



# The Herpetological Bulletin

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# THE HERPETOLOGICAL BULLETIN

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**Front Cover:** Red salamander (*Pseudotriton ruber*) taken near Berea, Kentucky.

A Natural History Note on this species can be found on page 28. Photograph credit: Lief Van Laar, Wikimedia Commons.

# Factors affecting body condition in a great crested newt *Triturus cristatus* population.

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**ABSTRACT** - Body condition index (BCI) was calculated for 942 individual great crested newts *Triturus cristatus* captured from four ponds 2007 - 2011. Body condition did not vary significantly between ponds. BCI varied significantly between years and seasons but in different ways between the sexes. Males exhibited lowest body condition in March followed by a rise in the period March to May then a fall in June in three out of five years. There was no significant change in female BCI March to April within each year. There was no significant relationship between BCI scores and mean winter minimum air temperature in both sexes. Findings indicate that BCI varies considerably due to a range of factors and that long-term studies are required to determine whether changes in winter climatic conditions significantly affect great crested newt body condition.

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## INTRODUCTION

The use of Body Condition Index (BCI) scores to assess the physiological state of an individual has been widely applied in many studies of animal populations including amphibians (Cooke & Arnold, 2003; Kopecký et al., 2010), seabirds (Le Bohec et al., 2007; Lormée et al., 2003) and mammals (Schulte-Hostedde et al., 2001). Body Condition Index scores, which are usually based on mass/length regressions, may give an indication of the fat reserves of an animal and reflect its underlying health and susceptibility to disease (Janin et al., 2011). BCI scores may also indicate past foraging success, fighting ability and the capacity to cope with environmental pressures, which may impact on reproductive success (Jakob & Marshall, 1996). In the British Isles, research by Reading (2007) and Griffiths et al., (2010) indicates that populations of common toad *Bufo bufo* and great crested newt *Triturus cristatus* respectively have lower body condition after milder winters. This may be because individuals expend more body fat reserves in higher temperatures due to an increased metabolism (Reading 2007), resulting in increased susceptibility to disease, lower reproductive success and subsequent population decline. The great crested newt is of conservation concern, and may be the fastest declining amphibian within Europe (Griffiths, 2001). Within the British Isles this species is fully protected as part of Schedule 5 of the Wildlife and Countryside Act 1981 and Annexes II and IV of the European Habitat Directive 1992 (English Nature, 2001). Although many population studies have been conducted in this species (e.g. Baker, 1999; Williams, 2000; Jarvis, 2010) few have examined factors affecting body condition indices and how this may impact on population size and recruitment.

This study examined the body condition index scores of

adult great crested newts from three breeding ponds over a five-year period from three ponds within Epping Forest, UK. Specific aims were to determine whether BCI scores varied between years, ponds and seasons as well as whether there was a relationship between winter temperatures and BCI scores of adults in the spring.

## MATERIALS AND METHODS

Adult *T. cristatus* were captured in funnel traps as described in Griffiths (1985) and Griffiths & Inns (1988) from three ponds within Epping Forest, UK (51° 39' 47" N, 0° 2' 35" E) in the period 2007 to 2011. Two of the ponds (Frog and Lily) were 25 m apart whilst the third (Oak Plain) was separated across deciduous woodland by approximately 500 m. All great crested newts captured were taken to an outside work station located by the pond of capture. Snout-vent length (SVL) was measured to the nearest 0.5 mm using callipers. Body mass was recorded to the nearest 0.01 g using digital pocket scales. To record belly markings individuals were placed on a small plastic transparent plate with raised sides and a sponge gently placed over the top to provide a uniform background. A compact digital camera was used to take photographs of belly pattern markings from underneath since each individual has a unique belly pattern marking (Arntzen et al., 1999) which can result in subsequent identification of individuals. Once all measurements and data had been collected all newts were released into the pond of capture.

### Data analysis

Body condition index (BCI) scores were calculated using residuals (y) from a Model II regression of mass against SVL after log transformation using the formula:  $BCI = (\text{Log}_{10} \text{ SVL}) / (\text{Log}_{10} \text{ mass})$  (Green, 2001). Male body

condition indices were calculated March to June each year 2007 to 2011. Female body condition indices were restricted from March to the end of April each year 2007 to 2011 due to a drop in the incidence of capture later in the breeding season and a lack of data from May to June. Therefore no analysis was conducted for females in May and June of each year. Male and female great crested newts were grouped separately for BCI calculation due to differences in size (Verrell & Halliday, 1985). An unbalanced two-way analysis of variance (ANOVA) was performed using the general linear model (GLM) facility in Minitab to determine whether there was a difference in BCI with year, pond and season, which were treated as fixed factors. Calculations for BCI were made just once (at first capture) for each individual (as identified by belly recognition, Artnzen et al., 1999) to avoid pseudoreplication. Individuals identified as recaptures were not included in analysis. Weather data were collected from an on-site meteorological station. Mean winter (November to February) minimum air temperature was calculated since adults are typically in their terrestrial phase during this period (English Nature, 2001; Baker et al., 2011). Linear regression was used to determine whether there was a relationship between winter temperature and the BCI of males and females the following spring.

## RESULTS

### Differences in body condition between ponds

A total of 942 individual adults (540 male, 402 female) were captured in the period 2007 to 2011 from three ponds. Neither male nor female body condition varied significantly between ponds.

### Differences in body condition between years

Adult male *T. cristatus* showed significant variation in Body Condition Index scores between years (Fig. 1) ( $F_{4,540} = 6.61$ ,  $P < 0.0001$ ). Post hoc ANOVA revealed a significant difference in BCI scores between the years 2007 to 2009 ( $F_{2,488} = 73.78$ ,  $P < 0.0001$ ) but not between 2009 and 2011 ( $F_{2,181} = 2.00$ ,  $P = 0.19$ ). Mean BCI scores were significantly lower in 2007 at 2.06, compared to a range of 2.78 to 3.74 in the period 2008 to 2009. BCI scores were highest in 2008 at 3.74.

Body Condition Index scores were significantly different across years in females (Fig. 2) ( $F_{4,402} = 19.52$ ,  $P < 0.0001$ ). Post hoc ANOVA revealed a highly significant difference in BCI scores between 2007 and 2008 ( $F_{1,171} = 27.68$ ,  $P < 0.0001$ ) as well as in the period 2008 to 2011 ( $F_{3,355} = 10.31$ ,  $P < 0.0001$ ). The pattern of BCI scores was different to that exhibited by males. The highest mean BCI score was in 2007 at 4.71, the opposite for that shown by males. Females in the period 2008 to 2011 had more consistent mean BCI scores ranging from 2.31 to 2.94. The lowest BCI score was in 2011 at just 2.31, after a cold and relatively dry winter. Body condition appeared to decline through the study period 2007 to 2011. It was not possible to determine whether this was a temporary trend.

### Differences in body condition between seasons

Within each year, male body condition varied significantly between months (Fig. 1) ( $F_{3,540} = 5.36$ ,  $P = 0.01$ ). Mean BCI scores increased through the months March to May 2007 to 2009. This pattern was not as evident in the remaining years 2010 and 2011. BCI scores also fell in June 2008, 2009 and 2011 but not the remaining years. These results show that BCI is highly variable between months but overall appears to show an increasing trend through the breeding season, often with a fall in June. There was no significant variation in female BCI March to late April (Fig. 2).

### Interaction effects of pond, year and season

Male BCI scores showed significant interaction between year and season ( $F_{12,540} = 2.65$ ,  $P = 0.002$ ) and year and pond ( $F_{8,540} = 2.25$ ,  $P = 0.02$ ), indicating that both seasonal and pond variation depend on year. There was no significant interaction between pond and season ( $F_{6,540} = 2.00$ ,  $P = 0.06$ ). Due to lack of data in some months, a combined interaction of pond, year and season could not be performed.

Female BCI scores showed a similar pattern to that shown by males with significant interactions between year and season ( $F_{4,402} = 2.90$ ,  $P = 0.02$ ) but not pond and season ( $F_{2,402} = 1.40$ ,  $P = 0.25$ ). This also suggests that seasonal variation depends on year. Due to lack of data, a combined interaction of pond and year, along with year, pond and season could not be performed.

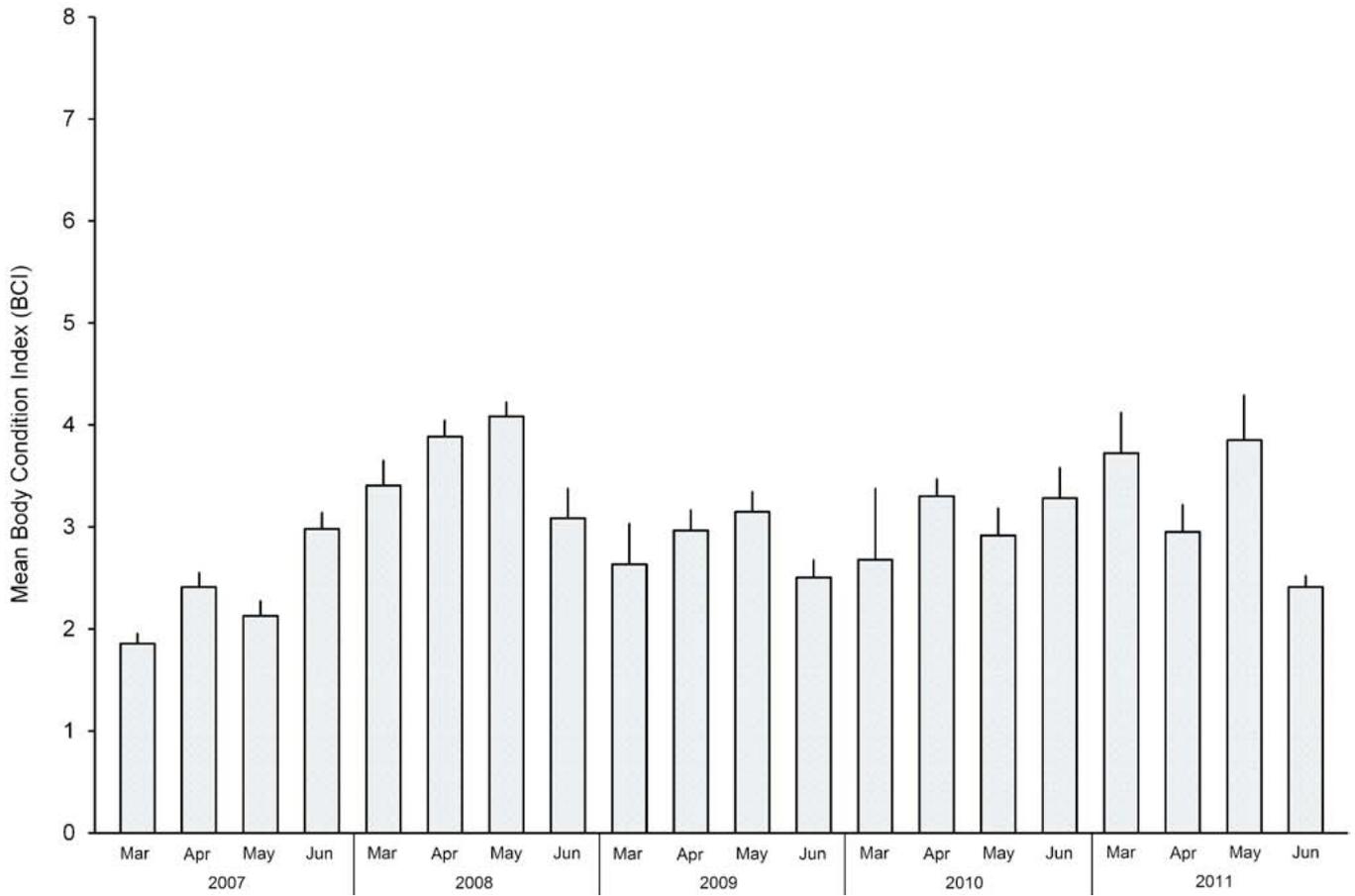
### Effects of winter temperature

There was no significant relationship between mean winter temperature each year and mean male or female body condition index scores the following spring. Therefore body condition in spring was not affected by winter temperature over the five year duration of the study.

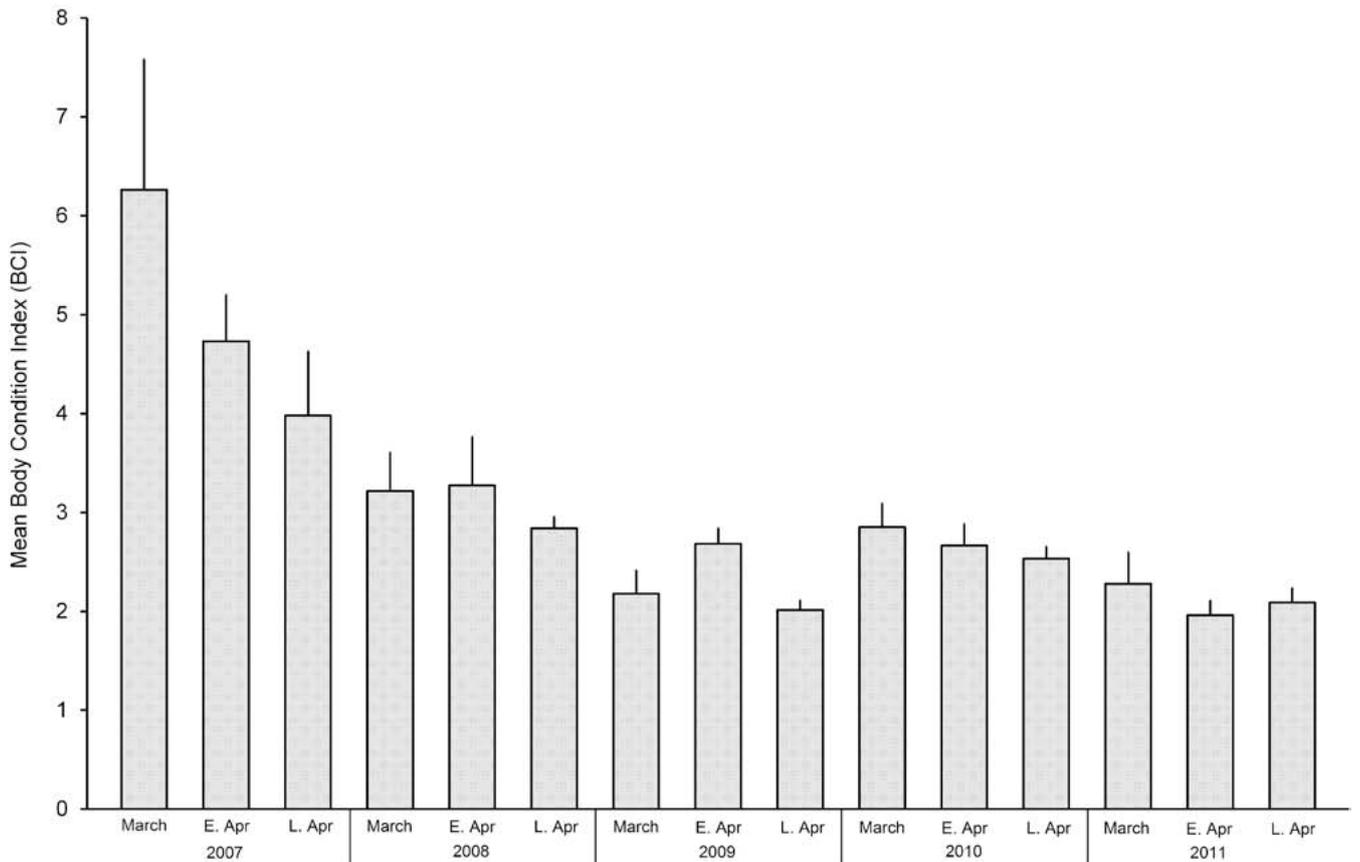
## DISCUSSION

Results from this five year study show that neither male nor female *T. cristatus* showed a significant difference in body condition between ponds. Previous studies on the common toad *B. bufo* have demonstrated that body condition indices vary between sites (Sztatecsay & Schabetsberger, 2006; Reading, 2010). However these studies were conducted at sites separated by a larger spatial scale than the ponds in this study. The ponds in the study area of this study were all within 500 m of each other such that microhabitat conditions such as light, temperature and vegetation cover, which may impact on feeding and therefore body condition, were very similar and not great enough to cause significant variations in BCI scores at breeding ponds.

