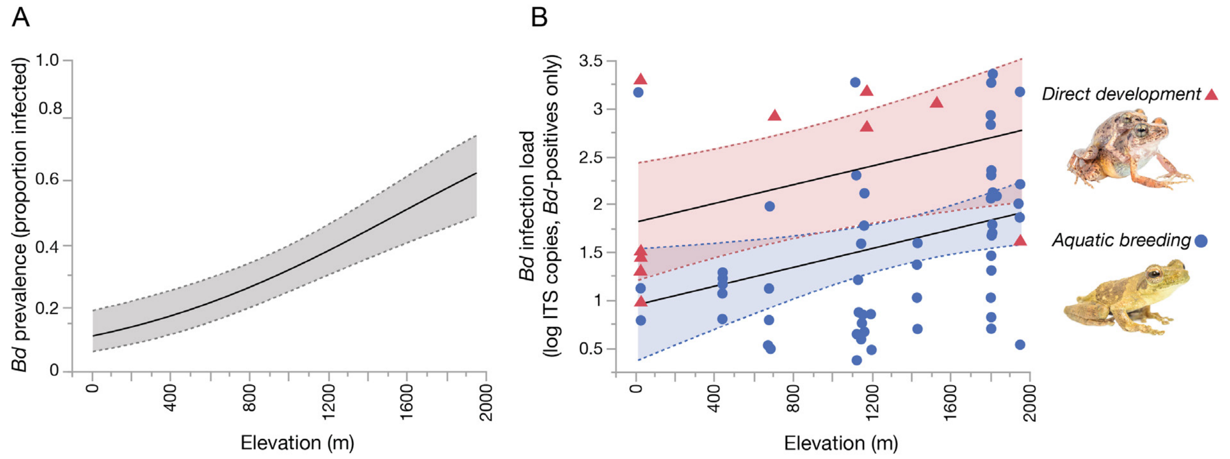

Figure 2. Effect of elevation (m) on both *Bd* prevalence (**A**; logistic fit) and infection intensity (**B**; gaussian fit) using data from the 2019 Bioko sampling season. 95 % confidence intervals are highlighted around the means (solid lines). Independent regression lines are shown for aquatic-breeding and terrestrial developing species in panel B.

to an increase in *Bd* prevalence (Whole Model Test: $\chi^2 = 31.955$, df = 1, $\beta = 0.001$, AICc = 207.210, $p < 0.0001$; Fig. 2A). Host species identity had a significant effect on *Bd* prevalence when included as a single explanatory variable ($\chi^2 = 68.845$, df = 22, AIC = 220.227, $p < 0.0001$), but this model had poorer AICc fit. Elevation and host life history were included in the most parsimonious data explaining *Bd* infection loads in the 2019 Bioko sampling data (whole model test: $F_{[2,55]} = 4.614$, $r^2 = 0.143$, AICc = 150.467, $p = 0.014$). In this model, elevation had a positive relationship with *Bd* infection loads ($\beta = 0.001$, $t = 2.54$, $p = 0.014$; Fig. 2B), and direct-developing frogs carried higher average *Bd* infection loads than aquatic breeders (Fig. 2B). When we included host species identity in our models instead of host life history (whole model test: $F_{[12,45]} = 2.561$, $r^2 = 0.405$, AIC = 158.284, $p = 0.011$), elevation remained a significant positive predictor of *Bd* loads ($\beta = 0.001$, $t = 2.89$, $p = 0.006$), but the model had poorer fit according to delta AICc. Although these relationships were stronger in the 2019 Bioko sampling data, the same patterns were found with combined 2019 Bioko and Rio Muni data (Prevalence: $\chi^2 = 49.172$, df = 1, $\beta = 0.001$, $p < 0.0001$; Infection Loads: $F_{[2,264]} = 31.891$, $r^2 = 0.195$, $p = 0.0001$). Models for 2011 Bioko sampling data did not reveal significant effects of life history, species identity or elevation on *Bd* prevalence, whereas host identity was a single strong predictor of *Bd* infection loads ($F_{[10,15]} = 3.951$, $r^2 = 0.725$, $p = 0.008$). Models using our Rio Muni data alone detected an effect of elevation on *Bd* prevalence ($\chi^2 = 4.548$, df = 1, $\beta = 0.002$, $p < 0.033$), and an effect of host life history on *Bd* infection loads, with direct-developing species carrying higher infections ($F_{[5,1]} = 10.346$, $r^2 = 0.674$, $p = 0.023$), although sample size was limited.