Both males and females showed significant differences in Body Condition Index scores between years with significant interaction between both years and seasons. However, only males showed a significant variation in BCI between seasons. The pattern of BCI scores across years was considerably different for each sex. Males had the lowest BCI in 2007, before scores rose in 2008 and levelled off in the period 2009 to 2011. In contrast, females had the highest BCI scores in 2007, followed by a drop in BCI



**Figure 1.** Mean Body Condition Index (BCI) of adult males March to June, 2007 - 2011. Error bars denote standard error.



**Figure 2.** Mean Body Condition Index (BCI) of adult females March to April, 2007 - 2011. Error bars denote standard error. Mar = March; E. Apr = Early April; L. Apr = Late April.

scores through the rest of the study period. The reasons for the observed differences in BCI between the sexes may be because BCI scores are likely to indicate fecundity in females and fat reserves in males. Individuals from different sexes are likely to be differentially affected by variations in biotic and abiotic factors in different years at breeding and overwintering habitats. In addition males and females exhibit different microhabitat utilisation in aquatic and terrestrial habitats, which may affect acquisition of fat reserves or development of eggs in males and females respectively. Therefore the differences in patterns of BCI in males and females may be due to differences in the way the sexes allocated food resources and fat reserves.

Other studies on amphibians have shown that BCI scores vary over time (Arntzen et al., 1999; Wheeler et al., 2003; Băncillă et al., 2010) but few have documented differences in sexes between years. Griffiths & Mylotte (1987) state that male *T. cristatus* probably do not feed on the way to breeding ponds, however they do appear to take prey on arrival at the aquatic environment (Griffiths & Mylotte, 1988). Griffiths (1986) recorded differences in prey captured by different co-habiting newt species but not between male and female great crested newts. Further research specifically examining how biotic factors affect BCI scores is required before more definite conclusions can be drawn.

Male, but not female, *T. cristatus* exhibited a significant change in body condition between seasons. Males showed low body condition at the start of the season in the period 2007 to 2009, followed by a rise until May within each of these years. Body condition declined in June in three of the five study years. This trend appears contrary to that found by Arntzen et al. (1999) and Sinsch (2003) who both found that great crested newts had highest BCI scores at the start of the year and declined as the breeding season progressed. Similarly, Băncillă et al., (2010) noted that yellow-bellied toads had highest body condition index scores in spring and lowest in the autumn. However, Verrell (1986) observed a decrease in body fat content in smooth newts *Lissotriton vulgaris* over the winter period. The reason why males had low BCI scores at the start of the season in this study may reflect specific microhabitat conditions in terrestrial habitats or differences in timings in emerging from hibernation and first breeding. Once males enter water, an increase in hydration may alter body mass and condition. Therefore BCI scores may vary seasonally due to differences in hydration levels of individuals. Males in this study exhibited a drop in BCI score in June in three years. Arntzen et al., (1999) state that this is due to high energy expenditure during the aquatic reproductive phase which lowers mass and therefore body condition.

There was no significant difference in female BCI across March to late April. A high BCI score at the start of the season would be expected in females due to individuals holding large numbers of eggs (Halliday & Tejedo, 1995). These will be lost through the breeding season, resulting in a drop of BCI score. However a drop in BCI across the breeding season was not observed in this population, perhaps due to analysis being restricted to the early and mid-parts of the breeding season (March to April). Further

data across over a longer seasonal time period are required to determine whether BCI in females changes with season in this population.

The effects of winter temperature revealed non-significant trends in both males and females. Therefore further data are required to ascertain whether trends become significant over a longer time period. The results from this research highlight that long-term studies over more than five years are required before significant inferences can be drawn from climatic data and its influence on body condition and survival.

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# Captive husbandry and breeding of the banded knob-tailed gecko (*Nephrurus wheeleri cinctus*) at Perth Zoo

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**ABSTRACT** - This paper describes the captive husbandry and breeding information gained from 11 wild-born banded knob-tailed geckos, *Nephrurus wheeleri cinctus*, held at Perth Zoo from May 2009 to June 2014. Geckos bred from late October through to late May. Females produced 2-4 clutches per season initially, with older females producing 5-6 clutches per season. Incubation lasted 51-60 days, with 33-63 day intervals between clutches, shortening to 23-46 days in the more fecund females. Regardless of the number of clutches produced each breeding season, the inter-season interval remained similar, at 225-268 days. Egg weights and dimensions are described, along with hatchling birth weights. Hatchling growth rates are provided for seven individuals, from hatching through to 300 days of age.

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## INTRODUCTION

The banded knob-tail gecko is found in the mid-west of Western Australia and has two sub-species; *Nephrurus wheeleri wheeleri* which is found in the Murchison and Goldfields regions and *N. w. cinctus* which is found in the Pilbara region and inhabits rocky outcrops and ranges supporting spinifex vegetation. Both sub-species are of similar appearance and can appear pink to reddish brown to sandy brown and ghostly grey. Their body and limbs are covered in rosettes; they have a broad dorso-ventrally compressed tail and broad dark brown bands on the body and tail (Storr & Johnstone, 1990; Wilson & Swan, 2003). The main difference in appearance between the two sub-species is that *N. w. wheeleri* has four bands across the neck, shoulder, rump and tail base while *N.w.cinctus* has five bands with the neck/shoulder band being broken in two, although this character has been noted to be quite variable (Swan, 2008).

*N. w. cinctus* is a relatively common species in the Pilbara region of Western Australia. Perth Zoo acquired 11 geckos in May 2009 from Department of Parks and Wildlife (DPaW) after these animals were taken from the wild illegally and subsequently confiscated by the regulating authority. It was determined by DPaW that these animals were unable to be returned to the wild. Of the 11 geckos, there were 10 adults and one juvenile (5 females and 6 males). This paper describes the husbandry methods used for successful care and breeding of this species at Perth Zoo.

## MATERIALS AND METHODS

### Husbandry Conditions

Adult *N. w. cinctus* were housed in 880 mm long x 550 mm wide x 600 mm high enclosures off-display. Due to

availability of space these animals were kept in a room that also housed other lizard and dragon species and as such they were kept on the normal day/night cycle. The enclosures were made of ply wood and had a perspex front/door and thin wire mesh on top. The enclosure was furnished with sand substrate to a depth of 30-50 mm; both red sand and brown coarse river sand have been used. This species burrows and excavates shallow depressions and if provided with deeper areas of sand (50-100 mm) they create their own tunnels and burrows. This however, can make it difficult to visually inspect and perform daily health checks as the animals bury themselves and have to be dug out on occasions. A 3 L clear/opaque plastic container with a small hole cut in the front filled with slightly moist sand was provided for them as a nest box and for them to burrow in. Other furnishings included flat rock pieces positioned on top of each other as well as bark and branches to create hides. Ply wood cut and fashioned to make tiered ledges were also used. This species seemed to prefer digging out small depressions in the sand under pieces of flat rock. A small shallow water dish was also provided in each enclosure (although no drinking from this was observed). All enclosures had Reptistar® 36w UV fluorescent lights during daylight hours and 60-80W heat lamps (Red® for night cycle) positioned at one end of the enclosure. Ambient room temperatures in summer range from absolute minima of 19-20° C to a maximum of 26° C with enclosure hot spots of 31°-35° C provided. Winter ambient temperatures recorded an absolute minimum of 15° C and maxima of 21°-22° C with hot spots of 24°-28° C.

Servicing these enclosures consisted of removing any faecal matter (this species tends to defecate in the same area on most occasions), providing fresh water, lightly misting down half or one side of enclosure every three days, checking the nest box if necessary, checking and recording temperature using min/max thermometers and Raytek® temperature reader.

All geckos were fed three times a week in spring-autumn (Sept-May) and twice a week in winter (June-August) on a staple diet of live crickets (Family: Gryllidae), with live mealworms (*Tenebrio molitor*) offered as a variation. Some individuals took dead wood roaches offered from tongs occasionally, although most refused. All insects offered were dusted in Repti-vite® vitamin supplement once a week and calcium powder twice a week. Each individual gecko was offered 2-3 medium-large crickets, and female geckos that had just laid eggs were offered more food for a short period to support good body condition. Live insects were scattered throughout the enclosures for the animals to catch themselves.

Four enclosures were set up for the geckos with a different sex ratio in each. This was done to determine if there would be any differences in breeding success or housing complications. Enclosure 1 had two males, enclosure 2 one male one female, enclosure 3 had two males one female and enclosure 4 had one male and two females.

**RESULTS**

**Husbandry**

Geckos settled into a captive environment quite well and took insects offered on tongs or long tweezers and this helped to ensure all animals received an adequate amount of food. *Nephrurus* spp. geckos are nocturnal or crepuscular in their activity, but since we kept our geckos on a normal day/night cycle, food was offered between 12-3 pm for ease of husbandry. It was noted in our collection that the females seemed to be more active feeders and when insects

were offered either live, or from tweezers, they would emerge from their hides and start hunting the insects almost immediately, regardless of time of day. All geckos were lightly misted with a fine mist of water every three days for hydration, with individuals observed licking water droplets off their eyes and lips.

Sloughing occurred quite regularly and the geckos would turn a ghostly grey colour 1-3 days before they sloughed. Care should be taken to check that all the slough comes off, especially around their toes. No problems have been noted at Perth Zoo with all geckos sloughing well.

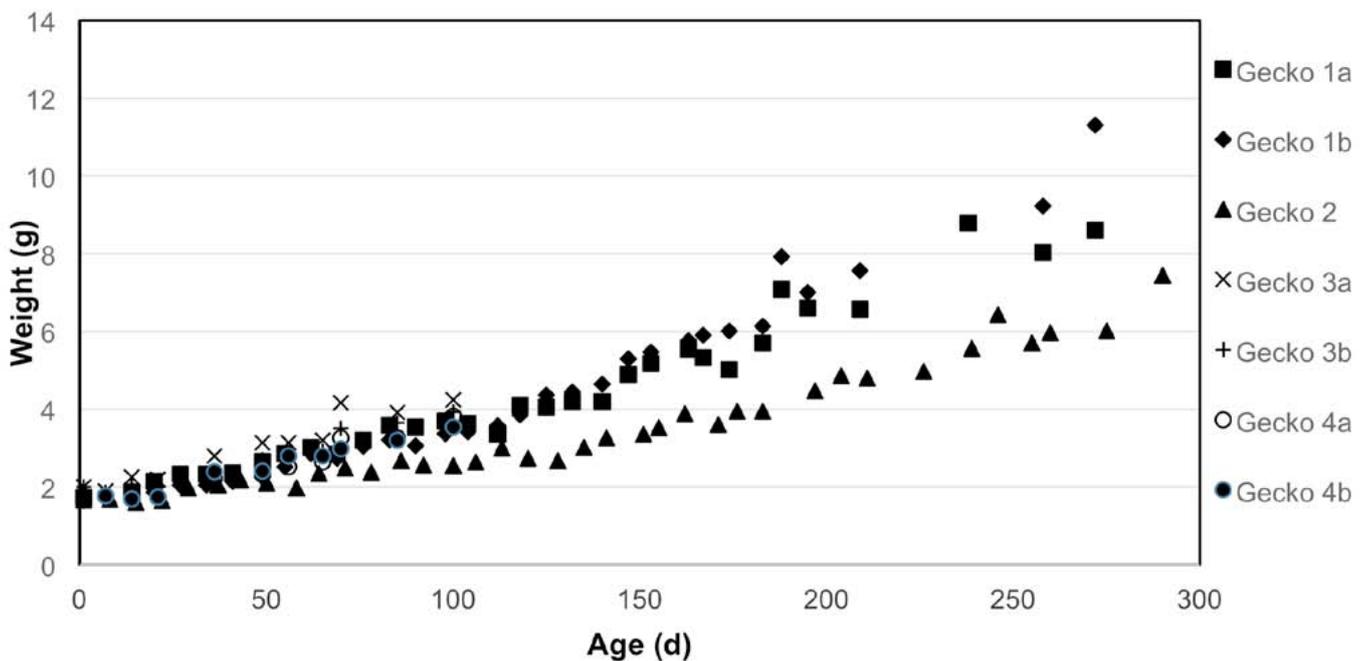
All geckos maintained a very good level of health and no issues arose. Only one adult animal has been lost with the cause of death unknown (wild caught animal, so age at acquisition was unknown). All individuals were weighed regularly and visual checks were performed daily.

Each housing set-up provided no complications, with breeding success recorded from all females and no fighting observed between males. Animals were observed sharing the same hide regularly and nesting together, and all fed very well and all animals maintained a healthy weight.

**Captive Breeding**

The breeding season for this species has been reported as commencing in early spring and extending into late summer/early autumn with females laying on average 3-5 clutches per season (Swan, 2008). The geckos at Perth Zoo arrived in May 2009 and no breeding occurred until mid-late autumn the following year (May-June 2010), with the late start to breeding presumably due to settling in to the captive environment. The first successful breeding resulted in one female laying two eggs in a shallow depression

**Figure 1.** Growth rate for seven *N. w. cinctus* hatched at Perth Zoo from July 2010-May 2011. Animal identification is as given in Table 3.



in the sand, and the following month a second female laid two eggs on top of the sand, one of which was not viable. The second egg appeared to be normal and was placed in the incubator along with the other clutch. The first two clutches were the only occasions where eggs have not been laid in the nest box provided. The clear/opaque containers used as a nest box are a reliable way of seeing where and when the eggs are deposited without disturbing the female prior to laying. The females all concealed their digging very carefully and in most cases it was difficult to determine if they had laid at all when viewed from the top of the container, so the ability to see through the nest box was a distinct advantage.

At the end of the winter cooling period the temperature in the enclosures was gradually increased over a few weeks and the food supply offered was also increased. Females started digging and test holing around mid-October to late-November, both in the nest boxes and surrounding areas. Females were also visually checked and confirmed to be gravid by gently holding a female and looking at her abdomen to see if developing eggs could be clearly seen through the ventral abdominal wall. Over the second breeding season all four females laid a second clutch within 52-66 days after laying their first clutch, with one female laying the very same day her first clutch hatched. From December-April three females laid two clutches and one female laid three clutches (Table 1).

After the first breeding season, eggs were laid between late-October and late-May, with the number of clutches per female increasing with each year. One female laid six clutches in a seven-month period. When 2-4 clutches were laid in a season the interval between clutches usually ranged from 33-63 days, when 5-6 clutches were laid the interval shortened to around 23-46 days (Table 1). This is within the inter-clutch interval reported for *N. wheeleri* by Brown (2012).

Incubation temperature of *N. w. cinctus* eggs at Perth Zoo has been 29°-30° C with 29.5° C used on most occasions. Incubation medium was perlite (vermiculite was used on one occasion) and both ratios of 2:1 and 1:1 of perlite/water w/w were used, with the dryer 2:1 ratio the most successful. Incubation lasted between 51-60 days (Table 2), which matches the 50-61 days reported by Treseder (2008), but generally shorter than that reported by Brown (2012). One clutch of eggs developed a fungal growth over the eggs halfway through incubation, this did not affect the eggs and both hatched and produced healthy individuals. On only one occasion has an egg failed to hatch, when a fully formed embryo was found dead inside the egg when it was manually opened after 58 days incubation. Despite some adult females producing more clutches in successive breeding seasons, the duration of each season remained fairly constant, with the interval between clutches being reduced (Table 1 and 2).

Eggs were ca. 22 x 11-12 mm in size and weighed 2.3-2.7 g when freshly laid. Hatchlings weighed 1.65-2.01 g at birth and had a snout-vent length of 42-45 mm (Table 3) which is larger (contra: 35 mm) than that reported by Brown (2012).

		2010/11	2011/12	2012/13	2013/14
Female 1	No. clutches	3	4	4	6
	Inter-clutch interval (d)	56.5±3.5, 54-59	51.5±4.9, 45-55	44.7±16.1, 33-63	39.8±14.7, 30-63
Female 2	No. clutches	3	3	4	5
	Inter-clutch interval (d)	57	54.0±9.9, 47-61	36.0±9.1, 26-44	35.5±9.0, 23-44
Female 3	No. clutches	2	2	-	-
	Inter-clutch interval (d)	66	54		
Female 4	No. clutches	2	2		
	Inter-clutch interval (d)	52	52		

**Table 1.** Number of clutches produced each season and inter-clutch interval (in days) (mean±s.d. and range) for *N. w. cinctus* at Perth Zoo during 2010/11-2013/14.

	Incubation period (d)			Interval between successive breeding seasons (d)	
	Mean±s.d.	Range	n	Mean±s.d.	n
Female 1	57.1±1.7	55-60	8	225.0±12.5	3
Female 2	51	-	2	233.5±26.1	2
Female 3	55.5±2.1	53-59	8	234.7±43.7	4
Female 4	52	-	2	268	1

**Table 2.** Incubation period (in days) (mean±s.d., range and sample size) and duration of the interval between successive breeding seasons for *N. w. cinctus* at Perth Zoo. Identification of breeding females matches that used in Table 1.

Clutch No.	Egg Wt	Egg dimensions (mm) L/W	Hatchling Wt (g)	Hatchling S-V Length (mm)
	(g)			
1a	2.73	-	1.72	-
1b	2.56	-	1.65	-
2	2.34	-	1.67	-
3a	2.50	22.5 x 11.5	2.01	42.0
3b	2.49	22.0 x 11.0	2.00	42.0
4a	2.41	22.0 x 11.0	1.78	45.0
4b	2.50	23.0 x 12.0	1.78	44.0

**Table 3.** Egg weight (g) and dimensions (mm), and hatchling weight (g) and snout-vent length (mm) for *N. w. cinctus* at Perth Zoo during 2010/2011.

### Hatchling Growth

Hatchlings were housed in small holding enclosures (600 mm L, 400 mm W, 400 mm H) made from the same materials as used for adult enclosures. The set-up was virtually identical to that for adults, just on a smaller scale with small hides made of flat rock and bark, sand substrate to a depth of 10-20 mm, a small shallow dish of water and heat lamp positioned at one end of enclosure. All hatchlings started eating within 4-7 days of hatching and small crickets were the preferred food. Two individuals began taking food offered from tongs within seven days of hatching, others within two months. To stimulate a drinking response, hatchlings were lightly misted with water every 3-4 days the same as adults, young geckos were observed

licking droplets of water off their eyes and lips and from the surface of rocks. All hatchlings sloughed 4-7 days after hatching.

Hatchling growth rates were steady and consistent during the first year, but the minor differences in body weight apparent at hatching increased with age, until there was up to 4 g difference in body weight between some individuals (Fig. 1). At 300 days of age young geckos were about 67 percent of adult body weight (mean 14.5 g; ISIS, 2014)

## DISCUSSION

The only records of keeping *N. w. cinctus* in zoo collections other than those from Perth Zoo, or institutions that received animals from this collection, are from Nakhorn Ratchasima Zoo in Khorat, Thailand. The four animals in that collection all died in 2008 and 2009, and there is no evidence of them having bred, and their origins are not given in the ISIS data set. Keeping *N. w. cinctus* in captivity has been relatively easy with few complications and minimal stimulation required to start breeding. Some of the more important points were to ensure they had an adequate area to dig and to lay their eggs, as well as a cooling period through winter. Ensuring the females stay in good condition and have plenty of food leading up to, and after egg deposition is also essential, especially since they lay multiple clutches in a season.

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# First data of natural recovery of any Orinoco crocodile *Crocodylus intermedius* population: Evidence from nesting.

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**ABSTRACT** - Since the end of commercial hunting in the 1960's, there were no signs of recovery of the Orinoco crocodile (*Crocodylus intermedius*) populations throughout its range. In this study, nest counts have been used to establish population trends in the Arauca Department, Colombia. From December 2014 to April 2015, we surveyed 166.7 km of rivers for nests. Twenty-four nests were located, 2.2 times more than recorded 13 years previously. Our results indicate that *C. intermedius* populations in the area are increasing. The awareness of local people, a reduction in fishing activities and use of the river for transport may explain the recovery.

## INTRODUCTION

The original populations of the Orinoco crocodile (*Crocodylus intermedius*) were depleted over its entire distribution area (Colombia and Venezuela) due to an intensive period of commercial hunting during the second third of the last century (Medem, 1981; Godshalk 1982). Since the end of commercial hunting 50 years ago, there has been no evidence of recovery in any of the remaining populations, either in Colombia or Venezuela. However, in Venezuela all the populations have been strengthened through the reintroduction of hundreds of captive-reared crocodiles but the two most important populations (Cojedes and Capanaparo Rivers) remain in decline (Mena et al., 2010; Moreno 2012). Antelo (2008). Antelo et al. (2010) describe the establishment of a new *C. intermedius* population in Venezuela, but in this case all the crocodiles were reintroduced. The reasons given for non-recovery are related to the human factors: contamination, habitat destruction, poaching, egg-robbing and the sale of hatchlings (Thorbjarnarson & Hernández, 1992; Seijas et al., 2010). Competition with the sympatric *Caiman crocodilus* has been also suggested (Thorbjarnarson & Hernández, 1992; Seijas et al., 2010).

The first census conducted in Colombia, from 1974 to 1975, showed that the most important *C. intermedius* population was in Arauca, with 180 individuals observed with the total estimated number in Colombia at 780 (Medem, 1981). Despite the ending of commercial hunting, 20 years later Lugo (1996) estimated the *C. intermedius* Colombian population at 123 crocodiles, 50 of them in Arauca. Six years later, Ardila et al., (2002), concluded that this population remained stable.

This study presents new information about *C. intermedius* population numbers by surveying for nest sites. Previously, Bonilla & Barahona (1999), Ardila et al. (2002) and Castro et al., (2012) presented some data concerning the population status and reproduction of *C. intermedius* in

Arauca. We add to this knowledge with new observations on reproductive biology including the results of the first ranching in Colombia with this species. Ranching is the main action proposed in *The National Program for the Orinoco Crocodile Conservation* (Ministerio del Medio Ambiente, 1998). The results presented are of great importance for the *C. intermedius* conservation in Colombia. Our results are based on nest counts, which we believe is a valid method to establish the population trends of *C. intermedius*. The method avoids the high variability of counts produced by traditional spotlight methods (e.g. Chabreck 1966; Woodward & Marion 1978; Hutton & Woolhouse 1989) due to the risks involved in night work at this area as a result of armed conflict.

## MATERIALS AND METHODS

The field study was conducted by one of us (L.F.A.) in three sections of the rivers Lipa, Ele, and Cravo Norte (Arauca Department). Five surveys were conducted: 1) from 19 December to 23 December 2014; 2) from 8 January to 11 January 2015; 3) from 16 January to 19 January 2015; 4) from 12 April 2015 to 17 April 2015 and 5) from 22 April 2015 to 24 April 2015.

A total of 166.7 km of the following rivers were surveyed for and the monitoring of *C. intermedius* nests: Lipa River (34 km, from 6°43'16.94"N; 70°53'54.42"W to 6°35'11.65"N; 70°43'23.68"W); Ele River (35.7 km, from 6°34'54.19"N; 70°47'1.26"W to 6°35'11.65"N; 70°43'23.68"W) and Cravo Norte River (97 km, from 6°30'53.00"N; 70°47'50.08"W to 6°23'24.00"N; 70°25'58.80"W). This territory is not under any form of protection, but there has been armed conflict between local guerrillas and the National Army of Colombia for the previous 50 years.

Surveys were conducted using a wooden boat powered by a 40HP outboard motor. Nests were located using footprints left in the sand by breeding females. A wooden stick was gently introduced into the sand to detect the eggs.

Once located, the nest was geo-referenced with a GPS and distance to water and vegetation was measured. Due to the lack of permission, the nests were not opened. Nests were monitored until they were harvested by local people or lost due to egg predation.

Simultaneously, in collaboration with Corporinoquia, the regional environmental authority, 150 eggs from four nests were collected from sites situated on the Ele River and transported to Wisirare Park (Orocué, Casanare) for controlled incubation. Transport and egg incubation is as explained in Antelo et al. (2010) and was carried out by local people on 30 January 2015. Eggs were transported 536 km on roads and paths after being issued with certificate of biodiversity No. 0560531, from the Corporinoquia.

## RESULTS

Twenty-four nests were found along the banks of the 166.7 km surveyed (0.14 nests/km): one at the Lipa River (0.03 nests/km), 10 at the Ele River (0.28 nests/km) and, 13 at the Cravo Norte River (0.13 nests/km). Nesting occurs during the early dry season, in January. We were able to determine the date of 20 nesting events (Table 1). The earliest clutch was laid 4 January and the latest on 24 January 2015. Nesting peaked in the third week of January

(12 nests), followed by the second week (5 nests). Wild clutches hatched between 3 April and 23 April 2015. The mean incubation time for seven wild clutches was 89.8 days, ranging from 88 to 97 days. In captivity, hatching took place between 4 April and 19 April. Natural and artificial incubation lasted between 79 and 85 days.

Twelve of the nests (50%) hatched normally, five (20.8%) were harvested by local people, four (16%) were collected for artificial incubation, two (8.3%) were flooded due to rising river levels and one (4.2%) was partially predated by feral pigs although some of the eggs hatched (Table 1). Average distance from nests to the water and gallery forest was 12.5 m (maximum 41m; minimum 3m; SD=9.1m) and 21.5m (maximum 86 m; minimum 3 m; SD= 20m) respectively. Median height above water level was 112.7 cm (maximum 220 cm; minimum 57 cm; SD = 38.8m). In nest number 5 (Table 1), six crocodiles had emerged from the nest and were found in the water without any signs of nest opening. Later we placed two camera-traps close to the nest to film nest opening by the female but after 5 days we achieved no results and we removed the cameras. However, later it was found the female had subsequently opened the nest.

Nest attendance was observed at 21 nests (87.5%). An adult crocodile (presumably the mother) was recorded

**Table 1.** Nests location and incubation result. "Hatchlings" indicates the maximum number of *C. intermedius* counted in the vicinities of the nest. NF= Not found; --- No data.

Nº	Latitude	Longitude	River	Result	Hatchlings
1	6°37'45.57"N	70°45'04.85"W	Lipa	Hatched	41
2	6°33'30.65"N	70°42'01.09"W	Ele	Hatched	59
3	6°33'25.46"N	70°41'53.40"W	Ele	Harvested	---
4	6°33'37.45"N	70°41'44.45"W	Ele	Hatched	NF
5	6°32'18.76"N	70°41'04.05"W	Ele	Hatched	23
6	6°32'06.73"N	70°41'05.31"W	Ele	Collected	8
7	6°32'05.04"N	70°41'07.37"W	Ele	Collected	34
8	6°32'03.82"N	70°41'08.02"W	Ele	Collected	22
9	6°31'57.20"N	70°40'27.92"W	Ele	Collected	19
10	6°32'27.22"N	70°40'58.08"W	Ele	Flooded	---
11	6°31'44.91"N	70°40'05.49"W	Ele	Harvested	---
12	6°31'40.99"N	70°48'30.98"W	Cravo Norte	Harvested	---
13	6°30'15.66"N	70°44'22.28"W	Cravo Norte	Hatched	42
14	6°29'15.28"N	70°40'26.48"W	Cravo Norte	Predated	4
15	6°28'50.17"N	70°39'30.30"W	Cravo Norte	Hatched	28
16	6°28'14.58"N	70°38'07.05"W	Cravo Norte	Hatched	59
17	6°27'50.22"N	70°38'51.33"W	Cravo Norte	Hatched	13
18	6°27'57.37"N	70°36'28.34"W	Cravo Norte	Hatched	42
19	6°28'00.66"N	70°37'21.07"W	Cravo Norte	Flooded	----
20	6°27'31.61"N	70°35'56.08"W	Cravo Norte	Hatched	39
21	6°26'43.40"N	70°31'15.83"W	Cravo Norte	Hatched	37
22	6°27'03.38"N	70°31'17.26"W	Cravo Norte	Harvested	----
23	6°26'03.48"N	70°28'29.19"W	Cravo Norte	Harvested	----
24	6°23'25.62"N	70°25'55.96"W	Cravo Norte	Hatched	40



**Figure 1.** Female *C. intermedius* (bottom right of picture) guarding her hatchlings at Ele River. Picture taken by camera trap at 10.35 am.

in the vicinity of the nest, but her behaviour was not aggressive towards the investigator. Based on the presence of sand tracks, seven females had continued to visit their nest even after they had been predated or collected. In 12 of 13 cases where the eggs hatched parental care was also observed; in the remaining nest no hatchlings were found. Hatchlings and females were located downstream on the same shore as the nests in 3 cases; downstream in the opposite shore of the nest in 8 cases and in 1 instance were found upstream in the opposite shore of the nest. Only two groups of hatchlings were protected by aquatic vegetation, the rest were found on bare banks. Size of the hatchlings groups observed ranged from 4 (in the partially predated nest) to 59 (Table 1).