DISCUSSION

We found a positive association between *Bd* prevalence, infection intensity, and elevation in amphibian communities on Bioko Island and mainland Equatorial Guinea. Significant effects of elevation on infection dynamics have been observed in several amphibian communities around the world. For instance, there are several documented *Bd*-driven population declines at high elevation sites in both tropical and temperate regions (Vredenburg et al., 2010; Walker et al., 2010; Catenazzi et al., 2011; Carvalho et al., 2017), and host elevational range predicted decline probability in a neotropical amphibian community (Lips et al., 2003). Likewise, several studies report increased *Bd* prevalence at high elevation sites (Brem & Lips, 2008; Kielgast et al., 2010; Piovia-Scott et al., 2011; Gründler et al., 2012) and increased *Bd* infection loads at higher elevation (Gründler et al., 2012). Our results indicate that elevation may also play an important role in *Bd* dynamics within Equatorial Guinea's amphibian communities.

One framework for explaining why amphibians at higher elevations have higher pathogen loads is the thermal mismatch hypothesis, in which hosts are predicted to be more susceptible to parasites when environmental conditions move away from the host's ideal thermal range (Cohen et al., 2017). In particular, amphibian exposure to warmer temperatures and temperature variability is known to reduce physiological fitness (Rome et al., 1992; Longhini et al., 2021), and increase disease susceptibility and mortality (Raffel et al., 2006; Rohr & Raffel, 2010; Neely et al., 2020). Tropical amphibians, which live at consistently higher temperatures than temperate amphibians, are generally more sensitive to temperature changes associated with global warming (Deutsch et al., 2008; Duarte et al., 2012) and have a narrower thermal range (Rohr et al., 2018). Additionally, several studies demonstrate that high elevation amphibians are more susceptible to temperature variability than low elevation amphibians (Kissel et al., 2019; Cohen et al., 2019). Thus, tropical, high elevation amphibian communities are particularly prone to a thermal mismatch as the climate warms. Furthermore, multiple studies have shown that as global temperatures continue to rise, *Bd*'s range will shift to cooler, higher elevations (Pounds et al., 2006; Cohen et al., 2019) that better match its optimal growth conditions (Woodhams et al., 2008; Voyles et al., 2012). Though *Bd* is less lethal under warmer environmental conditions (Fisher et al., 2009), pathogens generally have larger thermal performance curves than hosts, and thus if the temperature is under the lethal limit for the pathogen, a thermal mismatch will generally favour the pathogen over the host (Cohen et al., 2017; Rollins-Smith, 2017). Correspondingly, Neely et al. (2020) found higher mortality of cool-adapted frogs when both broad thermal range and high elevation, cool-adapted frogs were exposed to *Bd* in a simulated warming event. The combination of more optimal *Bd* growth conditions at higher elevations and the suboptimal immunity found in hosts experiencing a thermal mismatch may explain why high elevation Equatorial Guinea amphibian communities have higher *Bd* infection loads.

Our results support a significant effect of host developmental mode on *Bd* infection patterns. We found that species with direct-development exhibit higher *Bd* infection loads (considering only infected frogs) than aquatic breeders. However, both aquatic breeders and direct-developers showed surprisingly similar *Bd* prevalence, indicating that direct-developing frogs respond differently to *Bd* once they become infected. Differing host ecology and *Bd* epizootiology are known in other tropical amphibian communities. For instance, Gründler et al. (2012) documented similar findings where infection loads were significantly higher in terrestrial breeding species than lotic breeding species, and Mesquita et al. (2017) found that direct-developers acquired higher loads and had higher mortality than aquatic breeders after exposure to *Bd* in a lab experiment. Both studies were conducted with frogs from Brazil's Atlantic Forest where *Bd* is considered enzootic (Gründler et al., 2012; Rodriguez et al., 2014). This pattern of direct-developing species afflicted with *Bd* in enzootic conditions has also been documented in other tropical communities by Burrowes et al. (2004), Catenazzi et al. (2017) and Moura-Campos et al. (2021). In epizootic stages, however, aquatic breeding rather than direct-developing species show conspicuous declines, and this pattern shifts during the subsequent enzootic stage (Lips et al., 2003; 2006; Kriger & Hero, 2007; Brem & Lips, 2008; Catenazzi et al., 2011). Surviving aquatic breeding populations may subsequently become enzootic reservoirs of *Bd* for less resistant direct-developing species (Scheele et al., 2017; Catenazzi et al., 2017).

Terrestrial direct-developing species typically do not congregate around water bodies during the breeding season (Rowley & Alford, 2007); consequently, they are in contact with water less frequently than aquatic breeding species and are not exposed to *Bd* during developmental larval stages. As a result, it appears they may experience weaker selection for *Bd* resistance than aquatic breeding species (Mesquita et al., 2017). For instance, Becker et al. (2019) found that even low loads of *Bd* spillover from aquatic breeding species resulted in significantly higher *Bd* infection loads and increased mortality of a direct-developing species. Thus, in enzootic conditions direct-developing frogs may still be susceptible, and low load pathogen spillover in the environment and direct heterospecific transmission can result in chytridiomycosis and die-offs, even in relatively stable environments (Catenazzi et al., 2017; Becker et al., 2019). These dynamics may further be exacerbated by seasonal variation and changing environmental stressors. For instance, although our temporal sampling is limited, we found differences in both *Bd* prevalence and infection load between our 2011 (August–September; beginning of long wet season) and 2019 (April; short wet season) sampling periods, suggesting that both prevalence and infection load may vary seasonally and interannually in these communities. Drought conditions and even seasonal dry periods cause host behavioural changes in some direct-developing species that result in increased infection loads and mortality (Longo & Burrowes, 2010; Longo et al., 2010; Ruggeri et al., 2018; Moura-Campos et al., 2021). It is unclear whether similar dynamics are at play in afrotropical communities but the strong patterns we detected, particularly on Bioko Island, present an ideal setting for investigating spillover, seasonal variation, and disease risk in afrotropical direct-developers.

Currently, it is unclear whether *Bd*-related declines are occurring in afrotropical amphibian communities. Populations of high elevation spray toads in Tanzania began experiencing sharp declines in 2012, eventually becoming extinct in the wild. However, altered environmental conditions, as well as *Bd*, could have been contributing factors (Makange et al., 2014; Weldon et al., 2020). Studies in Cameroon originally failed to detect *Bd* in highland amphibian populations, but more recent studies have confirmed the presence of *Bd* and suggest a trend of *Bd*-related declines correlated with higher elevation (Baláž et al., 2012; Doherty-Bone et al., 2013; Hirschfeld et al., 2016). It is also unclear whether the presence of *Bd* in Cameroon, Equatorial Guinea, and other afrotropical communities is endemic versus novel or if these amphibian populations are naïve to *Bd*. Additionally, our understanding of *Bd* strains in central Africa is still quite limited, including information on genotypic diversity, current ranges, virulence, whether these strains are native or introduced, and whether co-infection and hybridisation occur (Zimkus et al., 2020). We have not observed die-off events or clear declines in Equatorial Guinea, but our results highlight the potential susceptibility of direct-developing species and high elevation communities. By focusing monitoring efforts on these high-risk amphibian communities, we will begin to better understand the role of host ecology, pathogen ecology, climate change and variability, and afrotropical environmental conditions on Central African *Bd* dynamics.

ACKNOWLEDGEMENTS

We thank Universidad Nacional de Guinea Ecuatorial and Instituto de Desarrollo Forestal y Manejo Del Sistema Nacional de Áreas Protegida (INDEFOR-AP) for research permits and permission to export samples for study (permits 0130, 020/2019, 0224). For logistical support we thank the Bioko Biodiversity Protection Program and for assistance in the field we thank N. Fernandez, A. Fertig, A. Motove, J. Mualeri, J. Nguema Mituy, Martin Nsue, P.B. Ona Owono Owono. For assistance in the laboratory we thank A. Longo. Thanks to C. Irian for the photographs used in Figure 1 (*Arthroleptis poecilonotus* and *Chiromantis rufescens*).

Authors' Contributions

PJM and RCB conceived of the project; PJM, JEM, LJB, LAS, CI and RCB performed the fieldwork; VMM, PJM and RCB performed the lab work; VMM and CGB analysed the data; VMM and RCB produced the figures; VMM and PJM wrote the first draft of the paper; all authors contributed to reviewing and revising the paper.

Data Accessibility Statement

The complete dataset of museum specimen vouchers and corresponding *Bd* loads is available in Table S1.

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Accepted: 2 April 2023

Herpetological Journal
SHORT NOTE
https://doi.org/10.33256/33.4.111114
Published by the British Herpetological Society

Morphological anomalies of tadpoles from a contaminated stream in the state of Tlaxcala, Mexico

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The contamination of aquatic systems by anthropogenic activities may impact amphibian populations by causing malformation and death in tadpoles. However, there often needs to be more information regarding environmental pollutants' effects on amphibians, such as contaminated systems from the state of Tlaxcala in Mexico. This study reports on morphological abnormalities observed in *Lithobates spectabilis* tadpoles found in a stream of three sites monitored in Tlaxcala. The tadpoles presented swollen heads, edema, intestinal hemorrhage, anomalies in the mouth, a deviated tail, and underdeveloped tail fins. We hypothesise that these abnormalities have been caused by exposure to contamination from nearby communities, including garbage and sewage pumped directly into the stream. This research provides the first report on the potential adverse effects of contaminant exposure on tadpole development and morphology in this region. Identifying malformations in tadpoles could help detect contaminants in aquatic systems for further analysis.