Median clutch size of the 4 collected nests was 40, ranging from 37 to 44 eggs/nest. Ten eggs of each of the 4 nests were measured and weighed. Median length, width and weight was respectively, 7.8 cm, 4.9 cm and 90.2g (n = 40). From the incubation of 150 eggs at Wisirare Park, 84 crocodiles hatched (56%), which will be reared in captivity until they reach at least 80 cm total length prior to release back to the wild.

## DISCUSSION

Previous works reported seven (Bonilla & Barahona 1999), 11 (Ardila et al., 2002), and 9 (Castro et al., 2012) nests. Bonilla & Barahona (1999) surveyed 168 km of Lipa, Ele and Cravo Norte Rivers from January to April 1995. Ardila et al. (2002) surveyed 100.2 km of Lipa, Ele and Cravo Norte Rivers for four months (November-December 2000 and March-April 2001). Castro et al. (2012) surveyed 185 km of Cravo Norte, Ele and Lipa Rivers in April 2012. Study areas of these works do not match exactly with ours, but are almost the same. In this study twenty four nests were observed, and consequently we report the highest nest density in the study area. In the Venezuelan populations, Antelo (2008) reported 31 breeding females for the El Frío Biological Station (3.8 nest/km) and Caño Guaritico Wildlife Refuge; Hernández et al., (2014) founded 25 nests at the Capanaparo River (0.25 nest/km) and 27 nests were

estimated by Espinosa & Seijas (2010) at the Cojedes River System (1.3 nests/km).

Reproductive chronology, nest, clutch, and egg characteristics are similar to those described for this region previously and for other wild and captive populations of *C. intermedius* (Thorbjarnarson & Hernández, 1993; Ramo et al., 1992; Seijas, 1994; Lugo, 1995; Colvée, 1999 and Antelo, 2008). In our sample, egg mass is lower than found in these other studies, perhaps a consequence of differences in sample sizes. Bonilla & Barahona (1999) observed nesting from December to January and Ardila et al., (2002) from January to February. In our study nesting was restricted to January.

Nest predation observed in our study (4.2%), is significantly lower than reported for the Cojedes River (11%) or the Biological Station El Frío (60%) (González-Fernández 1995; Antelo, 2008). Thorbjarnarson & Hernández (1993) noted that human predation was the only cause of nest loss at the Capanaparo River. In the same river, Hernández et al. (2014) observed that 4.5% and 53.8% of the Orinoco crocodile nests were predated and harvested respectively. Egg harvesting was previously noted in Arauca (Bonilla & Barahona 1999; Ardila et al., 2002; Castro et al., 2012), and seems to be a traditional issue in the area.

Antelo (2008) stated that Orinoco crocodiles were not able to leave the nest without the help of the mother, but in this study it was observed that at least the hatchlings placed in the upper side of the nest can emerge from the nest on unaided. Nest attendance (Fig. 1) observed (87.5%) is higher than reported for the Biological Station at El Frío (59%; Antelo 2008) and for the Cojedes River System (47.7%; Seijas & Chavez, 2000). Antelo (2008) described aquatic vegetation being employed as cover by hatchlings, but in our studies most of the hatchling groups were located in open areas, so risk of predation could be higher. Hatching success (56%) of collected eggs is low compared with other studies (Joanen & McNease 1987; Piña et al., 2005; Piña et al., 2007; Moreno et al., 2011 in Hernández et al., 2014), but in good agreement with certain other crocodilian studies (Webb et al., 1983; Whitaker 1987; Piña et al., 2003). Lack of experience of local people collecting the eggs and the long distance from the beaches to the incubator could explain these results.

## CONCLUSIONS

Our data suggest that the *C. intermedius* population in Ele, Lipa, and Cravo Norte Rivers has recovered in recent years without any kind of management. Another explanation is that the field effort made in this study is slightly greater than in previous studies. Nevertheless, increases in the number of adult crocodiles (> 2.5 m), estimated at 152 (Anzola, unpublished data), support the assertion that the population is indeed increasing. These results also demonstrate that the Arauca population is the most important in Colombia and one of the most important in entire range of *C. intermedius*. There are no records of any other instances of natural recovery of this species.

There could be several possible reasons this population

has recovered naturally. They include a) the awareness of the local people towards crocodile conservation; many, for example, do not consider the crocodile as a threat; b) a reduction in commercial fishing, which is no longer economically viable due to decreasing fish numbers and c) river navigation has decreased due to increased road building by oil companies, local and national governments. The number of nests located indicates that an adequate ranching program could substantially increase the number of available crocodiles for reintroduction in Colombia. Ranching of hatchlings should be a more effective strategy than egg ranching. Ranching should be done with the collaboration of local people, due to their deep knowledge about *C. intermedius* nesting behaviour in the area. The challenge is to change their harvesting tradition into a conservation activity. Between 10 to 20% of the crocodiles bred in captivity should be returned to the Arauca Rivers, with the remaining individuals used to establish new wild populations or strengthen existing ones.

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## Trunk vertebrae osteomyelitis in a spectacled caiman (*Caiman crocodilus*)

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**ABSTRACT** - Osteomyelitis is frequently reported in turtles, lizards and snakes and *Salmonellae* are increasingly reported as the causative organism. However, very little is known of this disease occurring in crocodylians. Crocodylians are shy, robust animals and often hide clinical symptoms, especially when submerged in water. Knowledge of disease in crocodylians is important, especially in zoos and farms where they are often kept in high densities. Here we report the first known case of trunk vertebrae osteomyelitis in the order Crocodylia. A six year old, captive raised spectacled caiman (*Caiman crocodilus*) was admitted for veterinary examination after developing a postural abnormality in the vertebral region, including spinal curvature and dorsal indentation. Radiographic and Computed Tomographic studies showed muscle wastage and multifocal vertebral osteolytic changes suggestive of osteomyelitis. The caiman was euthanized and post-mortem examination revealed coelomic serosanguinous effusion and a diffuse severe fibrinous coelomitis with firm visceral adhesions involving the liver and spleen. A 15 cm irregular mass was found within and greatly expanding a major blood vessel ventral to the trunk vertebrae. Examination of the mass revealed an organized thrombus and examination of the affected trunk vertebrae revealed severe osteolytic changes with extensive remodeling. *Salmonella enterica houtenae* was isolated from the vertebral lesions. *Salmonellae*, a common constituent of the reptilian gut microbiota, are potentially pathogenic and can become clinically important in times of stress. *Salmonella enterica houtenae* was previously isolated from reptiles and humans with pathological symptoms. However, this appears to be the first documented case in association with osteomyelitis in vertebrates.

### INTRODUCTION

Infectious osteoarthropathy is frequently reported in reptile groups such as lizards, snakes and turtles (Silverman, 2006; Jacobson, 2007; Souza et al., 2014), however, detailed reports of osteomyelitis in crocodylians are lacking in the scientific literature. Osteomyelitis is defined as an inflammation of the bone marrow, cortex, and possibly periosteum. Although osteomyelitis is most commonly of bacterial origin, other causes can include fungi, parasites, viruses, foreign bodies, and corrosion of metal implants. Microorganisms typically gain access to bone through the bloodstream, localised infection, or trauma (Fossum & Hulse, 1992). Despite injuries often inflicted on crocodylians resulting from social disputes with other individuals in the wild and captivity, recovery usually occurs without infection in healthy animals. This is likely because their innate immune system reacts aggressively towards pathogens (Siroski et al., 2009). However, due to their shy, robust nature it can be difficult to distinguish

sick individuals from healthy ones due to a lack of clinical signs, especially when submerged in water and as a result the animals often die before they can be treated (Leslie et al., 2011).

*Salmonella* bacteria are a common cause of osteomyelitis in reptiles (Souza et al., 2014). They are a common constituent of the reptilian gut microbiota and can be shed intermittently without displaying clinical signs. However, they are opportunistic facultative anaerobes that can become pathologically important to reptiles under stressful conditions (Jacobson, 2007). Reptiles living in captivity can experience stress more frequently than in wild populations, typically due to husbandry deficiencies.

Reports of *Salmonella enterica houtenae* date back to 1969 (Iveson et al., 1969) when it was isolated from a parasitic tick inhabiting the ear of a lizard. In 1978 it was isolated from the liver and air sac of a pet cockatiel (Phillips et al., 1978), and the authors suspected that it was transmitted from a wild reptile. They also refer to four samples submitted previously to the Veterinary Services Laboratories that were subsequently identified as

*S. enterica houtenae*. These samples were isolated from a snake in Texas and three snakes at the San Diego Zoo.

Salmonella bacteria have zoonotic potential. *S. enterica houtenae* has been implicated in a brain abscess in a 44 month old boy (Ma et al., 2003). The patient suffered a relapse but subsequently recovered. However, there was no verification of the patient or family coming into contact with any reptiles. In 2004, a 2.5 month old infant suffered meningitis (Wybo et al., 2004), *S. enterica houtenae* was reported as the pathogen responsible. This patient also suffered a relapse and later recovered. This family did, however, have two pet iguanas. Swabs were taken from the lizards but did not yield any pathogens. It is, however, possible that the lizards could have shed the bacterium at the time the swabs were collected. One lizard subsequently died.

Here, we report to the best of our knowledge the first known case of trunk vertebrae osteomyelitis in crocodylians. *S. enterica houtenae* was isolated from a direct swab of the vertebral lesions.

## CASE REPORT

A privately owned female *Caiman crocodylus* ca. six years old was individually housed indoors in a 1,000 litre pond with 1.7 X 0.75 metres of land area available for basking. Temperatures were thermostatically controlled with ambient temperatures ranging from ca. 25°C to ca. 30°C with basking temperature ca. 35°C. Water was filtered and maintained at 29°C. UV lighting was provided. The caiman was fed on a diet of gut loaded invertebrates, fish, mice, chicken flesh and fresh water crustaceans. Multivitamin supplements were used occasionally.

After observing swelling and an exudative superficial excoriation on the right dorsum with damaged scales on the dorsal region of the right thigh, it was surmised that the caiman may have injured herself against the frame of her enclosure. The lesions were treated with pharmacy obtained iodine solution each day for several days. Judging that it was on its way to recovery, no further treatment was provided other than regular feeding and allowing the animal to thermoregulate freely.

After four months, the caiman stopped eating and was observed basking for prolonged periods of time in the terrestrial part of the enclosure. Drinking fresh water while on land was observed on two occasions. Over the following weeks, a mid-body swelling was observed and due to her mature age and change of season, in addition to her behaviour (lack of appetite/ increased basking), we suspected she was gravid. Two months later the swelling began to decrease, revealing a slight arching in the region of the twelfth and thirteenth trunk vertebrae with apparent kyphosis. No eggs were produced. The right dorsum was raised and a depression appeared on the left dorsum. The animal was admitted for veterinary investigation at the University College Dublin Veterinary Hospital.

Dorso-ventral and lateral radiographs of the whole body were taken. A survey computed tomographic (CT) helical scan of the whole body was performed using

a four-slice CT unit with 1 mm slice thickness. The CT scan was reconstructed using bone and soft tissue algorithms (*Siemens Medical, Somatom Sensation 4, 2 Wittelsbacherplatz, 80333 Munich, Germany*). The animal was contained without sedation for all examinations. The radiographs and CT (Figs. 1a-1d) studies showed extensive muscle wastage and multifocal vertebral osteolytic changes with remodelling and spondylolisthesis between the twelfth and thirteenth trunk vertebrae. These findings were consistent with a multifocal disease with a pathological compression and fracture of the twelfth trunk vertebra. Based on the severity of the findings, the prognosis was considered poor and the caiman was euthanised.

The carcass weighed 5.2 kg, a decrease from ca. 8 kg in life, and appeared emaciated. Necropsy revealed 100 ml of free serosanguinous fluid within the coelomic cavity and a diffuse severe fibrinous coelomitis with firm adhesions between the intestines, liver and spleen. A 15 cm irregular mass with a firm exterior and bloody soft surface was found within and greatly expanding a major blood vessel ventral to the region of the twelfth and thirteenth trunk vertebrae. Examination of the vertebrae corroborated the severe osteolytic changes and remodelling as seen in the CT scan. The most severely affected vertebrae were the eleventh, twelfth and thirteenth (Figs. 1e & 1f). Histological examination of the twelfth trunk vertebra revealed a marked cellular infiltration of heterophils on a background of elongated cells with fusiform nuclei and indistinct cell borders (fibrosis). Macroscopic examination of the mass revealed an organised thrombus.

Bacteria were not detected on microscopic examination. This is common in bacterial osteomyelitis when endotoxaemia leads to thrombosis of metaphyseal vessels which results in ischemia and necrosis of bone (Silverman, 2006). However, a direct swab of the vertebral lesion was aseptically collected at necropsy and revealed *S. enterica houtenae*, *alpha haemolytic Streptococcus* and *Proteus* sp. The Salmonella strain was serotyped by slide agglutination and an analytical profile index was carried out to check the biochemical reactions revealing the antigenic formula 51:z4, z23. Further tests analysed the antimicrobial susceptibility of the strain and it was fully susceptible to all antimicrobials checked. For verification purposes, swabs were aseptically collected from the infected lesion, which was by this time, frozen and thawed during the post-mortem process. DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Irvine, California, USA). Universal bacterial primers were used to amplify the *16S rRNA* (Genewiz, USA). The isolates (five in total) were all identified using BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) as *Moellerella wisconsensis*, *Kurthia zopfii*, a *Vagococcus* sp., *Staphylococcus condimentii* and *Pseudomonas fragi*. There are no records of these bacteria causing osteomyelitis or disease in reptiles and it is likely that the reason they were detected after freezing was because the storage conditions may have altered the bacterial composition of the vertebrae.

## DISCUSSION

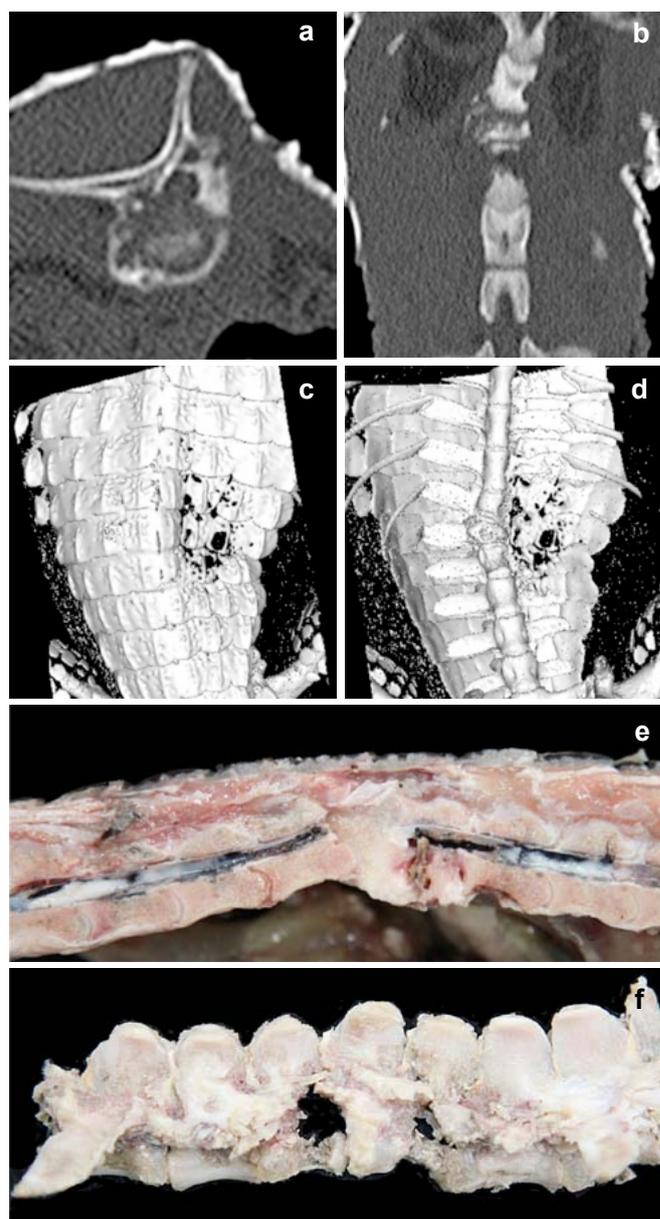
To the best of the author's knowledge, this is the first report describing trunk vertebrae osteomyelitis in the order Crocodylia and we suggest *S. enterica houtenae* to be the causative organism. Due to their shy, robust nature, sick individuals often hide clinical signs and as a consequence, they can be difficult to identify (Leslie et al., 2011). In the case presented here, the severity of the imaging findings was surprising considering that the patient had no obvious ambulatory dysfunction. The radiographic studies grossly underestimated the skeletal changes due to the artefacts generated by the keratinised skin. However, the CT studies accurately demonstrated the severity of the problem and the very poor prognosis.

*Salmonella enterica houtenae* was previously isolated from reptiles and humans with pathological symptoms (Iveson et al., 1969; Ma et al., 2003; Phillips et al., 1978; Wybo et al., 2004). However, this appears to be the first documented case in association with osteomyelitis in vertebrates. Salmonella was the only pathogen isolated from the infected lesion that has been previously identified as the most common, definitive aetiological agent of osteomyelitis in reptiles (Jacobson, 2007; Souza et al., 2014), and we believe it is the likely pathogen responsible for the infection presented here. The bacteria likely came into contact with the caiman as a result of its captive environment. Crocodylians often thrash their food about in the water which could further aid in the contamination of the ambient environment. In this case, the injury previously sustained could have facilitated translocation of bacteria to the vertebrae, leading to a focus for infection, and ultimately causing terminal disease (Silverman, 2006; Jacobson, 2007). Therefore, this case should be considered as a possible trauma-related infection.

Salmonella is an important zoonotic organism widely associated with farming livestock and reptiles in the pet trade and zoos (Jacobson, 2007). It is a common cause of food poisoning and a source of more severe cases including a brain abscess, osteomyelitis and meningitis (Canessa et al., 2011; Ma et al., 2003; Wybo et al., 2004). Reptiles are intermittent shedders and as a result, Salmonella can be difficult to detect amongst individuals, therefore strict hygiene practises should be maintained when working or interacting with animals known to be natural carriers.

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**Figure 1a.** Transverse CT image at the level of the twelfth trunk vertebra. The whole of the twelfth trunk vertebra and left lamina are remodeled with an amorphous shape and with patchy osteolytic changes. The spinous process is lytic and the left transverse process is missing. The depression of the epaxial muscle and the overlap of two areas of keratinized skin on the left side of the caiman are evident. **b.** Longitudinal 3D MPR reconstruction CT image of the caudal spine in the twelfth and thirteenth trunk vertebrae. There are severe osteolytic changes of the thirteenth trunk vertebra which shows an amorphous foreshortened and wedge shape. There is vertebral body lysis of the thirteenth trunk vertebra. The malalignment between the spine in the region of the twelfth and thirteenth trunk vertebrae is evident. **c.** Longitudinal 3D surface rendering reconstruction CT image of the caudal spine in the region of the twelfth and thirteenth trunk vertebrae. The depression of the left side of the paralumbar epaxial muscles and the curvature of the spine toward the left side are evident. **d.** Longitudinal 3D volume rendering reconstruction CT image of the caudal thoracic and lumbar spine. The vertebral body lysis of the last thoracic and first lumbar vertebral bodies are visible. There are lytic changes of the left transverse processes of the last three thoracic and first two lumbar vertebrae. The malalignment between the thoracic and lumbar spine is evident. **e.** Erosion of the vertebral bodies in the region of the twelfth and thirteenth trunk vertebrae. **f.** Obvious vertebral body erosion associated with infectious osteomyelitis.

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# Karyotype of a rare minute frog, *Oreophryne* cf. *anulata* (Anura: Microhylidae), in Agusan Marsh, Mindanao, Philippines

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**ABSTRACT** - The Philippine archipelago has a rich amphibian fauna but data on their karyology are scarce. A recent amphibian diversity assessment of one of the largest wetlands and a major site for biodiversity conservation in the Philippines (Agusan Marsh Wildlife Sanctuary) reported three rare endemic species of minute frogs which are suspected to be new, including two undescribed species of *Oreophryne*. Here we describe the karyotype of one of the *Oreophryne* species, which is a form of *Oreophryne anulata*. Mature frogs were obtained by acoustic and opportunistic sampling from a site bordering Terminalia forests, and then reared in an enclosure for one to three months before they were karyotyped. Analyzable metaphase spreads were routinely obtained from seven colchicine-treated frogs by squashing intestinal epithelial cells followed by vapor fixation, air-drying and staining with aceto-orcein. Chromosome analysis indicated a normal diploid karyotype of  $2n=22$  including four metacentric and seven submetacentric chromosome pairs, without distinguishable sex chromosomes. The karyotype differs from that of the other *Oreophryne* species that have been karyotyped so far, for example, *Oreophryne biroi* in the Australo-Papuan region. This difference has an interesting evolutionary implication, which could contribute to the understanding of the mechanisms and rates of speciation in genus *Oreophryne* and is vital to the taxonomy and conservation of the endemic *Oreophryne* species in Agusan Marsh.

## INTRODUCTION

The Philippines has currently more than 100 species of amphibians and the current rate of discovery of new species is high (Almeria and Nuñez, 2013; Diesmos et al., 2002). About 84 (78.6%) of these amphibian species are endemic but this figure is likely to increase when new species are formally described (Diesmos et al., 2002). Unfortunately, most of these species are poorly known, and the few studies are limited to species composition, diversity, endemism, abundance and threats (Almeria and Nuñez, 2013; Alcalá et al., 2012; Nuñez et al., 2010; Relox et al., 2010; Diesmos et al., 2002; Brown et al., 2000). Despite the value of karyologic and genetic studies in formal classification of suspected new species, taxonomic revision of those that are believed to be a complex of cryptic species (Thode and Alvarez, 1983), and assessment of the variability and conservation status of many species, such studies about Philippine amphibians are scarce (Kuramoto and Yong, 1992). This lack of information is of concern due to global rapid declines of amphibian populations (Woodruff, 2010; Bickford et al., 2010). The increasing number of critically endangered, endangered and vulnerable species in the Philippines (Alcalá et al., 2012) demands an urgent assessment of the status of amphibian diversity in the Philippines.

A recent species inventory of Agusan Marsh, one of the largest wetlands and one of the centres of biodiversity in the Philippines, revealed a high diversity of amphibians with 41% endemism including three rare endemic species of minute frogs suspected to be new to science (Almeria

and Nuñez, 2013). Two of these belong to the microhylid frog genus *Oreophryne* of the subfamily Astereophryne, which is widespread in the Indo-Australian archipelago between the southern Philippines and New Britain. This genus is most diverse in New Guinea and immediate adjacent islands, and is the largest component of the Papuan microhylid fauna with 54 currently named species (Frost, 2015).

There are two currently recognised species of genus *Oreophryne* in the Philippines, *O. anulata* and *O. nana*, about which little is known (Alcalá, 1986). Both can be found in the southern part of the Philippines, specifically on the islands of Biliran, Camiguin and Mindanao. The two species of *Oreophryne* in Agusan Marsh, which are suspected to be new, still await further studies that could aid in their taxonomic classification and conservation.

This paper describes the karyotype of a suspected new *Oreophryne* in Agusan Marsh that is a close form of *O. anulata* (Fig. 1). To date, there is only one published report on the karyotype of a species of *Oreophryne*. *O. biroi* is an Australo-Papuan species with a diploid karyotype of  $2n=26$  (Mahony et al., 1992). No karyologic studies of this genus have been done in other localities where it is distributed including Indonesia and the Philippines. The present information could help clarify the evolutionary history, assess genetic diversity, and improve the conservation status of the species. Furthermore, it could contribute to the understanding of the mechanism and rates of speciation in this genus (Vences et al., 2002). Aside from supplementing the scarce karyologic data on amphibians in the Philippines, this study can also be

useful in monitoring resources of genetic diversity in its amphibians (Chulalaksananukul et al., 1998).

## MATERIALS AND METHODS

### Collection, identification and husbandry of frogs.

Ten mature frogs were collected by acoustic and opportunistic approaches in Sitio Kaliluan, Campo 6, Neuva Era, Bunawan, Agusan del Sur beside Magsagasang creek (08° 09.825" North latitude and 125° 58.044 East longitude) at an elevation of 27–30 masl. Collection was made in a site bordering a Terminalia forest inhabited by the amphibians of interest based on a previous survey (Almeria and Nuñez, 2013). Samples were acclimatised and reared in an open-system customised frog enclosure at an ambient temperature of 23–25 °C following the protocol of Poole and Grow (2012) for one to three months prior to sacrifice for chromosome analysis. The enclosure was artificially lit for 12 hours daily using a lamp, and the frogs were fed every other day with captured fruit flies (*Drosophila melanogaster*). They were regularly provided with moisture by spraying rainwater at least twice a day. Waste material was drained by pouring water into the enclosure and then draining it through a hole at the bottom.

The frog samples were referred to and identified by Dr. Arvin Diesmos, curator of the Zoology division of the National Museum of the Philippines, as *Oreophryne* cf. *anulata* which exhibits distinct differences from the *O. anulata* samples that he collected from various areas of Mindanao (personal communication, 04/01/14). The samples were morphologically similar to, and were likely belonging to the same population, as the *Oreophryne* sp. 1 previously reported by Almeria and Nuñez (2013) as a candidate new species. The frogs were identified as male on the basis of their advertisement calls and their vocal sac whereas females were identified by the presence of eggs in their coelom during dissection. All institutional and national guidelines for the care and use of laboratory animals were followed.

### Chromosome analysis.

Due to the minute size of the frogs, metaphase arrest was done by keeping the frogs more or less immersed in 0.05% colchicine solution in a shallow plastic container 3–24 hours before sacrifice. They were subsequently euthanised, washed in running water, and then immediately dissected. The entire intestine from the rectum to the anterior end of the stomach was removed, cleaned, and incubated for 30 minutes in a 0.05 M KCl hypotonic solution. Initial fixation was done by soaking the intestine in a freshly prepared 3:1 ethanol-acetic acid solution for at least 10 minutes. Then, it was immersed in fresh fixative solution overnight in a refrigerator. The fixed intestine was trimmed into 2–3 mm portions, transferred to a clean glass slide with a drop of 45% acetic acid, and then stained with 1% aceto-orcein for 20 minutes. The slide was covered with a cover slip and thumb pressure was applied to squash the cells and induce chromosome spreading.

Five to twenty-two metaphase spreads from each frog

sample were examined under a compound microscope with oil immersion objective, and the chromosomes in each spread were counted. At least three well-spread metaphases with the modal chromosome number were analyzed and used to prepare representative karyograms for each frog. Chromosome spreads were photographed by a CANON Power Shot A3200 IS digital camera with 14.1 megapixels. The actual sizes of the metaphase chromosomes were measured using a calibrated micrometer eyepiece. The chromosomes were cut out from the photographs and arranged in pairs according to size following the usual layout of a karyogram using the Paint tool of Microsoft XP. The lengths of the short arm and long arm, and the total length of the chromosomes, were digitally measured using the downloadable virtual actual-sized ruler application for Windows 2010. The measurements were encoded in an Excel spread sheet, and the following formulas were used to describe the features of the karyotype:

- (1) Relative length = (Chromosome length / Total number of haploid genome) X 100
- (2) Arm ratio = (Length of long arm / Length of short arm) X 100
- (3) Centromere index = (Length of short arm / Total length of the whole chromosome) X 100
- (4) Fundamental number = Total number of short arms + Total number of long arms

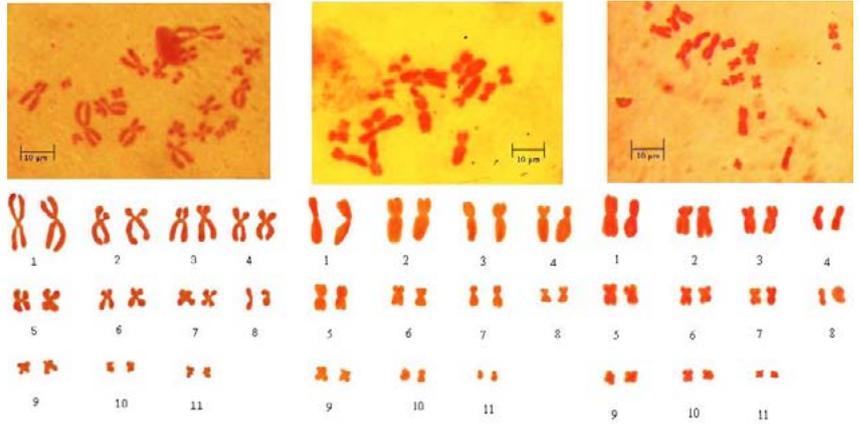
Based on the above measures, the chromosomes were classified according to the scheme of Levan and Tjio (1956), and an ideogram was constructed.