Keywords: *Lithobates spectabilis*, pollutants, anthropogenic, edema, abnormalities

Amphibian populations are in decline and at risk worldwide (Wake & Koo, 2018), and one of the leading causes is the presence of environmental contaminants in aquatic systems (Stuart et al., 2004). Contaminant runoff affects the different stages of development in amphibian species that inhabit permanent systems, particularly in waterbodies where the accumulation of pollutants is much greater compared to smaller waterbodies (Barrett et al., 1995; Hu et al., 2020).Tadpoles are particularly vulnerable to contaminants during the early stages of development since they start their life cycle as aquatic organisms (Cooke, 1981; Rosenshield et al., 1999). Contaminants such as metals, microplastics, agrochemicals and radioactive have been shown to negatively impact the morphology, physiology, and behaviour of amphibians that begin their life cycles in aquatic systems (Mann et al., 2009; Ouellet et al., 1997). Tadpoles are very susceptible to contaminants due to their permeable skin, however, some tadpoles are more vulnerable than others. This depends on the dose of the contaminant to which they are exposed and the organism's absorption, elimination, and biotransformation (Lotufo et al., 2015). In addition, it also depends on the octanol-water partition coefficient (Mackay et al., 2013). Therefore, some tadpoles that inhabit contaminated areas can experience harmful effects only if they are in constant exposure, preventing the concentrations of the contaminant in the tissue from persisting at levels that could cause a detrimental biological effect (Lotufo et al., 2015).

Environmental contaminants can directly affect external morphological structures, leading to locomotion problems (Araújo & Malafaia, 2020), bent tails (Pérez-Iglesias et al., 2015; Sunderman et al., 1991; Montalvão et al., 2018), malformation of the mouthparts (keratodons) and changes in skin colouration (Schuytema & Nebeker, 1998; Egea-Serrano et al., 2012). In addition, contaminants affect internal morphological structures, leading to edemas (swelling of the body with liquid) (Wells et al., 2005; Liendro et al., 2015), intestinal malrotation and reddish colouration of the visceral (Severtsova & Aguillón-Gutiérrez, 2013; Lefcort et al., 1998; Snodgrass et al., 2005; Gutiérrez & Bautista, 2015; Peltzer et al., 2013; Christopher et al., 1996). These anomalies could affect ecological aspects such as patterns of relative abundance, dominance, and richness, among others (Bridges & Semlitsch, 2000; Ficken & Byrne, 2013) and physiological mechanisms such as metabolic responses and alterations during metamorphosis (Wells et al., 2005; Severtsova & Aguillón-Gutiérrez, 2013).

Mexico ranks fifth in amphibians worldwide, and approximately 69.5 % of the species are considered threatened. Contamination affects a relatively high number of Mexican amphibians threatened by toxic chemicals (Frías-Alvarez et al., 2010). The malformations in tadpoles have generated scientific interest, with increased detection of malformations within the last decades (Araújo & Malafaia, 2020; Wells et al., 2005; Peltzer et al., 2013). However, in the state of Tlaxcala, Mexico, there has yet been no report of contamination and abnormalities in tadpoles that inhabit aquatic systems. In this study,

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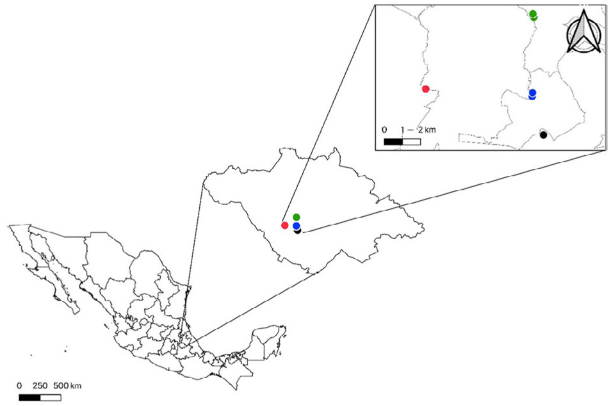

Figure 1. Capture sites located in three locations in the state of Tlaxcala, México. The pink and blue dots correspond to the capture sites in STH, the green and yellow dots in SAT, and the purple in the LTT. In addition, downtown Tlaxcala is marked with a black dot.

we report on morphological abnormalities in tadpoles of Rana manchada (*Lithobates spectabilis*) located in a contaminated stream.

Lithobates spectabilis tadpoles were captured during amphibian monitoring from 1 May 2021 to 30 April 2022, at five sites located in three communities (i.e. human settlements): one site in La Trinidad Tenexyecac (hereinafter LTT; 19° 21'8.37" N, 98° 18'30.79" W), two sites in San Ambrosio Texantla (hereinafter SAT; 19° 21'16.343" N, 98° 15'5.348" W) and two sites in San Tadeo Huiloapan (hereinafter STH; 19° 23'46.131" N, 98° 15'42.101" W). The capture sites are found in the municipalities of Ixtacuixtla and Panotla, in the state of Tlaxcala, Mexico (Fig. 1). The capture sites are located at a distance from the downtown of Tlaxcala of ~7.6 km for LTT, ~4.6 km for SAT and ~8.01 km for STH. This distance between the capture sites and the downtown of Tlaxcala was measured linearly using the Qgis software (Quantum GIS Development Equipment). The vegetation is composed of *Quercus* spp.

The tadpoles were captured using a fishing net in the stream, and with the permission of the wildlife department (SEMARNAT SGPA/DGVS/03662). A group of biologists from the Universidad Autónoma de Tlaxcala determined the identity of the species. In addition, the species was determined by utilising the identification guide for *L. spectabilis*. The tadpoles of *L. spectabilis* are of the stream type; it has a long and muscular tail with low fins. In addition, tadpoles of this species grow to be quite large, and individuals with a total length of over 100 mm are common (Hillis & Frost, 1985).

Once captured, the tadpoles were immediately transported to the laboratory in two tanks (51 x 29.5 x 26 cm) with water from their respective sites. We obtained the tadpole's body mass (Mb) using a digital balance with a precision of 0.1 g (Ohaus, Newark, New Jersey, U.S.A.), and snout-vent length (SVL) using a caliper to the nearest 0.1 mm (Truper, CALDI-6MP; 14388). Morphological characteristics were determined with a binocular stereoscope (Mitoc®, 1272ZH), according to criteria reported by Gutiérrez & Bautista (2015). Photographs were taken with a Canon-DS126491 camera.

Figure 2. Morphological anomalies in *Lithobates spectabilis* tadpoles located in the contaminated stream SAT, Tlaxcala, Mexico. **(A)** Contamination mainly by garbage and **(B)** car washing in the stream. **(C)** Tadpole without abnormalities with normal head (arrowhead), long tail (black arrow) and caudal fin with melanophores (red arrow). **(D)** Tadpoles with morphological abnormalities folded fin and without melanophores (red arrow). **(E)** In addition, they had small tails that were thin, curved, and slightly crooked (arrow), **(F)** edema, deformation of labial papillae (arrowhead), and **(G)** intestinal bleeding (arrowhead).

We captured 100 tadpoles of species *L. spectabilis* (LTT n = 30; SAT n = 40; STH n = 30). We observed that three tadpoles presented morphological abnormalities at one site in particular (SAT), which is a stream contaminated by anthropogenic activities (Fig. 2A & B). During surveys, we observed abundant evidence of contamination of streams at SAT, primarily solid waste material (polystyrene, aluminium, plastics, glass, detergents, oils) resulting from various anthropogenic activities. In addition, people visit the stream to generate garbage (Fig. 2A), and wash their cars in the stream (Fig. 2B).

We found tadpoles without abnormalities (Fig. 2C), and the tadpoles with abnormalities had a caudal fin that was not well-formed and lacked melanophores (Fig. 2D). Additionally, the abnormal tadpoles had small tails that were thin, curved, and slightly bent (Fig. 2E). The tadpoles had a swollen head (edema in the body with a transparent liquid), deformation of labial papillae (Fig. 2F) and intestinal bleeding (Fig. 2G).

Although we did not obtain enough data from the tadpoles with abnormalities to carry out a statistical test, we observed that the Mb of these was substantially greater (0.29 ± 0.1 g) when compared to tadpoles without abnormalities (0.19 ± 0.06 g). Furthermore, snout-vent length (SVL) of tadpoles with abnormalities was lower (17.7 ± 6.1 mm) concerning tadpoles without abnormalities (20.6 ± 2.0 mm) in SAT. While capturing the tadpoles with abnormalities, we observed a slow and unsynchronised swimming movement, which made capturing the tadpoles faster.

The present work is the first formal report of alterations in the morphology of tadpoles in a site that is part of the megalopolis of the Mexican highlands. Curiously, malformations were only observed in tadpoles found in the capture sites located in the SAT community, which is the closest to downtown Tlaxcala, unlike the other two study sites. The SAT site is constantly visited by the community people, so malformations in the tadpoles can be indicators of environmental health and habitat quality. Additionally, according to interviews with authorities, sewage is discharged directly from the community, without treatment, into this aquatic system. This finding may be important because surface-water discharge from industrial and municipal is one of the significant sources of contaminant mixtures in aquatic systems (Pal et al., 2010) and tadpoles exposed to these domestic residual releases result in developmental alterations, metabolic disruption, and behavioural effects (e.g. preference or avoidance of areas with high contaminants) (Melvin et al., 2016; Krishnamurthy et al., 2008; Pal et al., 2010). These results indicate the first study determining the effect of wastewater on tadpole development in the stream SAT and that the distance between SAT and downtown Tlaxcala could indicate more stream contamination and a possible increase in the frequency of abnormalities in the tadpoles.