## RESULTS

Analyzable metaphase spreads were obtained from seven out of ten frog samples: three males, three females, and one with unverified sex. The data on chromosome counts indicate that the modal chromosome number is 22 (Table 1). As shown by the representative karyotypes of the seven frog samples (Fig. 2–3), all the chromosomes are monocentric and biarmed, and there are no heteromorphic sex chromosomes. Based on the calculated relative length (RL) of the chromosomes (Table 2), the karyotype of the analyzed frogs include one large (chromosome pair 1; RL > 15%), three intermediate (chromosome pairs 2, 3 and 4; RL > 10%), and seven small pairs (chromosome pairs 5–11; RL < 10%) (Table 2, Fig. 1–2). Based on the calculated centromere indices and arm ratios (Table 2), chromosome pairs 1, 9, 10, and 11 are metacentric while chromosome pairs 2, 3, 4, 5, 6, 7, and 8 are submetacentric (Levan and Tjio, 1956). Since all of the 22 chromosomes in the diploid karyotype of the frog samples are biarmed, the fundamental number is therefore 44.

## DISCUSSION

Since the frogs used in this study were minute (mean snout-vent length = 16.27 mm), colchicine treatment was carried out using the immersion technique. This was based

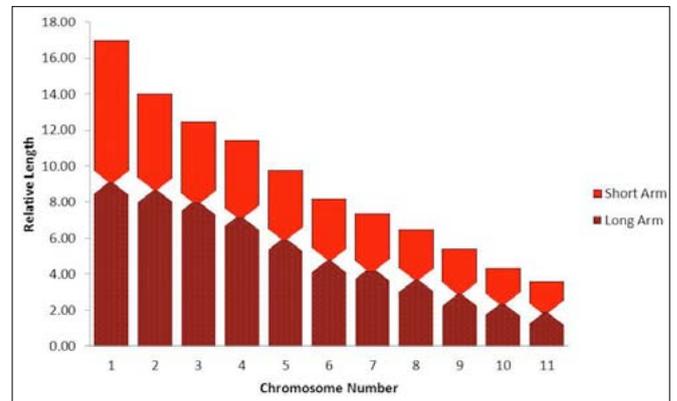


**Figure 1.** Adult female sample of *Oreophryne cf. anulata* on a Philippine one peso coin (diameter 24 mm).

**Figure 2.** Representative metaphase spreads (top) and karyograms (bottom) prepared from intestinal epithelium of three *Oreophryne cf. anulata* samples from Agusan Marsh, Bunawan, Agusan del Sur, Philippines.

Frog Sample	Sex	Chromosome Numbers										
		16	17	18	19	20	21	22	23	24	25	26
1	Male	2	1	-	2	2	2	12	-	-	-	1
2	Male	-	-	-	2	3	1	8	-	-	-	-
3	Male	-	-	1	1	3	1	6	-	-	-	-
6	Female	-	-	-	-	1	1	3	-	-	-	-
7	Female	-	1	1	-	-	-	4	-	-	-	-
8	Female	-	1	-	-	1	2	4	-	-	-	-
9	Unverified	-	-	1	1	1	-	8	-	-	-	-

**Table 1.** Frequency distribution of chromosome numbers observed in intestinal epithelial cells of seven *Oreophryne cf. anulata* samples from Agusan Marsh, Agusan del Sur, Philippines. The modal chromosome number is  $2n=22$ .



**Figure 3.** Ideogram of *Oreophryne cf. anulata* samples from Agusan Marsh, Bunawan, Agusan del Sur, Philippines.

Chromosome Number	Mean Arm Ratio	Mean Relative Length	Mean Centromere Index	Centromere Position
1	1.21	17.01	46.75	Nearly median
2	1.66	14.01	38.26	Sub-median
3	1.78	12.49	36.41	Sub-median
4	1.72	11.45	37.58	Sub-median
5	1.56	9.77	39.51	Sub-median
6	1.44	8.19	41.54	Sub-median
7	1.33	7.34	43.41	Sub-median
8	1.34	6.48	43.08	Sub-median
9	1.17	5.4	46.38	Nearly median
10	1.15	4.33	46.71	Nearly median
11	1.1	3.6	47.58	Nearly median

**Table 2.** Mean arm ratios, relative lengths, centromere indices, and centromere positions of chromosomes in intestinal epithelial cells of seven *Oreophryne cf. anulata* samples from Agusan Marsh, Agusan del Sur, Philippines.

on the fact that in frogs water intake is primarily through highly-vascularized “drink patches located on the posterior portion of their belly (Poole and Grow, 2012). However, the immersion technique did not yield a large number of analyzable metaphases. This could be due to a number of factors including insufficiency of colchicine that was actually absorbed through the skin, age, sex, physiological state of the amphibians, and effects of the hypotonic treatment and fixation. Although a heteromorphic pair of chromosomes was not detected, the presence of sex chromosomes cannot be discounted because the analyzed chromosomes were not differentially stained. For instance, the karyotype of *Bufo marinus* also lacks a heteromorphic pair of chromosomes but chromosome banding revealed a pair of sex chromosomes (Abramyan et al., 2009).

The karyotype of the frogs in this study differs considerably from that of the only other *Oreophryne* species that has been published, *O. biroi*, which is from the Australo-Papuan region. This has a diploid chromosome number of  $2n=26$  (Mahony et al., 1992). The latter’s karyotype consists of ten metacentric (1, 5-13) and three submetacentric (2-4) pairs, of which 1 is large, 3 are medium, and 9 are small pairs. This supports

previous studies of the relatively high chromosomal rearrangement in the family Microhylidae (Mahony et al., 1992; Kuramoto and Allison, 1989; Bogart and Nelson, 1976). The variations between members of the same genus may be due to several mechanisms including polyploidy and chromosomal rearrangements such as chromosome fusion, chromosome fission, and changes in composition of heterochromatin (De Mattos et al., 2014; Gruber et al., 2012; Vos et al., 2011; Wickbom, 1950). Morescalchi (1979) noted that rapid chromosomal evolution is associated with speciation in tropical habitats. On the other hand, Bogart (1981), proposed that chromosomal variability is correlated with the terrestrial habit (including parental behaviour, increased territoriality, and small clutch size).

According to Bogart and Nelson (1976) and Kuramoto and Allison (1989), the  $2n=26$  karyotype is “primitive” for Microhylidae. Mahony et al. (1992) suggested on the basis of the karyological data that the standard karyotype of the microhylid subfamily Asterophryinae (which includes the genus *Oreophryne*) found in New Guinea and Australia has  $2n=26$  chromosomes consisting of 5 large and 8 small pairs. The results of the present study show that a species of genus *Oreophryne* in the Philippines has a smaller diploid number of  $2n=22$ . All microhylids with  $2n=22$  chromosomes are said to be New World microhylid members of the Microhylidae (Bogart and Nelson, 1976).

Based on molecular studies, Kurabayashi et al. (2011) suggested that the colonisation route of *asterophryine microhylids* where the genus *Oreophryne* belongs was via Indo-Eurasia. According to their hypothesis, the family Microhylidae split into the subfamilies Asterophryinae, Microhylinae and Dyscophinae in India around 70 Ma. Microhylinae and Asterophryinae entered Eurasia while Dyscophinae proceeded to Madagascar. Asterophryinae split to Gastrophrynoidea in Southeast Asia around 48 Ma while the remaining Asterophryinae proceeded to New Guinea and Australia at around 25 Ma. However, the data of Mahony et al. (1992) and Kuramoto and Allison (1989) on the karyotype of genus *Oreophryne* in the Indo-Australian archipelago, and the karyological data on *O. cf. anulata* in the present study, do not support the suggested colonisation route of subfamily Asterophryinae via Indo-Eurasia. Instead, the data suggest that the origin of the Southeast Asian *Oreophryne* is from the Australo-Papuan population. This is illustrated by a reduced diploid chromosome number of 22 in *O. cf. anulata* from the Philippines as compared to the primitive diploid chromosome number of 26 in the Australo-Papuan *Oreophryne* species. The reduction in chromosome number from  $2n=26$  to  $2n=22$  might have resulted from chromosomal rearrangements such as fusion of smaller chromosomes in the “primitive” karyotype into larger chromosomes (De Mattos et al., 2014; Vos et al., 2011).

Since the karyological data presented in this study is the only report so far on an *Oreophryne* species, which is not from an Australo-Papuan form, further investigations of the karyology and molecular studies of the *Oreophryne* species present in the Philippines and the other Southeast Asian representatives are urgently needed to validate these initial findings.

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# Cannibalistic phenotype of the larvae of the Oita salamander *Hynobius dunni*

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## INTRODUCTION

The larvae of many temperate salamanders develop under conditions of high density, low productivity with associated low abundance of prey, and therefore strong competition (Wells, 2007). This has sometimes resulted in the evolution of facultative cannibalistic phenotypes, whereby some larvae develop morphology enabling them to consume conspecifics, at the cost of increased risk of disease (Pfennig et al., 1991). This is best studied in ambystomatid salamanders, particularly the *Ambystoma tigrinum* group (Hoffman & Pfennig, 1999), but has also been reported in other taxa (see Wells, 2007 and below). *Hynobius dunni* Tago, 1931 is distributed in lowland central Japan and breeds in early spring following snow-melt; its larvae develop in ponds where competition may be fierce (Kaneko & Matsui, 2004). This provides conditions under which cannibalistic morphs are likely to evolve, and cannibalistic larval phenotypes have been reported in allied taxa (see below). However, this adaptation has not been reported in *H. dunni*. The presence of this feature in *H. dunni* was investigated using a captive colony of salamanders.

## METHODS

Two pairs of *H. dunni* genetically originating in Beppu Shi, Oita Prefecture, Japan, were maintained in captivity and environmentally cycled throughout 2014-2015 to induce reproduction in early April 2015. One resulting larval clutch was maintained at an initial density of c.0.5 larvae/L (Low Density; LD; initial N = 42), and the other at an c.1 larva/L (High Density; HD; initial N = 73). Larvae were housed in mature, pond-style aquaria between 12 and 19°C and fed on aquatic invertebrates ad libitum. Developmental stages follow Iwasawa & Yamashita (1991). Larvae were photographed against a scale and measured using ImageJ (<http://imagej.nih.gov/>). Statistical analyses were performed in SPSS 22.0 for Windows.

## RESULTS AND DISCUSSION

Fifteen (26%) of HD larvae developed a cannibalistic phenotype (Fig 1) and there were no LD cannibalistic morphs. Measuring a subset of five larvae of each phenotype at Stage 51, cannibal larvae had wider heads

proportionately for their Snout-to-Vent Length (SVL) (mean maximum head width/SVL = 0.44) than normal larvae (mean maximum head width/SVL 0.35) and less pointed snouts (mean head width at eyes/max head width = 0.70 in normal larvae and 0.80 in cannibals). Larval duration, total length and mortality rates of HD and LD larvae are reported in Table 1. The cannibalistic phenotype differentiated at Stage 50 and such larvae were observed consuming conspecifics on three occasions; the corpses of the other 13 lost HD larvae were not found and these are presumed to have been cannibalised as well. Although only five of the 15 cannibals were measured, these were drawn randomly and were morphologically representative of the group as a whole. It was not practical to monitor the growth rates of larvae and so the developmental trajectory

Feature	High Density Larvae	Low Density Larvae
Larval duration (days post hatching)	74-113	70-84
Mean (N; SD) TTL at metamorphosis (mm)	72.3 (57; 4.0)	73.9 (41; 3.8)
Proportion of larvae with cannibalistic phenotype (%)	26	0
Mortality (no. dead/total)	16/73	1/42

**Table 1.** Larval duration, Total Length at metamorphosis, proportion of cannibalistic phenotype larvae and mortality in High- and Low-Density larvae of *Hynobius dunni*.

of cannibal and normal phenotypes in this species is not known.

Cannibalistic larval morphs have been reported in *H. retardatus* and *H. nebulosus tokyoensis* (Kusano et al., 1985; Nishihara, 1996; Michimae & Wakahama, 2001); more complete data for comparison are available for the former species. Irrespective of phenotype, *H. retardatus* larvae have proportionately broader heads (head width at eyes/max head width = 0.90 in cannibals and <0.86 in normal larvae, both at Stage 51; Michimae & Wakahama, 2001) than recorded here for *H. dunni*. However, there is a smaller disparity in this ratio between cannibal and normal larvae (difference = c. 0.04 in *H. retardatus* compared with c. 0.1 in *H. dunni*); i.e. *H. dunni* cannibals in this captive population are proportionately more distinct from normal



**Figure 1.** *H. dunni* larvae on 28/5/2015 (day 56 post hatching; Stage 51 sensu Iwasawa and Yamashita (1991)), showing normal (left) and cannibalistic (right) morphs.

larvae. The proportion of cannibalistic HD *H. dunni* larvae was within the range found in *H. retardatus* (Michimae & Wakahama, 2001). Like in *H. retardatus* (Nishihara, 1996), cannibalistic *H. dunni* larvae did not have the elongated vomerine teeth evident in *Ambystoma* cannibals (e.g. Lannoo & Bachman, 1984). Unlike in *H. retardatus*, there were no intermediate morphs and larvae conformed clearly to one of the two morphologies shown in Fig. 1.

Although unreplicated, these observations suggest that the development of cannibalistic morphs was triggered by higher stocking density, as is the case in *H. retardatus* and *H. tokyoensis* (Kusano et al., 1985 - describing *H. tokyoensis* as a subspecies of *H. nebulosus*; Nishihara, 1996; Michimae & Wakahama, 2001). HD larvae metamorphosed smaller (two-sample  $t_{96} = 1.933$ , 1-tailed  $p = 0.028$ ) and later (two-sample  $t_{96} = 8.82$ , 1-tailed  $p < 0.0001$ ) and mortality was substantially higher (see Table 1). This is indicative of density-induced larval stress in *Hynobius* (Kusano, 1981) and so is consistent with the development of cannibalistic larvae in response to competition. A single larva of the high density clutch maintained in isolation in 2L of water was able to grow to 86mm TTL at metamorphosis, suggesting that a simple genetic/maternal effect factor (as exists in *H. retardatus*; Michimae et al., 2009) is unlikely to alone have led to smaller size in the HD clutch. The cannibalistic phenotype developed despite larvae being fed ad libitum and so direct competition for food is unlikely to have been important; indeed in *H. retardatus*, cannibals develop in larvae held and fed separately from, but within the same aquarium as, conspecifics (Nishihara, 1996; Michimae & Wakahama, 2001), suggesting a chemical trigger may be involved.