The tadpoles showed edema, which can signify exposure to these particular chemicals. The plastics product of garbage generation (Rhodes, 2019), metals like aluminium (Ismail et al., 2019), and even different pesticides (Lenkowski et al., 2010) are widely present in the different aquatics system and causes different abnormalities such as edema. However, viruses, bacteria and fungi also can cause edema and other morphological abnormalities (Densmore & Green, 2007). On the other hand, intestinal hemorrhage affects their feeding and induces the death of individuals (Marco & Blaustein, 1999). The intestinal abnormalities are due to oxidative stress or necrosis in the cells (Wells et al., 2005; Liendro et al., 2015). The tadpoles with deformation of labial papillae have feeding problems (Pérez-Iglesias et al., 2015). Caudal fin malformation affects thrusting, bending, buckling, locomotion and escape from predators (Doherty et al., 1998). In addition, other researchers determined the curved and deflected tail may affect swimming activity and orientation (Cooke 1981; Rosenshield et al., 1999), just like foraging (Horat & Semlitsch, 1994).

Although we found damage to the morphology of the tadpoles, the usual concentrations of pollutants in the stream in SAT are unknown. Our results suggest that the anomalies found in the tadpoles may be a biological indicator of contamination in this small aquatic system. However, the specific contaminants responsible for these malformations were not identified. It is, therefore, essential to analyse the water and sediments of the aquatic ecosystems of Tlaxcala, since there currently needs to be more information on water quality. For this reason, it is critical to carry out more ecotoxicological research and provide adequate solutions to avoid contamination and possible threat status of aquatic species at this site. Monitoring tadpoles with morphological anomalies can be a vital strategy for identifying potentially contaminated sites in Tlaxcala, México.

ACKNOWLEDGEMENTS

We thank the authorities of San Tadeo, Huiloapan, San Ambrosio Texantla, and the Trinidad Tenexyecac community for their support in the logistics of this work. Likewise, to A. Popocatl and E. Chamorro for their support.

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Accepted: 2 April 2023

Volume 33 (October 2023), 115–118

Herpetological Journal

SHORT NOTE

https://doi.org/10.33256/33.4.115118

Published by the British Herpetological Society

Intensive and efficient egg-laying tempo of the parthenogenesis mourning gecko *Lepidodactylus lugubris*

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Although suffered from many evolutionary disadvantages, asexual species benefit from their high efficiency to build clonal populations in a novel habitat. Here we report that a parthenogenetic mourning gecko *Lepidodactylus lugubris* continuously laid 12 clutches within two years. The egg-laying period lasted from mid-April to late October, and the egg-laying interval ranged between 24 and 73 days with a mean of 34.4 days. Referring to this fecundity, a female gecko could reproduce up to 24 clonal female offspring every year, and expand the population size in an efficient way much faster than most bisexual species. The intensive and efficient egg-laying tempo, associated with the parthenogenetic breeding mode, are crucial factors which facilitated their successful colonisation into many islands.

Keywords: clonal population, cost of sex, invariant clutch size, invasive species

Figure 1. The mourning gecko *Lepidodactylus lugubris* collected from Guishan Island in this study, photographed in 2021.

From a long-term aspect, parthenogenetic species were regarded as an evolutionary deadend (Burt, 2000; Barraclough et al., 2003). Asexual species usually suffered from many disadvantages such as losing the opportunity of recombination, lack of the ability to adapt fluctuating environment and accumulation of deleterious mutations (Normark et al., 2003; Otto, 2009). However, asexual species are free from the "cost of sex" (Maynard Smith, 1971; Lehtonen et al., 2012). In addition to the advantages of saving time and energy in courtship behaviours, a straightforward benefit is the two-fold breeding efficiency because only daughters are produced. This advantage facilitates their clonal colonisation to new environments, especially on the islands (Baker, 1955; Cuellar, 1977; Ineich, 1999).