Although at Stage 51 *H. dunni* cannibals are overall similar in their morphological modifications to those of other *Hynobius* species, these traits had disappeared by Stage 68 (metamorphosis) and all individuals looked alike, which is also documented in *H. retardatus* (Nishihara, 1996). It was not possible to follow individuals and so the effect of cannibalistic phenotype on size at metamorphosis and larval duration in *H. dunni* is unknown but the two distinct morphological groups evident in the larvae were no longer present. It is possible that by the end of

larval development, increased temperatures and higher productivity in nature, as well as reduced larval density and slowing larval growth rates at this point in development (Kusano, 1981), alters the balance between the benefits and presumed costs (Pfennig et al., 1991) so that it is not advantageous to maintain the cannibalistic phenotype later in development.

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# Common lizards (*Zootoca vivipara*) and slow-worms (*Anguis fragilis*) are not found in coppiced small-leaved lime (*Tilia cordata*) areas of a Northamptonshire-Cambridgeshire nature reserve

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## INTRODUCTION

Coppicing is a method of management in deciduous woodland in temperate climates in Europe. Stems are cut down nearly to ground level at regular intervals; the regrowth is of relatively small, young stems which traditionally have been harvested for a number of uses, such as for charcoal. Because the canopy in coppiced woodland is often more dense than in mature stands of trees, coppicing has in recent years been used as a conservation tool. It is considered to increase diversity of both flora and fauna, particularly in the case of several bird species, often providing a wider range of structure, light-penetration and age groups of trees (Harmer & Howe, 2006).

Collyweston Great Wood and Eastern Hornstocks National Nature Reserve (coordinates: 52.59395,-0.507216 and National Grid Reference TF 013 006) consists of two adjacent woodlands with a total area of 155 ha that sit on the border of Northamptonshire and Cambridgeshire in the UK. It is under the management of Natural England. The reserve is designated a Site of Special Scientific Interest (SSSI) and is classified as broadleaved, mixed and yew woodland (Gardiner, 2011). The two major habitats are dominated by ash-maple and pedunculate oak: neither of these has been coppiced in this woodland. This study, however, focussed on areas dominated by coppiced small-leaved lime trees (*Tilia cordata*). These are unusual: coppiced trees in the UK are most commonly hazel, sweet chestnut or ash (Harmer & Howe, 2006). The limes were traditionally coppiced for centuries in order to make rope and firewood. There are also areas of open ground, which are maintained artificially through cutting. They are mostly made up of bracken (*Pteridium aquilinum*), tufted hair grass (*Deschampsia cespitosa*), creeping soft grass (*Holcus mollis*), wood sedge (*Carex sylvatica*) and stinging nettles (*Urtica dioica*). Brambles (*Rubus fruticosus*) are particularly dominant in four out of the six open sites used (Gardiner, 2011).

The reserve was chosen for the study for several reasons. Firstly, it is a fragmented part of an ancient woodland that has had reptiles recorded in various parts of it in the past. Secondly, there were enough coppiced areas

of appropriate size and age for this study, as well as open areas. Thirdly, in terms of accessibility, the reserves were easy to visit daily on foot or by bicycle. Finally, Natural England management at this reserve specifically wanted information on the effect of coppicing on slow-worms. The overall aim of the study was to compare numbers of common lizards (*Zootoca vivipara*) and slow-worms (*Anguis fragilis*) in coppiced and open areas of the reserve.

## METHODS

In each part of the reserve, three young coppiced sites (coppice under 2-6 years old) and three older coppiced sites (coppice over 9-17 years old) were selected. Due to the small number of homogeneously coppiced sites, there were restrictions to the sites available. The sites in each category were selected to have a relatively homogeneous level of coppicing and to be of similar ages to each other where possible. Three open areas were sampled in the Eastern Hornstocks and two open areas were sampled in Collyweston Great Wood, (due to there not being enough open areas of an appropriate size). Two weeks into sampling, another open area in Collyweston was included when it became apparent that reptiles were only being detected in open areas.

Numbers of common lizards and slow-worms were assessed using coverboards: these were chosen as refugia due to their reduced disturbance on animals and vegetation (Houze & Chandler, 2002; Wilson & Gibbons, 2009), their low cost (Ryan et al., 2002) and because they create suitable microhabitats for attracting these reptiles (Wilson & Gibbons, 2009). In addition to this, coverboards are less conspicuous and prone to vandalism (Reading, 1997) than other physical methods. Furthermore, coverboards have a higher detection probability than visual transects at detecting slowworms (Sewell et al., 2012). Ideally, it is best to use a combination of different survey techniques for maximum detection probability (Ryan et al., 2002), but this was beyond the scope of the project.

In each site selected, ten pieces of roofing felt and ten pieces of corrugated bitumen, all 50 square cms, were laid out in an array with an alternating grid of materials with 5 meters between them. Originally, corrugated tin was going

to be used instead of bitumen as this is one of the most common coverboard materials used in studies (Wilson & Gibbons, 2009) but the tin ended up being too difficult to source and get cut into standardised sizes, as well as being expensive. The coverboards were left at the sites for one week, for the local reptile population to acclimatise to them being there, as well as for them to sink further into the vegetation, as is standard with coverboard studies (Wilson & Gibbons, 2009). The sites were sampled 3-6 days a week for 8 weeks. Each day, alternately, one reserve was sampled in the morning and the other later in the day. The reserves were alternately sampled in the mornings (approximately 8-10am) and afternoons (approximately 2-5pm).

## RESULTS AND DISCUSSION

Overall, 41 common lizards and 102 slow-worms were found in the open areas during the 8-week survey period. None were found in either category of coppice during the same period.

The importance of this finding is largely a practical one: no attempt is made here to explain the apparent absence of common lizards and slow-worms from coppiced areas, which was unexpected, although there is abundant evidence that under some circumstances, the exact nature of canopy cover can have an effect on reptile and amphibian populations (e.g. Greenberg, 2001; Webb et al., 2005; Pike et al., 2011; Sutton, 2013). One possibility, which needs to be investigated, is that the coverboards are not effective in coppiced areas because there is too little penetration of infra-red radiation to allow them to be used for thermoregulation. If this were to be the case, their attractiveness to reptiles would simply be as refuges. It is possible that this is insufficient to make them effective tools for assessing the presence of the animals, but this explanation seems unlikely, although there is some evidence that the efficiency of coverboards can vary depending on microhabitat conditions (Chavel et al. 2012). For the present, therefore, it seems prudent to assume that common lizards and slow-worms are either absent, or present at much lower densities, in both types of coppice than in open areas of woodland. While management of complex environments usually has many objectives – some of which may be conflicting – the fact that the coppiced areas in small-leaved lime woodland do not seem favourable for reptiles is a factor which managers of this habitat will need to take into consideration. The work reported here strengthens the case for continuing the practice of clear felling and maintaining open areas in woodlands, which is almost always beneficial for a wide range of flora and fauna (for reptiles and amphibians, see for example Greenberg, 2001; Goldstein et al. 2005; Pike et al. 2011).

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## *Pseudotriton ruber* (Red salamander): Larval defensive posture

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Salamanders employ numerous strategies to deter predation, including toxic or noxious skin secretions, aposematic, pseudoaposematic, or cryptic colouration, vocalizations, antagonistic defences such as biting and tail lashing, flight (escape) behaviours, caudal autotomy, and a variety of immobile defensive postures (reviewed in Brodie, 1983). Most immobile defensive postures in salamanders either display aposematic signals or increase crypsis, thus reducing the risk of injury or mortality to the salamander (Brodie, 1977). Defensive posturing has been well documented for adult forms of many salamander species (e.g. Brodie, 1977; 1983); however, defensive postures of larval salamanders are rarely reported (but see Brodie et al., 1974). This note reports observations of defensive posturing in a larval *Pseudotriton ruber*, a medium-sized semi-aquatic salamander native throughout much of the eastern United States (Petranka, 1998).

On 08 May 2015 at 17:00h (United States Eastern Standard Time), the author observed a larval *P. ruber* (total length: 10 cm) in a small unnamed spring near Bloomsburg, Columbia County, Pennsylvania USA (41°1'24.3"N, 76°27'1.0"W; WSG84 grid; 150 m elevation). Upon initial detection, the larval *P. ruber* swiftly crawled ~25 cm along the gravel substrate and subsequently bent its head in a downward position against the substrate (Fig. 1). After assuming the posture, the salamander remained immobile for approximately 60 seconds. Following a brief capture for identification, the salamander was released and again swiftly crawled ~15 cm, bent its head downward against the substrate and remained immobile for approximately 30 seconds before retreating underneath leafy debris.

When threatened, adult *P. ruber* frequently assume a defensive posture with the body curled, rear limbs extended, and tail raised (Petranka, 1998). Additionally, salamanders of the genus *Pseudotriton* are known to elevate, coil or flip



**Figure 1.** Line drawing of a larval *Pseudotriton ruber* in defensive posture.

their bodies, assume immobile postures, or undulate tails in response to a predator (Brodie, 1977; 1983). However, to my knowledge no descriptions of defensive posturing have been reported for larval *P. ruber*. Salamanders often exhibit “flash” behaviours consisting of sudden, rapid movements prior to immobility, likely to misdirect potential predators (Brodie, 1977). The rapid locomotion observed prior to immobility of the larval *P. ruber* may be analogous with such behaviour.

Salamander antipredator postures generally position the body in such a way as to increase the chances of survival if attack occurs (Brodie, 1977). The bending of the head in a downward position against the substrate observed in the larval *P. ruber* prior to immobility may protect the individual’s head from damage if attack occurs. Further research should examine the frequency and effectiveness of antipredator postures in both larval *P. ruber* and other larval salamanders.

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## *Pseudophilautus popularis* (common shrub frog): Male territorial combat and vocal interaction in Sri Lanka

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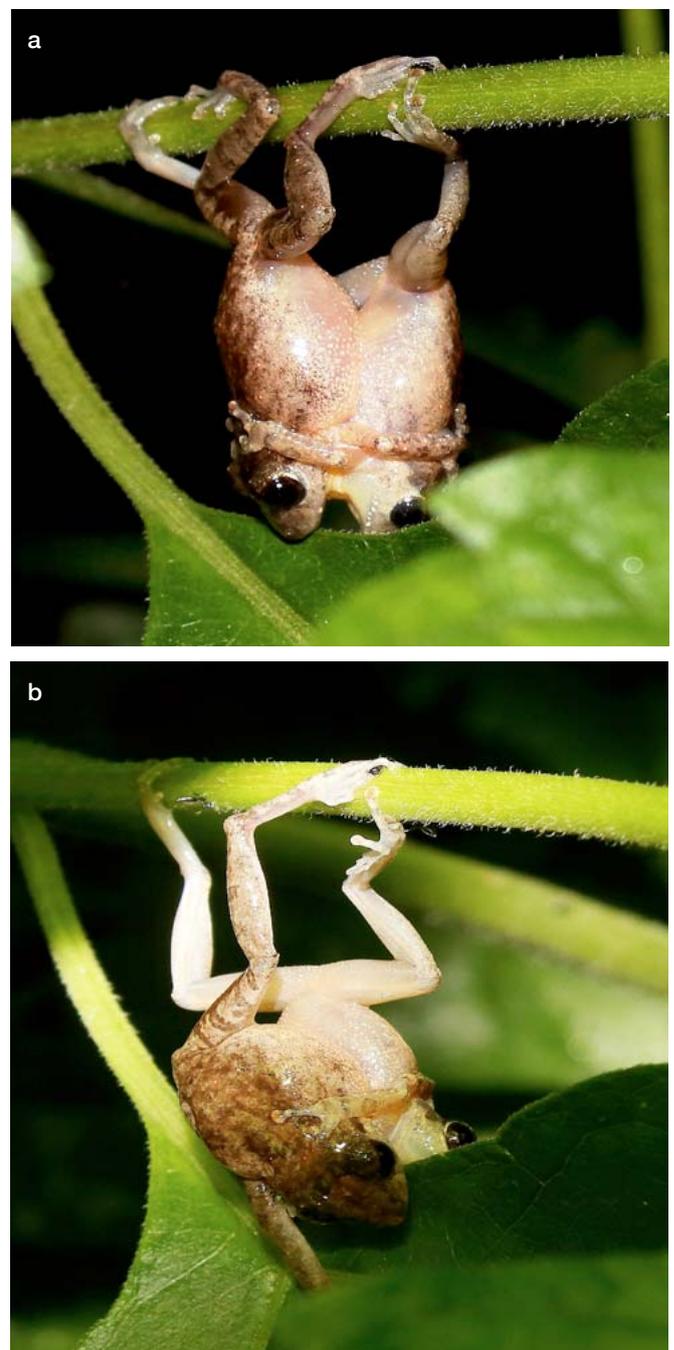
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The Common Shrub Frog, *Pseudophilautus popularis* (family: Rhacophoridae, Manamendra-Arachchi and Pethiyagoda, 2005) is a widely distributed species endemic to the low-county wet zone (up to 1060 meters above sea level) in Sri Lanka (Manamendra-Arachchi & Meegaskumbura, 2012). It is a synanthropic species that can be found near forest edges and grasslands (Manamendra-Arachchi & Pethiyagoda 2005; Karunarathna & Amarasinghe, 2010). Although *P. popularis* is commonly found within its distribution range, little is known about male-male interactions. Herein, we report the first ever observation of vocal interactions and combat behavior in a Sri Lankan amphibian, *P. popularis* (Manamendra-Arachchi & Pethiyagoda, 2005).

The observation was made at 2243 hrs on 14 June 2015 just after rain, in Hunuwela Rubber Estate, Ratnapura District, Sri Lanka (6.6375°N, 80.6000°E, WGS84, 280 m elevation). The site is surrounded by mature evergreen forests, including rubber and tea plantations, and is close to the southern slope of the central hills. Initially we encountered the advertisement calls of *P. popularis*, which sounded like “creek...creek...,” with the calls gradually sharpening as they progressed (see Samarasinghe, 2011). We observed the first male calling in a resting position on a leaf blade of *Chromolaena odorata* (family: Asteraceae) about 0.2 m above ground. The second male was also calling in a resting position about 0.3 m above ground on a branch of the same plant. Approximately one minute after our observations started, the first male approached the branch where the second male was sitting. At this point, the calls of both males change to a high-pitched call, which sounded like “tip...tip...” Each male called around 10 times while situated 3 cm apart on the same branch. Both frogs soon engaged in physical contact by grasping each other, presumably a sign of aggression, and by lifting their forelimbs and showing their swollen vocal sacs. A few seconds later, it appeared as if both males were about to fall off the branch but clung on



**Figure 1a.** Combat between two male *P. popularis*. **1b.** Photograph showing *P. popularis* male attempting to dislodge the second male *P. popularis*.

with their hindlimbs, while their forelimbs and upper bodies were locked in a tight clasp (Fig. 1a). Later, the first male was able to grasp a leaf of the plant and use it as an anchor and strike the second male with its remaining forelimb and both hindlimbs (Fig. 1b). Subsequently, the second male retreated off the branch and from the immediate region. The first male returned to making the high-pitch aggressive calls for a few more minutes. The event lasted for about eight minutes although no female frogs were observed in the vicinity.

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## *Natrix maura* (viperine snake) marine foraging.

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The viperine snake *Natrix maura* is native to south-western Europe and north-western Africa (Sindaco et al., 2013) where it is primarily an aquatic species that preys upon fish and amphibians (Braña, 1998; Rugiero et al., 2000). This species typically inhabits lentic and lotic freshwater habitats, but also tolerates waters with high salt concentration, in marshes and coastal pools (Steward, 1971; Schleich et al., 1996). However its presence in marine habitats is exceptional and has only been reported in a few cases, in Italy (Lanza, 1983), in southern Spain (Cabo & Olea, 1978) and in the Cies islands (western Spain; Galán, 2012). The populations of the Cies islands are adapted to the marine environment, feeding exclusively on marine fishes (e.g. *Lipophrys pholis*; Galán, 2012).

In September 2012, at the coordinates 41.41°N, 2.23°E (Sant Adrià del Besós, on the seashore in north-eastern Spain) we observed a *N. maura* on a rock feeding on an adult eel (*Anguilla anguilla*) (Fig. 1). The site of the observation is an artificial stone structure adjacent to the mouth of the River Besós but oriented to the open sea. This area is a fully saline environment, with little or no freshwater influence (because Besós is a small irregular river). We also captured another *N. maura* specimen that regurgitated a ringneck blenny *Parablenius pilicornis*. These observations extend the known geographical range of marine behaviour in *N. maura*. Moreover neither fish species was reported previously as prey of *N. maura*.

Populations of other European natricines are known to be associated with seashore/estuarine habitats (*N. natrix* and *N. tessellata*; Boulenger, 1913; Lanza, 1983; Lapini et al., 1999; Van der Meijden & Chiari, 2006; Strugariu et al., 2011; Baker, 2015). In Bulgaria, one coastal population of *N. tessellata* showed physiological tolerance to dehydration, which would allow these snakes to survive indefinitely in sea water (Brischoux & Kornilev, 2014).

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**Figure 1.** *N. maura* in the process of consuming an adult eel (*A. anguilla*) on the seashore (San Adrià del Besós, north-eastern Spain). Photographed by Miguel Ángel Fuentes.

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## *Trimeresurus malabaricus* (Malabar pit viper): Diving behaviour and underwater apnoea duration

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Pit vipers belong to the sub-family Crotalinae which includes about 231 species (The Reptile Database 2015). They are either arboreal or terrestrial with the exception of the Cottonmouth (*Agkistrodon piscivorus*), which is semi-aquatic in nature. Diving behaviour and apnoea (suspension of external breathing) have been well studied in aquatic elapids (Heatwole & Seymour 1975; Heatwole 1999; Ineich & Laboute 2002; Aubret et. al. 2007; Brischoux et. al. 2007), however, little is known about viperids occupying aquatic niches and their apnoea abilities.

Malabar pit vipers (*Trimeresurus malabaricus*) are often found in riparian habitat (Whitaker and Captain 2002; Ganesh et. al. 2010), however, diving behaviour has never previously been reported in this species. This note is based on two observations made on free ranging *T. malabaricus* diving voluntarily. Observations were made in the tropical moist evergreen forest of Agumbe, which is located in the central Western Ghats of Karnataka State in India (13° 31' 21" N, 75° 5' 17" E, elevation 650 - 850 m a.s.l.). This region receives heavy annual rainfall (7,500 to 10,000 mm) during the monsoon season (June to September). *T. malabaricus* is one of the most common venomous snake species found in this region.

On 10 April 2011 at 11 am, while following a King cobra (*Ophiophagus hannah*) during a telemetry project, which was moving along a shallow, slow flowing stream, an adult *T. malabaricus* (total length approximately 35 – 40cm) was seen on the same bank (13°31'24.68"N, 75° 6'18.57"E). After the *O. hannah* had passed through the area in which the *T. malabaricus* was sat unnoticed, the *T. malabaricus* proceeded to move into the stream. Swimming at a slow pace, the snake was observed to halt in the middle of the stream (approximate depth 35 – 45cm). The stoppage was followed by a short backward jolt of the leading half of the body, causing the snake to completely submerge 8 – 10 cm below the surface. After the backward jolt, the snake remained underwater for 5 - 6 seconds, of which approximately 3 - 4 seconds it was completely motionless. Resurfacing involved a slight forward progression during which only the head broke the surface for approximately 1 - 2 seconds, before repeating the backward jolt action. The snake repeated this action three more times, remaining submerged for 5 - 6 seconds each time. After surfacing for a fourth time, the snake swam a further 3 m downstream to a submerged horizontal branch. It then wrapped its tail



**Figure 1.** *T. malabaricus* coiled on a branch underwater with its body completely submerged.

around the branch and coiled its body in a typical ambush position, fully submerged approximately 5cm under the surface, with its head facing upwards (Fig. 1). Underwater tongue flicks with occasional slow sideward movement of the head were observed. The snake remained in this position without any attempt of surfacing to breath for approximately 20 minutes and then surfaced in a slow and controlled manner with tongue flicking, leaving the body still submerged and coiled. The snake was not visibly gasping or heavily breathing on surfacing. At this point the observation ceased due to the necessity of following the studied *O. hannah*.

In a further incident on 22 July 2015 at 10:20 pm, another adult *T. malabaricus* (total length approximately 35 – 40cm) was observed in an open concrete tank (80 cm wide, 250 cm long and 45 cm deep) at Agumbe Rainforest Research Station (13°31'3.76"N, 75° 5'21.39"E). This tank is a regular breeding site for Malabar gliding frogs (*Rhacophorus malabaricus*) during the monsoon season, which are common prey for *T. malabaricus*. The snake was initially observed floating, partially submerged, at the surface of the 30 cm deep water. The snake remained motionless with its head under the surface pointing downwards and floating in unusual sideward position due to an inflated body (Fig. 2). After approximately 1 minute the snake surfaced and swam a little distance. Once



**Figure 2.** The second *T. malabaricus* floating in a water tank with its head submerged. Notice the unusual sideward position due to inflated lung.

stopped, the snake retracted its body, with its head, once again, in a submerged downward position and kept floating. The snake repeated this action of partial submergence for four more times, remaining apnoeic for approximately 1 minute during each submergence event within 15 minutes of observation before it ceased due to heavy rain.

Aubret (2004) has shown that snakes may develop apnoea relative to the usage of an aquatic habitat. His study, along with our observations, raise questions about the diving and apnoea capabilities of all non-aquatic snake species. Furthermore, Heatwole (1977) suggests that all reptiles already possess advantageous fundamental physiology to allow survival in an aquatic environment if required. Further studies on the diving behaviour of *T. malabaricus* would shed more light on this topic.

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## Oophagy in the Smooth snake (*Coronella austriaca*)

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The smooth snake (*Coronella austriaca*) is a small colubrid that is relatively widely distributed throughout most of Europe. Its distribution becomes less continuous eastward where the species reaches western Kazakhstan, the Caucasus, northern Asia Minor and northern Iran (Sindaco et al., 2006; Corti et al., 2010; Drobenkov, 2014). Across its range, *C. austriaca* can be found in different habitats types ranging from forests to open meadows depending on latitude and altitude (Santos et al., 2009; Luiselli & Razzetti, 2010).

This non-venomous snake is known to be an ambush predator that is able to detect prey by chemical cues (Amo et al., 2004; Pernetta et al., 2009). The diet of *C. austriaca* mainly consists of lizards, small snakes and mammals; however, invertebrates including small amphibians are also predated (Luiselli, 1996; Reading & Jofré, 2013). Typology of consumed prey changes depending on snake size: larger snakes tend to feed mostly on larger prey (e.g., small mammals) than smaller snakes, which usually prefer Lacertids (Reading & Jofré, 2013). In this paper we report the first observation of oophagy in *C. austriaca*.

In May 2015, we were searching for *C. austriaca* in southern Tuscany (Central Italy) in order to take high quality pictures of this snake. In the late afternoon, an individual was found under a rock in a dry bushy open area bordering an oak wood, at an altitude of 90 m a.s.l. The total length of the snake was 35.5 cm and therefore considered as sub-adult. Sex recognition of *C. austriaca* at this life stage is quite difficult (Najbar, 2006; Van Gelder et al., 1988). Some pictures were taken placing the animal in a white plastic box. Probably due to the stress of being handled, the snake regurgitated six eggs most likely that of *Podarcis* lizards (Fig. 1). Both *P. sicula* and *P. muralis*, inhabit the surrounding area but it was not possible to ascribe the eggs to either species.

Evidence of feeding on eggs, either from faeces or stomach contents, is absent in *C. austriaca* despite the numerous individuals analyzed in previous studies (Luiselli, 1996; Reading & Jofré, 2013) although oophagy for the congeneric *Coronella girondica* has been reported (Luiselli et al., 2001).



**Figure 1:** *C. austriaca* regurgitating six eggs of an unknown *Podarcis* lizard.

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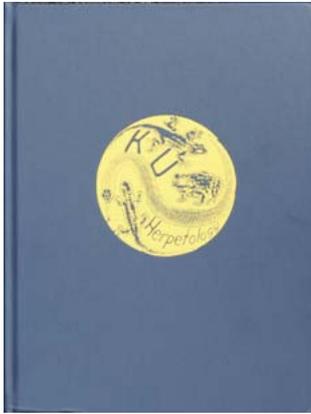
Accepted: 3 September 2015

# Herpetology at Kansas: A Centennial History

William E. Duellman (2015)

Publisher: Ithaca, New York, USA, Society for the Study of Amphibians and Reptiles (SSAR).

346 p. ISBN 978-0-916984-5



Those of us who live in the Old World often look rather condescendingly at our cousins in the New – we tend to assume, for example, that our institutions must all be much older than theirs. It comes as a surprise, therefore, to discover that the University of Kansas (KU) was founded in 1866, before most British “redbrick” universities had received their charters and almost a century before the

modern “plate glass and lavatory tile” genre had even been thought of. By 1901, KU had embarked on building a large, ornate Museum of Natural History. The collections quickly came to include amphibians and reptiles. It wasn’t until 1926, however, when Edward Taylor – who had made extensive herpetological collections in the Philippines and elsewhere – was appointed to the Faculty, that “herpetology at Kansas” might be considered to have been born.

This 346-page book was written by William Duellman, who was appointed Assistant Curator of Herpetology in 1959. He retired in 1997, having become (with Linda Trueb) the foremost authority on the amphibians of Central and South America, built up one of the biggest graduate programmes in herpetology in the world, and overseen the growth of the herpetological collection to nearly 300,000 specimens. The book was published to coincide with the 58th meeting of SSAR, held at KU in 2015, but it isn’t clear to me what the centenary was.

My first impression of this book was “this tells me more about the University of Kansas than I want to know”. But, although it’s a bit factual and – dare I say it – dull in places, a fascinating story emerges. As so often with successful human

endeavours, much hinged on the personalities of highly-motivated people. Sometimes they worked together in very productive ways – I lost count of the number of expeditions to South America and elsewhere mounted by staff and associates of the Museum. Sometimes they clashed: it was ever thus.

The volume is number 31 in the Contributions to Herpetology published by the Society for the Study of Amphibians and Reptiles. Like all the books in the series, it is beautifully produced, copiously illustrated with black-and-white photographs, has been edited with great skill and, so far as I can tell, is commendably free from errors and typographical mistakes.

So, a tour de force which is unique. I wrote that over-employed and often misused word advisedly – I don’t know anything which gives so much detail about the history of one institution in the annals of herpetology. It will stand as a monument, for many years to come, to the endeavours of a talented and devoted body of workers who built up an amazing organisation in what seems – condescension again – an unlikely place. This is a group of people who have made a seminal contribution to tropical herpetology. And as the book makes clear, they had a lot of fun doing so. Their parties were legendary, and many of the “big names” of academic herpetology were there. Gosh, how I wish that I could have been there too...

ROGER AVERY

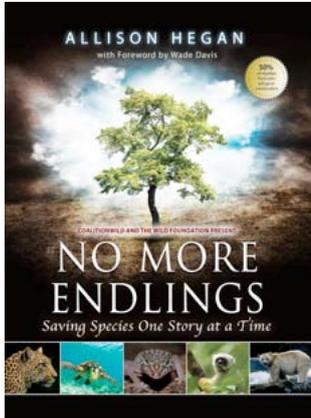
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*Accepted: 31 August 2015*

# No More Endlings, Saving Species One Story at a Time

Allison Hegan, Editor (2015)

Publisher: Motivational Press, Carlsbad, California. 429p. ISBN: 978-1-62-865198-0



No, there isn't a misprint in the title as printed above. An endling, apparently, is the last individual of a declining species or subspecies: when it dies, the taxon will be extinct. "Lonesome George" was a well-known herpetological example.

This is a most unusual book. The editor, Allison Hegan, has assembled 46 short accounts (they're mostly about 2000 words each) of animals, plants and in a few cases ecosystems, which are seriously declining or in danger of extinction. They're arranged geographically by continent. Each account follows a standardised but extremely original format. It starts with an anecdotal section dealing with some aspect of the subject, written in a popular vein. In my opinion, these sections are the best parts of the book. Have you ever tried, as an editor, to forge a unified set of texts from authors who come from a wide variety of backgrounds, have different standards and aspirations, and who write in very different ways? It's exceedingly hard to do, and Allison Hegan appears to have succeeded magnificently: there is a coherence about this book of a kind that is very rare in multi-authored works. Then there follows a short biography of the author(s) under the heading "Meet...". The formal classification is then given, followed by a more serious and factual account of the biology, threats and current efforts at conservation. Another novel feature is that each account also has a list of websites under the heading "To learn more and donate".

Reptiles and amphibians have eight entries: they are the barefoot banded gecko, *Coleonyx switaki*; the Antiguan racer snake, *Alsophis antiguae*; the Cuban iguana, *Cyclura nubila*; the hawksbill turtle, *Eretmochelys imbricata*; the Pebas stubfoot toad, *Atelopus spumarius*; the Mallorcan midwife toad, *Alytes muletensis*; the Malagasy rainbow frog, *Scaphiophryne gottlebi* and the false gharial, *Tomistoma schlegelii*. It would be easy to quibble about this list – barefoot banded geckos are not endangered over much of their range, for example, hawksbills are not the most endangered sea turtles and Cuban iguanas are not the most endangered iguanas – but that would be to miss the point. This is not a work of scholarship or for reference. It's propaganda – in the best sense of that word - written with passion and conviction in the clear hope that others will come to share the editor's and authors' belief that conservation is something that really matters. The whole thing is so well done that I'm sure it will succeed.

I haven't said anything about illustrations. That's because my review copy is an electronic one, and doesn't include them all. But the ones that I do have, like almost everything else about this volume, are very good indeed.

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Accepted: 3 November 2015



## BRITISH CHELONIA GROUP 2016 SPRING SYMPOSIUM

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For further information and directions visit: [www.britishcheloniagroup.org.uk](http://www.britishcheloniagroup.org.uk)

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