The mourning gecko *Lepidodactylus lugubris* (Duméril & Bibron, 1836) is the most well-known parthenogenetic Squamata which has increasingly expanded its range during the last century. This gecko was deduced to have a hybrid origin, while the central Micronesia is where two putative parental species overlap their distributional ranges (Radtkey et al., 1995; Ineich, 1999; Karin et al., 2021). It has been reported from multiple tropical and subtropical countries; most of which were colonised in recent years. For example, the species has reached several central and south American countries (Nania et al., 2020) during the last decades, established new invasive populations in Thailand (Lapwong & Juthong, 2018), and is continuing to spread (Hoogmoed & Avila-Pires, 2015). In the islands off the coast of east Asia (except the endemic populations on Daito islands), populations of *L. lugubris* occurred after WWII, including islands of Taiwan, Ryukyu and adjacent islets (Yamashiro et al., 2000; Ota et al., 2004; Lee et al., 2019).

The breeding ecology of *L. lugubris* has been studied in different aspects. For example, Brown & Murphy-Walker (1996), Yamashira & Ota (1998) and Röll & von

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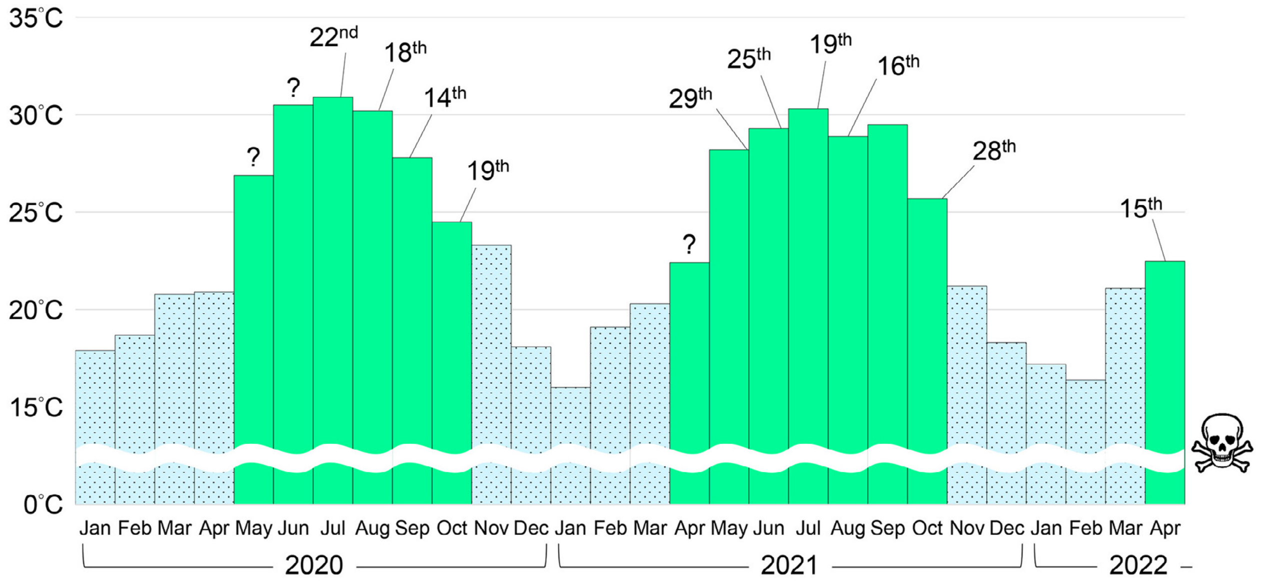

Figure 2. Month and date of the thirteen clutches produced by a young captive mourning gecko *Lepidodactylus lugubris* from 2020–2022. The green bars indicate the potential breeding season in Taiwan, and the question marks indicate that the date of the clutch were not confirmed. The gecko died of dystocia (egg-binding) on the last clutch on 15 April 2022.

Düring (2008) reported the rare cases of sterile males in this species. Brown & Sakai (1988) compared the impact of social experience on egg development; and Brown & O'Brien (1993) further compared the growth rate and fecundity of dominant versus subordinate females. The hatching success was evaluated under the influence of temperature and salty water (Brown & Duffy, 1992; Ota, 1994). In recent years, this gecko is further applied as a model species which contributes to a variety of fields of embryological research, including developmental morphology, sexual development or clutch size variation (Griffing et al., 2019a; 2019b; Sakai, 2021). However, most of these experiments were conducted in Hawaii or Okinawa, and their fecundity in other subtropical regions has never been studied.

Here we report a case of a captive mourning gecko which laid 12 clutches (24 eggs) within 2 years. This record helps to roughly estimate the fecundity of this parthenogenetic species under a subtropical environment.The young *L. lugubris* (snout-vent length = 24.6 mm; Fig. 1) was collected on 14 July 2019 from Guishan island (24.84167 N, 121.95315 E) (research permission No. 1110072285). Based on measurements from Brown & O'Brien (1993), the mean snout-vent length of newly hatched *L. lugubris* is 18.7 mm, and the growth rate is 3 mm per month in the first two months. Therefore, the gecko was deduced to be less than two months old which was hatched during middle to late May of 2019.

The individual was transferred to the laboratory in National Taiwan Normal University (25.00678 N, 121.53564 E) in Taipei and was kept in an acrylic enclosure (30 × 15 × 20 cm). The floor of the enclosure was covered with 2 cm of humic soil mixed with coco chip and coco fibre, and a pottery pot was provided as shelter. Because we did not use an air conditioner in the enclosure which housed the gecko, the room temperature and humidity were identical to the outdoor ambient environment with light and dark cycles following natural conditions. The climate of Taipei City (the captive site) and Guishan Island (the collection site) represented congruent temperature fluctuation (open data from the Central Weather Bureau, Taiwan; 2019–2022). In the breeding season, four to five newly hatched crickets *Gryllus bimaculatus* or cockroaches *Shelfordella lateralis* (mean mass = 0.0218 g each) were consumed every day, with calcium and vitamins provided once a week. Collection and husbandry of the gecko was approved by Institutional Animal Care and Use Committee (IACUC) of National Taiwan Normal University (license No. 110038).

The individual reached sexual maturity (> 40 mm snout-vent length) in spring of 2020 and laid the first and second clutches (both 2 eggs) in May and June, respectively. Since July, we started to record the precise egg-laying date. During 2020–2021, it produced 12 clutches (Fig. 2); 6 clutches per year and 2 eggs per clutch. Under the climate of Taipei, the egg-laying period lasted from mid-April to late October, only in months when the mean temperature was higher than 22.4 °C. The egg-laying interval (N = 7) ranged between 24 and 73 days, with a mean of 34.4 days. Among these eggs, two clutches hatched in 2020, but others failed because we did not maintain enough moisture in the enclosure. On 15 April 2022 the gecko died during its 13th clutch from dystocia (egg-binding).

As a member of Gekkonidae, *L. lugubris* exhibits an invariant clutch size (ICS): usually two eggs are laid in each clutch (occasionally one in some cases), which is a common phenomenon in the Gekkonidae, *Anolis* spp., some skinks and some *Takydromus* lizards (Kluge, 1967; Andrews & Rand, 1974; Shine & Greer, 1991; Vitt & Caldwell, 2014; Lin et al., 2020). In these cases, the lizards could compensate their fitness by producing repetitive and continuous clutches, as shown from this gecko.

Based on records in Hawaii, the time for egg development (the period from eggs > 1 mm to the egg-laying day) was usually 25–26 days (Brown & Sakai, 1988). However, the inter-clutch interval was more than 40 days (Brown & O'Brien, 1993). In this study, we did not record the egg development period; whereas the inter-clutch interval could be as short as 24 days, and more frequently to be 27 to 28 days (N = 4). Compared to Hawaii, the climate of northern Taiwan represents a much higher temperature oscillation among seasons, i.e. a hotter temperature in summers (mean maximum temperature 34 °C vs. 30 °C in July), and a cooler temperature in winters (mean minimum temperature 14 °C vs. 20 °C in January) (data source: https://tw.weatherspark.com/). In two previous works, Ota (1994) and Sakai (2016) indicated the temperature limitation of egg development in cooler climate condition. This might explain why the mourning gecko in Taiwan was not able to breed all year round. We suspect that the high seasonal variation in Taiwan might shrink the breeding season of the gecko, and the higher temperature in summer may accelerate the development of the eggs in the females' body.

Because *L. lugubris* could reach reproductive age in the second year (Brown & O'Brien, 1993; this study), they have the potential to build a clonal population soon after they arrive in a new habitat. In order to provide a simplified speculation on how fecund a parthenogenetic gecko could be, we estimated the upper bound of the gecko (although it could not be reached in a realistic situation). Assuming the most extreme situation that all the eggs successfully hatched and all offspring successfully survived, two females could expand the population size to 26 in the second year, and reach 338 in the third year. At the same time, a pair of bisexual gecko with the same fecundity (6 clutches per year) could only reach the population size of 98; only half of them are females if the sex ratio of the species is 1:1.

As one of several fastest spreading geckos in the world, the occurrence of *L. lugubris* in many tropical and subtropical regions seems inevitable. We conclude that the intensive and efficient egg-laying tempo, associated with the parthenogenetic breeding mode, have facilitated the colonisation of this gecko in many tropical and subtropical islands. Although suffered from many disadvantages under a long-term evolutionary aspect, the asexual breeding system has undoubtedly acquired advantages in this age with frequent human-mediated dispersal events.

ACKNOWLEDGEMENTS

This work was funded by Ministry of Science and Technology, Taiwan (MOST 110-2311-B-178-001-MY3). Thanks to Chung-Wei You for the photograph in Figure 1.

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Accepted: 8 April 2023

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